Strain Differences in the Hypolipemic Action of Dietary Calcium in Mature Male Rats[1]

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ABSTRACT

Two strains of rats, Holtzman and Wistar, were found to differ significantly in serum and fecal lipid response when fed a corn-soya diet containing 18% added cocoa butter or corn oil and 0.08% or 1.2% calcium. Interactions of strain with fat and with calcium were noted. The Holtzman rat usually had lower serum and tissue lipid levels and higher fecal lipid levels than the Wistar rat. The magnitude of the strain differences is sufficient to explain the incompatibility of results of the different investigators who have been studying lipid metabolism.

INTRODUCTION

CONFLICTING REPORTS have appeared on the effects of various fats on rat serum lipids. Hegsted et al. (1,2) and Aftergood et al. (3) found that oils high in di- and polyenoic fatty acids were hypocholesterolemic in rats whereas Okey at al. (4,5) and Best et al. (6) failed to find this effect. In experiments designed to elucidate possible mechanisms for the hypolipemic action of elevated levels of dietary calcium in man (7) and rats (8-10) different rat strains and different fats were employed. The experiments to be reported here compare lipid metabolism in two strains of rats.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar strain and male Holtzman strain albino rats, approximately 400 days of age, were housed two in a cage; four cages were used for each dietary treatment. The animals were fed the experimental diet and distilled water ad libitum. The rats were weighed weekly. Three-day fecal collections were made by cage during the second and third weeks of the experiment. The experimental period was 21 days.

Dietary Treatment

The basic composition of the corn-soya experimental diet has been reported previously (10). The experimental diet contained 2% endogenous fat to which 18% fat, either as cocoa butter or corn oil, and 2% cholesterol were added. The diets contained either 0.08% endogenous calcium or 1.20% calcium; 1.12% was added in the form of calcium carbonate. The added calcium replaced an equal weight of washed sand, which was employed to keep the diets isocaloric and isonitrogenous. The diets were calculated to contain 15.6% protein and 1.18% fiber.

Analytical Procedure

After 21 days on diet the rats were anesthetized with Nembutal and bled by heart puncture with disposable syringes. The serum was immediately separated and assayed. Eighteen hours prior to bleeding, food was removed. The rats were bled in an order designed to negate the effect of diurnal changes, such as reported by Rosenfeld and Lang (11). Livers were removed immediately, washed free of blood with water, and stored frozen until assayed.

The analytical procedures employed and the analytical standard errors have been reported previously (8,10). Statistical analysis was done by the analysis of variance.

RESULTS

Feed consumption and weight gains for all groups were comparable. Mean initial weight of the Wistar rats was 530 g, and of the Holtzman rats, 520 g. Feed consumption was determined both by feed weigh-back and, in those diets in which sand was an ingredient, by the determination of sand. The mean feed consumption, corrected for feed wastage, was 17.1 g per rat per day.

Fecal Lipid Changes

The results of fecal analysis are given in Table I. Fecal total lipids and fecal bile acids were significantly lower in the Wistar rats than in the Holtzman rats under comparable conditions of dietary fat and calcium, $P<0.01$.

[1]Presented at the 57th AOCS Annual Meeting, Los Angeles, May 1966.

TABLE I

Fecal Analysis in Two Strains of Rats Fed Cocoa Butter or Corn Oil at Two Calcium Levels[a]

Strain	Holtzman				Wistar			
Fat fed	Corn oil		Cocoa butter		Corn oil		Cocoa butter	
Calcium level (%)	0.08	1.2	0.08	1.2	0.08	1.2	0.08	1.2
Fecal excretion								
Wet (g)	8.13 ± .23	8.29 ± 1.12	7.88 ± 0.40	9.24 ± 0.10	7.58 ± 0.66	6.90 ± 0.65	4.92 ± 0.22	6.67 ± .37
Total lipid (mg)	670 ± 33	978 ± 44	962 ± 68	1968 ± 78	490 ± 33	615 ± 35	583 ± 54	1664 ± 97
3-β-Hydroxysterols (mg)	407 ± 26	417 ± 26	355 ± 15	506 ± 13			216 ± 19	337 ± 50
Bile acids (mg)	6.3 ± 1.2	23.2 ± 0.6	10.2 ± 0.5	27.1 ± 0.5	4.7 ± 0.5	11.9 ± 0.4	5.5 ± 0.9	10.5 ± 1.0
Lipid phosphorus as phospholipid (mg)	9.9 ± 0.4	8.0 ± 1.8	11.2 ± 0.3	15.9 ± 0.4	6.3 ± 0.5	18.2 ± 0.9	6.8 ± 0.1	6.5 ± 0.8

[a] Results given in mean weight per rat per day ± standard deviation of mean.

TABLE II

Serum and Liver Lipids in Two Rat Strains Fed Cocoa Butter and Corn Oil at Two Calcium Levels[a]

Strain	Holtzman				Wistar			
Fat fed	Corn oil		Cocoa butter		Corn oil		Cocoa butter	
Calcium level (%)	0.08	1.2	0.08	1.2	0.08	1.2	0.08	1.2
				Mg per 100 ml				
Serum								
Total lipids	438 ± 44	400 ± 27	600 ± 89	400 ± 40	613 ± 26	688 ± 43	519 ± 23	494 ± 12
Phospholipids	113 ± 5	92 ± 9	172 ± 4	119 ± 3	117 ± 2	167 ± 12	126 ± 4	111 ± 6
Cholesterol	126 ± 2	73 ± 2	168 ± 7	93 ± 2	181 ± 14	119 ± 4	186 ± 17	128 ± 4
Triglycerides	126 ± 15	70 ± 5	126 ± 16	66 ± 3	288 ± 4	283 ± 5	131 ± 20	72 ± 4
				Mg per Liver				
Liver								
Total lipids			1822 ± 64	1653 ± 143			2438 ± 291	2099 ± 250
Cholesterol			511 ± 35	540 ± 47			621 ± 75	610 ± 65
Triglycerides			395 ± 105	276 ± 55			422 ± 66	337 ± 75

[a] Results are presented as mean ± standard deviation of mean.

Similarly the fecal excretion of 3-β-hydroxysterols was lower in the Wistar rats fed cocoa butter, at comparable calcium levels, than in the Holtzman rats, $P<0.01$. The excretion of lipid solvent-soluble phosphorus was higher in the Holtzman rats fed cocoa butter, at comparable calcium levels, $P<0.01$, and increasing dietary calcium increased fecal lipid solvent-soluble phosphorus in the Holtzman rats fed cocoa butter, $P<0.01$, but not in the Wistar rats. When the fat was corn oil, the reverse was noted in that increasing dietary calcium increased fecal lipid solvent-soluble phosphorus in the Wistar rats, $P<0.01$, but not in the Holtzman rats.

Serum Lipids are shown in Table II. Serum cholesterol was significantly higher in the Wistar rat than in the Holtzman rat with corn oil at both calcium levels, $P<0.01$, and with cocoa butter at the 1.2% calcium level, $P<0.01$. Although serum cholesterol was lowered by corn oil when compared with cocoa butter in the Holtzman rats at the 0.08% calcium level, no such effect was noted in the Wistar rat.

Whereas serum triglycerides were significantly higher in the Wistar rat than in the Holtzman rat when fed corn oil, $P<0.01$, no significant difference was noted with the cocoa butter diet. Elevated dietary calcium lowered serum triglycerides in Holtzman rats fed corn oil, $P<0.01$, but no lowering was noted under comparable conditions in the Wistar rat.

An examination of the data on serum phospholipids indicates a significant interaction between strain and diet. If the effect of corn oil and cocoa butter, averaged over the two calcium levels, is compared, the mean serum phospholipids were 103 and 146 mg per 100 ml for corn oil and cocoa butter respectively in the Holtzman rats and 142 and 119 mg per 100 ml respectively in the Wistar rats. Thus, in the Holtzman rats, corn oil lowered serum phospholipids as compared with the cocoa butter, and in the Wistar rats the corn oil caused an elevation in serum phospholipids as compared with cocoa butter. This interaction between strain and diet was significant at $P<0.01$. Although the serum phospholipids were lowered with increasing dietary calcium, $P<0.01$, this lowering was most pronounced in the Holtzman rats fed cocoa butter.

In serum total lipids there was an interaction between strain and calcium, $P<0.01$. Thus elevated dietary calcium significantly lowered serum total lipids in the Holtzman rats fed cocoa butter but did not do so in the Wistar rat. Interactions were also noted between dietary fat and calcium, $P<0.01$, and between dietary fat and strain, $P<0.05$. Corn oil thus caused a lowering of serum total lipids in the Holtzman rat but elevated it in the Wistar rat.

Liver Lipids

Although individual comparisons of the various liver lipids were not significant at $P<0.05$ (Table II), the mean total lipids, cholesterol, and triglycerides of the liver were always lower in the Holtzman rat than in the Wistar rat.

DISCUSSION

The data indicate a significant difference between the two strains, both in their response to calcium and to dietary fat. This type of difference could account for the conflicting results of different investigators. It is particularly noticeable in the excretion of cholesterol metabolites, fecal bile acids, and fecal-3-β-hydroxysterols since the Wistar strain invariably excreted less. This difference in cholesterol metabolite excretion is reflected in the significantly higher serum cholesterol levels noted in the Wistar strain when compared with the Holtzman strain under comparable dietary conditions of fat and calcium. Similarly the lowered excretion of total lipids by the Wistar strain, which in large part is accounted for by free fatty acids (12), is reflected in the higher serum triglycerides of the Wistar strain.

Strain-dependent serum cholesterol levels in rats have been reported previously by Kohn (13), who noted that hypophysectomy negated these strain differences although at an elevated cholesterol level. More recently Moore and Williams (14) reported similar differences in plasma lipid composition and degree of aortic atherosclerosis in different strains of New Zealand White rabbit. Similarly Clarkson et al. (15), working with pigeons, found breed differences in the degree of and even incidence of intimal lesions in the thoracic aorta. The results reported in this paper are in agreement with those of Kohn (13) with respect to serum cholesterol but also show that the other serum lipids are strain-dependent.

Based upon these results, it becomes imperative that the rat strain and previous dietary history be specified when reporting results of lipid metabolic studies. These strain differences could be particularly important in

comparing results of different investigators and in explaining ostensibly conflicting data.

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Chloroplast Pigments of the Marine Dinoflagellate *Gyrodinium resplendens*

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ABSTRACT

The photosynthetic marine dinoflagellate, *Gyrodinium resplendens,* was grown axenically and harvested during logarithmic growth for analysis of its lipid-soluble pigments. Chlorophyll *a* and 8 carotenoids were isolated from the methanol and acetone extract by column and thin-layer chromatography. Chlorophyll *c* was isolated by partitioning the total extract between saline aqueous acetone and hexane.

Absorption spectra taken in hexane, ethanol, methanol and carbon disulfide confirm the presence of β-carotene, peridinin, dinoxanthin and diadinoxanthin as major carotenoids.

Four new minor xanthophylls are also described, one of which, named *pyrrhoxanthin,* resembles an alkali-labile keto-epoxide. At least one of the minor xanthophylls occurs as an ester.

Diadinoxanthin from *Gyrodinium* and antheraxanthin from *Euglena gracilis* seem to be identical with respect to absorption curves, polarity, number of 5, 6-epoxy groups and lack of allylic hydroxyl groups; however, co-chromatography of stereoisomers after iodine-isomerization showed slight differences.

Most of the carotenoids are further characterized here by their partition ratios between hexane and 95% methanol. Several of the carotenoids were tested for the presence of 5, 6-epoxy and allylic hydroxyl groups. Four of the pigments, comprising 91% of the total carotenoids are revealed as 5,6-monoepoxides by their instability toward dilute acid. One carotenoid resembles a diepoxide.

INTRODUCTION

SCHÜTT (1) WAS THE FIRST to examine the carotenoid pigments of the Pyrrhophyta or dinoflagellates. It was he who identified the major carotenoid, peridinin (= sulcatoxanthin), which has since been found in the majority of photosynthetic species investigated. All of these dinoflagellates contain other xanthophylls as well. The same or similar carotenoids have also been extracted from certain marine invertebrates containing symbiotic dinoflagellates or "zooxanthellae."

In the first thorough study of dinoflagellate pigments, Strain et al. (2) identified β-carotene, chlorophylls *a* and *c*, and peridinin and described two new xanthophylls, dinoxanthin and diadinoxanthin. These workers, as well as Pinckard et al. (3), also noted the presence of unrecognized carotenes and xanthophylls. The present study deals particularly with these undescribed pigments.

Other investigations of dinoflagellate pigments and their spectral features are summarized in Table I.

PROCEDURE

Cellular Material

Gyrodinium resplendens Hulburt, 1957, was isolated from a plankton sample from a marine slough near Long Beach, California and raised as an axenic, clonal culture in a supplemented sea water medium (Table II). This clone has been deposited in the Culture Collection of Algae, Indiana University. The cultures were grown in fernbach flasks (2.8 liter size) each containing 1 liter of medium, for 15 days, when a concentration of 1.21×10^4 cells per milliliter was reached. Both cool and warm white fluorescent (1:2) lamps furnished constant illumination at an intensity of 500 to 700 ft-c. Nineteen liters were harvested during the logarithmic growth phase by a Sharples continuous centrifuge yielding 19 g of wet cells.

Extraction of Pigments

Pigments were extracted by soaking wet cells repeatedly in fresh volumes of methanol and finally in acetone; this was done over a period of several hours in the dark at approximately 23C under a nitrogen atmosphere. The dry weight of the extracted cells was 2.66 g (14% of the wet weight). Pigments in the combined methanol and acetone extracts were transferred to diethyl ether following a several-fold dilution with a saturated sodium chloride solution. The aqueous phase was extracted exhaustively

TABLE I

Absorption Maxima Reported for Known Dinoflagellate Pigments
(Natural sources other than dinoflagellates are noted below)

Pigment	Solvent	Absorption maxima (mμ)	Natural source	Reference
Chlorophyll *a*	Acetone	663, 615, 580, 535, 430, 410	Various angiosperms	(4)
	Acetone	665, 618, 580, 535, 430, 412	*Gymnodinium* sp.	(5)
	Diethyl ether	662, 615, 578, 535.5, 430, 410	Stinging nettles	(6)
	Methanol	657, 612, 571, 532, 504, 494	Dinoflagellate symbiont of *Anemonia sulcata* (Anthozoa)	(7)
Chlorophyll *c*	Diethyl ether	626, 577.5, 443.6	*Nereocystis luetkeana* (Phaeophyta)	(6)
	Diethyl ether	633, 581, 450	*Gonyaulax polyedra*	(8)
	Diethyl ether	625, 580, 445	*Gymnodinium* sp.	(5)
	Diethyl ether	627, 578, 447	*Exuviaella* sp.; *Amphidinium carterae*	(9)
	Methanol	635, 588–9, 450	*Gonyaulax polyedra*	(10)
	Methanol	633, 584, 450	*Amphidinium carterae*	(9)
Dinoxanthin	Ethanol	471, 441.5, 416	*Peridinium cinctum;* dinoflagellate symbiont of *Anthopleura xanthogrammica* (Anthozoa)	(2)
	Ethanol	475, 446[a]	*Gymnodinium* sp.	(5)
	Ethanol	475, 445[a]	*Peridinium trochoidium*	(11)
Diadinoxanthin	Hexane	477–8, 447–8	*Exuviaella* sp.; *Amphidinium carterae*	(9)
	Ethanol	478, 448, 423	*Peridinium cinctum;* dinoflagellate symbiont of *Anthopleura xanthogrammica* (Anthozoa)	(2)
	Ethanol	475, 446[a]	*Gymnodinium* sp.	(5)
	Ethanol	475, 445[a]	*Peridinium trochoidium*	(11)
Pigment G	Ethanol	456, 430, 407[b]	*Amphidinium carterae*	(12)
Neodinoxanthin	Ethanol	446, 438	*Peridinium cinctum;* dinoflagellate symbiont of *Anthopleura xanthogrammica* (Anthozoa)	(2)
		(no spectra)	*Gymnodinium* sp.	(5)
		(no spectra)	*Peridinium trochoidium*	(11)
Neodiadinoxanthin	Petroleum ether	472, 442	*Peridinium trochoidium*	(11)
	Ethanol	471, 442	*Peridinium cinctum;* dinoflagellate symbiont of *Anthopleura xanthogrammica* (Anthozoa)	(2)
Peridinin	Hexane	490, 457	*Prorocentrum micans* (red tide)	(3)
(= Sulcatoxanthin)	Hexane	485, 454	*Exuviaella* sp.; *Amphidinium carterae*	(9)
	Petroleum ether	485, 455	*Peridinium cinctum;* dinoflagellate symbiont of *Anthopleura xanthogrammica* (Anthozoa)	(2)
	Ethanol	475	*Peridinium cinctum;* dinoflagellate symbiont of *Anthopleura xanthogrammica* (Anthozoa)	(2)
	Ethanol	470[a]	*Gymnodinium* sp.	(5)
	Methanol	462–3	*Gonyaulax polyedra*	(10)
	Carbon disulfide	516, 482, 450	Dinoflagellate symbiont of *Anemonia sulcata* (Anthozoa)	(7)
	Carbon disulfide	516, 483	*Peridinium cinctum;* dinoflagellate symbiont of *Anthopleura xanthogrammica* (Anthozoa)	(2)
	Carbon disulfide	516.7, 481.9	marine mud (Gulf of California)	(13)
	Carbon disulfide	518, 482, 449	*Prorocentrum micans* (red tide)	(3)
	Benzene	502, 465	*Prorocentrum micans* (red tide)	(3)
Neoperidinin	Ethanol	464	*Peridinium cinctum;* dinoflagellate symbiont of *Anthopleura xanthogrammica* (Anthozoa)	(2)
	Ethanol	470[a]	*Gymnodinium* sp.	(5)

[a]Believed impure by original authors.
[b]Chromatographic position and absorption maxima indicate diadinoxanthin-furanoid-oxide.

with ether. The combined ether extracts were dried by filtration through anhydrous sodium sulfate and the ether removed under reduced pressure in a rotary evaporator. The oily residue was redissolved in hexane.

Chromatography

All chromatography of pigments was carried out in dim artificial light, or in darkness when possible. Exposure of pigment solutions to light and atmospheric oxygen was kept to a minimum. A variety of adsorbents was used for chromatographic isolation of pigments. Dry-packed columns of Silica Gel H: Celatom (diatomaceous earth) in 1:1 ratio gave preliminary separation of all zones. Pigments were eluted with dilutions of 5-20% acetone in hexane. Each was purified by column chromatography on Silica Gel H, magnesium oxide or calcium carbonate, each mixed with an equal weight of Celatom. Separation of all major pigments was repeated by thin-layer chromatography (TLC) on Silica Gel G (250 μ layer). Zones bearing the adsorbed pigment were scraped individually from the plate and eluted immediately with methanol. The silica gel was retained on a sintered glass filter. Pigment solutions were evaporated under a stream of dry nitrogen at less than 50C.

Absorption Spectra

Absorption spectra were taken on a Model 505, Bausch and Lomb recording spectrophotometer. The opal glass technique (15), for living cell absorption, was used with a Cary Model 14 recording spectrophotometer. Absorption spectra were taken in Eastman Kodak "Hexane" (bp 68-69C); other solvents were of reagent grade and were used without further purification.

Partition Coefficients

The optical density of pigment solutions in hexane were read before and after partitioning (by shaking 20 times) with an equal volume of 95% methanol freshly equilibrated with hexane. This procedure when applied to known carotenes and xanthophylls was found to duplicate within 2% the results of Petracek and Zechmeister (16). Following their method, the hexane phase is also pre-equilibrated with 95% methanol.

Saponification

Hydrolysis of esters was carried out overnight in 4% solution of sodium hydroxide in 95% ethanol in the dark under nitrogen at room temperature.

TABLE II

Enriched Sea Water Soil Extract Medium for the Culture of Marine Dinoflagellates

Filtered sea water (Whatman No. 1)	750 ml
Glass distilled water	200 ml
Soil extract (autoclave 1 part soil with 1 part water; filter and use the liquid)	15 ml
KNO_3	2 mM
K_2HPO_4	0.2 mM
Thiamin HCl	1 mg
Vitamin B_{12}	1 μg
Biotin	2 μg
Trace element solution	30 ml
pH of medium not adjusted	

Composition of the Trace Element Solution

Final concentrations in the culture medium (not including elements present in the sea water and soil extract) when using 30 ml of the stock solution per liter of medium. P II metals (14).

EDTA (Na, H_2O)$_2$	80.6 μM
H_3BO_3	553 μM
$FeCl_3.6H_2O$	5.4 μM
$MnCl_2.4H_2O$	21.8 μM
$CoCl_2.6H_2O$	0.5 μM
$ZnCl_2$	2.3 μM
pH of trace element stock solution	7.5

Epoxide Detection

A drop of 9% hydrochloric acid was added to several milliliters of a pigment solution in either ethanol or methanol. A hypsochromic shift of the absorption maxima of 5,6-epoxides was complete after 5 min. The change to the 5,8-furanoid-oxide was accompanied by a slight decrease in extinction.

Carbonyl Reduction

The pigment was dissolved in several milliliters of dry diethyl ether and reduced by adding a few milligrams of lithium aluminum hydride. After 5 min the resulting yellow precipitate was decomposed by careful addition of ethanol.

Methylation

For each milliliter of pigment solution in methanol one drop of 9% hydrochloric acid was added and the mixture allowed to stand for 1 hr in the dark under nitrogen at room temperature.

All of the above reactions were carried out on carotenoid solutions of optical density <1 (light path 1 cm).

Iodine-Catalyzed Isomerization

An equilibrium mixture of isomers was prepared by illuminating (intensity 175 ft-c) with cool white fluorescent lights a spectrophotometrically determined weight of the pigment in hexane with 1-2% by weight of iodine for 3 hr or until equilibrium was reached. Equilibration of isomers occurred only if the hexane

TABLE III

Spectral Characteristics of *Gyrodinium* Pigments

(Pigments are listed in order of elution. Absorption minima are given in parentheses.)

Compound	Absorption maxima (minima) in mμ				
	n-hexane	Ethanol	Methanol	Carbon disulfide	Other solvents
β-Carotene	475 (465) 448, ~427				~485, 460 (Chloroform)
Gy 443 (a + b)	473 (460) 443, ~420	473 (461) 444, ~420		502 (488) 470, ~444	
Pyrrhoxanthin	487 (477) 459	471		512, 586	
Pyrrhoxanthin-furanoid-oxide	463 (455) 438				
Gy 439	469 (455) 439 (421) 416	470 (458) 441 ~419		497 (483) 466 ~441	
Chlorophyll *a*					661, 616, 572, 528 428, ~411 (diethyl ether)
Dinoxanthin	469 (456) 439 (424) 416	470 (459) 441 (422) 419	467 (455) 438 (420) ~416	498 (485) 467 ~441	
Dinoxanthin-furanoid-oxide			445 (436) 419 (406) 396		
Diadinoxanthin	475 (462) 445 ~421	476 (463) 446 ~424		506 (492) 474 ~449	
Diadinoxanthin-furanoid-oxide	456 (444) 429 ~408				
Peridinin	484 (471) 454 ~431	472	467	512 (499) 480 ~454	
Peridinin-furanoid-oxide	461 (449) 433 ~411		448		
Gy 442	468 (460) 442 ~429		469 (458) 440 ~417		
Chlorophyll *c*					663, 582, 447 (90% acetone)

was first dried by filtration through a silica gel column.

RESULTS

Fig. 1-6 are absorption spectra in various solvents of the isolated pigments of *Gyrodinium*. In some cases the identification of *cis* peaks is questionable due to lipid contaminants which absorb in the near UV. Table III summarizes the absorption maxima of these pigments. Further information on partition behavior and on the results of epoxide tests are given in Table IV. The following carotenoids are discussed in order of their elution from Silica Gel H-Celatom columns prior to saponification. Chlorophyll *a* followed pigment Gy 439 in this sequence.

β-carotene

A single carotene comprised 2% of the total carotenoids and was the first pigment eluted during chromatography of the total hydrolyzed or unhydrolyzed pigments. In absorption curve and partition ratio (100:0) it resembles partially isomerized β-carotene. In thin-layer co-

TABLE IV

Chemical Characteristics of *Gyrodinium* Carotenoids

(Pigments are listed in order of elution from Silica gel H-Celatom columns; elutant 5-20% acetone in hexane.)

Carotenoid	Relative abundance	5,6-Epoxide test	Partition ratio between hexane and 95% methanol		
			Unsaponified	Saponified	After Methylation
β-carotene	ca. 2%	....	100:0	100:0	....
Gy 443 *a*	Trace	Negative	93:6	30:70	....
Gy 443 *b*	Trace	Negative	80:20	....	....
Pyrrhoxanthin	Trace	Positive	77:23	Base-labile	75:25
Gy 439	ca. 3%	Negative	28:72	19:81	....
Dinoxanthin	ca. 18%	Positive	7:93	7:93	15:85
Diadinoxanthin	ca. 33%	Positive	6:94	6:94	9:91
Peridinin	ca. 40%	Positive	2:98	Base-labile	15:85
Gy 442	ca. 3%	Positive	....	....	....

Relative abundance was determined spectrophotometrically. The approximate weight was calculated using the extinction coefficient of β-carotene: $E^{1\%}_{1\ cm}$ at 451 mμ in light petroleum = 2505 (17)

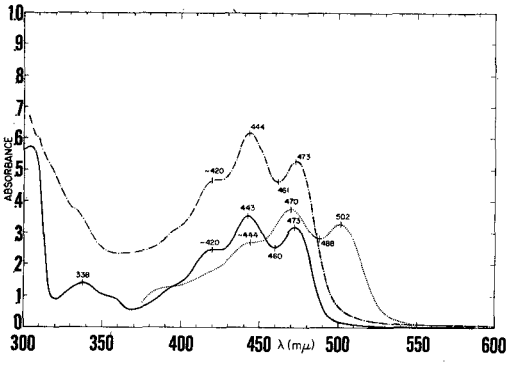

FIG. 1. Gy 443. Solid line: hexane. Dash-dot line: ethanol. Dotted line: carbon disulfide.

chromatography (solvent hexane) with synthetic α and β-carotene this pigment was inseparable from the latter.

GY 443

A trace of this yellow xanthophyll was eluted from the column with 5% acetone in hexane. The change to predominantly hypophasic character after saponification of the natural ester suggests the presence of 2 hydroxyls. Re-chromatography of this pigment on Silica Gel G plates yielded a slight separation into 2 yellow zones, *a* and *b* of absorption maxima (hexane): *(a)* 472, 442, ~ 419, 337 and *(b)* 470, 441, ~ 418, 337, respectively. The close similarity of spectra and exact coincidence of the *cis* peaks suggests they are isomers. The partition ratios before hydrolysis were: *a*, 93:6 and *b*, 80:20. Lack of sufficient pigment prevented comparison of *b* with *a* after hydrolysis of the esters. Neither *a* nor *b* was affected by dilute acid, indicating an absence of 5,6-epoxy groups. Both zones were completely stable on Silica Gel G thin-layer plates. Fig. 1 shows the absorption spectra of GY 443 in hexane, ethanol and carbon disulfide.

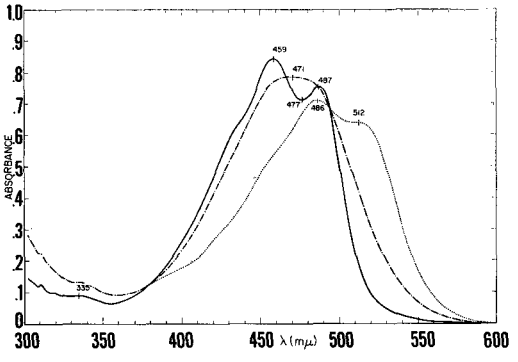

FIG. 2. *Pyrrhoxanthin*. Solid line: hexane. Dash-dot line: ethanol. Dotted line: carbon disulfide.

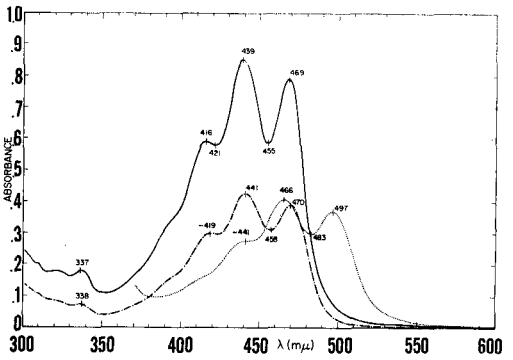

FIG. 3. Gy 439. Solid line: hexane. Dash-dot line: ethanol. Dotted line: carbon disulfide.

Pyrrhoxanthin (GY 459)

Trace amounts of this new orange xanthophyll, here named *pyrrhoxanthin*, were eluted from the column with 7% acetone in hexane. The absorption maxima as recorded in hexane were reduced to a single maximum in ethanol or methanol displaced toward longer wavelengths; this solvent effect is well known for carotenoids containing conjugated carbonyl groups. After an attempted methylation in acidic methanol for 1 hr, the observed spectral shift indicated a 5,6-monoepoxide. Reaction of the resulting furanoid-oxide with lithium aluminum hydride gave a hypophasic product with absorption maxima shifted 50-60 mμ toward shorter wavelengths. The reaction-product was not isolated. *Pyrrhoxanthin* was stable on Silica Gel G thin-layer plates, but was labile toward alkali. No methylation occurred with unhydrolyzed *pyrrhoxanthin*, indicating absence of free allylic hydroxyl groups. Absorption spectra of *pyrrhoxanthin* in hexane, ethanol and carbon disulfide are given in Fig. 2.

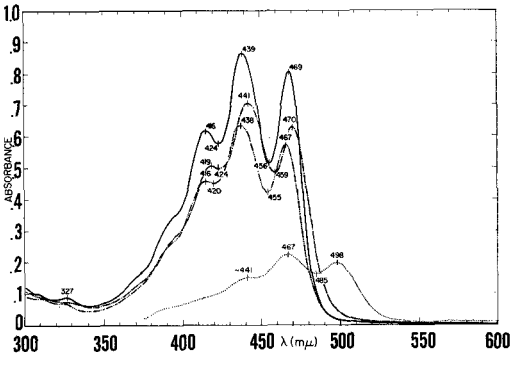

FIG. 4. Dinoxanthin. Solid line: hexane. Dash-triple-dot line: methanol. Dash-single-dot line: ethanol. Dotted line: carbon disulfide.

TABLE V

Spectral Properties of Antheraxanthin and Diadinoxanthin

Carotenoid	Absorption maxima in mμ			Reference
	Light petroleum	Ethanol	Carbon disulfide	
Antheraxanthin (all-*trans*)	473, 444, 423	477, 447, 424		(17)
		479, 449	510, 478	(21)
		477, 447, 424, 338		(22)
		477, 447		(23)
			512.5, 481, 448	(24)
		476, 450, ~420	510, 479	(25)
Antheraxanthin (*cis*-isomer)		472, 445, ~418	506, 476	(25)
Diadinoxanthin (*cis/trans*)	475, 445, 421 339[a]	476, 446, 424 338	506, 474, 449	Present work

[a]In *n*-hexane.

GY 439

This xanthophyll was eluted with 10% acetone in hexane. Upon mild acidification of its methanolic solution, a partial degradation of the absorption spectrum had occurred after 5 min though this change was not a normal epoxide shift of the absorption spectrum. On saponification GY 439 seemed to remain stable. The moderate difference in the partition ratios of this pigment before and after hydrolysis is enigmatic, but may indicate that a short chain ester was present. In spectral character GY 439 resembles ε-carotene although the *cis* peaks of these do not coincide: GY 439, 338; ε-carotene, 328 (18). Fig. 3 gives the absorption spectra of GY 439 in hexane, ethanol and carbon disulfide.

Dinoxanthin

Dinoxanthin was eluted with 15% acetone in hexane as a major yellow band immediately following chlorophyll *a* on silica gel or calcium carbonate columns. A spectral shift, characteristic of a 5,6-monoepoxide, was produced by a trace of acid. There was some spontaneous change of the epoxide to the furanoid-oxide on silica gel plates; the latter trailed slightly behind the epoxide. Dinoxanthin occurs here in an unesterified form; its partition ratio of 7:93 is in accord with a dihydroxy-monoepoxide structure (19). The absorption maxima and fine structure of the dinoxanthin spectral curve (in hexane) are consistent with a chromophore of 9 aliphatic double bonds, as in neurosporene. The failure of dinoxanthin to methylate shows that the hydroxyl groups are nonallylic. Chromatography of freshly extracted dinoxanthin on Silica Gel G plates gave no trace of neodinoxanthin, which was reported by Strain et al. (2). Absorption spectra of dinoxanthin in hexane, ethanol, methanol and carbon disulfide are shown in Fig. 4.

Diadinoxanthin

This pigment, like dinoxanthin, occurs unesterified and is not base-labile. The neodiadinoxanthin of Strain et al. (2) was not observed during TLC. The epoxide nature of diadinoxanthin, previously determined (9, 20), was confirmed here. Upon attempted methylation of diadinoxanthin-furanoid-oxide the partition ratio changed from 6:94 to 9:91. This small change in polarity suggests the possibility of

TABLE VI

Properties of the Iodine Catalyzed Equilibrium Mixtures of Antheraxanthin[a] and Diadinoxanthin[b]

Carotenoid	Stereoisomer	R_f value[c]	Absorption maxima in mμ (acetone)	% of total carotenoids
Antheraxanthin-furanoid-oxide	Neo A	0.67	457, 429, ~406	18.4
	Trans	0.63	457, 429, ~407	44.0
	Neo U	0.60	454, 427, ~406	25.4
	Neo V	0.51	452, 425, ~403	12.2
Diadinoxanthin-furanoid-oxide	Neo A	0.67	455, 428, ~406	19.1
	Trans	0.63	456, 429, ~407	33.0
	Neo U	0.60	454, 427, ~406	30.0
	Neo V	0.51	451, 425, ~403	17.9

[a]ex *Euglena gracilis*

[b]ex *Gyrodinium resplendens*

[c]Chromatographed on Schleicher and Schuell No. 966 paper, elutant: 15% acetone in hexane. Multiple chromatography (2 hr).

some elimination of the epoxy group by the acid, rather than methylation. It is concluded that the 2 hydroxyl groups present are nonallylic to double bonds. In spectral character and polarity, diadinoxanthin most closely resembles antheraxanthin (3,3'-dihydroxy-5,6-epoxy-β-carotene). Table V compares absorption maxima of antheraxanthin isolated from various sources, with absorption maxima of diadinoxanthin isolated from *G. resplendens*. Antheraxanthin shows a partition ratio of 5:95 between hexane and 95% methanol (19). The relative polarities of 2.23 and 2.26 determined by Krinsky (20) for diadinoxanthin and antheraxanthin, respectively, suggest a dihydroxy-5,6-epoxy structure for diadinoxanthin that is isomeric with antheraxanthin. The partition ratio and the results of the allylic hydroxyl test further support this proposed structure.

The identity between diadinoxanthin and crystalline antheraxanthin was further tested by comparison of their stereoisomeric sets prepared by iodine catalysis. The 2 equilibrium mixtures of isomers gave identical cochromatograms on a Silica Gel H plate, an Eastman Chromagram Sheet (silica gel), and on Schleicher and Schuell No. 966 (silica gel impregnated) paper. Isomers were best resolved on the latter by multiple elutions with 15% acetone in hexane. Pigment zones were cut out and eluted with acetone; the ratios of isomers are given in Table VI.

Fig. 5 gives the absorption spectra of diadinoxanthin in hexane, ethanol and carbon disulfide.

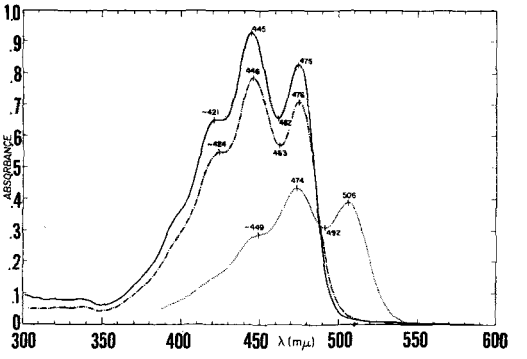

FIG. 5. Diadinoxanthin. Solid line: hexane. Dash-dot line: ethanol. Dotted line: carbon disulfide.

Peridinin

This, the major carotenoid of *G. resplendens*, was eluted with 17% acetone in hexane. Elemental analysis of peridinin (= sulcatoxanthin) (7) showed a molecular formula of $C_{40}H_{52}O_8$. Upon exposure to 4% sodium hydroxide in ethanol, peridinin is decolorized within a few seconds; Strain et al. (2) had determined previously that peridinin was sensitive to alkali. Recently, a unique photosynthetic dinoflagellate apparently lacking peridinin has been reported (26), but it is not clear whether the pigment extract was initially saponified, thereby destroying this base-labile pigment.

Although peridinin is hypophasic, it is sufficiently nonpolar to suggest that two or more of its oxygen atoms contribute to ester linkages, as is the case in fucoxanthin (27). The diminished fine structure of the peridinin spectral curve in alcohol compared to that in hexane indicates the presence of a conjugated carbonyl group (2). However, the amount of fine structure present in the spectral curve in hexane suggests that only one conjugated carbonyl group is present. An epoxide test on peridinin gave no spectral shift after 5 min. However, after attempted methylation of peridinin for 2 hr its absorption maximum was shifted 20 mμ toward shorter wavelengths, suggesting that a 5,6-epoxy group is present; though the compound is unusually stable toward acid. Methylation produced 2 new pigments which were easily separated by TLC from the remaining trace of peridinin. Both products gave spectral curves in hexane and methanol which are very similar in shape to those of peridinin. Peridinin and its 2 derivatives are compared in Table VII in order of their elution with 35% acetone in hexane (thin layer chromatography). The polarity of these furanoid derivatives, A and B, suggests that they are isomeric and result from partial methylation of peridinin.

Reaction of peridinin with lithium aluminum

TABLE VII
Absorption Maxima of the Methylation Products of Peridinin

	Absorption maxima (minima) in mμ		Partition ratio
	n-Hexane	Methanol	(*n*-hexane: 95% methanol)
Peridinin-furanoid-oxide A	461 (449) 434 ~ 411, *cis* 312	447	15:85
Peridinin-furanoid-oxide B	460 (448) 433 ~ 411, *cis* 312	448	15:85
Peridinin	484 (471) 454 ~ 431, *cis* 325	472	2:98

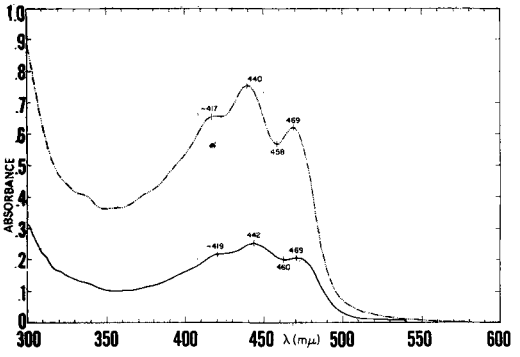

FIG. 6. Gy 442. Solid line: hexane. Dash-dot line: methanol.

hydride yielded a product with absorption maxima at 390, 364, 345, 329 mμ in methanol. The reaction appears to have shortened the chromophore to approximately 5 conjugated double bonds.

After several months in storage (–8C, dark) peridinin gave rise to 2 red chromatographic zones which followed the unaltered compound on a calcium carbonate column. Perhaps one of these derivatives is the neoperidinin previously described (2). However, freshly extracted peridinin was resolved on silica gel plates as a single zone and gave a characteristic absorption curve.

GY 442

This yellow pigment was recovered in trace amounts by chromatography of the whole extract on Silica Gel G plates. An epoxide test in acidic methanol yielded a hypsochromic shift of 43 mμ in absorption maximum, characteristic of a di-epoxy xanthophyll. Its extreme polarity also indicates 2 free hydroxyl groups. However, the appearance of other maxima at shorter wavelengths suggested that some decomposition products were formed by the acid.

Absorption spectra of GY 442 in hexane and methanol are given in Fig. 6.

Chlorophyll a and c

The absorption spectra of the living cells in culture medium and of the total pigments in acetone from *G. resplendens* in the 600 to 700 mμ region indicate the presence of 2 chlorophylls. Chorophyll *a* was isolated on thin-layer plates while chlorophyll *c* was obtained by partitioning the total extract between saline aqueous acetone and hexane (28). The hypophasic chlorophyll gave absorption maxima of chlorophyll *c* (Table III). Table VIII gives quantitative data on the chlorophylls and carotenoids of *G. resplendens* based on the equations of Parsons and Strickland (28), Parsons (28) and those of Humphrey and Jeffrey (29). Comparative values for other dinoflagellates may be found in Parsons et al. (30) and McAllister et al. (31)

DISCUSSION

The characteristic dinoflagellate pigments, peridinin, dinoxanthin and diadinoxanthin, were isolated from an axenic, clonal culture of *Gyrodinium resplendens*. The latter 2 yellow pigments are more abundant here than in other dinoflagellates studied; living cells of this alga are distinctly yellow. The only carotene present appears to be β-carotene; some workers have reported the presence of other unrecognized carotenes. Four new xanthophylls present in minor amounts are described here; GY 443, *pyrrhoxanthin*, GY 439 and GY 442. At least 91% of the carotenoids of *G. resplendens* are 5,6-monoepoxides. Pigment GY 442 (3%) appears to be a di-epoxide.

Photosynthetic dinoflagellates have a greater percentage (94%) of epoxy-xanthophylls than do other algal divisions where epoxides are also predominant: Chloromonadophyta, 87% (23); Euglenophyta, 89% (22); Xanthophyta, 88% (21). Of the 4 groups only the dinoflagellates have yielded a di-epoxide.

The *G. resplendens* epoxides are unstable to varying degrees when adsorbed on silica gel thin-layer plates. The epoxide slightly precedes its furanoid-oxide derivative in chromatography. However epoxides appear stable on silica gel columns where the pigment-adsorbent complex is at all times immersed in solvent.

The identity between diadinoxanthin from diatoms (Bacillariophyta), and antheraxanthin from *Euglena* (Euglenophyta) was sug-

TABLE VIII
Pigment Ratios in *Gyrodinium resplendens*

	Method of estimation		
	Trichromatic (28)	Dichromatic (29)	Partition (28)
Chlorophyll *a* (μg/10^6 cells)	21.9	25.8	.....
Chlorophyll *c* (μg/10^6 cells)	14.0	9.03	13.3
Carotenoid (μg/10^6 cells)	37.0	.....	.....
Chlorophylls (μg/10^6 cells)	35.9	34.8	.....
Chlorophyll *a*/*c*	1.56	2.86	.....
Carotenoid/Chlorophyll	1.03	.....	.....

gested by Krinsky (20). In the present work the furanoid-oxides of chromatographically pure diadinoxanthin (*Gyrodinium*) and crystalline antheraxanthin (*Euglena*) were not distinguishable by their absorption curves, partition behavior, or instability toward acid. Upon iodine catalysis both yielded equilibration mixtures of isomers which had identical R_f values. However, the proportions of isomers within each set were significantly different (Table VI). Spectral curves of the corresponding isomers from each set were alike, except for the isomer designated *trans*; this isomer of diadinoxanthin showed slightly less fine structure in its spectral curve than that of antheraxanthin. The lack of crystalline diadinoxanthin prevented a more favorable comparison of these 2 pigments. In the event of synonymy, antheraxanthin would take precedence as the older name.

Diadinoxanthin from *Gyrodinium* is most similar spectrally to a natural *cis*-isomer of antheraxanthin isolated in crystalline form from *Lilium candidum* (25). The presence of a *cis*-peak was always noted here in chromatographically pure diadinoxanthin, but partial isomerization of carotenoids during extraction is always likely.

The presence of conjugated carbonyl groups in peridinin and pyrrhoxanthin is indicated here by their dissimilar absorption curves in hexane and ethanol (2, 32). Reaction of these pigments with lithium aluminum hydride caused extreme shortening of the chromophore (50-100 mμ). Notably, hydride reduction has a similar effect on fucoxanthin (33), though not on conventional epoxy-carotenoids (34).

The change in partition behavior of GY 443 after saponification indicates that at least one of the minor xanthophylls of *Gyrodinium* occurs as an ester. The qualitative results of this study have confirmed a preliminary analysis (unpublished) of the pigments of a stationary culture of *G. resplendens*.

Addendum. Recently, a marine dinoflagellate was reported to contain fucoxanthin instead of peridinin as the major pigment (35).

ACKNOWLEDGMENTS

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The Total Synthesis and Metabolism of 4-Decenoate, Dodeca-3,6-Dienoate, Tetradeca-5,8-Dienoate and Hexadeca-7,10-Dienoate in the Fat-Deficient Rat

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ABSTRACT

Methyl 4-decenoate (10:1ω6), methyl dodeca-3,6-dienoate (12:2ω6), methyl tetradeca-5,8-dienoate (14:2ω6) and methyl hexadeca-7,10-dienoate (16:2ω6) were prepared by total synthesis. Rats raised on a fat-deficient diet for 2½ months received 100 mg per day of one of the experimental acids or methyl linoleate for a period of 16 days. The liver lipids were extracted, converted to methyl esters and analyzed by gas-liquid chromatography. Neither 10:1ω6 nor 12:2ω6 served as biosynthetic precursors for linoleate. Small amounts of 14:2ω6 were converted to linoleate while 16:2ω6 served as an efficient precursor for linoleate and longer chain ω6 acids. None of the short chain ω6 acids were incorporated directly into liver lipids.

INTRODUCTION

STUDIES IN SEVERAL LABORATORIES have established that dietary linoleate (18:2ω6) is the initial precursor for the biosynthesis of a series of polyunsaturated fatty acids (PUFA) of the ω6 configuration (1-4). Although linoleate is generally recognized as the parent compound in this biosynthetic sequence, recent studies by Klenk (5) have shown that the rat is able to convert hexadeca-7,10-dienoate (16:2ω6) to linoleate and subsequently to longer chain PUFA of the ω6 family. Conversely the shortest chain ω6 acid, 2-octenoate (8:1ω6), is not converted to linoleate by the rat (6) although apparently this conversion does take place in the chicken (7). The present study was undertaken to define in more detail the minimum structural features required in a short chain ω6 acid before conversion to linoleate can occur.

EXPERIMENTAL

Fatty Acid Synthesis

The synthesis of methyl tetradeca-5,8-dienoate (14:2ω6), methyl hexadeca-7,10-dienoate (16:2ω6), methyl dodeca-3,6-dienoate (12:2ω6) and methyl 4-decenoate (10:1ω6) was carried out according to Diagram I.

Diagram I

A. Methyl tetradeca-5,8-dienoate (14:2ω6) and methyl hexadeca-7,10-dienoate (16:2ω6)

$$\underset{\text{I}}{R\text{–}C\equiv C\text{–}CH_2Br} + \underset{\text{II } x=3;\ \text{III } x=5}{HC\equiv C\text{–}(CH_2)_x\text{–}COOH} \xrightarrow{EtMgBr} \underset{\text{IV } x=3;\ \text{V } x=5}{R\text{–}C\equiv C\text{–}CH_2\text{–}C\equiv C\text{–}(CH_2)_x\text{–}COOH}$$

$R = CH_3\text{–}(CH_2)_4\text{–}$

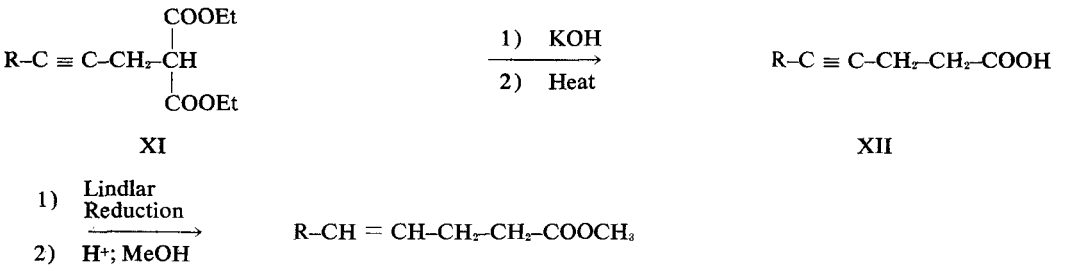
$$\xrightarrow[2)\ H^+;\ MeOH]{1)\ \text{Lindlar Reduction}} \underset{\text{VI } x=3;\ \text{VII } x=5}{R\text{–}CH=CH\text{–}CH_2\text{–}CH=CH\text{–}(CH_2)_x\text{–}COOCH_3}$$

B. Methyl dodeca-3,6-dienoate (12:2ω6)

$$\underset{\text{VIII}}{R\text{–}C\equiv C\text{–}CH_2\text{–}C\equiv C\text{–}CH_2\text{–}CH_2OH} \xrightarrow{CrO_3} \underset{\text{IX}}{R\text{–}C\equiv C\text{–}CH_2\text{–}C\equiv C\text{–}CH_2\text{–}COOH}$$

$$\xrightarrow[2)\ H^+;\ MeOH]{1)\ \text{Lindlar Reduction}} \underset{\text{X}}{R\text{–}CH=CH\text{–}CH_2\text{–}CH=CH\text{–}CH_2\text{–}COOCH_3}$$

C. Methyl 4-decanoate



XI XII XIII

All melting points were run on a Fisher-Johns melting point apparatus and are uncorrected. Ultraviolet measurements were made in spectro-grade methanol on a Beckman DK-2 spectrophotometer. Infrared spectra were run in carbon disulfide on a Beckman IR-8 grating instrument using sodium chloride cells with a light path of 0.1 mm. Mass spectra were run at 70 ev using a Hitachi-Perkin Elmer Model RMU-6D mass spectrometer.

1-Bromo-2-Octyne (I). The synthesis of 1-bromo-2-octyne was carried out according to the general procedure of Gensler and Thomas (8) as previously described (9).

5-Hexynoic Acid (II). 1-bromo-3-chloropropane, obtained from K and K Laboratories, was treated with sodium acetylide in liquid ammonia as described by Campbell and Campbell (10). The 1-chloro-4-pentyne, obtained in 80% yield, had bp 112-114 with n_D^{17} 1.4484; literature value n_D^{20} 1.4448 (11). The 1-chloro-4-pentyne was converted to 5- hexynoic acid using the procedure of Gunstone and Sykes (12). The product, obtained in 70% yield, had bp 129-131/16 mm with n_D^{17} 1.4503; literature value n_D^{17} 1.4500 (13).

7-Octynoic Acid (III). Preparation was carried out as previously described (9).

Tetradeca-5,8-diynoic Acid (IV). The 5-hexynoic acid and 1-bromo-2-octyne were coupled according to the general procedure of Osbond et al. (14). To 14.58 g (0.6 mole) of Mg turnings in 150 ml of tetrahydrofuran (THF) in a 1-liter 3-neck flask in an ice bath was added 72.0 g (0.66 mole) of ethyl bromide in 70 ml of THF in the course of an hour. The Grignard complex was stirred for about an hour at room temperature after which time 33.6 g (0.3 mole) of 5-hexynoic acid in 100 ml of THF was added at ice bath temperatures. The reaction was stirred at room temperature for 2 hr after which time 1.5 g of CuCN was added followed by 28.35 g (0.15 mole) of 1-bromo-2-octyne in 50 ml of THF. The reaction mixture was stirred, under nitrogen, at room temperature for 24 hr and poured into 2 N H_2SO_4 and extracted 3 times with ether. The combined ether extracts were washed until neutral to pH paper and dried over anhydrous Na_2SO_4. The ether and residual THF were removed under reduced pressure. The resulting oily brown residue was dissolved in petroleum ether (bp 30–60C) and crystallized and recrystallized at –20C to yield 11 g (33% yield) of amber crystals with mp of 27–28C. Reduction of an aliquot with platinum oxide in methanol gave a product which had a melting point and mixed melting point with authentic myristic acid of 51–52C.

Methyl Tetradeca-5,8-dienoate (VI.) Aliquots of 2–3 g of tetradeca-5,8-diynoic acid in 100 ml of purified hexane containing 2 ml of a 5% solution of synthetic quinoline in hexane were reduced with Lindlars catalyst (15) at atmospheric pressure. The product was isolated by filtering off the catalyst, adding 100 ml of water containing 2 ml of HCl and extracting 3 times with petroleum ether. The methyl ester was formed by stirring with 5% anhydrous methanolic-HCl overnight at room temperature. The methyl ester, obtained after extraction with petroleum ether, was dark yellow in color. It was purified by silicic acid column chromatography eluting with petroleum ether-ether 92:8 (v/v). The resulting product had n_D^{20} 1.4585. The methyl ester, $C_{15}H_{26}O_2$, requires O, 75.58; H, 10.99 and O, 13.42%: found C, 75.51; H, 10.83 and O, 13.55%. No *trans* double bond was present as measured by absorption at 965 cm^{-1} in the infrared spectrum. The ultraviolet spectrum shown $\lambda_{max.}$ at 230 mu, $E_{1cm}^{1\%}$ 15, with shoulders at 266 μ and 277μ. The mass spectrum showed a parent ion peak with m/e of 238 in agreement with the calculated molecular weight of methyl tetradeca-5,8-dienoate. Fig. 1 shows that the

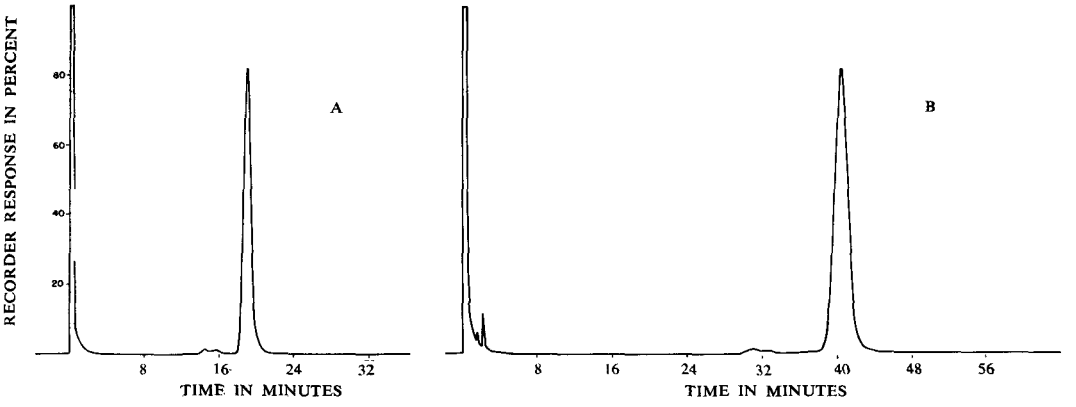


FIG. 1. Gas-liquid chromatography of *A*, methyl tetradeca-5,8-dienoate and *B*, methyl hexadeca-7,10-dienoate. Chromatography was carried out with an F&M Model 810 gas chromatograph equipped with a flame ionization detector on a 10 ft by ¼ in. I.D. stainless steel column packed with 20% ethyleneglycol succinate on 80-100 mesh Gas-Chrom P. The oven temperature was 150C with a helium flow rate of 50 ml/minute.

methyl tetradeca-5,8-dienoate was 98% pure as determined by gas-liquid chromatography (GLC).

Hexadeca-7,10-diynoic Acid (V). Coupling of 1-bromo-2-octyne and 7-octynoic acid was carried out as described for the synthesis of IV except that the reaction was refluxed for 24 hr instead of stirring at room temperature. The product, after crystallization and recrystallization from petroleum ether, was obtained in 47% yield and had a melting point of 35–36C. Reduction of an aliquot with platinum oxide in methanol gave a compound with a melting point and mixed melting point with authentic palmitic acid of 61–62C.

Methyl hexadeca-7,10-dienoate (VII). Hexadeca-7,10-diynoic acid was reduced with Lindlar's catalyst, converted to the methyl ester, isolated and purified as described above. The resulting product had n_D^{20} 1.4596. Methyl hexadeca-7,10-dienoate, $C_{17}H_{30}O_2$, requires C, 76.64; H, 11.36 and O, 12.01%: found C, 76.64; H, 11.45 and O, 12.04%. Infrared analysis showed the presence of less than 2% isolated *trans* double bond. The ultraviolet spectrum showed $\lambda_{max.}$ 231 μ, $E^{1\%}_{1cm}$ 17 with shoulders at 263 and 277 μ. The mass spectrum had a parent ion peak at m/e of 266 in agreement with the calculated molecular weight. Fig. 1 shows that the methyl hexadeca-7,10-dienoate was 95% pure when analyzed by GLC.

Dodeca-3,6-diyne-1-ol (VIII). Dodeca-3,6-diyne-1-ol was prepared by coupling 3-butyn-1-ol, obtained from K and K laboratories, with 1-bromo-2-octyne as described by Stoffel et al. (16). The product, recovered in 83% yield by distillation, had bp 110–115 C/0.3 mm with n_D^{20} 1.4850; literature value n_D^{20} 1.4850 (16).

Dodeca-3,6-diynoic Acid (IX). The dodeca-3,6-diyne-1-ol was converted to dodeca-3,6-dynoic acid by chromic acid oxidation as described by Stoffel et al. (16). The product, after crystallization and recrystallization from petroleum ether, was obtained in 45% yield and had a melting point of 64–65C; literature value 65C (16). Reduction of the product with platinum oxide in methanol gave a product with melting point and mixed melting point with authentic lauric acid of 41–42C.

Methyl dodeca-3,6-dienoate (X). Dodeca-3,6-diynoic acid was reduced with Lindlar's catalyst, isolated, purified and converted to the methyl ester as described above. The purified methyl ester had n_D^{20} 1.4563. The methyl dodeca-3,6-dienoate, $C_{13}H_{22}O_2$, requires C, 74.24; H, 10.55 and O, 15.22%: found C, 74.14; H, 10.55 and O, 15.07%. Only trace amounts of isolated *trans* double bond was present as measured by infrared analysis at 965 cm^{-1}. The ultraviolet spectrum showed only end absorption. The mass spectrum had a parent ion peak at m/e of 210 in agreement with the calculated molecular weight. GLC analysis, as depicted in Fig. 2 shows that the methyl dodeca-3,6-dienoate was 96% pure.

Diethyl 2-octynylmalonate (XI). Synthesis was carried out by adding 1-bromo-2-octyne to the sodium salt of diethyl malonate in

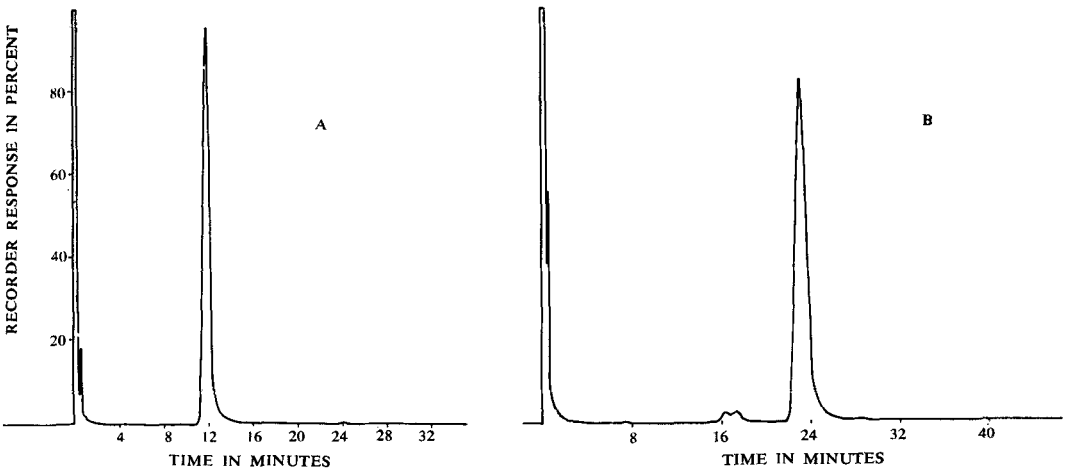


FIG. 2. Gas-liquid chromatography of A. methyl 4-decenoate and B. Methyl dodeca-3,6-dienoate. Chromatography was carried out with an F&M Model 810 gas chromatograph equipped with a flame ionization detector on a 10 ft by ¼ in. I.D. stainless steel column packed with 20% ethyleneglycol succinate on 80-100 mesh Gas-Chrom P. The oven temperature was 130C with a helium flow rate of 50 ml/minute.

ethanol as described by Kennedy et al. (17). The reaction was refluxed for 6 hr, the ethanol was removed under reduced pressure, water was added and the product was recovered by ether extraction. The product, obtained in 63% yield, was recovered by distillation with bp 121–128C/0.3 mm; literature value bp 124–128C/0.3 mm (17).

4-Decynoic Acid (XII). The diethyl 2-octynylmalonate was saponified by refluxing for 3 hr in ethanolic KOH according to Kennedy et al. (17). The reaction mixture was cooled and filtered. The collected precipitate was dissolved in water, acidified with 2 N H_2SO_4 and extracted 3 times with ether. The combined ether extracts were washed with water and dried over Na_2SO_4. The ether was removed under reduced pressure. The 2-octynylmalonic acid, without additional purification, was decarboxylated by heating in an oil bath at 160C for 2 hr. Crystallization of the residue from petroleum ether gave 4-decynoic acid in 60% yield with mp 35–36C; literature value 36.5–37.0C (17). Hydrogenation of the product with platinum oxide gave a compound which had melting point and mixed melting point with authentic decanoic acid of 29.5–30C.

Methyl 4-decenoate (XIII). 4-Decynoic acid was reduced with Lindlar's catalyst, isolated, converted to the methyl ester and purified as described above. The methyl 4-decenoate had n_D^{20} 1.4398. Methyl 4-decenoate, $C_{11}H_{20}O_2$, requires C, 71.69; H, 10.94 and O, 17.37%; found C, 71.87; H, 10.85 and O, 17.56%. No *trans* double bond was detected in the infrared spectrum. The ultraviolet spectrum showed only end absorption below 220μ. The mass spectrum had a parent ion peak at m/e of 184 in agreement with the calculated molecular weight. The methyl 4-deconate was 99% pure when analyzed by GLC analysis as shown in Fig. 2.

Feeding Studies

Male Spraque Dawley weanling rats, 20 days old, were maintained on a fat-deficient diet, purchased from General Biochemicals, for 2½ months. The rats were then divided into 6 groups. One group was maintained on the fat-deficient diet while the other groups received either 100 mg per day of one of the synthetic methyl esters or methyl linoleate which was purchased from the Hormel Institute. The esters were administered orally with a 100 μl blunt syringe for a period of 16 days.

Lipid Isolation and Analysis

The rats were sacrificed by ether anesthesia and the livers were stored frozen in isotonic saline until they could be extracted.

The lipids were extracted with chloroform-methanol 2:1 (v/v) as described by Folch et al. (18). The lipids were interesterified by refluxing with 5% anhydrous methanolic-HCl according to Stoffel et al. (19). The methyl

TABLE I

Fatty Acid Composition of Total Liver Lipids (Area Percent on GLC)[a]

Component	Fat Free (11) Controls	4-Decenoate (6) (10:1ω6)	Dietary Acid Dodeca-3,6-dienoate (5) (12:2ω6)	Tetradeca-5, 8-dienoate (6) (14:2ω6)	Hexadeca-7, 10-dienoate (6) (16:2ω6)	Linoleate (6) (18:2ω6)
14:0	0.9 ± 0.3	0.6 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.3	0.4 ± 0.1
16:0	23.3 ± 1.6	25.6 ± 1.6	24.2 ± 3.0	23.1 ± 2.5	26.3 ± 0.9	22.0 ± 2.7
16:1	6.4 ± 0.8	6.3 ± 1.8	5.9 ± 1.2	5.1 ± 1.0	5.2 ± 1.5	4.6 ± 1.6
18:0	13.8 ± 1.3	13.1 ± 2.1	14.2 ± 1.2	15.2 ± 0.6	15.0 ± 0.9	15.3 ± 2.3
18:1	35.3 ± 3.0	37.3 ± 2.7	34.3 ± 2.6	31.3 ± 2.0	24.8 ± 1.8	25.3 ± 4.5
18:2	1.2 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	2.6 ± 0.4	5.2 ± 0.3	6.7 ± 0.9
20:3ω9	13.8 ± 1.6	11.7 ± 2.6	15.1 ± 3.1	12.9 ± 1.9	6.4 ± 0.8	6.5 ± 1.2
20:3ω6	.....	.....	.....	1.1 ± 0.3	1.6 ± 0.5	1.8 ± 0.6
20:4	3.1 ± 0.6	2.5 ± 0.5	2.9 ± 0.8	6.2 ± 1.0	12.3 ± 1.6	14.1 ± 2.6
20:5ω3	0.5 ± 0.1	0.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
22:4ω6	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.4 ± 0.1	0.2 ± 0.1
22:5ω6	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	1.1 ± 0.3	1.2 ± 0.4
22:5ω3	Trace	Trace	Trace	Trace	Trace	Trace
22:6ω3	1.0 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	1.5 ± 0.7

[a] Results expressed as the mean ± the standard deviation.

The number of animals on each dietary acid is indicated by the number following the dietary acid.

esters were recovered by extracting with redistilled petroleum ether (bp 30-60C).

The rat liver methyl esters were analyzed on a F&M Model 810 gas chromatograph. The stainless steel column, 10 ft long by ¼ in. diameter, was packed with 20% ethylene glycol succinate on 80-100 mesh Gas-Chrom P. Methyl esters up to and including arachidonate were chromatographed at an oven temperature of 185C. Esters with longer retention times were chromatographed at 200C. The flow rate of helium, the carrier gas, was about 60 ml/min at an inlet pressure of 60 psi.

Methyl esters were identified by comparing retention times with authentic standards and by comparing equivalent chain lengths with published values (20).

The methyl ester composition was measured by triangulation. All results are therefore expressed as area percent.

RESULTS

The fatty acid composition of total liver lipids is given in Table I. The fatty acid composition of the animals maintained on the fat-deficient diet is similar to that reported by other investigators (21-23). The content of 18:2 and 20:4 was depleted with a corresponding increase in the level of 20:3ω9. In those animals receiving linoleate the expected increase in ω 6 fatty acids was observed along with a reduction in 20:3ω9 content. In those animals receiving either 10:1ω6 or 12:2ω6 the liver lipid fatty acid composition was similar to the fat-deficient controls. Failure to observe an increase in either 18:2 or 20:4 accompanied by a depression in the level of 20:3ω9 indicates that these two short chain ω6 acids were unable to serve as biosynthetic precursors for longer chain ω 6 acids.

When 14:2ω6 was the dietary variable small but measurable conversion to longer chain ω 6 acids occurred. The levels of 18:2 and 20:4 increased respectively 2.1 and 2.0 times over the fat-deficient controls. The content of 20:3ω9 however remained at approximately the same level as found in the fat-deficient controls. These findings suggest that although 14:2ω6 can serve as a precursor for longer chain ω 6 fatty acids the small amount so converted is insufficient to depress the level of 20:3ω9 during the 16-day feeding period used in these studies.

In agreement with the findings of Klenk (5) feeding 16:2ω6 results in appreciable conversion to linoleate and therefore also to longer chain ω6 metabolites. Feeding 16:2ω6 is almost as effective in altering the fatty acid composition as feeding linoleate itself. When 16:2ω6 was the dietary variable the levels of 18:2 and 20:4 increased respectively 4.2 and 4.0 times over the fat-deficient controls, whereas the respective increases in 18:2 and 20:4 content were 5.5 and 4.7 times when linoleate was the dietary variable. The level of 20:3ω9 was reduced to about the same extent when either 16:2ω6 or linoleate was fed.

In the GLC analysis attempts were made to look for the short chain ω6 dietary acids. Failure to detect any of the dietary short chain acids indicates that they either were not incorporated into liver lipids or that the amount incorporated was so small it was below the level of detection used in these experiments.

DISCUSSION

The results obtained in the studies reported here are in general agreement with the concepts of fatty acid biosynthesis. If 10:1ω6 had served as a biosynthetic precursor for linoleate presumably the conversion would have taken place by successive malonate condensation steps to yield 12-octadecenoate. Microsomal oxidative desaturation (24) at the 9-position would then yield linoleate. However, studies by Fulco and Mead (25) have already established that the rat is unable to convert 12-octadecenoate to linoleate by desaturation at the 9-position. Alternatively if 12-octadecenoate was formed it could be incorporated directly into tissue lipids. In the present study it is unlikely that 12-octadecenoate would be resolved from oleate in the GLC analysis. However, failure to see an alteration in 18:1 content when compared with the fat-deficient controls suggests that this conversion also does not occur. Studies by Brenner et al. (6) and recently confirmed by Anderson and Reiser (26) have shown that 8:1ω6 is similar to 10:1ω6 in that it is not converted to linoleate by the rat. It is now well established that α, β-unsaturated fatty acids are intermediates in both de novo saturated fatty acid biosynthesis (27) and in the microsomal chain elongation pathway (28). Since 2-octenoate is an α, β-unsaturated fatty acid this compound is more likely to serve as an intermediate in saturated rather than unsaturated fatty acid biosynthesis. Inconsistent with the above considerations is the surprising observation by Reiser et al. (7) that the chicken is able to convert *cis*-2-octenoate to linoleate.

Failure of 12:2ω6 to serve as a precursor for linoleate is consistent with the type of PUFA found in rat tissue lipids. No PUFA have been described in rat tissue lipids in which the first double bond is at the 3 position. Recent in vitro studies by Brenner and Peluffo (24) have shown that no acids are formed in which the first double bond is at the 3 position. Apparently the rat is not only unable to desaturate fatty acids at the 3 position, but even when supplied via dietary supplement, is unable to convert acids with the first-double bond at the 3 position to longer chain PUFA. In contrast the first double bonds in 14:2ω6 and 16:2ω6 are located respectively at the 5 and 7 positions. If the position of the first double bond in an unsaturated fatty acid determines whether that compound can serve as an active metabolite for PUFA biosynthesis one would predict that both 14:2ω6 and 16:2ω6 would be active precursors. For example arachidonate, in which the first double bond is in the 5 position, is converted to 22:4ω6 (3). Likewise 22:4ω6, in which the first double bond is in the 7 position, is converted to 22:5ω6 (3) and probably to 22:4ω6 (29).

The results reported here are in general agreement with a recent study by Stoffel and Scheid (30) on the metabolism of various ω6 acids in HeLa cells. These investigators showed that 12:2ω6 is neither elongated nor incorporated into phospholipids. In contrast 14:2ω6, 16:2ω6, and 16:3ω6 were all converted to longer chain ω6 acids and incorporated directly into phospholipids. The results reported here differ from those reported by Stoffel and Scheid in that none of the short chain ω6 acids were detected in total liver lipids. Failure to detect these short chain compounds in liver lipids may possibly be explained by the specificity of the rat liver acylating enzymes since Stoffel et al. (31) have shown that 16:3ω6 is incorporated into lysolecithin to yield lecithin at a slower rate than longer chain PUFA.

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Phospholipid Distribution in Blood and Tissues of Some Submammalian Species

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ABSTRACT

The pattern of phospholipid distribution in blood and tissues such as liver, heart, and kidney of four representative species of fish, toad, turtle, and pigeon has been studied. The percentage of phosphatidylcholine in plasma was similar, but in erythrocytes the difference was striking. Ethanolamine and serine plasmalogens were absent in the plasma of all the species. In erythrocytes the highest concentration of phosphatidylethanolamine was noted in the toad. The greatest difference in sphingomyelin and plasmalogen concentrations was found between toad and turtle erythrocytes.

In the liver, phosphatidylcholine accounted for more than 55% of the total lipid phosphorus. The percentage of all individual phospholipids except sphingomyelin in kidney was comparable in all the species.

INTRODUCTION

PHOSPHOLIPIDS as a class of compounds are universally present in all cellular organisms. Distribution of these compounds vary not only from species to species but also from one tissue to another in the same species. Studies on the comparative aspects of phospholipid distribution in the nervous tissue of various species of animals have been carried out (1-4). Although the distribution of phospholipids in some other mammalian tissues has been worked out by different authors (5-10,14,15), comparative data on the distribution of various phospholipid components in the tissues of submammalian species of Indian origin are scanty. As the pattern of tissue phospholipid distribution is likely to be influenced by various nutritional and physiological factors, it was thought worthwhile to investigate the pattern of phospholipid distribution in plasma, erythrocytes, and certain tissues of *Heteropneustes fossilis, Bufo malanostictus, Kachuga Smithi,* and *Columba livia intermedia.*

EXPERIMENTAL

Materials

The animals (fish 140-160 g, toad 200-300 g, turtle 1,500 g, and pigeon 400-500 g) were obtained from the local market. The fish *(H. fossilis)* were kept in laboratory aquaria for seven days before experiments were started and were fed with live earthworms and goat-liver slices. The toads *(B. malanostictus)* were reared in the froggery and were allowed to subsist on insects and earthworms ad lib. The turtles *(K. Smithi)* and pigeons *(C. livia intermedia)* were kept in laboratory cages for a period and were given frog and fish flesh and pulses respectively.

All the animals were fasted overnight before they were sacrificed on the eighth day. After they were anesthetized with ether, the liver, heart, and kidney were removed, weighed accurately on a chemical balance, and extracted for phospholipids. Only the whole brain tissue of the fish was also taken for study. Blood samples (5-10 ml) from fish were collected in heparinized centrifuge tubes by cutting the caudal vein and allowing the blood to drain in the tube. With toad, turtle, and pigeon the blood samples were collected by direct heart puncture. The samples were centrifuged at 2,500 rpm for 10 min. The volume of supernatant plasma was noted and pipetted out. The volume of packed erythrocytes, washed twice with 5 ml of 0.9% NaCl, was also noted and put aside for phospholipid extraction.

Extraction and Fractionation of Phospholipids

Dawson's method of extraction and fractionation of phospholipids was adopted with slight modifications. Tissues were extracted with 7 vol of methanol and 14 vol of chloroform for 3 hr at room temperature. The extract was filtered and washed twice with 0.9% NaCl solution to remove water-soluble phosphate impurities. The washed extract was taken to dryness in vacuo. The residue was redissolved in 10 ml of chloroform. One ml of this was put aside for the estimation of total lipid phosphorus. For phospholipid fractionation the remaining 9 ml of extract were dried in vacuo at a temperature below 45C. The residue, dissolved in 0.8 ml of CCl_4, was hydrolyzed by 0.25 ml of 1N NaOH at 37C for 20 min. The hydrolyzed material was cooled and neutralized with 0.4 ml of ethylformate. The mixture was dried in vacuo below 60C. The residue was mixed with 1 ml of the aqueous layer and

2 ml of the organic layer from isobutanol-chloroform (1:2) mixture, equilibrated with 1 vol of H_2O. After being centrifuged for 10 min at 2,500 rpm, the upper aqueous layer was pipetted out as completely as possible with a micropipette and was stored at 0C for chromatography.

The lower organic layer was hydrolyzed by 10% TCA at 37C with constant shaking for 30 min. Two ml of light petroleum were added. After being centrifuged for 10 min at 2,500 rpm, the lower aqueous layer was washed twice with 5 ml of a chloroform-ether mixture (1:4) and was neutralized with ammonia vapor, then stored at 0C for chromatography.

The combined upper organic layer was hydrolyzed in a boiling water bath with 1.7N methanolic HCl in sealed tubes for 4 hr. The seal was broken, the contents were concentrated to a suitable volume in vacuo, and stored at 0C for chromatography.

Paper Chromatography

Measured quantities of the sample were loaded on Whatman No. 1 filter paper which had been previously washed with 0.2N acetic acid and distilled water. The papers were developed for 18 hr with water-saturated phenol-acetic acid-ethanol (100:10:12). After the papers were dried in air, the spots were detected by spraying 0.25% ninhydrin in absolute acetone and subsequently heating the papers at 80C for 3 min. The spots were cut from the chromatogram and were digested with perchloric acid (72%) for phosphate estimation by the method of Fiske and Subbarow (11).

RESULTS AND DISCUSSION

Phospholipid distribution in the plasma and erythrocytes in four different submammalian species is presented in Table I. The percentage of phosphatidylcholine in the plasma was more or less similar in all the species examined. Except for a trace of ethanolamine plasmalogen found in toad plasma, ethanolamine and serine plasmalogens were absent in the plasma of all species. All other phospholipid components followed a similar pattern except that the percentage of choline plasmalogen was somewhat higher in the toad plasma.

In the erythrocyte membrane the difference in phospholipid distribution was more striking. The highest percentage of phosphatidylcholine was found in pigeon erythrocytes; the value in erythrocytes of the turtle was lower by about 22%. The fish and toad erythrocyte membrane contained almost similar quantities of phosphatidylcholine. Phosphatidylethanolamine concentration was however greatest in the toad erythrocytes and constituted about 14% of the total lipid phosphorus. In the turtle the corresponding value was only 4.5%. Sphingomyelin in the turtle erythrocytes constituted about 26.2% of the total lipid phosphorus; in fish, toad, and pigeon the values were 22%, 18.2%, and 20.9% respectively. The highest difference in respect to sphingomyelin was therefore found between amphibian and reptilian erythrocyte membranes.

TABLE I

Phospholipid Distribution in Erythrocyte Membrane and Plasma of Some Submammalian Species
Results as Percentage of Total Lipid Phosphorus, Average of 10 Analyses on Each Tissue

	Phospholipid Percentage of Total Lipid Phosphorus							
	Heteropneustes fossilis		Bufo malanostictus		Kachuga Smithi		Columba livia	
	Plasma	Erythrocytes	Plasma	Erythrocytes	Plasma	Erythrocytes	Plasma	Erythrocytes
Phosphatidyl-choline	78.0±2.1	28.5±2.5	80.2±3.5	28.7±1.8	75.3±3.4	22.5±4.2	79.2±2.3	32.4±3.1
Phosphatidyl-ethanolamine	1.8±0.2	5.4±1.2	2.2±0.09	14.0±1.2	2.5+0.06	4.5±1.05	1.5±0.07	12.8±1.6
Phosphatidyl-serine	trace	12.8±1.4	—	8.0±1.2	—	8.5±1.4	trace	5.0±1.5
Phosphatidyl-inositol	2.0±0.4	1.2±0.1	1.5±0.08	0.9±0.02	1.8	1.0±0.05	2.1±0.6	2.8±0.5
Ethanolamine plasmalogen	—	1.2±0.4	trace	4.2±0.5	—	3.5+1.12	—	1.3±0.7
Serine plasmalogen	—	2.0±0.6	—	2.5±0.5	—	1.5±0.2	—	0.9±0.03
Choline plasmalogen	2.3±0.25	7.0±1.2	5.8±0.9	8.1±1.3	3.5±0.7	10.2±1.4	2.2±0.7	4.6±0.03
Sphingo-myelin	11.2±1.5	22.0±2.3	11.5±1.4	18.2±1.6	15.2±1.3	26.2±2.1	12.5±1.2	20.9±2.4
Unidentified	2.0±0.1	3.0±0.5	1.0±0.06	3.5±1.1	1.5±0.05	3.8+0.8	3.0+0.4	2.5±0.2

TABLE II

Phospholipid Distribution in the Tissues of Submammalian Species
Average of 10 Analyses on Each Tissue

Species	P-choline	P-inositol	P-ethanolamine	P-serine	Choline plasmalogen	Ethanolamine plasmalogen	Serine plasmalogen	Sphingomyelin
Heteropneustes fossilis								
Brain	37.0±3.4	4.2±0.8	24.8±2.9	15.0±1.7	—	1.2±0.3	—	12.5±1.9
Liver	58.5±3.1	1.0±0.3	17.5±2.0	6.2±0.7	trace	0.5±0.08	trace	7.0±1.0
Heart	26.6±1.4	1.0±0.3	16.0±2.5	5.3±1.0	6.2±1.2	2.7±0.7	—	16.2±2.6
Kidney	33.5±1.8	1.5±0.2	19.3±3.1	8.0±1.0	1.5±0.15	3.2±0.6	—	18.0±3.0
Bufo malanostictus								
Liver	56.8±4.2	1.2±0.4	18.5±2.0	7.3±1.2	—	1.2±0.3	trace	8.3±1.5
Heart	28.5±2.3	1.0±0.7	20.0±2.5	7.5±1.4	6.8±0.5	3.3±0.3	—	20.2±2.3
Kidney	33.6±1.2	0.8±0.03	19.8±3.2	9.6±1.03	1.0±0.01	3.0±0.1	—	19.5±0.09
Kachuga Smithi								
Liver	55.2±3.6	1.6±0.06	18.0±1.9	5.8±0.09	trace	0.4±0.01	trace	9.2±1.2
Heart	25.8±2.3	1.0±0.08	18.5±2.1	7.0±0.34	3.5±0.12	4.2±0.09	—	17.2±1.87
Kidney	33.7±	2.0±0.04	21.5±2.2	7.2±0.82	1.2±0.03	4.5±1.01	—	16.3±2.1
Columba livia intermedia								
Liver	60.2±2.6	1.2±0.04	19.5±1.9	5.8±0.07	—	0.4±0.01	trace	9.2±1.02
Heart	35.4±2.6	0.7±0.01	20.5±1.8	7.5±0.5	3.0±0.09	2.0±0.12	—	18.0±1.5
Kidney	32.2±2.9	1.5±0.01	21.6±2.1	5.3±0.7	1.0±0.01	2.5±0.06	—	23.8±2.1

The distribution of plasmalogens was also found to follow a similar pattern in the same species. Comparable values in ethanolamine plasmalogens were obtained from fish and pigeon, from toad and turtle. Serine and choline plasmalogen values were comparable in fish, toad, and turtle, but in pigeon the values were lower by about 50%. It is evident from the results that, except for plasmalogens, the same types of phospholipids are present in erythrocytes and plasma. Turner et al. (12) reported a high phosphatidylcholine:sphingomyelin ratio in the red cells of dog, guinea pig, and dingo and also reported the absence of phosphatidylcholine in the red cells of goat, sheep and ox. These results seem to agree well with those obtained by Hanahan et al. (13) in human and pig red cells.

Phospholipid distribution in the liver, heart, and kidney of the four species is summarized in Table II. It is seen that, in liver, phosphatidylcholine accounted for more than 55% of the total lipid phosphorus in all the species and that the remaining 35% was constituted by phosphatidylinositol, phosphatidylserine, plasmalogens, and sphingomyelin. The percentage of hepatic phospholipids was found to be comparable in all these species. Values for p-choline, p-serine, p-inositol, and plasmalogens in the kidneys of different species tallied but differed in respect to sphingomyelin, which in fish, toad, turtle, and pigeon was 18%, 19.5%, 16.3,% and 25% respectively. The values of cardiac sphingomyelin, though much higher compared with those in liver, were found to be quite comparable in the four species of animals.

A more systematic and comprehensive study in more species of animals is however required to gain an insight into the possible relationship between the phospholipid pattern and the functional complexity of the organism and to form any conclusion regarding the evolutionary significance of individual phospholipids.

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Positional Distribution of Fatty Acids in Depot Triglycerides of Aquatic Animals

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ABSTRACT

Stereospecific triglyceride analyses were performed on fats of the following animals: five aquatic invertebrates, five freshwater fish, six marine fish, three marine birds, two amphibia, two seals, a whale, and a marine turtle. The distribution of fatty acids was asymmetrical in most cases. A formula is presented which describes the general tendencies of fatty acid distribution in many animal fats, and some special rules which modify this formula are stated.

INTRODUCTION

THE POSITIONAL DISTRIBUTION of fatty acids in triglycerides of animals is nonrandom: different acids tend to accumulate in different positions of the glycerol. The principles that regulate the distribution are not known although there have been speculations (1,2), but when the fatty acids in the positions 1, 2, and 3 (1,2,3-triacyl-L-glycerol) of a triglyceride are compared (2), it becomes clear that some general patterns are repeated in the fats of related animals. The analyses of several mammalian fats, for instance, could be summarized in a formula which described the tendencies in the proportional distribution of fatty acids (2).

A general formula of fatty acid distribution might be useful for predicting the patterns of new fats, but it would be more valuable as the basis for inquiries into the biochemical history of fats; it would be helpful, certainly, to recognize the general rules of distribution before probing into their origins. The formula for mammals (2) is unfortunately not a universal one. It does not even accommodate all mammalian fats; lard and seal oil are known exceptions. However, even though no universal pattern may be apparent, general rules valid for different groups of animals may still exist. Such an expectation seems reasonable since all animal fats contain the same four to ten fatty acids (minor components neglected), and all are synthesized, as far as known, through the same pathway via phosphatidic acid. It may even be possible to understand the different patterns as variations of a fundamental one, dependent on species, family, or class, and on the diet of the animals. It is with this intention that stereospecific analyses have been collected on the triglycerides of 25 aquatic animals. Since the fatty acids of aquatic, and especially marine, fats have a wide range of chain lengths and numbers of double bonds, the present survey is more comprehensive than a previous one (2).

MATERIALS AND METHODS

The origins of the fats which are analyzed are listed in Table I. The fats were extracted with hexane, and the triglycerides were purified by chromatography on silicic acid and aluminum oxide. The fatty acid compositions in positions 1, 2, and 3 (1,2,3-triacyl-L-glycerol) were determined by "stereospecific analysis" of the triglycerides according to a revised procedure (4). In this analysis the triglyceride is degraded to a racemic mixture of α,β-diglycerides by the action of a Grignard reagent. The diglycerides are converted to phospholipids, which are then resolved by the stereospecific enzyme phospholipase A (EC 3.1.1.4). The fatty acids in position 3 cannot be directly analyzed in this manner, but their composition can be calculated in two independent ways; the agreement of these calculations measures the accuracy of the analysis. This aspect of the method has been discussed at length in previous publications (4,5). Since the analyses of aquatic fats proved to be more difficult than those of fats of terrestrial animals, previous standards of accuracy (4,5) have been relaxed, and analyses have been accepted when the discrepancy between the two calculations of position 3 was as large as 15%, relative, for a major ($>10\%$) fatty acid or as large as 3%, absolute, for a minor one. A value of 30% for an acid in position 3 may therefore be the average of 27.8% and 32.2%, and a value of 7% the average of 5.5% and 8.5%. In general however, the agreement was much better.

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TABLE I
Origin of Aquatic Animal Fat Triglycerides

Category	Sample number	Common name	Genus and species	Origin	Tissue extracted
Fish (fresh-water.	1	Burbot (maria)	*Lota lota*	Lake Winnipeg	Liver
	2	Sheepshead (fresh water drum)	*Aplodinotus grunniens*	Lake Erie	Whole animal
	3	Speckled trout A[a]	*Salvelinus fontinalis*	Laboratory animal fed on beef liver and trout chow	Whole animal
	4	Speckled trout B	*Salvelinus fontinalis*	Laboratory animal fed on beef liver and trout chow	Whole animal
	5	Goldfish[a]	*Carassius auratus*	Laboratory animal fed on commercial fish food	Whole animal
Fish (marine)	6	Herring	*Clupea harengus*	North Atlantic Ocean	Whole animal
	7	Mackerel A	*Scomber scombrus*	North Atlantic Ocean	Whole animal
	8	Mackerel B	*Scomber scombrus*	North Atlantic Ocean	Whole animal
	9	Barn-door skate	*Raja laevis*	Nova Scotia coast	Liver
	10	Cod A	*Gadus morhua*	Nova Scotia coast	Liver
	11	Cod B	*Gadus morhua*	Nova Scotia coast	Liver
Invertebrates	12	Squid (sea arrow)	*Illex illecebrosus*	Newfoundland coast	Liver
	13	Periwinkle	*Littorina littorea*	Nova Scotia coast	Whole animal
	14	Lobster A[a]	*Homarus americanus*	Nova Scotia coast	Hepatopancreas
	15	Lobster B	*Homarus americanus*	Nova Scotia coast	Hepatopancreas
	16	Scallop	*Platopecten magellanicus*	North Atlantic Ocean	Hepatopancreas
Mammals	17	Harbor seal[b]	*Phoca vitualina*	Nova Scotia coast	Blubber
	18	Harp seal	*Pagophilus groenlandicus*	Gulf of St. Lawrence	Blubber
	19	Sei whale	*Balaenoptera borealis*	Antarctic Ocean[c]	Blubber
Birds	20	Herring gull	*Larus argentatus*	Nova Scotia coast	Subcutaneous and intestinal fat
	21	Grey gull (black-backed gull)	*Larus marinus*	Nova Scotia coast	Subcutaneous and intestinal fat
	22	Cormorant	*Phalacrocorax auritus*	Nova Scotia coast	Subcutaneous and intestinal fat
Amphibia	23	Mudpuppy	*Necturus maculosus*	Wisconsin[d]	Whole animal
	24	Frog[a]	*Rana pipiens*	Wisconsin[d]	Whole animal
Reptiles	25	Leatherback turtle	*Dermochelys coriacea*	Nova Scotia coast	Dermal fat

[a] Taken from (3).

[b] Taken from (4).

[c] Whale oil was obtained from DE-NO-FA og Lilleborg Fabriker, Fredrikstad, Norway. The sample came from the 1965-1966 Antarctic catch, which totaled 17,563 sei whales and 2,318 fin whales. Comparison of this sample with the fatty acid compositions of sei whale and fin whale blubber oils published by Sano et al. (11) indicates that the sample received is almost exclusively of sei whale origin.

[d] Purchased from Steinhilber and Company, Oshkosh, Wis.

It was also found that, when an analysis had to be repeated, the new averages would be very close to the old ones even if these had been obtained from data with unacceptable discrepancies. The explanation is that errors during the analysis (caused, for instance, by oxidation of unsaturated acids) will appear in opposite directions in the two calculations of position 3 (5). The data have been normalized to full percentage in Table II and may be taken as correct to ±1% absolute for minor, ±8% relative for major components.

RESULTS AND DISCUSSION

A first examination of Table II threatens to dispel any hopeful expectations that some simple and general rules might be found to describe all depot fats. It might even seem that the fats of animals of the same species will not fit into one formula; the two lobsters (No. 14, 15) are a glaring example. However, the patterns of the two cod (No. 10, 11) agree very well despite the difference in fatty acid composition, and so do the patterns of the trout (No. 3, 4) and the mackerel (No. 7, 8) except for discrepancies in the distribution of 20:1.

Since two samples of human fat and two of lard also showed good agreement (2), it is only in one pair of analyses (the two lobsters) out of six, that the patterns have been found incompatible. If this case is set aside, it would seem that every species has its own more or less distinct arrangement of fatty acid (perhaps this is true only for vertebrates). Furthermore, closely related animals, such as the two seals (No. 17, 18) or the two gulls (No. 20, 31), may have similar patterns. An attempt will be made to draw some general conclusions from the data, first about the different groups of animals, and then about the different fatty acids. To reach the following conclusions use has been made of a graphical presentation in

TABLE II

Fatty Acid Distribution in Animal Depot Fats

	Position	Fatty Acids (mole percentage) 14:0	16:0	16:1	18:0	18:1	18:2	20:1	22:1	20:5	22:5	22:6	Others
Fish, fresh water													
1. Burbot	1	3	16	28	6	38	1	11	1	2	1	1	
	2	5	17	31	4	14	2	1	1	10	1	4	
	3	1	3	17	2	50	4	2	1	9	3	2	
2. Sheepshead	1	3	15	25	4	29	5	5	2	2	1	1	
	2	4	28	22	1	11	5	5	2	4	5	6	
	3	2	8	20	1	38	6	6	4	8	1	1	
3. Trout A	1	2	13	8	7	24	6	11	9	4	2	3	
	2	3	6	14	1	35	11	7	2	4	2	9	
	3	4	13	8	8	25	5	12	9	6	2	1	
4. Trout B	1	3	24	10	14	37	5	1	2	1	1	1	
	2	1	3	17	1	45	12	3	4	1	3	7	
	3	2	19	11	12	36	7	6	5	1	1	1	
5. Goldfish	1	2	17	8	7	32	16	7					
	2	4	32	8	3	20	22	4					
	3	1	9	7	4	38	24	7					
Fish, marine													
6. Herring	1	6	12	13	1	16	3	25	14	3	1	1	
	2	10	17	10	1	10	3	6	5	18	3	13	
	3	4	7	5	1	8	1	20	50	4	1	1	
7. Mackerel A	1	6	15	11	3	21	2	8	18	5	1	2	
	2	10	21	6	1	9	1	5	5	12	3	20	
	3	2	5	4	2	21	2	19	24	10	1	5	
8. Mackerel B	1	6	15	12	3	19	2	16	14	4	1	1	
	2	11	30	8	1	9	1	7	5	10	2	11	
	3	4	7	10	2	27	2	9	25	6	1	4	
9. Skate	1	2	19	12	5	30	1	12	8	4	1	5	
	2	3	15	7	1	9	1	8	5	6	7	37	
	3	1	6	6	1	28	2	19	11	11	2	11	
Fish, marine													
10. Cod A	1	6	15	14	6	28	2	12	6	2	1	1	
	2	8	16	12	1	9	2	7	5	12	3	20	
	3	4	7	14	1	23	2	17	7	13	1	6	
11. Cod B	1	2	12	11	5	35	1	14	12	3	1	3	
	2	5	14	8	1	9	1	8	8	11	3	32	
	3	2	6	9	1	25	2	18	11	11	1	12	
Invertebrates													
12. Squid	1	4	28	8	4	21	4	6	3	12	1	4	
	2	2	2	4	1	7	1	7	5	28	2	38	
	3	4	12	9	2	26	1	17	13	5	1	10	
13. Periwinkle	1	2	13	6	4	27	5	18	9	6	1	4	
	2	9	17	9	1	12	5	13	3	16	2	7	
	3	3	4	10	1	21	5	16	9	8	2	9	
14. Lobster A	1	3	13	10	3	22	2	11	7	8	<1	3	
	2	4	12	7	1	17	2	10	2	13	<1	15	
	3	4	13	10	3	25	2	12	8	8	<1	5	
15. Lobster B	1	2	31	10	4	29	1	7	5	5	1	1	
	2	2	8	5	1	19	2	11	6	20	4	17	
	3	2	8	9	3	42	2	10	7	11	1	2	
16. Scallop	1	3	19	5	6	19	3	7[a]	8[b]	22	<1	5	
	2	6	5	5	1	9	2	2[a]	10[b]	33	1	29	
	3	10	12	10	1	10	2	5[a]	14[b]	22	1	8	
Mammals													
17. Harbour seal	1	4	11	15	1	29	1	18	8	3	2	3	
	2	11	13	30	1	30	3	3	1	1	1	1	
	3	1	4	14	1	26	1	16	7	8	6	10	
18. Harp seal	1	1	7	9	1	27	1	17	4	6	4	15	
	2	6	9	27	2	36	5	4	1	2	1	3	
	3	1	5	11	1	20	2	7	1	12	11	26	
													18:4 / 20:4 ω3
19. Sei whale	1	3	13	3	4	14	1	33	10	3	1	6	1 / 5
	2	12	6	12	1	29	5	10	2	5	1	3	4 / 6
	3	4	6	2	2	7	1	28	16	6	3	16	1 / 3

(Continued)

	Position	14:0	16:0	16:1	18:0	18:1	18:2	20:1	22:1	20:5	22:5	22:6	Others
Birds													
20. Herring gull	1	2	22	4	13	41	7	7	3	<1	<1	<1	
	2	3	15	3	9	48	11	6	4	<1	<1	<1	
	3	2	17	5	7	46	9	7	5	<1	<1	<1	
21. Gray gull	1	4	17	7	7	33	6	15	8	<1	<1	<1	
	2	4	12	5	7	39	6	15	11	<1	<1	<1	
	3	2	20	10	5	29	7	16	9	<1	<1	<1	
22. Cormorant	1	3	34	7	14	21	1	3	3	5	1	3	
	2	2	21	10	6	37	2	4	4	4	2	3	
	3	2	21	11	10	30	1	4	5	7	2	3	
Amphibian													
23. Mudpuppy	1	5	31	23	5	20	2	3[c]	3	1	1	1	
	2	2	6	21	1	25	18	9[c]	3	1	3	5	
	3	2	7	15	2	38	5	7[c]	5	4	4	5	
													18:3
24. Frog	1	3	26	10	4	27	12						9
	2	2	17	8	1	30	25						13
	3	2	18	8	3	35	16						9
Turtle													12:0
25. Turtle	1	18	24	7	12	12	1	5	5	1	1	1	7
	2	10	4	13	2	45	1	3	3	2	3	5	2
	3	14	10	8	5	19	1	11	6	4	2	2	9

[a] Unknown.

[b] 18.4.

[c] 20:1 + 18:3.

which the fatty acids are plotted according to their proportion per position (2). Such graphs made comparisons much easier, but since they are not indispensable, only three are reproduced as examples (Table III). It must be remembered that the following statements are no more than tentative generalizations.

Invertebrates, Amphibia, Turtle

Polyenoic acids prefer position 2; the saturated acids, 16:0 and 18:0, position 1; longer acids position 3 (exception:scallop). The accumulation of 12:0 and 14:0 in the α-positions of the turtle fat is peculiar.

Birds

The fatty acid distribution has a more random appearance in these fats; all proportional percentages are between 20 and 47, most of them between 25 and 40. The patterns are nearly symmetrical in the gulls, reminiscent of the fats of three domestic birds (3).

Fish

Position 1 attracts saturated and monounsaturated acids; position 2 polyunsaturated and short acids; position 3 long acids. All fish accumulate 16:0 in position 2 except the trout; the trout also show an exceptionally symmetrical pattern.

Mammals

In the seal oils the shorter fatty acids accumulate in position 2, but the polyenoic acids occupy position 3, and then 1. The same pattern has been found in the fat of a polar bear (4), and it seems that it is not peculiar to these animals but that any mammal fed with marine oil will lay down such a fat (unpublished results). The marine mammals should therefore be included in any general formula for mammals. In the sei whale oil there is a partial reappearance of polyenoic acids in position 2, reminiscent of the pattern in pilot whale, *Globicephala malaena* (2).

In a dicussion of the different fatty acids earlier results (2) will be included.

Saturated Acids

Myristic acid, 14:0, if it is a major component, is most often found concentrated in position 2 in fish and mammals (2). Stearic acid, 18:0, is found in 1 and 3 in all the animals except one gull (No. 21). Palmitic acid, 16:0, always a major component, can serve to divide animal depot fats into two groups. In the first one there is a deficit of this acid in position 2. This group includes most mammals (2), the birds, three out of four invertebrates, the two amphibia and the turtle, but only two (closely related) fish, the trout and the salmon (7). In the second group palmitic acid is prominent in position 2: in most fishes and in the pig and its relative, the peccary (8). The seals should not be included in this group but in the first one since the 16:0 in their fats seems to collect in position 2 by default only, together with 14:0, 16:1, 18:1, because the long-chain acids preempt the α-positions. The rule that unsaturation is favored in position 2

TABLE III

Proportional Distribution of Fatty Acids in Triglycerides in Percentage of Fatty Acid per Position[a]

Triglyceride	Position	10	20	30	40	50	60	70	80
Cod A	1			14:0 16:0 18:1 16:1 20:1 22:1				18:0	
	2		18:1 20:1	16:1 22:1	16:0 14:0 20:5		22:5	22:6	
	3		14:0 16:0 22:6	16:1	18:1 22:1	20:1 20:5			
Squid	1	22:6	22:1 20:1	20:5	14:0 16:1 18:1			16:0 18:0 18:2	
	2		18:1 16:1 20:1 22:1				20:5	22:6	
	3	20:5	22:6	16:0	14:0 16:1	18:1	20:1 22:1		
Herring Gull	1			22:1 18:1 20:1 14:0 16:1 18:2	16:0 18:0				
	2			16:0 22:1 16:1 18:0 20:1	18:2 14:0 18:1				
	3			18:0 16:0 14:0 18:1 18:2 20:1	16:1 22:1				
		10	20	30	40	50	60	70	80

[a] Fatty acids occurring in traces (<3%) are omitted from this table.

(2) is still followed in seals: palmitoleic acid outweighs palmitic. In fish and in pig, by contrast, 16:0 is singled out for preferential location in 2 whereas the distribution of 16:1 is normal. In both groups the distribution of 16:0 is usually asymmetrical, and if it is, more of the acid is always found in position 1 than in 3; this seems to be a universal feature.

Monoenoic Acids

The monoenoic acids in Table II are mixtures of isomers. In a typical marine oil (6), the 16:1 fraction was a mixture of the Δ^7 and Δ^9 isomers (minor components neglected), and 18:1, 20:1, and 22:1 were mixtures of Δ^9 and Δ^{11} isomers. Each positional isomer may be distributed differently, but the present analyses give only the distribution of the combined isomers.

Table II indicates that 16:1 is rather randomly distributed. In terrestrial mammals (2) and seals this is also true for 18:1 whereas 16:1 is found concentrated in position 2 in accordance with the formula mentioned in the introduction. In most fish 18:1 is α-bound. The fractions 20:1, and especially 22:1, aggregate in the α-positions in fish and in the marine mammals though there is no fixed pattern of distribution between positions 1 and 3. There is no consistency of patterns in the invertebrates.

The monoenoic acids are probably in part endogenous, formed in the animals by dehydrogenation of saturated acids in the Δ^9 position (6,9). This process can be very rapid, and it is dependent on the nutritional state of the animal (10). Perhaps animals employ this mechanism in emergencies to synthesize "average" fatty acids. Swamped with satur-

ated acids and unable to produce polyenoic ones, they may use the monenoic acids ("average" in chemical and physical behavior) as stopgaps in any position of a phospholipid or a triglyceride. Future analyses, then, might reveal that the distribution patterns for the Δ^9 monoenoic acids are determined by the quantity and the distribution of the saturated and polyenoic acids, in a complemental manner, but that the Δ^{11} isomers, which are more likely to be exogenous, follow simple and rigid patterns.

Polyenoic Acids

Linoleic acid, 18:2, is usually the major polyenoic acid of nonmarine animals. It is found accumulated in position 2. The concentration in 3 is the second highest and may sometimes approach that in 2 (rabbit, goldfish) (2). The same rules hold for the long-chain polyenoic acids, 20:5 and 22:6, in marine fish and invertebrates, but in the triglycerides of mammals these acids cannot take the place of 18:2 although they do so in the phospholipids (unpublished observations). Instead they occupy position 3, then position 1 (seals, polar bear) (4).

CONCLUSION

If from the 40 available analyses an all-inclusive formula is extracted, it must be partly buried again under qualifications.

Tendencies of fatty acid distribution in animal fats

Position 1. saturated
2. short, unsaturated
3. long

The formula should be read in terms of the proportional distributions of fatty acids. It is too general to have much value in predicting the pattern of any particular fat, but it serves rather well to describe the tendencies of distribution of most acids in most fats. For instance, it is usually found that in position 1 the proportion (not the concentration) of 18:0> 18:1> 18:2; and 16:0> 16:1; and 20:1, 22:1> 20:5, 22:6. For position 2, it is usually true that 16:1> 16:0 (but see below); 18:2> 18:1> 18:0; 20:5, 22:6> 20:1, 22:1; and also 14:0> 16:0> 18:0, and 16:1> 18:1. In position 3 are generally found 22:1, 20:1> 18:1> 16:1, but the exceptions are many. Analyses separating all the isomeric acids might bring order into this seeming confusion.

Deviations from the proposed pattern are found in most fats; the following ones are rules in their own right:

Pigs, most fishes: 16:0 in position 2.

Mammals: 20:5, 22:5, 22:6 in position 3, also 1. (It is perhaps more logical to state that these acids are barred from position 2. Their distribution between 1 and 3 then follows from the general formula.)

Birds: nearly random or symmetrical distributions.

These rules can be stated with some confidence. As for other classes of animals, the solitary analyses available at this time can only be registered and filed.

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The Structure of Rat Liver Triglycerides[1]

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ABSTRACT

The fatty acid compositions at the 1-, 2-, and 3-positions[3] of rat liver triglycerides were determined by using pancreatic lipase and diglyceride kinase. The distribution of acids between the 1- and 3-positions is not random; rather each position has a characteristic composition. The relative abundance of species and positional isomers in the triglyceride mixture was predicted by using values from the stereospecific analysis and assuming that the composition of each position is independent of the other two. The total triglyceride was resolved into species by using TLC with silver nitrate and Silica Gel G, and the relative amounts corresponded closely with those predicted on the basis of this assumption.

The major species were isolated, and the distribution of their fatty acids among the three glyceride positions was determined. From these data the relative amount of each positional isomer was calculated. The results indicate that the esterification of fatty acids at each position proceeds with a specificity that is not correlated with the composition of the other positions of the molecule.

The relative abundance of the different liver triglyceride species is also found to be related in part to the composition of the 1,2-diglyceride units found in the lecithins of this tissue.

INTRODUCTION

THE METABOLIC STEP in which the 3-position of triglycerides is acylated may influence the over-all fatty acid composition of the triglyceride fraction in two ways: by preferentially incorporating certain 1,2-diglyceride units and by selecting particular acyl groups for esterification. Weiss et al. (2) and Goldman and Vagelos (3) attempted to show specificity for certain diglycerides in the synthesis of triglycerides but were hampered by the difficulty of dealing with insoluble subtrates. Gøransson (4) showed that the ratio of incorporated oleate to palmitate in rat liver triglycerides was much higher than that of the diglycerides, suggesting that a preferential esterification of oleate relative to palmitate occurs at the 3-position in vivo.

A recent finding (5) that, in rat liver, the fatty acid composition at the 3-position varies dramatically from that at the 1-position reinforces the suggestion of a 3-acylating system which has some specificity for the acid to be esterified. The present study deals with the analysis of the distribution of fatty acids among the three positions in individual triglyceride species.

The stereospecific analysis is summarized in Fig. 1. Triglycerides are incubated with pancreatic lipase, which hydrolyzes the primary ester linkages to yield a mixture of 1,2- and 2,3-diglycerides, which are further degraded to 2-monoglycerides. The reaction may be stopped at a stage of partial hydrolysis and the mixed diglycerides recovered and incubated with ATP and the diglyceride kinase from *Escherichia coli*. This enzyme phosphorylates only the 1,2-diglyceride to yield a phosphatidic acid which contains the fatty acids originally at the 1- and 2-positions of the triglycerides. The composition of the 1-position is found by the differences between the phosphatidic acid and the monoglyceride fractions. The composition at the 2-position is that of the isolated monoglyceride, and the composition of the 3-position is found by the differences between the triglyceride and the phosphatidic acid fractions.

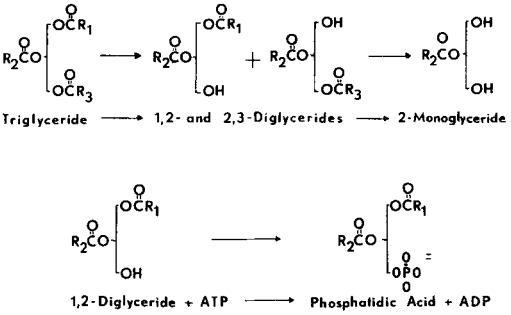

FIG. 1. Stereospecific analysis of triglycerides.

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[3]The stereospecific numbering system recommended by Hirschmann (1) is used throughout this paper.

EXPERIMENTAL PROCEDURES

Preparation of Enzymes

Lipase was prepared as described previously (5). The preparation used for routine triglyceride analysis had a specific activity of 270 units/mg, as measured by a micro-adaptation of the method of Desneulle (6). One unit of activity equals 1 meq of ester hydrolyzed per minute per milliliter. Before each lipase preparation was used, its activity under the conditions used for triglyceride analysis was determined. Triglyceride samples (5 mg) were treated with lipase for 0, 1, 2, 3, 4, or 5 min. Aliquots of the lipid extracts were chromatographed on silicic acid plates to determine the diglyceride content. The time which gave the maximum yield of diglyceride was then used for the stereospecific analysis.

Diglyceride kinase was prepared as described by Pieringer and Kunnes (7). After heat treatment it was stored at –10C in 0.01 M sodium phosphate –0.1% cysteine hydrochloride buffer. No loss of activity was observed after three to four months.

Chromatographic Methods

Plates coated with Mallinckrodt Reagent Grade Silicic Acid, 200 mesh, without binder, were used for the separation of neutral lipid and phospholipid classes.

Separation of triglyceride species or methyl esters of fatty acids of different degrees of unsaturation was done on plates coated 0.5-mm thick with 16% silver nitrate in Silica Gel G. The plates were dried in air in the dark for approximately 2 hr, activated at 125C for 2 hr, and stored over P_2O_5 in the dark.

Methyl esters were analyzed with a Barber-Coleman Model 10 gas chomatograph, equipped with a hydrogen flame detector and a 1/4-in by 6-ft column packed with 10% diethylene glycol succinate on Gas-Chrom P. The column temperature was 195C, and the flow of carrier gas (argon) was approximately 60 ml per minute.

Preparation of Triglycerides

Male albino rats weighing 190 to 200 g were obtained from Rawley Farms, maintained in the laboratory on Rockland Rat Diet for at least one week, and used before attaining a weight of 250 g. Animals were killed by decapitation. The livers were removed and cooled on ice.

The livers were homogenized in a Waring Blendor in chloroform-methanol (1:2), 4 ml per gram of wet tissue, for 2-3 min. A small amount of Santoquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline), approximately 1 mg per 50 grams of tissue, was added to retard oxidation of polyunsaturated fatty acids. While the Blendor was still running, enough chloroform was added to make the final ratio of chloroform-methanol (2:1), followed after 1 to 2 min by water (1 ml per gram of tissue).

The mixture was filtered through glass wool. The residue was washed with chloroform-methanol (2:1), and the combined filtrates were centrifuged. The cloudy chloroform layer was separated, cleared by the addition of methanol; the solvents were evaporated. To remove the water carried into the chloroform solution, the lipid was redissolved in chloroform-methanol (in some cases benzene-ethanol was used), and the solvent was evaporated. This was repeated until a clear oil was obtained as a residue.

Triglycerides were isolated from the total lipid extract by a modification of the procedure of Carroll (8). The lipid from approximately 100 g of liver was put on 200 g of Florisil (deactivated with 7% water) in hexane (Phillips technical grade, 99% hexane). Elution with 500 ml of 4% diethyl ether in hexane removed some pigments and cholesterol esters. Triglycerides were then eluted with 1,250 ml of 8% diethyl ether in hexane. The later fractions of triglyceride from this column were contaminated with a more polar lipid. They were combined and rechromatographed on a 30-g column, and the triglycerides were eluted with 500 ml of 7% diethyl ether in hexane. The pure triglyceride fractions from the first and second columns were combined; the solvent was evaporated, and the triglycerides were dissolved in hexane. Final concentration was about 20 mg/ml.

Separation of Triglyceride Species

The triglycerides, isolated as described above, were applied to a silver nitrate-Silica Gel G plate. Then 1-2 mg of the triglyceride fraction was used for the determination of the distribution of species. For preparative work 8-10 mg was loaded on each 20 × 20 cm plate. The average load was 0.5 mg per cm length of band. Two solvent systems were used in determining the distribution of species; diethyl ether-petroleum ether-benzene-methanol, 7.5:30:60:1, or chloroform-isopropanol, 98:2. The first system was used for all preparative work. After development the plates were sprayed with dichlorofluorescein in methanol and viewed under ultraviolet light. The bands were scraped into test tubes. A solution of 2% NaCl in 20% water in methanol was added until the char-

acteristic rose color of the dichlorofluorescein-silver complex disappeared. Diethyl ether was added (ca. 10 times the volume of NaCl solution used), and the slurry was mixed on the Vortex mixer. The mixture was centrifuged and the ether decanted. The precipitate was extracted again with ether, and the ether extracts were combined for analysis.

Positional Analysis of Triglycerides

The positional distribution of fatty acids was determined by using pancreatic lipase, followed by selective phosphorylation of the 1,2-diglycerides by diglyceride kinase as described previously (5). The following minor changes were made in the procedure. After lipase hydrolysis and thin-layer chromatography of the mono-, di-, and triglycerides the bands were made visible by spraying one edge of the plate with dichlorofluorescein while the rest of the plate was masked. This change was made after it was observed that spraying with I_2 led to some loss of the polyunsaturated acids. The diglycerides were eluted from the part of the plate not sprayed with dichlorofluorescein and used as substrate for the kinase reaction. The previous method (5) of calculating the amount of each methyl ester present (by using gas chromatography) was modified as recommended by Brandt and Lands (9). The retention times were measured from that point behind the real injection point where the width at half-height was actually zero.

The methyl esters of 14:0, 16:0, 18:0, 20:0, 18:1, and 18:2 were identified by comparison of their retention times with those of the standards obtained from the Hormel Institute, University of Minnesota, Austin, Minn. A plot of the logarithm of these retention times vs. the number of carbons in the fatty acid chain was made, and the equivalent chain-lengths of the other components were determined. They were compared with those found by Hofstetter et al. (10), and the acids were tentatively identified.

A mixture of methyl esters from the total triglyceride was then chromatographed on a silver nitrate-Silica Gel G plate to separate the components according to the number of double bonds in the chains. The positions in which the esters were found on the silver nitrate plate corroborate the identifications made by equivalent chain-lengths for all the acids named in Table I. Four components were found which have 20 or 22 carbons, as shown by hydrogenation, and four or more double bonds. These components were still present after purification of the methyl esters on silicic acid plates. They were not found in blanks or in samples of Santoquin or of the residues remaining after evaporation of several liters of the solvents used. Their equivalent chain-lengths do not correspond well to any reported values, and they are indicated in this study as unidentified.

RESULTS

The distribution of fatty acids found for a sample of rat liver triglycerides is shown in Table I. The triglycerides contained up to 10% of fatty acids with 18 carbons and more than two double bonds, or with 20 or 22 carbons and from one to six double bonds. The sample shown in Table I contained 7.3% of such acids.

TABLE I

The Distribution of Fatty Acids Among the Positions of Rat Liver Triglycerides

	Composition (mole %)					Distribution (%) Position		
	I	II	III	IV	V	1 (V-III)	2 III	3 (IV-V)
Acid	TG	PA	MG	TG x 3	PA x 2	3	3	3
14:0	1.4	1.4	1.1	4.2	2.8	0.6	0.4	0.5
16:0	27.1	35.4	8.6	81.3	70.8	21	2.9	3.5
16:1	3.0	1.6	4.0	9.0	3.2	0	1.3	1.9
18:0	2.2	3.1	1.0	6.6	6.2	1.7	0.3	0.1
18:1	30.0	28.9	39.6	90.0	57.8	6.1	13	11
18:2	25.1	27.8	42.1	75.3	55.6	4.5	14	6.6
18:3 (n-6) } 20:0 {	0.1		0.1	0.3				
18:3 (n-3)	1.2	0.2	0.9	3.6	0.4			
20:1	0.6		0.1	1.8				
20:2	0.5		0.2	1.5				
20:3 (n-9)	0.2		0.1	0.6				
20:3 (n-6)	0.2		0.2	0.6				
20:4 (n-6)	1.5	0.9	1.2	4.5	1.8			
20:5 (n-3)	0.2		0.1	0.9				
22:4 (n-6)	0.6		0.3	1.8				
Unidentified	2.1	0.6	0.7	6.3	1.2			

TABLE II

Distribution of Acids in Several Samples of Rat Liver Triglycerides[a]

Sample	Acid	Distribution (mole percentage) Position 1	2	3
I	16:0	18	2.2	3.5
	16:1	1.2	0.7	0.4
	18:0	2.4	0.2	0
	18:1	6.6	13	12
	18:2	4.0	16	13
II	16:0	17	1.8	5.4
	16:1	0.3	0.6	2.0
	18:0	1.0	0.2	1.1
	18:1	8.0	12	12
	18:2	7.5	17	7.3
III	16:0	20	1.4	6.7
	16:1	0.3	0.9	3.1
	18:0	2.4	0.2	0.4
	18:1	5.6	13	14
	18:2	4.7	17	9

[a] The analysis of each sample was performed three times.

Individually these components are present in amounts too small to analyze reliably through all the steps of the procedure; therefore they were not routinely reported in the determination of the distribution of acids.

Results from nine separate analyses, shown in Table II, indicate the reproducibility of the methods used. The values represent the averages of three separate analyses for each of the three different preparations of rat liver triglycerides. In all cases the saturated acids are found predominantly in the 1-position. The 2-position contains predominantly unsaturated acids, as has been shown before (11). In the 3-position, unlike the 1-, about 80% of the acids are unsaturated. The results were considered to be sufficiently consistent for the conclusions made in this paper.

TABLE III

Distribution of Fatty Acids Among the Three Positions of the Species SM_2

Acid	Position 1	2	3
14:0	0.7	0.3	0.4
16:0	22.9	3.6	3.7
18:0	0.6	0.3	1.0
	24.2	4.2	5.1
16:1	0.2	1.7	2.1
18:1	9.2	26.8	25.2
	9.4	28.5	27.3

Positional isomer			Amount in whole triglyceride
SMM	24.2		10.0
MSM	4.2	$\times \left(\frac{13.9}{33.5}\right)$	1.7
MMS	5.1		2.1

The values shown are averages of duplicate analyses of three different samples.

TABLE IV

Distribution of Acids Among the Three Positions of the Species SD_2

Acid	Position 1	2	3
16:0	24.1	1.4	2.4
18:0	1.5	0.2	0.8
	25.6	1.6	3.2
16:1	1.0	0.3	0.2
18:1	0.1	1.8	3.0
	1.1	2.1	3.2
18:2	6.9	29.2	25.0

Positional isomer			Amount in whole triglyceride
SDD	25.6		9.5
DSD	1.6	$\times \left(\frac{11.3}{30.4}\right)$	0.6
DDS	3.2		1.2

The values shown are averages of duplicate analyses of three different samples.

The total liver triglycerides were separated into species on plates containing silver nitrate, and the distribution of fatty acids in the major species was determined. Table III shows the distribution found for the species containing one saturated and two monounsaturated acids residues per molecule (the SM_2 species). The relative amount of each positional isomer can be found from the limiting amount of saturated acids in the 1-, 2-, and 3-positions respectively. Thus the SM_2 species represents 13.9% of the whole triglyceride fraction, and the relative amount of each positional isomer in the whole triglyceride is 10.0, 1.7, and 2.1% (Table III).

The analysis of the SD_2 species (one saturated and two diunsaturated esters) is shown in Table IV. The SD_2 species contain 11.3% of the whole triglyceride. The isomer SDD is predominant, 9.5% of all the triglycerides.

The SMD species (one saturated, one monounsaturated, and one diunsaturated ester per molecule) was found to consist principally of the SMD and SDM isomers (Table V).

DISCUSSION

For the species SM_2 and SD_2 the relative amount of each positional isomer present is obvious from the amount of S at each position. This is not the case for the species SMD (Table V), as Brockerhoff has pointed out (12). In these experiments it was possible to estimate the relative amounts of the positional isomers of SMD without experimentally separating the pairs of enantiomers (13) because the total amount of one of these pairs, MSD

and DSM, is limited by the very low amount of S in the 2-position. If the values of 0.5 and 0.7 are assumed for MSD and DSM respectively, the amount of SMD is equal to the amounts of D at the 3-position minus the amount of D contained in MSD, or 9.9. Assuming the limiting possible values of MSD, 0 or 1.2, would give values of 10.4 or 9.2 respectively of SMD; that is, the value obtained for SMD is correct to ± 0.7 regardless of the values assumed for MSD. The same reasoning applies to the values obtained for SDM.

The results shown in Tables I-V suggest that the system which acylates the 3-position uses 18:1 more effectively than 18:2 and either of these more effectively than 16:0. It may also preferentially esterify diglyceride units which contain certain fatty acids. The data on the relative amounts of each isomer make it possible to determine whether the acyl specificity is correlated to diglyceride composition or not. The consequences of correlative and noncorrelative specificity are summarized in Figure 2.

Consider that some acyl-CoA, X, can be esterified to an SM diglyceride with a rate constant k_{SMX} for the formation of SMX. Similarly X is added to SD diglycerides with a rate constant k_{SDX}, so also for the addition of a different acyl group, Y, to the SM and SD diglycerides. Noncorrelative specificity exists if $k_{SMX}/k_{SDX} = k_{SMY}/k_{SDY}$. In this situation the ratio SMX/SMY will equal the ratio SDX/SDY, and this will equal the ratio of X in the 3-position of the triglyceride mixture over-all. In other words, the SM diglyceride will be distributed between SMX and SMY the same way that the SD diglyceride is distributed between SDX and SDY, and this distribution is the same as that of X and Y in the 3-position of all the triglycerides.

If, however, the acyl specificity were correlated to diglyceride composition, the distribution of X and Y between the SM derivatives would be different from the distribution for the SD derivative, or that of X and Y in the over-all mixture.

It seems clear that the synthesis proceeds with a noncorrelative specificity for the observed distributions are as follows:

$$\frac{SMM}{SMD} = 1.3 \qquad \frac{SDM}{SDD} = 1.3 \qquad \frac{M_{III}}{D_{III}} = 1.5$$

Assuming noncorrelative specificity for the acylation of each position, the observed distribution of fatty acids in the total mixture was

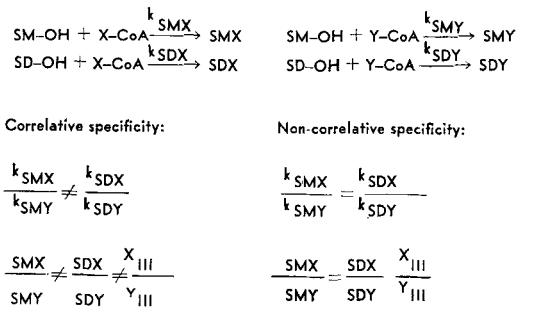

FIG. 2. Correlative vs. noncorrelative specificity.

used to predict the relative amount of each species that could be isolated. For comparison, the same prediction was made assuming 1,3-random-2-random acylation (14,15), as shown in Table VI. Although the two hypotheses are opposed on a fundamental point, the metabolic equivalence of the 1- and 3-positions, the predictions made using them are not strikingly different. The principal difference is noted in species containing two saturated residues, which would require at least one saturate at either the 2- or 3-position, an unfavorable situation. Vander Wal indicated recently (16) that the calculated percentage composition of the triglycerides in a mixture would be relatively

TABLE V

Distribution of Acids Among the Three Positions of the Species SMD

Acid	Position 1	2	3
16:0	25.2	1.1	3.6
18:0	0.5	0.1	1.2
	25.7	1.2	4.8
16:1	1.1	0.8	0.4
18:1	3.7	11.8	17.0
	4.8	12.6	17.4
18:2	2.4	19.4	10.6

Distribution corrected for amount of M_3 and M_2D present

Acid	Position 1	2	3
S	25.7	1.2	4.8
M	3.8	11.7	16.4
D	2.2	19.3	10.4

Positional isomer			Amount in whole triglyceride
SMD	9.9		7.9
SDM	15.8		12.6
MSD	0.5	$\times \left(\frac{25.5}{32.0}\right)$	0.4
MDS	3.3		2.6
DSM	0.7		0.6
DMS	1.8		1.4

The values shown are averages of duplicate analyses of three different samples.

TABLE VI

A Comparison of Observed and Predicted Distributions of Species in Triglycerides

Species	Relative Abundance: Predicted 1,3-Random 2-Random	Predicted Noncorrelative specificity	Observed[a]
S_3	1.0	0.7	0.6
SM_2	7.9	6.1	5.7
S_2M	11.4	13.5	13.9
S_2D	8.3	6.0	4.8
M_3	4.6	3.9	3.3
SMD	20.2	24.6	25.5
M_2D	11.3	9.8	7.6
SD_2	8.5	10.3	11.3
MD_2	9.2	8.0	5.0
Polyunsaturated	17.6	17.1	22.0

[a] The observed values are averages of analyses of eight different samples.

TABLE VII

A Comparison of Predicted and Observed Distribution of Positional Isomers of Triglycerides

Species	Isomer	Distribution: Predicted 1,3-Random 2-Random	Predicted Noncorrelative specificity	Observed
SM_2	SMM	5.3	11.6	10.0
	MSM	0.8	0.6	1.7
	MMS	5.3	1.3	2.1
SD_2	SDD	4.1	8.9	9.5
	DSD	0.3	0.3	0.6
	DDS	4.1	1.1	1.2
SMD	SMD	3.5	7.7	7.9
	SDM	6.1	13.6	12.6
	MSD	0.5	0.4	0.4
	MDS	6.1	1.5	2.6
	DSM	0.5	0.5	0.6
	DMS	3.5	0.9	1.4

little affected by large deviations from the 1,3-random hypothesis.

The difference in the two assumptions is much more apparent however, when the relative abundance of the isomers is considered (Table VII). The isomers which are mirror images of each other are present in quite different amounts. The close agreement between the calculated and observed contents gives further support to the applicability of the concept of a noncorrelative specificity to rat liver triglycerides.

The actual combination of fatty acids which are used in a given reaction is governed both by the selectivities of the acyl transferases involved and by the composition of the pool of acyl-CoA's available for transesterification. The data obtained in this study do not allow one to assess the quantitative importance of each of these factors in determining triglyceride composition. It is possible that the acyl-CoA pool is compartmentalized, i.e., one set of acyl-CoA's is available for esterification to phospholipids and a different set for acylation of the 3-position of triglycerides. Therefore it has seemed more appropriate to use the term "system" in this discussion, including both acyl transferase specificity and availability of substrates, than to attribute the observed differences in composition entirely to differences in enzyme specificity.

Finally, the fatty acid distribution data enabled the authors to calculate the relative amounts of different 1,2-diglyceride units. The known interconversions of glycerolipids in liver are summarized in Figure 3 (17). Some of the diglyceride units may be derived directly from phosphatidic acids. However, no one has yet been able to demonstrate that the specificities of the acyl transferases which form phosphatidic acid are sufficient to account for the observed isomeric compositions of the derived phospholipids (18,19). But the specifi-

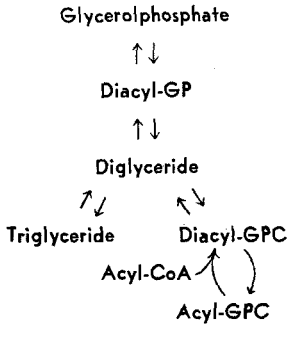

FIG. 3. Interconversions of glycerolipids in liver.

TABLE VIII

The Distribution of 1,2-Diglyceride Species in Liver Diglycerides, Triglycerides, and Phospholipids

Species	Distribution: Triglyceride	Diglyceride[a]	Lecithin[b]
00	4	5	1.8
01	24	38	11.4
11	8	12	2.0
02	28	14	28.0
12	14	5	6.5
22	3		
03			2.1
13			2.0
04	19	23	34.9
<4 double bonds		4	9.6

[a] Elovson (21).

[b] Lands and Hart (22).

cities observed for the acyl transferases which modify diacyl GPC do account adequately for the observed composition.

Bjornstad and Bremer (20) have shown that in rat liver the CDP-choline:diglyceride cholinephosphotransferase reaction is readily reversible in vivo. This result leads one to wonder to what extent the diglyceride pool is influenced by the composition of the lecithins. Table VIII shows a comparison of the distribution of 1,2-diglyceride units in triglycerides, diglycerides, and lecithins. Some species have quite similar relative contents in the three different types of glyceride. For some of the species which are not similar, the relative amount in the diglyceride pool is greater than or less than in either the triglyceride or lecithin. This may be attributable simply to the fact that the data for diglycerides were obtained in another laboratory on a different strain of animals. There is, however, the alternative possibility that both systems which put substituents on the 3-position discriminate against, or use preferentially, certain diglyceride units.

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Caltha palustris L. Seed Oil. A Source of Four Fatty Acids with *cis*-5-Unsaturation

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ABSTRACT

The seed oil of *Caltha palustris* L. yields two unusual polyunsaturated components, all-*cis*-5,11,14-eicosatrienoic acid (23%) and all-*cis*-5,11,14,17-eicosatetraenoic acid (1%). The C_{18} monoene fraction (26%) is a mixture of *cis*-5- and *cis*-9-octadecenoic acids (2:1). The C_{20} monoene fraction (12%) is a mixture of *cis*-11- and *cis*-5-isomers (3:1).

INTRODUCTION

CALTHA IS A GENUS of herbaceous plants in the family Ranunculaceae. Bailey (1) describes them as "beautiful, hardy-blooming marsh plants; though naturally bog-plants, they succeed admirably in a border in rather rich soil." *Caltha palustris* L. occurs naturally from the Carolinas to Canada and westward (1).

Previous work at this Laboratory and elsewhere has revealed some unusual features in certain seed oils of the Ranunculaceae. Fatty acids containing isolated double bonds in the 5,6-position have been found in seed oils of various species of *Thalictrum* and *Aquilegia*, all members of this family (2). Another of the Ranunculaceae, *Delphinium hybridum*, produces a seed oil containing 44% of a C_{20} fatty acid—*cis*-11-eicosenoic acid (3).

Results which were obtained in a continuing screening program at this Laboratory indicated that the fatty acid composition of *Caltha palustris* seed oil is quite unusual. This paper reports the structural determination of the major fatty acid of this oil.

EXPERIMENTAL PROCEDURES AND RESULTS

General Methods

Esterifications and transesterifications were carried out as follows, except where specified. Samples were refluxed 1 hr in a large excess of methanol containing 1% sulfuric acid (v/v). In each case, resulting mixtures were diluted with water, chilled in an ice bath, and then extracted repeatedly with petroleum ether (bp 30-60C). Combined extracts were dried over sodium sulfate and evaporated in vacuo.

Gas-liquid chromatographic (GLC) analyses of methyl esters were carried out by the method of Miwa and co-workers (4). GLC analyses of ozonolysis products were conducted as described below.

Analytical thin-layer chromatography (TLC) was performed on plates coated with 20% silver nitrate-impregnated silica, as described by Barrett et al. (5). For preparative TLC, layers 1-mm thick were used. Either benzene or benzene-ethyl ether (3:1) was used as the developing solvent. The preparative plates were impregnated with dichlorofluorescein as an internal indicator, and bands were visualized under ultraviolet (UV) light. Spots on the analytical plates were visualized by charring with sulfuric acid-chromic acid.

Infrared (IR) spectra were determined with a Perkin-Elmer Model 337 instrument, on 1% solutions in carbon tetrachloride. Nuclear magnetic resonance (NMR) spectra were obtained with a Varian HA-100 spectrometer on deuterochloroform solutions.

Permanganate-periodate oxidations were carried out by von Rudloff's method, specifically according to the modification in which *t*-butyl alcohol is used as a co-solvent (6).

Ozonolyses were performed on methyl esters essentially as described by Beroza and Bierl (7) except that an ozonizer described by Bonner (8) was used. GLC analyses of the cleavage fragments were done on both Apiezon L and LAC-2-R 446 columns with an F&M Model 402 gas chromatographic apparatus temperature which was programmed from 100 to 200C at 5C/min. GLC peaks were identified by a linear plot of retention times versus carbon-chain length. All GLC data reported are given as area percentages. Relative amounts of positional isomers were determined from these figures after computation of mole percentages of cleavage products by a variation of Onkiehong's C-factor (9,10).

Preparation of Mixed Methyl Esters

Coarsely ground seeds of *Caltha palustris* L. (16.77 g) were extracted overnight in a Soxhlet apparatus with petroleum ether (bp 30-

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

60C). Upon evaporation of the solvent 4.96 g of oil was obtained.

A 4.73-g portion of the oil was converted to a mixture of methyl esters by acid-catalyzed transesterification. According to GLC analysis, these esters had the composition indicated in Table I. Examination of these esters by TLC on silver nitrate-impregnated silica revealed distinct spots attributable to saturates, monoenes, dienes, and trienes. No *trans*-double bond absorption (10.35 μ) was observed in the IR spectrum of these esters.

TABLE I

GLC Analysis of Mixed Methyl Esters Derived from *Caltha palustris* Seed Oil
(Area percentage of Methyl ester)

Ester[a]	ECL[b] on R-446 column	Percentage
$C_{16}S$	16.06	6.5
$C_{18}S$	18.00	3.7
$C_{18}I$	18.14	22.1
$C_{18}II$	18.80	26.3
$C_{18}III$	19.58	5.2
$C_{20}S$	20.00	0.9
$C_{20}I$	20.24	11.5
$C_{20}II$	20.46	
Unknown	20.80	22.6
$C_{20}III$	20.96	
$C_{20}IV$	21.80	1.2

[a] Significance of symbols: S = saturated; I = one double bond; II = two double bonds; III = three double bonds; IV = four double bonds.

[b] ECL = equivalent chain-length (4).

Countercurrent Distribution of Methyl Esters

Countercurrent distribution (CCD) of *Caltha* mixed methyl esters was carried out with an acetonitrile-hexane system by the general procedure of Scholfield et al. (11). A 4.20-g portion of the mixed esters was divided among the first six tubes of a 200-tube Craig-Post apparatus. Ten ml of upper phase and 40 ml of lower phase were used throughout the distribution. After the 200 transfers of the fundamental series had been completed, upper phases were decanted into a fraction collector; two transfers were combined per tube. The weight distribution thus effected was as follows: Tubes 0-20, 0.08 g; 21-60, 1.63 g; 61-70, 0.19 g; 71-110, 2.05 g; 111-170, 0.32 g. Results of GLC analysis (R-446 column) of selected fractions are summarized in Table II.

Isolation of C_{20}-Trienoic Ester by Preparative TLC

Esters derived from CCD Tubes 80, 90, and 100 were examined by TLC on silver nitrate-impregnated silica plates (analytical) with the solvent system benzene-ethyl ether (3:1). Only two spots, corresponding to C_{18} diene and C_{20} triene, were revealed by the charring procedure; these were moderately well separated. Subsequently esters from CCD Tubes 86, 87, and 88, totaling 208.5 mg, were subjected to preparative TLC on silver nitrate-impregnated silica with the same solvent system. Bands of silica were removed from the plates with a Goldrick-Hirsch aspirator (12), and samples were eluted from the silica with ethyl ether. The faster-moving band yielded 94.5 mg of C_{18} diene, 98.5% pure by GLC. The slower-moving zone afforded 78.2 mg of C_{20} triene, 99.9% pure by GLC. The total weight recovery was 82.5%.

The IR and NMR spectra of the C_{20} triene were quite similar to those of methyl all-*cis*-5,9,12-octadecatrienoate (13). The similarity

TABLE II

Composition of CCD Fractions
(Results determined by GLC and expressed as percentage; some minor, unidentified components omitted)

Tube number[a]	Transfers[b]	Ester[c]						
		$C_{16}S$	$C_{18}S$	$C_{18}I$	$C_{18}II$	$C_{18}III$	$C_{20}I$	$C_{20}III$
25[d]	250		40.0				54.6	
30	260	1.9	29.8				67.2	
40	280	18.8		55.0			16.6	
50	300	15.6		69.1				
78[e]	340				38.7			47.2
80	360				50.1			47.7
90	380				48.9			49.9
100	400				49.5			49.1
150[f]	500					87.7		

[a] Numbers of tubes used to collect decanted upper phases. Two were combined in each tube.

[b] Number of transfers completed when upper phase was introduced into the tube indicated.

[c] Significance of symbols: S = saturated; I = one double bond; II = two double bonds; III = three double bonds; IV = four double bonds.

[d] This tube also contained 5.4% $C_{20}S$.

[e] This tube also contained 10.6% $C_{16}I$.

[f] This tube also contained 12.3% $C_{20}IV$.

TABLE III

Fractions Isolated by Preparative Thin-Layer Chromatography After Hydrazine Reduction of C_{20} Triene (in Order of Decreasing Rf)

Band	Weight, mg	Composition[a] (area percent of methyl ester by GLC)		ECL of major components (R-446 column)[b]
I	34.3	97%	$C_{20}S$	20.0
II	11.8	98%	$C_{20}I$	20.4
III	7.9	98%	$C_{20}I$	20.3
IV	6.1	98%	$C_{20}II$	20.8

[a] Significance of symbols: S = saturated; I = one double bond; II = two double bonds.

[b] ECL = equivalent chain-length (4).

of the IR spectra of the two esters in the 8.0-8.6 μ region was particularly striking since both have peaks at 8.0, 8.3, and 8.6 μ that differ in position and relative intensity from those observed for typical fatty methyl esters (e.g., oleate). No *trans*-double bond absorption (10.35 μ) was present in the IR. The pure C_{20} triene was cleaved oxidatively with permanganate-periodate; the resulting cleavage products were converted to methyl esters and analyzed by GLC. The major dibasic acid components (as methyl esters) were pentanedioic (11.4%) and hexanedioic (21.7%).

Partial Hydrazine Reduction of C_{20} Triene; Isolation and Characterization of Reduction Products

A 60.0-mg portion of pure C_{20} triene was dissolved in 15 ml of absolute ethanol, 0.05 ml of 64% hydrazine was added, and the mixture was purged with air and kept at 54C. After 48 hr the reaction was terminated by acidification with hydrochloric acid. The reduction products were isolated by repeated extraction with ethyl ether. Combined, dried, ether extracts were evaporated, and the residue was chromatographed on preparative silver nitrate-impregnated silica plates with benzene as the developing solvent. Separated bands were removed from the plate by means of a Goldrick-Hirsch aspirator (12). Samples eluted from the silica with ethyl ether and isolated by evaporation of solvent in vacuo are described in Table III.

Permanganate-periodate cleavage of Band II revealed the following composition of the resulting cleavage products (as methyl esters): nonanoate, 13.8%; pentadecanoate, 3.4%; undecanedioate, 27.6%; and tetradecanedioate, 48.3%. Other unidentified products were present.

Ozonolysis of Band III and GLC analysis of the products revealed the following cleavage products: C_{15} aldehyde, 56.6%; C_5 aldehydo-ester, 15.0%; and smaller amounts of other products of uncertain identity.

Hydrogenation of Mixed Methyl Esters

Esters derived from Tube 41 (39.1 mg) were hydrogenated with a platinum oxide catalyst at 1 atmosphere and ambient temperature. The hydrogenated product, isolated by filtration and evaporation, was a soft white solid. As determined by GLC, it contained the following straight-chain saturated methyl esters: C_{16} (17.0%), C_{18} (69.7%), and C_{20} (13.3%).

Oxidative Cleavage of C_{18} Monoene Concentrate

Material isolated from Tube 51 was cleaved oxidatively with permanganate-periodate. GLC analysis of the cleavage products, as methyl esters, showed: pentanedioate, 3.5%; nonanoate, 6.1%; nonanedioate, 22.9%; tridecanoate, 39.8%; and hexadecanoate, 15.1%.

A sample isolated from Tube 52 was fractionated by preparative TLC. The monoene portion was cleaved by reductive ozonolysis. GLC analysis of cleavage products revealed: C_5 aldehydo-ester, 14.8%; C_{13} aldehyde, 25.0%; C_9 aldehydo-ester, 14.3%; C_9 aldehyde, 12.9%; and other components. The calculated proportions of C_{18} monoene isomers were 65.1% Δ^5 and 34.9% Δ^9.

Oxidative Cleavage of C_{20} Monoene Concentrate

Material derived from CCD Tube 25 was cleaved oxidatively with permanganate-periodate. GLC analysis of the cleavage products, as methyl esters, indicated the following composition: nonanoate, 16.9%; undecanedioate, 26.2%; pentadecanoate, 11.4%; and octadecanoate, 44.4%.

A sample isolated from Tube 26 was cleaved by reductive ozonolysis. GLC analysis of cleavage products showed: C_5 aldehydo-ester, 5.1%; C_{11} aldehydo-ester, 37.2%; C_{15} aldehyde, 10.0%; and C_9 aldehyde, 29.4%. Other products, including some C_{18} saturated ester, were present. The calculated proportions of C_{20} monoene isomers were 25.5% Δ^5 and 74.7% Δ^{11}.

Oxidative Cleavage of C_{18} Diene-C_{20} Triene Mixture

Material isolated from CCD Tube 91 (57.0 mg) was cleaved with permanganate-periodate. GLC analysis of the cleavage products, in the form of their methyl esters, revealed the following composition: hexanoate, 2.7%; pentanedioate, 6.2%; hexanedioate, 7.1%; and nonanedioate, 75.0%.

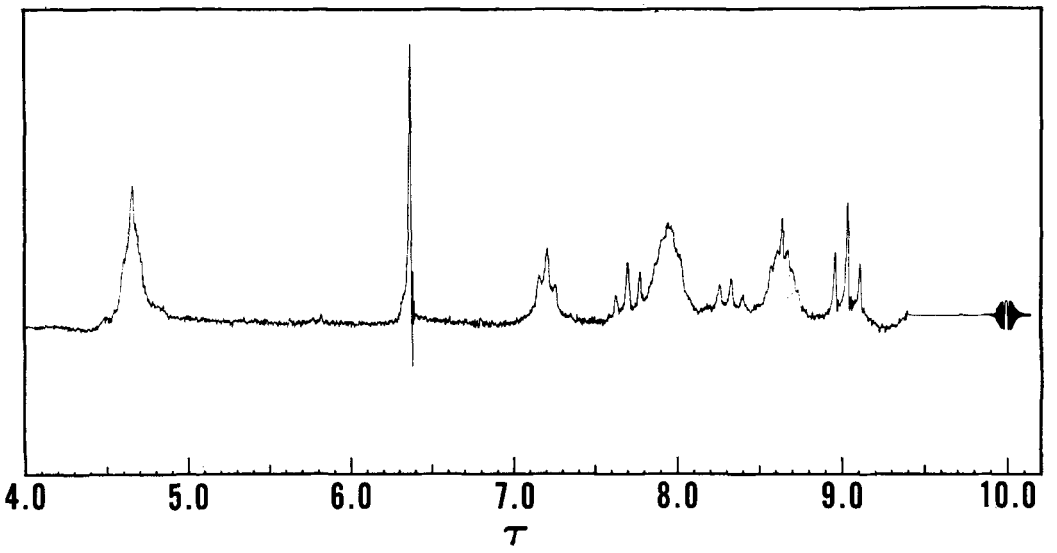

FIG. 1. NMR spectrum of methyl all-*cis*-5-11,14,15-eicosatetraenoate.

Isolation and Characterization of C_{20} Tetraene

Esters from combined CCD fractions 110-170 (189 mg) were separated into C_{18} triene and C_{20} tetraene components by preparative GLC. The separation was effected by means of an Aerograph "Auto-prep" apparatus. A 3-ft × 1/4-in. column, packed with 5% Apiezon L on 100/120 mesh silanized Chromosorb W, was used isothermally at 190C. A yield of 20 mg of tetraene was thus provided. The 34 protons of the tetraene methyl ester were distributed in the NMR spectrum (Fig. 1) as follows:

τ4.7 (multiplet), 8 vinylic protons; τ6.4 (singlet), 3 methoxyl protons; 7.2 (triplet), 4 diallylic methylene protons; τ7.75 (triplet), 2 protons α to carboxyl group; τ8.0 (multiplet), 8 protons α to double bond; τ8.3 (apparent triplet), 2 protons β to double bond and carboxyl; τ8.7 (multiplet), 4 shielded methylene protons; and τ9.1 (sharp triplet), 3 protons on methyl β to double bond. The IR spectrum showed no maximum at 10.35 μ (*trans* C = C).

The C_{20} tetraene was ozonized and analyzed, as described above (9), except that GLC analyses were conducted on products isolated at intervals of 30, 60, 120, and 210 sec after the start of ozonolysis (9). The following cleavage products were identified by GLC: C_3 aldehyde; C_6 unsaturated aldehyde; C_9 unsaturated aldehyde; C_5 aldehydo-ester; C_{11} unsaturated aldehydo-ester; and C_6 dialdehyde.

DISCUSSION

Since only straight-chain saturated acids were formed when mixed *Caltha* methyl esters were hydrogenated, it was concluded that no carbon-chain branching is associated with the fatty acids of *Caltha palustris* seed oil. Oxidative cleavage products of the C_{18} diene-C_{20} triene concentrate indicated that the C_{18} diene is conventional linoleate and that the C_{20} triene has one or more of the following distributions of double bonds: 5,8,14; 5,11,14; 6,9,14; or 6,11,14. IR spectral evidence showed that all the double bonds are *cis*.

Partial hydrazine (di-imide) reduction has been used extensively to determine double bond positions in nonconjugated polyunsaturated compounds (13-16). This procedure has been shown to reduce nonconjugated double bonds fairly randomly. Intermediate reduction products therefore yield definitive information concerning double bond positions in the starting material. Cleavage fragments derived from the two monoene reduction products (Bands II and III, Table III) formed during hydrazine reduction of the pure C_{20} triene placed the double bond at the 5-, 11-, and 14-positions. Thus the triene is *cis*-5,*cis*-11,*cis*-14-eicosatrienoic acid. This acid was found first in *Podocarpus nagi* (family Taxaceae) seed oil (14).

The C_{18} diene that is mixed with the C_{20} triene is the conventional linoleate according to the oxidation products which originate from

the C_{18} diene-C_{20} triene mixture. The material from Tubes 110-170, representing a fairly symmetrical though broad peak, is mainly C_{18} triene. However another ester is present, the ECL values (4) of which suggested a C_{20} tetraene.

The ozonolysis fragments obtained from the supposed tetraene are consistent only with a structure having double bonds in the 5-, 11-, 14-, and 17-positions of a C_{20} carbon chain. The NMR spectrum (Fig. 1 and Experimental Section) strongly supports this structural assignment. Particularly significant features of this spectrum include a sharply defined triplet at τ9.1 and a rough triplet at τ8.3. The τ9.1 triplet indicates that the terminal portion of the molecule must contain a methyl group β to a double bond, as in methyl linolenate. A terminal methyl group remote from a double bond, as in a typical fatty acid, gives rise to a distinctive NMR signal which is a rather distorted triplet (17). A τ8.3 triplet of the sort found in the C_{20} tetraene spectrum has been ascribed previously to a methylene group β to a double bond (17). On the basis of NMR spectra of Δ^5 acids and that of methyl *cis*-5,*cis*-13-docosadienoate (18,19), we believe that this triplet is associated uniquely with a methylene group β to both a double bond and a carboxyl group. Thus it is a useful criterion for the presence of Δ^5 double bonds in fatty acids. Since the IR spectrum of the C_{20} tetraene shows no *trans*-double bond (10.35 μ), its complete structure as the methyl ester of all-*cis*-5,11,14,17-eicosatetraenoic acid can be specified. The occurrence of this acid in the lipids of *Ginkgo biloba* (20) and in the seed oil of *Ephedra campylopoda* (21) has been reported. Arachidonic acid, the isomeric tetraene that is so important physiologically in the animal kingdom, has been demonstrated in plants thus far only in certain ferns and mosses (20).

Results obtained by oxidative cleavage of CCD fractions which contained the C_{18} monoenes as the predominant unsaturated components indicated the presence of both 5- and 9-isomers in a ratio of 2:1. Since the IR spectra of these esters show no *trans*-double bond absorption (10.35 μ), both isomers have the *cis*-double bond configuration. Similarly it was shown that the C_{20} monoene fraction is a mixture of *cis*-5 and *cis*-11 isomers. The 11-isomer predominates by a ratio of 3:1.

cis-5-Eicosenoic acid was first reported as a constituent of *Limnanthes douglasii* seed oil (22). This acid, as well as *cis*-5-octadecenoic acid, was identified as a constituent of *Thalictrum venulosum* seed oil (23). Since their discovery in *Limnanthes* (18,22), several diverse sources of fatty acids with isolated Δ^5 double bonds have been encountered, including various seed oils (14,18,20-25), leaf lipids of *Ginkgo* (20), slime molds (26), mosses (20), certain bacteria (27), and tall oil (28). Fatty acids of this class have been found in seed oils of a number of species in the Ranunculaceae (2), and thus it appears that isolated Δ^5-unsaturation may be a characteristic of lipids of that plant family.

When their structures were established, the C_{20} triene and the C_{18} diene esters would have been expected to overlap during CCD in the hexane-acetonitrile system. The polarity of a fatty methyl ester, as judged by its distribution coefficient in this solvent system, is affected about equally by the addition of one double bond or removal of two methylene groups (29,30).

The cleavage fragments from Bands II and III (Table III) demonstrated that the monoene reduction products from the triene were partially resolved by TLC. Band III contained mainly the Δ^5-monoene whereas Band II yielded principally the other two isomers (Δ^{11} and Δ^{14}), which migrated together on the preparative plate. This separation was not entirely unexpected since resolution of oleate and petroselinate (*cis*-6-octadecenoate) by TLC on silver nitrate-impregnated silica has been demonstrated (31). The *cis*-isomers with double bonds closer to the carboxyl groups are retarded more by complexing with Ag^+ than those with double bonds relatively farther away from the carboxyl.

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Search for New Seed Oils. XV. Oils of Boraginaceae[1]

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ABSTRACT

In a search for a preferred source of γ-linolenic (all-*cis*-6,9,12-octadecatrienoic) acid, seed oils of 33 species of Boraginaceae were examined. The desired triene was found primarily in the subfamily Boraginoideae in amounts ranging from 0.2 to 18%. Oils of this subfamily also contain 0.2 to 15% of the tetraene, all-*cis*-6,9,12,15-octadecatetraenoic acid. Total unsaturation and the relative proportions of the common acids varied widely in oils of the family. Monoene predominated in the subfamily Cordioideae, diene in Heliotropioideae, and a diverse composition among the Boraginoideae; seven had iodine values of 200 or above. *Cordia verbenacea* seed oil was unique among those examined in having 43% of C_{20} acids and 23% of components more volatile in gas chromatography than the usual triglycerides.

INTRODUCTION

MANY SEED OILS of the family Boraginaceae contain, in adition to the common fatty acids, all-*cis*-6,9,12-octadecatrienoic acid and all-*cis*-6,9,12,15-octadecatetraenoic acid (1-4). In recent years the 6,9,12-triene has become of interest as a starting material for the synthesis of all-*cis*-8,11,14-eicosatrienoic acid, which is then converted to prostaglandin E_1 by incubation with a particulate fraction of sheep vesicular glands (5). Other prostaglandins can be formed under altered conditions of incubation or with different tissues as the enzyme source (6). The 6,9,12,15-octadecatetraene may be amenable to analogous synthetic procedures.

In this paper we present data on the composition of seed and seed oils from 33 species of borages, only two of which have been reported previously (1).

MATERIALS AND METHODS

Samples were obtained, prepared, and analyzed as described in earlier papers of this series (3,7). In addition, the intact seed oils were evaluated for composition of triglycerides and accompanying constituents by gas chromatography, essentially by the method of Litchfield et al. (8).

RESULTS AND DISCUSSION

Of the borages examined, all species that provided seed oils with 1% or more 6,9,12-triene or 6,9,12,15-tetraene are in the subfamily Boraginoideae (Table I). All oils of the subfamily contain this triene (0.2 to 18%), and all but two, the tetraene (0.2 to 15%). Seed oils from *Onosmodium occidentale* and *Lithospermum purpurocaeruleum* contained the highest concentration of the 6,9,12-triene (18%); *Echium rubrum* and *Rochelia disperma* had the highest percentage of tetraene (15%). These levels of Δ^6 triene and tetraene are below the maxima reported earlier for other species (1,3,4). As sources for the 6,9,12-triene, the most desirable oils would appear to be those from *Anchusa azurea* (13%) and *Gastrocotyle hispida* (16%), as well as those of *Borago officinalis* (20%) and *Symphytum officinale* (27%) referred to previously (3), because only small amounts of linolenic acid and tetraene are present. The tetraene occurs only in the presence of larger amounts of one or both of the C_{18} trienes.

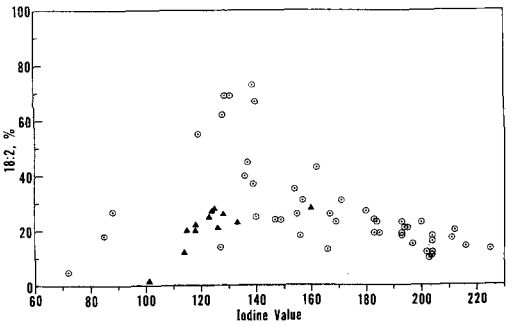

FIG. 1. Relation between iodine value and content of C_{18} diene. Triangles represent oils of the tribe Cynoglosseae. Data from Ref. 3 are included.

[1] Presented at AOCS meeting, New Orleans, May 1967. Earlier papers of this series carried the running title "Search for New Industrial Oils." The revised title is more appropriate for the basic chemical compositional data reported, that can be useful in a broader context than originally implied.

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TABLE I

Analytical Data on Boraginaceae Seeds and Oils

Source	Seed analysis: Component[a]	Wt./1,000, g	Protein N X 6.25 %, D.B.	Oil %, D.B.	Oil properties: Iodine value Wijs	Iodine value Calcd.	Refractive index n_D^{40}	Composition of methyl esters, % (area percentage by GLC): 16:0	18:0	18:1	18:2	18:3	18:3 Δ6,9,12	18:4	20:1	22:1	Other components: No.	Other components: Total, %
Cordioideae																		
Cordia salicifolia Cham.	S	67.0	28	58	85	86	1.4626	13	4	62	18	0.1	—	—	0.2	—	5	1
Cordia verbenacea DC.	S + P	24.4	19	38	72	73	1.4620	4	1	40	5	0.7	—	—	31	2	4	16[b]
Heliotropioideae																		
Heliotropium amplexicaule Vahl	S + P	4.6	17	5	—	140	—	9	4	16	66	4	0.8	—	0.2	—	2	0.5
Heliotropium curassavicum L.	S + P	1.0	9	3	—	113	—	14	4	29	50	0.8	—	—	—	—	7	2
Heliotropium strigosum Willd.	S + P	1.6	15	9	129	133	1.4677	12	3	16	69	0.3	—	—	Trace	—	4	Trace
Heliotropium supinum L.	S + P	3.3	11	14	128	131	1.4684	7	4	26	62	0.2	—	—	0.2	—	3	0.1
Boraginoideae																		
Cynoglosseae																		
Cynoglossum creticum Mill.	S–SC	12.8	25	42	101	101	1.4645	5	0.9	63	2	11	0.2	0.8	6	12	4	0.5
Cynoglossum lanceolatum Forsk.	S + P	0.8	18	25	118	116	1.4666	11	4	39	20	2	13	0.8	3	4	4	2
Lindelofia anchusoides (Lindl.) Lehm.	S + P	9.8	23	23	126	129	1.4671	9	2	42	21	7	9	3	4	3	6	1
Mattiastrum cristatum (Schreb.) Brand	S	15.6	36	26	118	116	1.4667	6	1	48	22	5	4	1	5	8	6	0.2
Rindera lanata (Lam.) Bunge	S	20.0	35	27	115	121	1.4665	6	2	48	18	8	4	3	5	6	1	0.3
Rindera umbellata (W.K.) Bunge	S	20.7	41	21	124	123	1.4676	7	2	38	27	6	8	0.2	5	6	8	0.4
Trichodesma indicum R.Br.	S + P	12.3	19	26	160	155	1.4708	8	4	26	28	29	2	1	0.7	—	3	0.9
Eritrichieae																		
Amsinckia tessellata Gray	S + P	5.4	24	26	156	157	1.4705	12	3	29	18	15	10	9	1	0.4	3	0.6
Cryptantha barbigera (Gray) Greene	S	0.04	32	33	183	176	1.4735	9	3	19	24	36	4	4	0.1	—	5	2
Gastrocotyle hispida Bunge	S + P	4.3	16	28	136	139	1.4678	10	2	28	40	1	16	—	2	2	2	Trace
Hackelia jessicae (McGreg.) Brand	S	1.1	—	25	—	147	—	12	3	28	21	11	9	8	4	2	6	2
Lappula barbatum (Bieb.) Guerke	S + P	2.0	19	23	202	202	1.4758	7	2	19	12	40	4	14	2	0.1	2	0.1
Anchuseae																		
Anchusa angustifolia L.	S + P	5.6	18	16	147	138	1.4706	10	2	32	24	13	11	3	2	2	4	0.6
Anchusa azurea Mill.	S + P	16.4	16	21	137	137	1.4684	9	2	24	45	Trace	13	—	3	4	3	Trace
Anchusa leptophylla Roem. & Schult.	S + P	7.1	17	21	140	143	1.4682	10	2	31	25	9	14	3	3	2	4	1
Anchusa officinalis L.	S	2.8	19	23	167	168	1.4725	8	3	22	26	20	11	5	2	1	5	0.5
Nonnea pulla DC.	S + P	8.3	21	34	154	153	1.4690	8	3	23	35	11	12	2	3	0.7	3	0.4
Lithospermeae																		
Arnebia griffithii Boiss.	S + P	2.5	10	15	193	191	1.4744	7	3	14	23	45	3	4	—	—	3	0.6
Lithospermum arvense L.	S + P	9.2	13	17	216	216	1.4774	5	2	11	14	43	14	10	1	—	2	0.5
Lithospermum purpurocaeruleum L.	S + P	16.6	14	14	200	202	1.4755	7	3	10	23	31	18	7	0.3	—	2	0.2
Onosma auriculatum Auch.	S + P	16.0	21	20	169	169	1.4719	7	3	28	23	29	6	4	0.2	—	—	—
Onosma cinerea Schreb.	S + P	14.6	24	29	180	184	1.4730	8	2	19	27	25	12	7	Trace	—	1	Trace
Onosmodium occidentale Mackenz.	S–SC	18.5	12	17	183	185	1.4743	7	3	21	19	26	18	6	0.8	—	2	Trace
Echieae																		
Echium rubrum Jacq.	S + P	1.5	18	15	212	217	1.4775	8	2	8	20	34	14	15	0.2	—	3	Trace
Echium vulgare L.	S + P	4.6	14	22	204	202	1.4758	4	3	16	18	37	11	10	0.8	—	—	—
Harpagonelleae																		
Rochelia disperma (L.) Wettst.	S	2.8	14	18	203	205	1.4766	6	3	17	10	39	5	15	3	0.7	3	0.6
Rochelia stylaris Boiss.	S + P	3.8	16	21	204	205	1.4760	6	2	18	12	40	5	14	2	0.4	1	Trace

[a] S = Seed; S + P = seed plus pericarp; S-SC = seed minus seed coat.

[b] Includes 12% of 20:0.

This sample of *Anchusa azurea* is distinctly different from that reported by Craig and Bhatty (1). It contained essentially no linolenic acid and C_{18} tetraene whereas their sample contained both.

For most of the borage family the interrelationship of oleic, linoleic, and linolenic acids is similar to those found in the mint family (9). The tribe Cynoglosseae deviates from the pattern in that its species, except for one, produce oils that contain less linoleic acid than expected from the iodine value (Fig. 1) and more C_{20} and C_{22} monoenoic acids.

The Δ^6 triene is found in most of the oils with iodine value above 110; the largest amounts of tetraene occur in oils with iodine values over 200. The quantities of these Δ^6 compounds are not obviously related to the percentages of any single common unsaturated acid.

Agreement between Wijs IV and those calculated from the GLC results (most differences range from −3 to +5) indicates that the degree of unsaturation of all significant components is properly assigned by their GLC characteristics, that the area percentage is essentially the same as weight percentage, and that there is no significant loss of polyunsaturated acids in the GLC column.

Titration of the oils with hydrogen bromide, as in the method for oxirane oxygen, showed less than 2% of reactive materials (calculated as epoxyoleic acid) except for *Heliotropium curassavicum*, which contained 5%. The reactive component(s) was not identified. Infrared absorption of thin films of the oil indicated no unusual constituents.

Direct GLC of the borage oils reveals that most contain primarily the expected C_{52}-C_{56} triglycerides (8). However many include small amounts of materials that have retention times similar to those of acetotriglycerides (usually C_{36}-C_{38} triglycerides) and of esters of pentacyclic triterpene alcohols (equivalent to C_{24}-C_{50} triglycerides) (10). Seed oil of *Cordia verbenacea* contains the largest amount encountered, about 23%, in several components with elution times similar to those of C_{38}-C_{44} triglycerides. This oil is also unique in the borage family because of its high proportion of C_{20} acids, 31% of C_{20} monoene and 12% of arachidic acid among the total fatty acids.

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The Occurrence and Biosynthesis of gamma-Linolenic Acid in a Blue-Green Alga, *Spirulina platensis*

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ABSTRACT

The acyl-lipid and fatty acid composition of six blue-green algae, namely, *Spirulina platensis, Myxosarcina chroococcoides, Chlorogloea fritschii, Anabaena cylindrica, Anabaena flos-aquae, and Mastigocladus laminosus* is reported.

All contain major proportions of mono- and digalactosyl diglyceride, sulfoquinovosyl diglyceride, and phosphatidyl glycerol, but none possess lecithin, phophatidyl ethanolamine, or phosphatidyl inositol. Trans-3-hexadecenoic acid was absent from all extracts.

The analyses provide further evidence that there is no general chemical or physical requirement for any specific fatty acid in photosynthesis.

S. platensis is unique among photoautotrophic organisms so far studied, containing major quantities of γ-linolenic acid (6,9,12-octadecatrienoic acid). This acid is synthesized by the alga by direct desaturation of linoleic acid and is primarily located in the mono- and digalactosyl diglyceride fractions.

The possible phylogenetic relationship between *S. platensis* and other plant forms is discussed.

INTRODUCTION

OF NATURALLY OCCURRING octadecatrienoic acids, the two most widely distributed varieties are α-linolenic acid (9,12,15-octadecatrienoic acid) and γ-linolenic acid (6,9,12-octadecatrienoic acid).

α-Linolenic acid is not synthesized by animals but is characteristic of higher plants and many algae, often accumulating in particularly high proportions in the photosynthesizing lamellae (1). Yet γ-linolenic acid is more typical of "animal" metabolism (2) and is synthesized by vertebrates (3), ciliated protozoa (4), and several classes of zoo-flagellates (5, 6). Some classes of fungi (e.g., the phycomycetes) synthesize γ-linolenic acid whereas others (ascomycetes and basidiomycetes) contain α-linolenic acid (7). There is no known fungal species which synthesizes both acids. γ-Linolenic acid is also present in some plant tissues, namely, mosses (8) in relatively small amounts, certain oil seeds (9, 10) and in phytomonads and chrysomonads (11).

In this paper are described studies on the cellular lipid composition and metabolism of a blue-green alga, *S. platensis*. This alga is unique in containing substantial quantities of γ-linolenic acid, which apparently plays the role normally fulfilled in other algae by α-linolenic acid.

The fatty acid and acyl lipid composition of five other species of blue-green algae is also reported.

MATERIALS AND METHODS

Algae

S. Platensis, Strain No. 1475/4 from the Cambridge Collection of Algae and Protozoa, was cultured in the light in an inorganic medium one liter of which contained NaCl (5 g) KCl (5 g), Na_2CO_3 (8 g), KNO_3 (1 g), $MgSO_4$ (0.25 g), K_2HPO_4 (0.1 g), Na citrate (0.2 g) "Metals 45" (0.02 g) (12), vitamin B_{12} (4.0 μg), and soil extract (100 cc.) This last was prepared from an alkaline (pH 7.5) garden loam. During growth the culture vessel was gently agitated and aspirated with 5% CO_2 in air. Microscopic examination of the cells used in this work showed only slight contamination by bacteria.

C. fritschii, A. cylindrica, A. flos-aquae, and *M. laminosus* were cultured on the standard medium for nitrogen-fixing algae (13) except that *C. fritschii* was grown in the absence of additional CO_2, and the pH of the medium for *M. laminosus* was maintained above 8.5.

^{14}C-Labeled Metabolites

2-^{14}C-sodium acetate and 1-^{14}C-linoleic acid were obtained from the Radiochemical Centre, Amersham. The linoleic acid was checked for chemical and radiochemical purity by radiochemical gas-liquid chromatography (radio-GLC). The positional specificity of the label in this sample was confirmed by radio-GLC

analysis of the products of chemical α-oxidation of the reduced methyl ester (14).

Incubation of S. platensis with ^{14}C-Labeled Metabolites

Cultures of *S. platensis* were harvested by centrifugation, and the cells were shaken with a small volume of the culture medium to give a thick suspension. Five μc of either of the labeled metabolites were added to separate 100-cc portions of this suspension, and the mixtures were then incubated aerobically in the light (4 × 40W white strip lamps at 8 in.) for the desired period. After incubation the suspensions were centrifuged and the residues washed with distilled water; after recentrifugation the cells were extracted and analyzed, as described below.

Extraction and Fractionation of Acyl Lipids

Algal pellets, obtained by centrifugation of the culture suspensions, were suspended in about 300 volumes of chloroform-methanol (2:1 v/v), and the mixture was allowed to stand at room temperature for about 3 hr. The suspension was filtered, the residue was re-extracted in a similar fashion; the combined filtrates were concentrated almost to dryness under reduced pressure at 45-50C. The concentrate was redissolved in chloroform-methanol (2:1 v/v), and water-soluble impurities were removed by the method of Folch et al. (15). Lipid analyses were obtained by thin-layer chromatography (TLC) on silicic acid and by column chromatography on DEAE cellulose, as described elsewhere (16).

Fatty Acids

Fatty acid methyl esters were obtained by refluxing the relevant lipid fractions with a mixture of methanol, benzene, and concentrated sulfuric acid (20:10:1 v/v) for 90 min. After dilution with water the reaction mixtures were extracted with hexane, and the extracts were dried over anhydrous sodium sulfate. The methyl esters were analyzed on a Pye 104 gas chromatograph connected to a flame ionization detector, using either polyethylene glycol adipate (PEGA) or silicone SE-30[1] as stationary phase.

Location of Double Bonds

After isolation of pure methyl esters by a combination of argentation-TLC and preparative GLC (17), the polyenoic acids were partially reduced to a mixture of monoenoic and saturated acids with hydrazine (18). The location of double bonds in the monoene mixture was established by the method of Davidoff and Korn (19).

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RESULTS

TLC on silica gel and column chromatography on DEAE cellulose showed that major acyl lipids of *S. platensis, M. chroococcoides, C. fritschii, A. cylindrica, A. flos-aquae,* and *M. laminosus* were mono-galactosyl diglyceride (MGDG), digalactosyl diglyceride (DGDG), sulfoquinovosyl diglyceride (SQDG), and phosphatidyl glycerol (PG). Phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol were not detected.

Fatty acid analyses of the total lipids extracted from the algae are given in Table I. *Trans*-3-hexadecenoic acid could not be detected in any of the extracts even when the respective phosphatidyl glycerol fractions were isolated and analyzed.

The Octadecatrienoic Acids of S. platensis

The methyl ester of the major C_{18} acid from *S. platensis* had retention volumes on PEGA and SE-30 identical with that of γ-linolenate methyl ester. When mixed with a sample of authentic γ-linolenic acid methyl ester obtained from the seeds of the Evening Primrose *(Oenothera biennis),* the two esters were inseparable by GLC on either stationary phase. Perfectly gaussian peaks with no broadening were obtained.

Oxidation of the unknown ester and of the products of its partial reduction with hydrazine indicated unsaturation at the 6, 9, and 12 positions, confirming that the original compound was 6,9,12-octadecatrienoic acid.

A combination of argentation-TLC and GLC on PEGA columns showed that both the neutral glyceride and monogalactosyl diglyceride fractions contained a small quantity of a C_{18} trienoic acid which co-chromatographed with α-linolenic acid on PEGA columns. Insufficient material was available to confirm the location of the double bonds in this fraction.

When *S. platensis* was incubated with 1-^{14}C-linoleic acid, rapid conversion of the substrate to γ-linolenic acid occurred; 25% of the linoleic acid was desaturated in 3 hr. Partial α-oxidation (20) of the product from this conversion showed that all of the label had been retained in the 1-position, indicating that the major mechanism involved is one of direct desaturation rather than a general breakdown of the precursor, followed by synthesis of γ-linolenic acid from the breakdown products.

TABLE I

Percentage Fatty Acid Composition of Some Blue-Green Algae

	Fatty acid							
	16:0	16:1	16:2	18:0	18:1	18:2	9,12,15–18:3	6,9,12–18:3
Spirulina platensis	43.4	9.7	t	2.9	5.0	12.4	t	21.4
Myxosarcina chroococcoides	38.2	8.6	1.2	4.0	6.8	9.2	33.3	—
Chlorogloea fritschii	42.3	4.9	t	5.4	14.3	17.2	15.8	—
Anabaena cylindrica	46.0	6.4	5.6	3.6	6.0	24.0	11.2	—
Anabaena flos-aquae	39.5	5.5	4.3	1.0	5.2	36.5	10.7	—
Mastigocladus laminosus	38.5	42.5	—	t	16.8	2.1	—	—
Anacystis nidulans[a]	47.0	38.8	—	1.4	10.0	—	—	—

[a] Reproduced from Holton *et al.* (33)

In 3 hr no chain elongation of the linoleic acid to C_{20} acids occurred.

The fatty acid composition of the individual acyl lipid classes of *S. platensis* is given in Table II. C_{20} acids containing more than three double bonds were not detectable in any fraction.

DISCUSSION

The acyl lipid compositions of the six blue-green algae were similar to those previously reported for two other blue-green algae (21) in that the major components were the four lipids generally associated with chloroplast lamellae, viz., MGDG, DGDG, SQDG, and PG. Blue-green algae do not possess such subcellular structures as mitochondria and nuclei, which are present in plant leaves and higher algae, and this probably explains the absence of lecithin, phosphatidyl ethanolamine, and phosphatidyl inositol from the more primitive organisms.

Spirulina platensis differs from all other photosynthetic tissues, so far studied, in containing a high proportion of γ-linolenic acid, and this acid appears to have a function analogous with that fulfilled by α-linolenic acid in many other algae and in the leaves of higher plants. For example, it is primarily located in the galactosyl diglycerides; a slightly greater proportion is present in the monogalactoside than in the digalactoside. It appears in the two other lamellae lipids in much lower concentrations (Table II). The location of γ-linolenic acid in the galactosyl diglycerides in such high proportions suggests that the acid is largely confined to the photo-

TABLE II

Percentage Fatty Acid Composition of the Major Acyl Lipids from *Spirulina platensis*

Fatty acid	R_V relative to 16:0 on PEGA	Lipid fraction					
		Total lipid	Neutral lipid[a]	MGDG	DGDG	SQDG	PG
16:0	1.00	45.6	29.7	45.0	42.8	48.8	45.2
16:1	1.14	10.5	17.8	1.9	9.4	3.0	4.0
?	1.38	t	2.3	t	t	—	t
?	1.56	t	4.1	—	t	—	t
18:0	1.94	2.9	9.3	3.1	2.0	2.0	2.0
18:1	2.17	5.0	13.2	1.3	7.9	4.5	9.4
18:2	2.66	13.4	11.7	5.6	4.8	39.6	37.8
18:3 (6, 9, 12)	3.06	21.5	6.4	42.9	33.7	1.6	2.1
18:3 (9, 12, 15)?	3.50	t	5.1	4.9	—	—	—
20:2	5.06	t	—	t	t	t	t
20:3	5.77	t	t	t	t	t	t

[a] A mixture of neutral glycerides, mainly triglyceride.

Scheme I

$$18:0 \rightarrow 18:1[9] \rightarrow 18:2[9,12] \rightarrow 18:3[6,9,12] \rightarrow 20:3[8,11,14] \rightarrow 20:4[5,8,11,14]$$
$$\downarrow$$
$$18:3[9,12,15]$$

Scheme II

$$18:2[9,12] \rightarrow 20:2[11,14] \rightarrow 20:3[8,11,14] \rightarrow 20:4[5,8,11,14]$$

synthesizing lamellar structures typical of the blue-green algae. Its synthesis by direct desaturation of linoleic acid also parallels that of α-linolenic acid in other photosynthetic tissues.

Despite these analogies however it must be remembered that γ-linolenic acid possesses a very different molecular shape from that of α-linolenic acid, and a consideration of data (Table I) now available for numerous blue-green algae (22) emphasizes the wide variety of fatty acid compositions which occurs among the different members of this group of organisms.

Thus within the blue-green algae, at least, there appears to be no general physical or chemical requirement for any particular type of fatty acid structure for normal photosynthetic function if it is assumed that the ubiquity of palmitate is an uncritical one. The absence of *trans*-3-hexadecenoic acid from all the blue-green algae appears to be another aspect in which this class of organism differs from other algae and the photosynthetic tissue of higher plants.

The presence of γ-linolenic acid in photoautotrophically cultured cells of *S. platensis* may be of considerable phylogenetic significance. Erwin and Bloch (23) studied the lipids of several ciliated protozoa and observed that their over-all fatty acid compositions resembled those of the few blue-green algae which had been studied up to that time except that the octadecatrienoic acid of the former group was γ-linolenic whereas that in the latter was α-linolenic. From the point of view of the total cellular fatty acid composition therefore, *S. platensis* resembles some of the ciliates particularly closely. However such resemblances are not reflected in the acyl lipid composition of the cells. The major lipids of the ciliates studied by Erwin and Bloch were triglyceride, phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl serine. *S. platensis* contains none of these lipids.

Similar arguments apply to comparisons between *S. platensis* and the fungi. In over-all fatty acid composition the alga closely resembles some of the phycomycetes (7), but the acyl lipids present in the two organisms are entirely dissimilar.

It seems more likely that *S. platensis* is related to the marine algae (which it resembles in preferring a growth medium of high salt content), diatoms, mosses, and ferns. These latter organisms contain the acyl lipid classes common to blue-green algae, and many of them contain small quantities of γ-linolenic acid (8,24) in addition to arachidonic acid, which is present in most of them. In these tissues it seems likely that γ-linolenic acid is an intermediate in the biosynthetic sequence leading to the C_{20} acid according to Scheme I. Nevertheless lack of experimental data demands that one cannot rule out the route established by Korn (25), Stoffel (26), and Hulanicka *et al.* (27) for soil amoeba, rat liver, and *Euglena* respectively, as in Scheme II. No evidence was obtained for the existence of any part of this pathway in *S. platensis.*

If the former, more common pathway is assumed, it is only necessary to propose the development of enzymes for the chain elongation of γ-linolenic acid, followed by further desaturation to arachidonate, to support the concept that some of the higher algae which contain C_{20} polyunsaturated acids are evolved from this class of blue-green alga. A sequence consistent with other biochemical and morphological data (28) would be:

red algae
↑
(some) blue-green algae→green and brown marine algae→mosses, ferns

In those cases where sufficiently detailed study has been made of the fatty acid distribution within the different cellular components of these higher algae and mosses, arachidonic and related acids have been located in the chloroplast or chloroplast lipids (29, 30), particularly the galactosyl diglycerides (30). This represents another point of resemblance to *S. platensis* in which γ-linolenic acid is specifically located in the galactosyl diglycerides. In this respect *S. platensis* and the marine algae differ from *Euglena gracilis.*

In the latter organism, little or no arachidonic acid is found in the plastid galactosyl diglyceride fractions (31,32), and the synthesis of this acid by *Euglena* appears to result from the animal-like metabolism associated with its heterotrophic growth rather than the photosynthetic metabolism which is operative during photo-autotrophic growth (31).

Thus from the point of view of lipid and fatty acid metabolism, *Spirulina platensis* would appear to resemble the marine algae more closely than *Euglena*, ciliated protozoa, or fungi.

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Metabolism of Alkyl Glyceryl Ethers in the Rat

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ABSTRACT

The metabolism of ^{14}C- and ^{3}H-labeled alkyl glyceryl ethers after intraperitoneal injections was examined in the liver and intestine of the rat. Additionally, in vitro experiments were conducted with intestinal homogenates and intestinal contents.

From these investigations it was concluded that the liver and the intestine metabolize the alkyl glyceryl ethers very differently. Intestinal contents can alter α-batyl alcohol, as indicated by preliminary experiments, and intestinal cells contain enzyme systems which convert the alkyl glyceryl ethers to the mono- and di-acyl derivatives. Very little esterified glyceryl ethers were found in the liver lipids. The intestine contains an enzyme system which, although it has a greater specificity for chain length and for isomeric position of the ether than that of the liver system, does cleave the glyceryl ethers.

From in vivo studies of intestinal tissue it was concluded that all of the injected glyceryl ethers were converted intact to the ethanolamine, serine, and choline alkyl glyceryl ether phospholipids; with the use of α-batyl alcohol, the phosphatidyl ethanolamine fraction contained most of the labeled glyceryl ether phospholipid; with β-batyl alcohol, α-chimyl, and β-chimyl alcohols, the phosphatidyl choline fraction contained most of the labeled alkyl glyceryl ether phospholipid. No significant amount ($< 1\%$) of labeled alkyl glyceryl ether phospholipids was found in any of the rat-liver lipids.

INTRODUCTION

THE ORIGIN AND METABOLIC significance of the alkyl glyceryl ethers in nature are unknown. Thompson and Hanahan (1), by using bone marrow, found that the radioactive label from 6-^{14}C-glucose 1-^{14}C-sodium palmitate and tritiated water was incorporated into the glyceryl ethers of the ethanolamine and choline phospholipids. The major part of the radioactivity from glucose was located in the α-prime carbon of the glycerol moiety of the ethers, suggesting that α-glycerophosphate may be a direct precursor. Gilbertson et al. (2) reported that rat epididymal fat pads catalyzed the incorporation of radioactive acetate and palmitate into alkyl and alk-1-enyl (plasmalogens) glyceryl ethers. Ansell and Spanner (3), using ^{32}P-orthophosphate, and McMurray (4,5), using cytidine diphosphate-^{14}C-ethanolamine, have shown that glyceryl ether phospholipids are formed in developing rat brain.

Early investigators (6-8) reported from in vivo experiments that the ether bond of chimyl alcohol was cleaved and that the cetyl alcohol moiety was oxidized to palmitic acid. The exact mechanism for this cleavage of the ether linkage is unknown at the present time. Tietz et al. (9) investigated a pteridine-requiring enzyme system in rat liver that oxidized batyl alcohol to the fatty acid and glycerol. They postulated that the ether was converted to a hemi-acetal, which then spontaneously gave rise to the long-chain aldehyde. Recently Snyder and Pfleger (10) reported that the liver was apparently the primary site of glyceryl ether catabolism; the major products of the ether cleavage were long-chain fatty alcohols and fatty acids.

The purpose of the present investigation is to study the metabolism of labeled alkyl glyceryl ethers which have been introduced into the animal by intraperitoneal injections. This route of injection would minimize direct contact of the ethers with the intestinal flora. In addition, the metabolic alterations of glyceryl ethers by in vitro systems which contain intestinal homogenates and intestinal contents are investigated.

METHODS

Preparation of Labeled Glyceryl Ethers

Labeled glyceryl ethers (alkyl glyceryl ethers in which there is no double bond in the *alpha-beta* position adjacent to the ether linkage) were prepared as described previously (11).

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The radiopurity of the labeled ethers was greater than 99%, as determined by thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) (10,11).

Solubilization of Labeled Palmitic Acid and Glyceryl Ethers

Though glyceryl ethers are readily solubilized by MIJR 52 (a polyoxyethylene stearate, Atlas Powder Company, Wilmington, Del.), it was found advantageous to use sodium taurocholate (Pfanstiehl Chemical Company, Waukegan, Ill.) as a solubilizing agent. Sodium taurocholate did not exhibit pathological or inhibitory effects whereas MIJR 52 inhibited the in vitro incorporation of labeled palmitate into phospholipids in a manner similar to the Tweens (12,13).

Sodium Taurocholate Solution of Palmitic Acid. To a chloroform solution containing 2.6 mg of 1-^{14}C-palmitic acid (0.10 mc) 0.01 ml of 5% NaOH was added. The chloroform was carefully removed by heating, and the sodium salt was dissolved with warming in 5 ml of sodium taurocholate solution (10 mg/ml). A ratio of fatty acid to bile salt of 1:5 (w/w) is needed to produce solubilization. The solution was maintained at 37C until the time of injection.

Sodium Taurocholate Solution of Glyceryl Ethers. The labeled glyceryl ether, in a small amount of chloroform, was mixed with an equal amount (w/w) of sodium taurocholate solution (10 mg/ml). The 1:1 ratio of glyceryl ether to bile acid produced a stable solution. Chloroform was removed by heating, and the solution was maintained at 37C until the time of injection.

Amount of Labeled Material Administered Intraperitoneally

In the in vivo studies 250 μg (20 μc) of 1-^{14}C palmitic acid were injected.

For the time studies, with α-1-^{14}C-batyl alcohol, 7.24 mg of the glyceryl ether (15.52 μc) were injected per animal; with β-1-^{14}C-batyl alcohol, 10.37 mg of the glyceryl ether (22.30 μc) were injected; with 2-^{3}H-α-1-^{14}C-α-1-^{14}C-2-^{3}H-chimyl alcohol, 5.60 mg of glyceryl ether were injected (25.45 μc of ^{3}H and 23.69 μc of ^{14}C); and with β-1-^{14}C-chimyl alcohol, 6.30 mg (22.65 μc) were injected.

Design of Metabolic Experiments

In Vivo. Forty male albino Sprague-Dawley rats (200-300 g) were used. Rats were anesthetized lightly with ether, and 1.00 ml of the sodium taurocholate solution, containing the labeled material, was injected intraperitoneally. After the desired period of time the animals were anesthetized and decapitated. The entire liver and small intestine were removed, and the lipids were extracted according to the procedure of Galanos and Kapoulas (14).

In Vitro—Intestinal Contents. Contents of the entire small intestine of a rat were removed and dispersed in standard fluid thioglycolate culture medium (Baltimore Biological Laboratory, Baltimore, Md.). Duplicate 5-ml aliquots, with and without intestinal contents, were transferred to screw-cap tubes. To all tubes 0.10 ml of sodium taurocholate solution, containing 120 μg of α-1-^{14}C-batyl alcohol (2.9 μc), was added. The tubes were then incubated at 37C for 20 hr. The lipids were extracted according to the procedure of Bligh and Dyer (15), and aliquots of the lipid extract were analyzed by TLC as described.

Intestinal Homogenates. The entire small intestine of the rat was removed, washed thoroughly with cold water to remove the contents, blotted dry, and weighed. The tissue was then homogenized in 0.25 M sucrose by using a Potter-Elvehjem homogenizer with a motor-driven pestle. The homogenate was filtered through cheesecloth. The volume was such that 1 ml of filtrate was derived from 200 mg of tissue. Each incubation tube contained 0.40 ml of 0.25 M Tris buffer, pH 7.4; 1.00 ml of 1:5 (w/v) rat intestinal homogenate; and water to a final volume of 2.00 ml.

After equilibration 0.10 ml of sodium taurocholate solution, containing 40 μg of α-1-^{14}C-batyl alcohol (0.10 μc), was added. The tubes were incubated at 37C for the desired time. The reaction was stopped by the addition of 4.0 ml of chloroform-methanol (2:1 v/v), and the lipids were extracted (14).

Deacylation with Sodium Methoxide

Aliquots of lipids were deacylated with sodium methoxide according to the procedure of Marinetti (21). The percentage of conversion, determined by using rat liver lipids labeled with palmitate and by using synthetic labeled glyceryl ether mono- and di-esters (11), was 100% in 20 min as reported (21).

Acetolysis of Phospholipids

The procedure of Thompson (22) for the acetolysis of phospholipids was modified by using sodium methoxide as the final deacylating agent. After acetolysis and deacylation, aliquots of the material were chromatographed by TLC.

The percentage of conversion of the acetolysis reaction was 88 ± 8%. Since there was a large degree of variation in this method, the acetolysis procedure was used only for fractions that contained large quantities of alkaline stable phospholipids.

Thin-Layer Chromatography

For neutral lipid separations, 5 × 20-cm plates of 500 μ thickness were prepared with Silica Gel G and activated at 110C for 30 min. Basic Silica Gel H plates of 500 μ thickness, prepared from a slurry of Silica Gel H and 0.001 M Na_2CO_3, were used for all phospholipid separations.

Approximately 250-350 μg of labeled total lipids with the appropriate carrier and standard mixtures were applied to the absorbant, and the plates were developed in the desired solvent system.

Routinely three solvent systems (A, B, and C) were used for the TLC separation of natural lipid mixtures.

By using hexane-diethyl ether- acetic acid (90:10:1 v/v/v) (16), free fatty acids, triglycerides, glyceryl ether diesters plus aldehydes, and cholesterol esters were fractionated. With this same system, after the lipid mixture had been treated with sodium methoxide, free fatty acids and total esterified fatty acids (as methyl esters) were determined.

By using a solvent system devised by the authors' laboratory consisting of hexane-diethyl ether- methanol- acetic acid (85:20:3:3 v/v/v/v), total phospholipids, monoglycerides, free glyceryl ethers, cholesterol plus alcohols and diglycerides, free fatty acids plus glyceryl ether monoesters, free aldehydes, triglycerides plus glyceryl ether diesters, and cholesterol esters were fractionated. This same system, used after sodium methoxide hydrolysis, permitted the separation of the total alkaline stable phospholipids, glyceryl ethers, cholesterol plus alcohols, free fatty acids, free aldehydes, and total esterified fatty acids (as methyl esters).

By use of the modified solvent system of Skipski et al. (17), reported by Snyder et al. (18), chloroform-methanol-acetic acid- 0.01 M NaCl (50:25:8:4 v/v/v/v) separation of the lysolecithin, sphingomyelin, phosphatidyl choline, phosphatidyl serine, and phosphatidyl ethanolamine fractions was obtained.

After the plates had air dried and had been sprayed with 0.2% 2,7-dichlorofluorescein in 95% ethanol, the lipid spots were visualized with ultraviolet light. The corresponding spots from two labeled lanes were then scraped into a scintillation vial for counting. The recovery of label from the TLC plates was 91.9 ± 4.6%.

Counting of TLC Fractions

To each scintillation vial containing Silica Gel and labeled material from the TLC separations, 10 ml of BBOT scintillation solution (19) were added. The samples were counted in a Packard Tricarb Model 314 EX liquid scintillation spectrophotometer. The ^{14}C counting efficiency was 67-69%. The simultaneous counting efficiency of ^{3}H and ^{14}C was 9% and 64% respectively (20).

For simplicity the data which were obtained from all metabolic experiments are expressed as mean values of analyses in duplicate or triplicate. The maximum standard deviation from the reported mean value was ± 9% of the mean.

RESULTS AND DISCUSSION

In Vitro Glyceryl Ether Metabolism

Most in vivo investigations with labeled glyceryl ethers have been concerned with metabolism after oral administration (6,7,8). Snyder et al. (23) reported that a significant cleavage

TABLE I

Alteration of α-1-^{14}C Batyl Alcohol by Rat Intestinal Contents (After 20 Hours of Incubation at 37C)

Incubation conditions	Distribution of ^{14}C-label (% of total lipid label)						
	Phospho-lipids	Mono-glycerides	Glyceryl ethers	Diglycerides + Alcohols + Cholesterol	Free fatty acids + Glyceryl ether monoesters	Free aldehydes	Solvent front + Glyceryl ether diesters + Triglycerides + Cholesterol esters
Without intestinal contents	0.86	0.91	95.45	0.70	0.48	0.07	1.55
With intestinal contents	3.80	1.51	79.52	5.22	6.43	0.70	2.83

of the ether bond occurred in the intestinal cells and contents. Large quantities of label found in the intestinal contents were not associated with the ether linkage but with catabolic products of the ether. Earlier Brohult (24) had found that glyceryl ethers promoted the growth of *Lactobacillus lactis*. Therefore it would appear that enzyme systems which may have their origin either in bacteria or in intestinal cells may catabolize glyceryl ethers.

In order to study the capability of intestinal contents to alter α-batyl alcohol, the preliminary experiment described first in the in vitro section on methods was conducted. The results of this experiment are given in Table I. It is apparent from the comparison of the glyceryl ether values, obtained with and without intestinal contents, that as much as 15% of the labeled glyceryl ether was altered by the intestinal contents during the period of incubation.

At least 10% of the label was found as esterified fatty acid and distributed in the phospholipids, diglycerides plus alcohols, and in free fatty acids plus glyceryl ether monoesters. These findings indicate that rat intestinal contents are capable of cleaving the ether bond. It is concluded that enzymes, either of bacterial or intestinal origin, which are present in the intestinal contents, can alter alkyl glyceryl ethers.

An additional set of in vitro experiments were carried out to determine if intestinal cells would cleave α-1-^{14}C-batyl alcohol. Rat intestinal homogenates were incubated with the labeled glyceryl ether, and the distribution of radioactive label was examined. No significant cleavage of the ether linkage was obtained after 1½ hr of incubation; in other words, no significant amount of labeled free fatty acid, alcohol, or aldehyde was found. In 90 min, 8% of the glyceryl ether was esterified with endogenous fatty acids to form the glyceryl ether monoesters. These results are similar to those reported by Sherr and Treadwell (25) for incubations of everted rat intestine with β-batyl alcohol. The major product corresponded to an acylated derivative which was equivalent to a diglyceride, and no oxidation of the ether was reported for the experiments with the intestinal sacs.

In Vivo Glyceryl Ether Metabolism

From earlier investigations concerned with the cleavage of the ether linkage (6-10), it is apparent that the fatty acid is the major catabolic product of the glyceryl ethers. In order to predict the labeling pattern that would be obtained for fatty acids derived from the ether

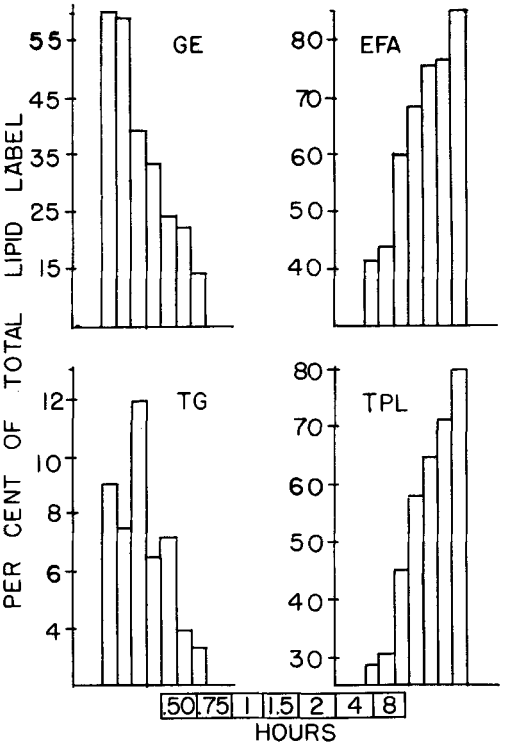

FIG. 1. Incorporation of Intraperitoneally Administered α-1-^{14}C-Batyl Alcohol into Rat Liver Lipids. Eight hours after administration, 24.5% of the injected dose was present in the liver lipids. GE- glyceryl ethers; EFA- esterified fatty acids; TG- triglycerides; TPL- total phospholipids.

linkage, a sodium taurocholate solution of sodium -1-^{14}C-palmitate was injected intraperitoneally. After 2 hr about one-third of the esterified fatty acid label was located in the neutral lipids. About 90% of the neutral lipid label was located in the triglycerides. Of the remaining two-thirds of labeled esterified fatty acid, about 68% of the label was located in the phosphatidyl choline fraction of the liver lipids. No significant amount of radio-active label was associated with liver lipids which were resistant to alkaline hydrolysis. Only about 5% of the injected dose was located in the liver lipids 4 hr after injection. In addition, the specific activity of the liver lipids decreased from 6000 DPM/mg lipid for 2 hr to 3500 DPM/mg lipid in 12 hr. The neutral lipid label decreased and the phospholipid label increased with time. Similar results were reported by Dittmer and Hanahan (26,27) for oral administration of labeled palmitate. They also reported that the labeling pattern for palmitic and stearic acids was not the same; the turn-over rate for stearic

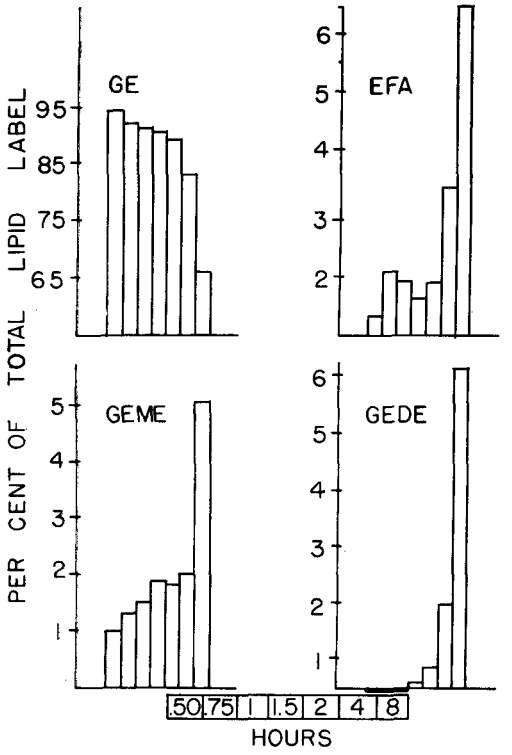

FIG. 2. Incorporation of Intraperitoneally Administered α-1-^{14}C-Batyl Alcohol into Rat Intestinal Neutral Lipids. Eight hours after administration, 7.5% of the injected dose was present in the intestinal lipids.

GE- glyceryl ethers; EFA- esterified fatty acids; GEME- glyceryl ether monoesters; GEDE- glyceryl ether diesters.

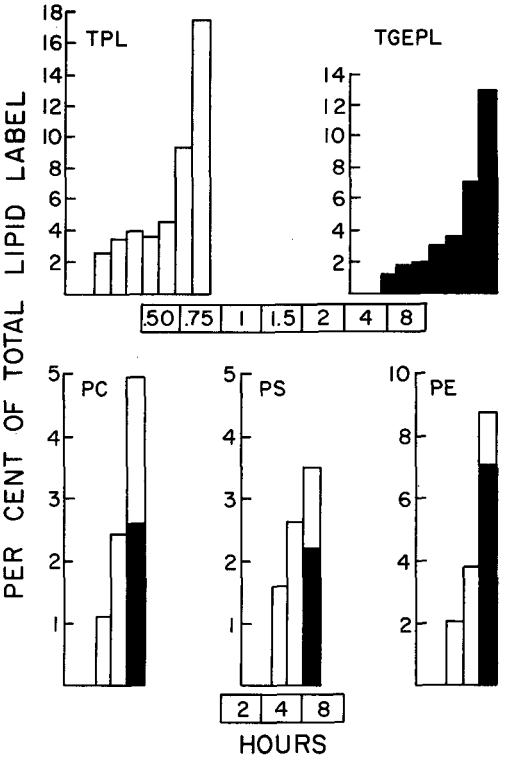

FIG. 3. Incorporation of Intraperitoneally Administered α-1-^{14}C-Batyl Alcohol into Rat Intestinal Phospholipids. The darkened area of the figure represents the amount of radio-active label which was located in the intact alkyl side-chain of the glyceryl ether.

TPL- total phospholipids; TGEPL- total glyceryl ether phospholipids; PC- phosphatidyl choline; PS- phosphatidyl serine; PE- phosphatidyl ethanolamine.

acid was much lower than that of palmitic, oleic, or linoleic acids (27). From the above preliminary results and from the reports of Dittmer and Hanahan (26,27) it was concluded that each fatty acid and, more importantly, each glyceryl ether, regardless of chain length or isomeric position of the ether linkage, must be considered separately. The time at which the animals are sacrificed after injection of the labeled material may determine the labeling pattern of the tissue lipids.

Time Studies and Labeling Patterns. Since there was a decrease in the amount of lipid label which was derived from intraperitoneally administered material, it was necessary to carry out time studies for the glyceryl ethers. Lipids of the liver and of the entire small intestine were examined. The liver and intestinal total lipid fractions contained the same amount of radioactivity per milligram of lipid for all time periods.

The distribution of label which was derived from α-batyl alcohol in rat liver lipids is shown in Fig. 1. After 8 hr only about 15% of the liver lipid label was found as the intact free glyceryl ether. All of the liver lipid label was located as esterified fatty acid except that of the remaining free glyceryl ether which had been injected. No significant free fatty acid label was observed for any of the liver lipid fractions.

The labeling pattern for the liver lipids after intraperitoneal injections of α-1-^{14}C-batyl alcohol is similar to that reported by Snyder and Pfleger (10) for intravenous injections. They reported that, 6 hr after intravenous injection of labeled batyl alcohol, 6.35 ± 1.35% of the injected dose was present in the liver and that only about 6% of the liver lipid label was found in the glyceryl ether fraction. In the

authors' laboratory, 8 hr after intraperitoneal injection, about 25% of the injected dose was located in the liver and only 15% of the liver lipid label was found as glyceryl ether.

A different labeling pattern was found for the intestinal lipids. As is shown in Fig. 2, 8 hr after administration of labeled α-batyl alcohol, 65% of the intestinal lipid label was present as free glyceryl ether, and the small intestine contained about 8% of the injected dose of label. Only 7% of the label was found as esterified fatty acids. In contrast to the liver, both mono- and diacyl derivatives of the glyceryl ether were present in the intestine. The glyceryl ether monoesters contained significant labels during all time periods (1-5%). Only after 2 hr were any significant labeled glyceryl ether diesters found.

As shown in Fig. 1 and 2, which compare the results obtained with α-batyl alcohol in the liver and intestine, there is little cleavage of α-batyl alcohol by the intestine even after 8 hr. These in vivo experiments substantiate the in vitro findings with intestinal homogenates. Less than 10% of the ether was cleaved in vivo, as measured by the total esterified fatty acid label. It is possible that, as soon as the ether was cleaved, the fatty acid which was formed in the intestine was then mobilized to some other tissue.

Glyceryl Ether Phospholipids. The major portion of the radioactive label in the intestine, which was derived from intraperitoneally administered α-1-^{14}C-batyl alcohol, was located in the phospholipids. About 18% of the total lipid label after 8 hr was associated with the phospholipids. The distribution of the label in the intestinal phospholipids is given in Fig. 3.

The phospholipid label of the intestine was stable to alkaline hydrolysis. Of the 18% radioactivity which was associated with the intestinal phospholipids, 8 hr after injection, about 77% of this label was stable to alkaline hydrolysis. Repeated hydrolysis of the intestinal lipids with sodium methoxide, from 20 min to 1 hr, resulted in little loss of label as esterified fatty acids. It seems that the intestine synthesized an alkaline stable phospholipid from the labeled glyceryl ether.

Radioactivity from α-1-^{14}C-batyl alcohol was found in the choline, serine, and ethanolamine phospholipid fractions: the phosphatidyl ethanolamine fraction contained the greatest amount of label, the choline and serine phosphatides contained about equal amounts of the label, and no significant label was found in the sphingomyelin fraction. From these results it was concluded that the intestine synthesized glyceryl ether phospholipids.

TABLE II

Incorporation of Intraperitoneally Administered Labeled Glyceryl Ethers into Glyceryl Ether Phospholipids of the Rat Intestine

Time (hours)	Labeled glyceryl ether injected	Glyceryl ether phospholipids formed (% of total lipid label ^{14}C)	Major labeled phospholipid fraction
4	α-1-^{14}C-Batyl alcohol	6.93	PE
8	α-1-^{14}C-Batyl alcohol	12.86	PE
4	β-1-^{14}C-Batyl alcohol	2.92	PC
8	β-1-^{14}C-Batyl alcohol	4.53	PC
4	2-^{3}H-α-1-^{14}C-Chimyl alcohol	16.50	PC
8	2-^{3}H-α-1-^{14}C-Chimyl alcohol	11.51	PC
4	β-1-^{14}-Chimyl alcohol	6.93	PC
8	β-1-^{14}-Chimyl alcohol	11.68	PC

PE = phosphatidyl ethanolamine.
PC = phosphatidyl choline.

By using the acetolysis procedure of Thompson (22), the distribution and isolation of the original glyceryl ether in the intestinal phospholipids was investigated. The acetolysis procedure promotes cleavage of the alk-l-enyl ethers (plasmalogens) to aldehydes (21). The results of this experiment are given in Figure 3. The darkened area of the figure represents the amount of radioactivity of labeled α-batyl alcohol present in the intestinal phospholipids. The phosphatidyl ethanolamine fraction contained about 80% glyceryl ether label and 20% esterified fatty acid label. No significant amount of labeled aldehydes, which would have been formed by cleavage of plasmalogens, was observed.

From these results it was concluded that the rat intestine can esterify and phosphorylate the intact glyceryl ether. It appears that the labeled glyceryl ether is esterified to the β-mono-ester-α-ether. By systems similar to those reported by McMurray (4,5), using cytidine diphosphate ethanolamine, the monoacyl glyceryl ether could be converted to the phosphatidyl ethanolamine glyceryl ether. Only in the intestinal lipids were the intact glyceryl ether phospholipids found. No significant quantity of labeled glyceryl ether phospholipids was found in the liver lipids.

Additional experiments were carried out with intraperitoneal injections of β-1-^{14}C-batyl, 2-^{3}H-α-1-^{14}C-chimyl, and β-1-^{14}C-chimyl alcohols to determine if these materials would also be converted directly to alkyl glyceryl ether phospholipids. The results are given in Table II. All of the labeled glyceryl ethers were converted to alkaline stable glyceryl ether phospholipids in the intestine. The β-ethers were

TABLE III

Distribution of Label from Intraperitoneally Administered 2-^{3}H-α-1-^{14}C-Chimyl Alcohol in Various Lipid Fractions

Time (hours)	Tissue	Lipid fraction	Ratio $^{14}C/^{3}H$
....	....	GE as injected	0.93
4	Intestine	GE[a]	0.93
4	Intestine	Total lipid	1.28
4	Intestine	TGEPL[a]	0.69
4	Intestine	Lysolecithin[a]	0.74
4	Intestine	Triglycerides	1.61
4	Liver	Triglycerides	4.31
4	Liver	Total lipid	5.80
8	Liver	Total lipid	9.75

[a]This fraction was determined after treatment with sodium methoxide. GE- glyceryl ethers; TGEPL- total glyceryl ether phospholipids.

not incorporated into the glyceryl ether phospholipids as rapidly as the α-ethers.

The difference in incorporation of the β-glyceryl ethers might be explained by the fact that in most acyl phospholipids the fatty acid which is attached to the secondary carbon of glycerol usually is unsaturated (26-28). Much to the surprise of the authors the major labeled phospholipids of the intestine after injection of β-1-^{14}C-batyl alcohol were the choline phosphatides (Table II). As discussed previously, the ethanolamine phosphatides were the major labeled fraction in the intestine after α-batyl alcohol injections (Figure 3). This difference in the distribution of labeled glyceryl ether phospholipid was caused supposedly by the isomeric position of the saturated ether group. For both the α- and β-chimyl alcohols, the phosphatidyl choline was the major labeled intestinal fraction. Less than 1% of alkaline stable glyceryl ether phospholipids was detected in any of the liver lipid extracts.

In order to substantiate the fact that the glyceryl ether was converted directly to the glyceryl ether phospholipids, animals were injected intraperitoneally with double-labeled α-chimyl alcohol (Table III). After 4 hr very little tritium was found in the total liver lipids; however, in the intestine, tritium was present in significant quantities. The difference in the amount of tritium label found in these two tissues demonstrates the degree of cleavage of the ether linkage. Treatment of the double-labeled chimyl alcohol with sodium methoxide did not alter the label ratio.

In the intestine it was found that the triglycerides contained a significant tritium label. In the liver triglycerides there is no significant tritium. It should be pointed out that the total glyceryl ether phospholipids (TGEPL) of the intestinal tissue seem to be more highly labeled with tritium than would be expected. The ratio of $^{14}C/^{3}H$ for the injected glyceryl ether was 0.93 as compared with 0.69 for the alkaline stable glyceryl ether phospholipids. The difference in ratio from the expected value (0.93) was caused partially by the fact that no water-soluble hydrolysis products were removed after sodium methoxide treatment. The $^{14}C/^{3}H$ ratio for the lysolecithin fraction (0.74), which was obtained by hydrolysis of the total lipids containing choline glyceryl ether phospholipids, was also less than the expected value. The divergence of the $^{14}C/^{3}H$ ratio from the expected value for the intestinal lipids may have been caused by the sample size or counting efficiency for tritium. It is also possible that biological exchange of the tritium may have taken place. However the quantity of tritium label which was found in the intestinal triglyc-

TABLE IV

Effect of Chain Length and of Isomeric Position on the Incorporation of Intraperitoneally Administered Glyceryl Ethers

Time (hours)	Tissue	Labeled glyceryl ether injected	% of Total lipid label		
			GE	GEDE	TG
4	Liver	α-1-^{14}C-Batyl alcohol	22.05	0.30	3.96
4	Intestine	α-1-^{14}C-Batyl alcohol	83.53	2.02	0.61
8	Liver	α-1-^{14}C-Batyl alcohol	14.00	0.57	3.38
8	Intestine	α-1-^{14}C-Batyl alcohol	65.95	6.55	1.39
4	Liver	β-1-^{14}C-Batyl alcohol	9.76	1.10	14.08
4	Intestine	β-1-^{14}C-Batyl alcohol	91.36	2.21	1.00
8	Liver	β-1-^{14}C-Batyl alcohol	5.92	1.52	2.95
8	Intestine	β-1-^{14}C-Batyl alcohol	81.02	6.95	1.83
4	Liver	2-^{3}H-α-1-^{14}C-Chimyl alcohol	2.21	0.78	28.42
4	Intestine	2-^{3}H-α-1-^{14}C-Chimyl alcohol	58.83	4.07	4.57
8	Liver	2-^{3}H-α-1-^{14}C-Chimyl alcohol	1.60	0.38	27.83
8	Intestine	2-^{3}H-α-1-^{14}C-Chimyl alcohol	44.98	0.68	8.32
4	Liver	β-1-^{14}C-Chimyl alcohol	5.53	2.25	36.31
4	Intestine	β-1-^{14}C-Chimyl alcohol	77.46	4.70	4.25
8	Liver	β-1-^{14}C-Chimyl alcohol	3.67	2.68	15.43
8	Intestine	β-1-^{14}C-Chimyl alcohol	26.99	11.31	15.26

GE- glyceryl ethers; GEDE- glyceryl ether diesters; TG- triglycerides.

erides and not in the liver triglycerides cannot be explained at the present time.

Cleavage and Esterification of Glyceryl Ethers. As discussed previously, there seemed to be very little cleavage of the ether linkage of α-batyl alcohol by the intestine. The question arose: could the small amount of labeled esterified fatty acids (7%) found in the intestinal lipids have been formed by another tissue and then transported to the intestine? To help answer this question, experiments were carried out with labeled β-batyl, α-chimyl, and β-chimyl alcohols. As is shown in Table IV, similar results were obtained for the α- and β-batyl alcohols by using intestinal tissue. The intestine cleaves the α-ethers to a greater extent than the β-ethers, confirming earlier work by Swell et al. (29). However, in contrast to earlier results reported (29) for α- and β-batyl alcohols, there is no detectable difference in the degree of esterification of these glyceryl ethers in the intestine. The intestine cleaved the chimyl alcohols to a much greater extent than the batyl alcohols. The liver cleaved all of the α-chimyl alcohol in 4 hr after injection whereas 15% of α-batyl alcohol remained after 8 hr.

In all of the experiments concerned with the metabolism of labeled glyceryl ethers after intraperitoneal injections, less than 1% of the radioactivity was detected in each of the following: free fatty acids, long-chain alcohols, cholesterol esters, monoglycerides, and long-chain aldehydes.

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Autoxidation of Saturated Fatty Acids. I. The Initial Products of Autoxidation of Methyl Palmitate

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ABSTRACT

A highly purified methyl palmitate free of all detectable impurities was oxidized by aeration at 150C. Monohydroperoxide was shown by thin-layer chromatography (TLC), spot and spray test, and polarography to be the initial autoxidation product.

INTRODUCTION

THE REACTIONS THAT TAKE PLACE between saturated fatty acids and their esters and atmospheric oxygen proceed at a rate which is slow in comparison with that observed for the unsaturated products. Stirton et al. (16) noted that methyl stearate with an iodine number of zero absorbed oxygen at 100C at a rate which was 11 times slower than methyl oleate. Peroxide values were determined only at the end of the oxidation period, and peroxidic oxygen accounted on the average for about 70% of the absorbed oxygen.

Most of the published studies of oxidation of model systems consisting only of saturated lipids are based on catalyzed oxidations (7, 10,11,13) or on reactions carried out at temperatures of 180C-200C (1-4,15). Under these conditions, complex mixtures of carbonyl, hydroxyl, and carboxylic compounds have been identified as products of thermal oxidation. From the analysis of the reaction products, Ramanathan et al. (15) and Endres et al. (2,3) concluded that dehydrogenation was the first step in the thermal oxidative reaction of methyl esters and triglycerides of saturated fatty acids. The resultant unsaturated molecule was then oxidized with the formation of hydroperoxides according to the schema of methyl oleate oxidation. Crossley et al. (1) also surmised that hydroperoxides were formed during oxidation of tricaprin at 190C. However none of these oxidized samples were found to contain a measurable peroxide concentration.

Zvidema (23) postulated that oxidation of paraffins at 100C-200C takes place primarily at the β-carbon atom, with an additional attack on the γ-atom. The initial product of this reaction is a monohydroperoxide which, in turn, yields a β-ketone and water. Twigg (20) however found that, when *n*-decane was oxidized to the extent that four moles of oxygen were absorbed per 100 moles of decane, about 10% of the hydrocarbon molecules oxidized were already attacked twice. The proportion of di-hydroperoxides, keto-hydroperoxides, and diketones was considerably higher than that expected statistically at this level of oxidation.

The course of peroxide accumulation during oxidation at 120C and 150C, as measured iodometrically, was found by Thaler and Saumweber (18,19) to follow closely the pattern observed by Knight et al. (8) for methyl oleate. The nature of these formed peroxides was not determined. Stearic acid with an iodine value of zero was shown by these researchers to undergo measurable peroxide formation within less than one day during oxidation at 60C, thus indicating that autoxidation of saturated esters did apparently take place even at moderate temperatures.

Thus, although the presence of peroxides in oxidized saturated fatty acids has been reported, the nature of the compounds or the role of pro-oxygenic impurities in their formation has not been established.

The present study was undertaken to determine whether or not highly purified saturated fatty esters undergo noncatalytic reactions with oxygen, also to establish the chemical nature of the primary stable products of such a reaction.

EXPERIMENTAL SECTION

Materials

Methyl palmitate and methyl palmitoleate were obtained commercially. The purity of the methyl palmitate was 99.5% and that of the methyl palmitoleate was 95%, as determined by gas chromatography. The 99.5% pure methyl palmitate contained 0.4% methyl pentadecanoate and two smaller impurities of unknown identity.

Methyl palmitate free of all detectable un-

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saturation, peroxides, or other measurable impurities was used to study the initial products of the autoxidation. This ester, hereafter referred to as "highly purified," was prepared by means of preparative-scale gas chromatography. The purity of this ester was determined by gas chromatography and by TLC.

Five μl samples of a 5% solution of the purified ester, dissolved in redistilled hexane, were examined chromatographically, and the relative areas under the peaks were computed. The area under the purified methyl palmitate peak averaged about 450,000 units of area in a system capable of detecting as few as 10 such units. No other peaks were noticed on the chromatogram. Hence the purity of this ester was greater than 99.99%.

Autoxidation of Esters

Oxidation of the purified ester was carried out at 60C, 150C, and 200C. At 60C three other samples consisting of methyl palmitate with different prooxygenic compounds, as well as a sample of methyl palmitoleate, were oxidized simultaneously in the dark with the highly purified ester. Five samples of 5 g each were weighed into standard active oxygen method tubes. One tube each of 95% methyl palmitoleate and 99.5% methyl palmitate were oxidized as received. "Highly purified" methyl palmitate was placed in the third tube; 99.5% methyl palmitate with added 0.25 g of lauroyl peroxide[2] 97% and 0.25 g of methyl linoleate 99% were placed in the last two tubes, respectively. The tubes were oxidized in an oil stability apparatus maintained at 60C ± 0.1C. Oxygen (USP grade) was freed from moisture, carbon dioxide, carbon monoxide, and hydrogen by means of a purification train and bubbled through the samples at a rate of 4 ml per minute. The purification train consisted of a quartz tube, packed with copper oxide brought to red heat to convert carbon monoxide to carbon dioxide and hydrogen to water, and absorption tubes containing Anhydrone (anhydrous magnesium perchloride) and Ascarite (sodium hydrate-asbesto absorbant) to remove moisture and carbon dioxide.

Five μl samples were withdrawn at suitable time-intervals for analysis of oxidation products by TLC and GLC.

The "highly purified" methyl palmitate was also oxidized at 150C and 200C. The oxidation flask consisted of a 10-mm I.D. × 170-mm tube with a side arm and a standard tapered joint neck fitted with a 5-mm O.D. × 10-mm filter stick that reached to within 5 mm from the bottom. Oxygen, purified as previously described, was brought into contact with the ester through the filter stick at the bottom of the oxidation tube at a rate of 12 ml per minute. The tube was submerged in a Tecam[3] fluidized bed sand bath that was maintained at 150C ± 1C and oxidized for 3 hr. The highly purified ester was also oxidized in the same system for 1 hr at 200C ± 1C. The oxidized esters were examined before, as well as after, reduction of the peroxides with sodium borohydride (9).

Analysis of Oxidized Esters

The esters were examined qualitatively for the presence of peroxides by TLC, following the method of Oette (12), and by polarography according to Willits et al. (22). The presence or absence of peroxides in a sample was determined by a modification of the TLC method, hereafter referred to as the "spot and spray test." Two μl of the ester were spotted on filter paper by means of a capillary tube and sprayed with the potassium iodide and starch indicators. The sensitivity of the reaction in oxidized methyl esters was better than 0.25 meq peroxide per kg of the sample.

Samples were also examined by infrared absorption spectroscopy and flame ionization gas chromatography with 6-ft × ⅛-in. columns containing DEGS with 2% phosphoric acid, SE-30, Apiezon-L, and ECNSS-S liquid phases.

The peroxide content of the esters oxidized at 150C and 200C were examined by a modification of the iodometric method of Wheeler (21).

RESULTS AND DISCUSSION

Oxidation at 60C

The course of peroxide formation in the methyl esters of the fatty acids during the autoxidation at 60C is given in Table I. All the samples except for the "highly purified" methyl palmitate showed positive peroxide formation during the course of the autoxidation. Presence of a monohydroperoxide was detected in these esters by means of TLC. Analysis of the "highly purified" methyl palmitate by gas chromatography failed to reveal any changes in the purity of the ester even after 56 days of oxidation at 60C.

In the solvent system used for TLC (65:45 petroleum ether-diethylether + 2% ammonium hydroxide), monohydroperoxides of methyl esters of fatty acids were readily separated

[2] USS Peroxygen Corporation.

[3] Tecam (Cambridge) Ltd., Duxford, England.

TABLE I

Peroxide Formation in Methyl Esters of Fatty Acids During Autoxidation at 60C

Days of oxidation	"Highly Purified" methyl palmitate	99.5% Methyl palmitate with added —	Lauroyl peroxide	Methyl linoleate	95% Methyl palmitoleate
0	—	—	++	—	++(T)
1	- -	—	++	+	++(T)
4	- -	—	++(T)	++	+++(T)
6	- -	+	++(T)	++(T)	+++(T)
9	- -	+	++(T)	++(T)	+++(T)
14	- -	++	+++(T)	+++(T)	+++(T)
20	- -	++	+++(T)	++(T)	+++(T)
27	- -	++(T)	+++(T)	+++(T)	+++(T)
37	- -	++(T)	+++(T)	++(T)	++
45	- -	++(T)	++(T)	++(T)	++
56	- -	++(T)	++(T)	++(T)	++

— Negative spot and spray test.
+ Weakly positive spot and spray test.
++ Positive spot and spray test.
+++ Strongly positive spot and spray test.
(T) Presence of a hydroperoxide (detected by TLC).

from more polar peroxides of the esters and highly peroxidized products of lipid oxidation (12). The retention factor for the monohydroperoxides was determined by thin-layer examination of samples of oxidized methyl oleate and methyl palmitoleate since monoethenoic compounds have been clearly shown to form monohydroperoxides on autoxidation (14,17).

Hydroperoxides were present in the sample of 95% palmitoleate at the start of the oxidation. The concentration, as well as the nature of the peroxides, as judged by TLC, increased during the first two weeks of the oxidation. On further oxidation the concentration of the hydroperoxides decreased, and after one month at 60C, all the monohydroperoxide was decomposed to more polar peroxidic compounds.

Methyl palmitate 99.5% with added lauroyl peroxide gave a positive spot and spray test and three distinct spots on TLC even at zero time. The three components seen on TLC were considerably more polar than the monohydroperoxides of the methyl ester and had retention factors of 0.2, 0.4, and 0.5 as contrasted with an R_f of 0.8 for the hydroperoxides of methyl palmitoleate. It was possible, therefore, to follow the formation of monohydroperoxides of methyl palmitate oxidation in the ester with added lauroyl peroxide by TLC. The first clear indication of the presence of monohydroperoxides in this sample was observed after four days of oxidation at 60C. As lauroyl peroxide was not likely to oxidize to monohydroperoxide, it must be concluded that these compounds were formed in the course of the oxidation of the 99.5% methyl palmitate.

Oette (12) showed that autoxidation of methyl linoleate yields at first a single spot with the same R_f value as the monohydroperoxide of methyl oleate, followed on further oxidation by more polar products of secondary oxidation. No such spots were observed in the sample of methyl palmitate with added 99% methyl linoleate at the start of the oxidation. After six days of oxidation at 60C clear indications for the presence of hydroperoxides in the ester were obtained by TLC. Some weak spots suggesting the presence of additional, more polar peroxides were also noted by TLC. All these peroxides might have been initially the products of autoxidation of methyl linoleate. However, as these peroxides persisted even long after all the palmitoleate was oxidized in the control tube, it would seem that at least a part of them were the products of oxidized methyl palmitate.

The 99.5% methyl palmitate was comparatively stable under the conditions of this experiment. The peroxides that were eventually formed as a result of autoxidation of the ester were readily identified on the basis of their R_f value on TLC as monohydroperoxides. That the impurities in this ester were more susceptible to oxidation at 60C was suggested by the fact that no noticeable peroxides were found in their absence in the "highly purified" sample. There is no reason to doubt that even the "highly purified" methyl ester would eventually have oxidized with the formation of hydroperoxides. By reducing the proportion

of relatively pro-oxygenic molecules in the sample, the probability of enough molecules reaching the energy level necessary for the initiation of autoxidation would appear to be also reduced.

Ingold (5) suggested that thermal initiation of autoxidation of saturated hydrocarbons may involve the reaction:

$$[1]\ RH + O_2 \rightarrow \dot{R} + H\dot{O}_2.$$

This reaction requires approximately 30-45 kcal/mole. It would be more likely that free radicals from another reaction such as decomposition of peroxides [reaction (2)] or a reaction of oxygen with a weaker RH bond [reaction (3)], such as in branching, would provide initiation.

$$[2]\ ROOR' \rightarrow R\dot{O} + \dot{O}R'$$

The following reaction will also involve less energy than equation (1) above:

$$[3]\ RH + O_2 + HR \rightarrow \dot{R} + H_2O_2 + \dot{R}.$$

These free radicals could be involved in the abstraction of an H from the saturated molecule leading to the formation of a monohydroperoxide in the same manner as occurs in the autoxidation of unsaturated acids.

Kinetically, therefore, the presence of a peroxide or any compound containing a C-H bond that is weaker than the C-H bonds of the saturated fatty ester increases the likelihood of thermal initiation of the autoxidation. This was clearly demonstrated by the samples of 99.5% pure methyl palmitate with the added lauroyl peroxide and those of the ester with added methyl linoleate (which has a central reactive methylene group), that autoxidized at a faster rate than the sample containing only the ester.

Based on these observations, one may postulate that, at 60C in a stream of pure oxygen, molecules of methyl palmitate held in the dark are essentially inert to thermal initiation of autoxidation.

These results are in agreement with the observation of Thaler and Saumweber (18) that the rate of autoxidation of saturated fatty acids at room temperature is a function of their purity. During 69 hr of autoxidation at 60C these workers noted that the peroxide value of methyl palmitate increased from 1.04 to 1.18 meq/kg. Although the esters used by Thaler and Saumweber in their study were only of limited purity, they did confirm that autoxidation, once initiated, would be propagated even at room temperature. The results of these workers also suggest that peroxides accumulate during the course of autoxidation of saturated fatty esters. It was impossible however to determine the chemical nature of these peroxides by the iodometric method of peroxide determination used in that study.

The results of the TLC and spot and spray test studies of samples of methyl palmitate autoxidized at 60C indicate that, once initiated, the reaction will continue through propagation. As shown in Table I, monohydroperoxides were formed in 99.5% methyl palmitate with added lauroyl peroxide. These peroxides are not present in lauroyl peroxide; hence, they must be products of methyl palmitate oxidation. The monohydroperoxides persisted in this sample, as well as in the ester with added methyl linoleate, even after all the monohydroperoxides of methyl palmitoleate decomposed.

Hence it would appear that autoxidation, once initiated, will continue through propagation. This reaction leads to the formation of monohydroperoxides, which are apparently the first stable products of autoxidation of saturated esters under the conditions of this study. Because of the slow reaction rate, the larger samples of peroxidase necessary for their identification by methods other than TLC could not be obtained from esters oxidized at 60C. Moreover the ability of "highly purified" methyl palmitate to undergo the initiation reaction during oxidation at 60C could not be established.

Oxidation at 150C and 200C

"Highly purified" methyl palmitate oxidizes readily at 150C. A sample of the ester oxidized in the dark for 3 hr at that temperature remained white, crystalline at room temperature, and odorless. Iodometric examination showed however that the oxidized ester contained approximately 660 meq of peroxides per kg.

TLC confirmed the presence of a peroxide with an R_f value of 0.8 and some other very weak spots of more polar peroxides (Fig. 1). The retention factor of the peroxide suggests that this compound is a monohydroperoxide, and its relative intensity clearly indicates that the initial oxidative attack was limited to one peroxide per molecule of methyl palmitate since the formation of dihydroperoxide would have been indicated by a second strong spot at a lower R_f.

These observations are in agreement with those reported above for the product of oxidation at 60C of 99.5% pure methyl palmitate. It can be concluded therefore that at 150C the initiation reaction of the autoxidation of

"highly purified" methyl palmitate does occur readily. The extent of oxidation of this ester, as measured by the iodometric peroxide method, was at least as large as that noted for a sample of 99.5% methyl palmitate. This observation would suggest that the ester itself is subject to the initiation reaction during oxidation at 150C because the presence of the pro-oxygenic impurities in the less pure ester did not measurably accelerate the reaction.

To confirm the nature of the peroxides formed during autoxidation at 150C, oxidized samples of the "highly purified," 99.5% pure, were subjected to polarographic analysis. A typical polarogram is shown in Fig. 2. The presence of a major polarographic wave with a half-wave potential of –0.9 volt was noted in all samples. This wave was within the reducing range for hydroperoxides noted by Willits et al. (22), and its intensity was proportional to the chemically determined peroxide value for the sample. A minor wave was also observed with a half-wave potential of –1.7 volts. Kalbag et al. (6) suggested that this wave was attributable to secondary products of oxidation. Lack of any polarographic peaks in the region of 0.00 - –0.19 volt rules out the presence of chemical peroxides in the samples (22).

As the monoxidized ester did not produce polarographic waves and the only major wave observed in the oxidized samples corresponds to that of a hydroperoxide, these results support the hypothesis that monohydroperoxides are the initial products of autoxidation of saturated fatty esters.

No peroxides were detected in the ester oxidized at 200C. Gas chromatographic analysis indicated however the presence of numerous decomposition products even after 1 hr of oxidation. These results are in agreement with Ramanathan et al. (15), Crossley et al. (1),

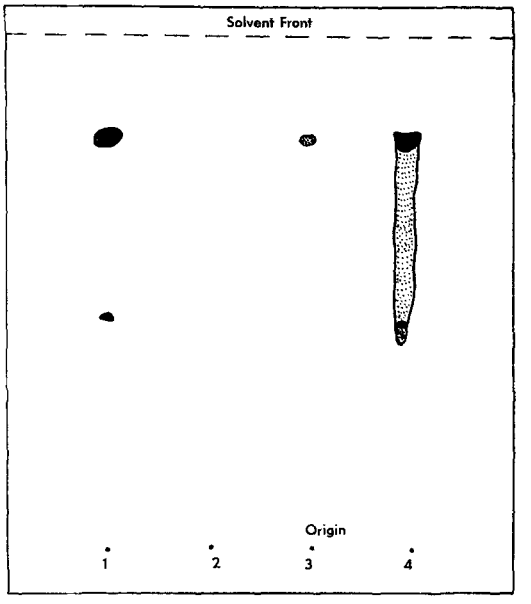

FIG. 1. Composite thin-layer chromatograph of oxidized methyl esters on Silica Gel G.
Developing solvent: 45% diethyl ether—petroleum ether (NH_3): peroxides indicated by KI—starch reaction.
Identification: *1*, 95% palmitoleate, 50 meq peroxide/kg.
2, "highly purified" palmitate, oxidized six weeks at 60C.
3, 99.5% palmitate, oxidized six weeks at 60C, 10 meq/kg.
4, "highly purified" palmitate, oxidized 3 hr at 150C, 660 meq/kg.

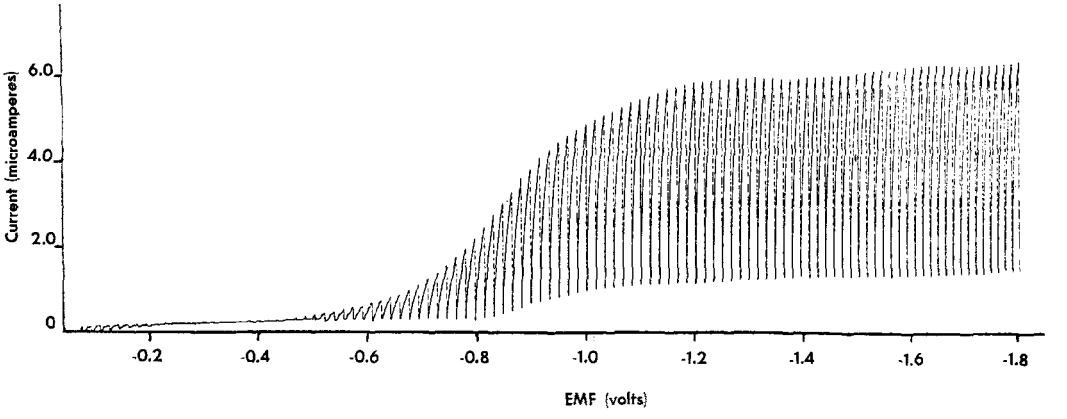

FIG. 2. Typical polarogram: methyl palmitate, 99.5% pure, oxidized ½ hr at 150C; peroxide content 170 meq/kg.

and Endres et al. (2), who observed complex mixtures of carbonyls, hydroxyls, and fatty acids and postulated that peroxides were formed during the course of the reaction but did not find any peroxides in the products of thermal oxidation of saturated esters.

Reduced Products of Autoxidation at 150C

Reduction of the products of oxidation at 150C of "highly purified" methyl palmitate with sodium borohydride yielded a colorless and odorless liquid. The reduced ester contained no measurable chemical peroxides and did not show the characteristic hydroperoxide wave on the polarograph.

Examination of the ester by gas chromatography indicated the presence of more than 10 major components in addition to the unoxidized methyl palmitate in the ester oxidized for 3 hr at 150C. After reduction however only a single major peak was observed in addition to the methyl palmitate. On the basis of infrared and mass spectroscopy, this peak was assigned to the methyl ester of hydroxy palmitate. The peaks observed in the oxidized sample can be attributed to the instantaneous thermal degradation of the hydroperoxides in the hot injection block of the gas chromatograph. The absence of dihydroxy esters in the reduced samples provides additional evidence that monohydroperoxides are the initial stable product of autoxidation of esters of saturated fatty acids.

ACKNOWLEDGMENT

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Autoxidation of Saturated Fatty Acids. II. The Determination of the Site of Hydroperoxide Groups in Autoxidizing Methyl Palmitate

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ABSTRACT

The monohydroperoxides of methyl palmitate oxidized at 150C were concentrated, purified, and reduced to yield a mixture of isomers of monohydroxy palmitate. No evidence of unsaturation in these molecules could be obtained, and no direct method for the resolution or identification of the individual isomers was found. Nuclear magnetic resonance (NMR) spectroscopy of the reduced esters suggested that the hydroxy groups were not located at either end of the chain of the fatty ester.

To establish the location of the initial oxidative attack on saturated esters, the reduced esters of oxidized methyl palmitate were chemically cleaved at the location of the hydroxy group by means of chromium trioxide oxidation and by the Beckmann transformation. The resulting mono- and dibasic fatty acids were methylated and analyzed by gas chromatography. This analysis indicated that the oxidation of methyl palmitate did not occur selectively at one location along the ester. Although all carbon atoms can apparently be oxidized, preferential oxidation was observed toward the center of the molecule.

INTRODUCTION

THE STRUCTURE AND location of hydroperoxides formed during the auto-oxidation of unsaturated fatty acids have been studied extensively. The isolated initial products of autoxidation of the methyl esters of oleic, linoleic, and linolenic acids were shown (7, 15,16) to be formed in conformance with the hydroperoxide theory of Farmer (6).

Paquot and de Goursac (14) studied the catalytic oxidation of saturated fatty acids. Oxidation of palmitic acid in the presence of 1% nickel phthalocyamine resulted in a mixture of all the even-numbered acids whereas, when 20% of sodium or potassium salts were added, no acids shorter than lauric were found upon oxidation. These results, and the isolation of small amounts of methyl ketones with an uneven number of carbon atoms, suggest that β-oxidation was the dominant form in saturated fatty acids of lower but even carbon number. Alternatively ketonic scission yields methyl ketones with an odd carbon number. Very small amounts of lactones were also found, indicating the occurrence of some γ- and Δ-oxidations.

Makalets (10) carried out oxidations of the first six members of the homologous series of acids of normal structure which were labeled with ^{14}C in the carboxyl group. Oxidation was carried out at 142C and at a pressure of 6 atmospheres with air enriched with oxygen (50%). The rate of separation of radioactive carbon dioxide was followed and was found to be independent of the chain length for acids containing three to six carbon atoms. The rate of oxidation for acetic acid was however markedly slower than that observed for the other acids. From this study Makalets concluded that decarboxylation was a major route of the oxidative reaction.

Thermal oxidation of methyl laurate and methyl stearate at 200C was studied by Ramanathan et al. (17). The presence of nonanoic acid at the end of 1 hr of oxidation of methyl stearate was explained by the specific susceptibility to oxidative attack at 200C of the carbon-carbon linkage between 9 and 10 carbons. Dehydrogenation, causing unsaturation in the molecule, was suggested as the first step in the oxidation, followed by the formation of hydroperoxides according to the mechanism of thermal oxidation of methyl oleate.

The course of peroxide accumulation during the oxidation of lauric and stearic acids at 120C and 150C, and their esters, was followed iodometrically by Thaler and Saumweber (20,21). The nature of the peroxides was not determined, but their accumulated decomposition products were analyzed by paper chromatography. Methyl laurate yielded, upon oxi-

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dation, the homologous series of dicarboxylic acids from ethanedioic acid to decanedioic acid, and probably undecanedioic acid. In the monobasic series the absence or noticeably lower concentration of heptadecanoic, pentadecanoic, and tridecanoic acids among the oxidation products of methyl stearate was noticed. The oxidation was therefore believed to have occurred simultaneously at all the methylene groups of the fatty acid chain, with some preference for the β-carbon atoms.

Tripalmitin and other synthesized triglycerides were oxidized at 200C by Endres et al. (4,5). None of the oxidized samples were found to contain peroxides. Dehydrogenation of the saturated triglycerides, giving rise to unsaturation in the molecule, was suggested as the initial step in thermal oxidation of tripalmitin. Subsequent oxidation results in the formation of hydroperoxides. The oxidative attack was believed to have occurred more or less at random along the carbon chain.

Oxidation of tricaprin at 190C was found by Crossley et al. (3) to yield complex mixtures of breakdown products. From these isolated products it was surmised that the initial step in the reaction was the formation of hydroperoxides. Oxidative attack was found to be concentrated at the α- and β-carbons; progressively diminishing oxidation also occurred farther along the chain.

It has been demonstrated that monohydroperoxides form as the initial stable products of autoxidation of methyl palmitate at 60 and 150C (1). Farmer's theory is however not applicable for saturated fatty acids, and the location of the initial oxidative attack in these esters is still a matter of speculation. The purpose of the present study was to investigate the mode of formation of monohydroperoxides during the autoxidation of esters of saturated fatty acids. Whether or not unsaturation is a step in the mechanism of autoxidation was also examined.

EXPERIMENTAL

Autoxidation

Forty grams of methyl palmitate, 99.5% pure, was oxidized in the dark in a stream of oxygen at 150C to a peroxide value of approximately 400 meq/kg. The oxidation was carried out in the apparatus previously described (1) except that a 50-ml flat-bottom flask was used as the reaction flask instead of the 10-mm I.D. tube. The course of the oxidation was followed by iodometric peroxide determinations.

Concentration of Oxidized Products

Preliminary concentration of the polar products of the autoxidation was achieved by counter-current extraction between hexane and 80% ethanol in five 125-ml separatory funnels. Twenty grams of the oxidized methyl palmitate were partitioned between 50 ml of each of hexane and 80% ethanol. The polar extracts were re-extracted with 50-ml portions of fresh hexane until they were found by gas chromatographic analysis to be nearly free of unoxidized methyl palmitate.

The peroxide-containing 80% ethanol was evaporated under vacuum at room temperature, and the peroxides were extracted from the residual water with ether. The ether extract was dried over sodium sulfate, filtered, and stored at -40C. The peroxide value of the ether extract was in excess of 2,500 meq peroxide per kg.

Reduction of the Concentrated Hydroperoxides

The concentrated hydroperoxides were reduced for 2 hr with sodium borohydride according to the previously described modification of the method of Matic and Sutton (11). Although not specific to hydroperoxides, this reaction was shown by Frankel et al. (7) not to affect unsaturation in the hydroperoxides. The products of the reduction had a peroxide value of zero.

Liquid-Partition Chromatography

The liquid-partition chromatography method of Frankel et al. (8) was used to separate the reduced hydroperoxides of methyl palmitate from the other polar secondary products of autoxidation.

Fission of Methyl Esters of Hydroxy Palmitate

The purified esters were fissioned at the hydroxy location by a modification of the chromium trioxide-acetic acid oxidation method of Smith et al. (19) and by hydrolysis of the amides formed by the Beckmann transformation of Christie et al. (2).

The oxidative cleavage of the purified hydroxy methyl palmitate with chromium trioxide dissolved in acetic acid was carried out for 1 hr at 20C. For a single isomer this reaction leads to the production of two mono- and two dibasic acids. A 99.5% pure methyl palmitate blank was oxidized with each sample.

Careful oxidation of hydroxy methyl palmitate with dilute chromium trioxide in acetic acid led to the formation of the corresponding keto esters. These esters were then converted into oximes, transformed to amides,

and hydrolyzed to free fatty acids and amides. For a single isomer of the hydroxy methyl palmitate, a single mono- and a single dibasic acid, as well as two amides, would be formed. A 99.5% pure methyl palmitate blank was oxidized simultaneously with each sample as a control.

Methylation of the Cleaved Hydroxy Methyl Palmitate

Boron trifluoride methanol reagent[2] was used for rapid esterification of the free fatty acids produced by fission of the hydroxy methyl palmitate. The method of Metcalfe and Schmitz (13) was used to esterify rapidly and completely both mono- and dibasic acids except that 4 ml of the reagent was used per 100 mg of fatty material, and the esterification was carried out in boiling water for 2½ min.

Analysis of the Products of Oxidation

The presence of unsaturation in micro- and ultramicro samples was investigated by means of a Brown Micro Hydro Analyzer.[3] Infrared, mass, and nuclear magnetic resonance spectroscopy, also gas chromatography were used for the analysis of oxidized esters and fission products from the methyl esters of hydroxy palmitate. Gas chromatographic columns were 6 ft x ⅛ in. SE-30, Apiezon-L ECNSS-S, and DEGS with 2% phosphoric acid.

Identification of the various peaks were accomplished by comparing their retention times on different columns with those of authentic compounds and by adding a mixture of these compounds to a sample of the oxidized ester and rechromatographing to confirm these retention times.

Quantitative analysis of the mono- and dibasic esters was based on the normalized peak areas of each type of these esters. Individual peak areas were calculated as the height of the peak times width at half height.

The procedure for the determination of the location of the autoxidative attack is as follows:

A. Autoxidation of methyl palmitate.

B. Concentration of the products of autoxidation.

C. Reduction of the concentrated hydroperoxides to methyl hydroxy palmitate isomers.

D. Liquid-partition purification of the methyl hydroxy palmitate isomers.

E. Fission of the methyl hydroxy palmitate adjacent to the hydroxy group and methylation of the acid fragments: a) chromium trioxide-acetic acid oxidation, b) Beckmann transformation of oximes formed from the hydroxy esters.

F. Gas chromatographic analysis of the acid fragments.

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RESULTS AND DISCUSSION

Dehydrogenation of Saturated Fatty Acids During Autoxidation

No evidence of unsaturation could be found in the molecules of oxidized methyl palmitate by either infrared spectroscopy or by micro hydrogenation analysis. Knight, Eddy, and Swern (9) showed that a *cis-trans* isomerization occurred in molecules of methyl oleate simultaneously with the attack by molecular oxygen. This isomerization can be observed in infrared spectra by an increased absorption at 980 cm^{-1}. No such increase in absorption could be observed in the autoxidized methyl palmitate either before or after reduction of the ester with sodium borohydride.

The Brown Micro Hydro Analyzer utilizes sodium borohydride as both a source of hydrogen and as an activator for the catalyst used in the hydrogenation. Sodium borohydride however reduces hydroperoxides. Testing for micro-unsaturation with this unit could therefore not be carried out on esters containing hydroperoxides. Frankel et al. (7) demonstrated that reduction of peroxides by sodium borohydride did not affect the unsaturation of the oxidized methyl linoleate; hence a test for unsaturation was carried out on samples of the reduced monohydroperoxide, and this analysis was meaningful. A sample containing 1 mmole oxidized methyl palmitate absorbed less than 0.01 mmole of hydrogen, indicating no measurable unsaturation in the oxidized molecules.

These results are not in agreement with Ramanathan et al. (17), who postulated a mechanism for the formation of hydroperoxides during the autoxidation of saturated fatty acids at 200C and suggested that unsaturation of the molecule occurred as the initial step in the reaction. Based on the results of this study, it seems that the oxidative attack on saturated fatty esters at 150C does not result initially in unsaturation of the molecule. The formation of a free radical R· is apparently the initial step in the autoxidation of saturated fatty acids at 150C, and the first stable products of this oxidation are monohydroperoxides (1).

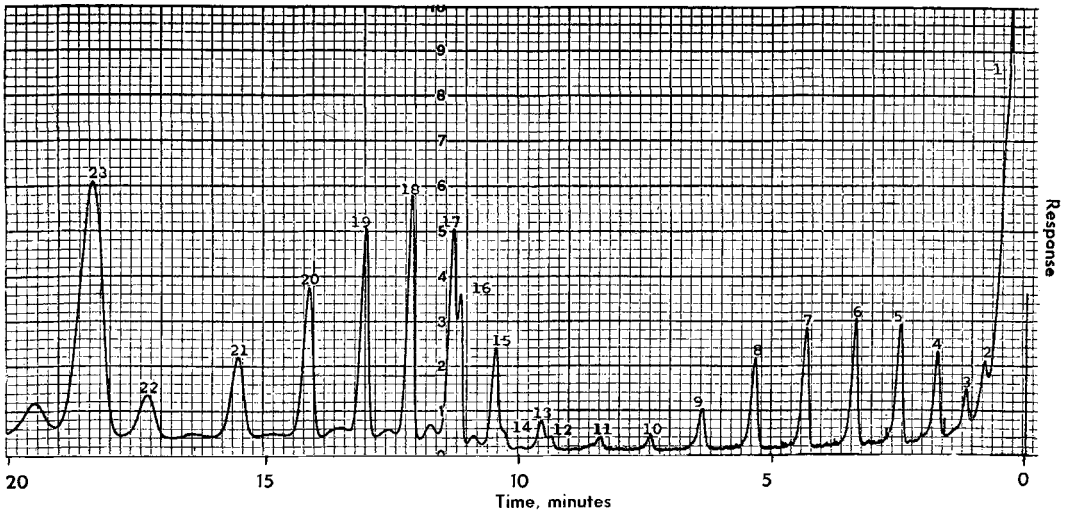

FIG. 1. Typical chromatogram of methyl esters obtained by oxidation of the methyl esters of hydroxy palmitate with chromium trioxide.

Identification: *1*, solvent and possible shorter chains; *2*, n-butanoate; *3*, n-pentanoate; *4*, n-hexanoate; *5*, n-heptanoate; *6*, n-octanoate; *7*, n-nonanoate; *8*, n-decanoate; *9*, n-undecanoate; *10*, n-dodecanoate; *11*, n-tridecanoate; *12*, n-tetradecanoate; *13*, n-heptanedioate; *14*, n-pentadecanoate; *15*, n-octanedioate; *16*, n-hexadecanoate; *17*, n-nonanedioate; *18*, n-decanedioate; *19*, n-undecanedioate; *20*, n-dodecanedioate; *21*, n-tridecanedioate and *22*, n-tetradecanedioate (estimated from log plot of retention time); *23*, methyl keto-palmitate (from infrared data).

Location on the Molecule of the Initial Oxidative Attack

In the case of unsaturated esters, the technique commonly used to locate the site of oxidative attack depends on the relative susceptibility of the double bond to oxidative cleavage (7,9,15,16). Obviously this technique cannot be utilized for saturated esters. The approach selected for this study was based on the concentration of the peroxides, followed by their quantitative conversion to the corresponding hydroxy esters. These more stable compounds were analyzed by NMR spectroscopy and then subjected to selective cleavage of the carbon-carbon bond adjacent to the hydroxy group and gas chromatographic analysis of the fragments. These methods of cleavage were found to be less selective than those used in the studies of unsaturated esters. They do however point out the general pattern of autoxidation in saturated esters.

The initial oxidative attack in methyl palmitate was found to occur all along the molecule. No single location was found to be exclusively susceptible to the formation of hydroperoxides, as is the case in unsaturated fatty acids. The center of the ester, between carbons 5-11, appears to be relatively more readily oxidized than those nearer to the two ends.

a) NMR spectroscopy of the hydroxy esters confirmed the absence of unsaturation in the oxidized sample. No absorption was noted in the region 7.0-5.0 ppm in which olefinic CH= CH protons occur.

Multiplets in the region of 2.0-2.5 ppm were produced by $-CH_2-\mathit{CH}_2-\overset{O}{\overset{\|}{C}}-O\mathit{CH}_3$. Integration of the peaks showed CH_2/CH_3 to be at a size ratio of 3 to 2. Therefore there could not have been a significant amount of *alpha*-hydroxy ester in the sample since this would not have given absorption in that range.

Terminal methyl groups (CH_3-) give a signal at approximately 1.0 ppm whereas $HO-CH_2-CH_2$ give a complex spectrum at approximately 3.4 ppm. No signal was obtained at 3.4 ppm, ruling out a terminal hydroxy group. In the region around 1.0 ppm a triplet appears to form. This in the methyl region would indicate that the terminal structure is CH_3-CH_2-, but $CH_3-\underset{\underset{OH}{|}}{CH}$ would be indicated by a doublet. It would seem therefore that the end of the ester chain in at least most of the molecules was of the form CH_3-CH_2-.

Analysis of the NMR spectra confirmed the previously stated results regarding the absence of unsaturation in the oxidized molecules of

TABLE I

Gas Chromatographic Retention Times of Esters of Mono-and Dibasic Acids Formed by Chromium Trioxide Oxidation of the Methyl Esters of Hydroxy Palmitate

	Retention Time, Minutes			
	GC analyses on DEGS w/acid		GC analyses on Apiezon L	
	RT of unknown	RT of authentic compound	RT of unknown	RT of authentic compound
Monobasic ester				
$CH_3(CH_2)_2COOCH_3$	0.8		2.8	
$CH_3(CH_2)_3COOCH_3$	1.1		3.2	
$CH_3(CH_2)_4COOCH_3$	1.7	1.6	4.9	4.8
$CH_3(CH_2)_5COOCH_3$	2.4		6.7	
$CH_3(CH_2)_6COOCH_3$	3.3	3.3	8.5	8.5
$CH_3(CH_2)_7COOCH_3$	4.3	4.3	10.3	10.4
$CH_3(CH_2)_8COOCH_3$	5.3	5.3	12.0	12.0
$CH_3(CH_2)_9COOCH_3$	6.3	6.3	13.8	13.8
$CH_3(CH_2)_{10}COOCH_3$	7.4	7.4	17.2	17.4
$CH_3(CH_2)_{11}COOCH_3$	8.4	8.5		23.8
$CH_3(CH_2)_{12}COOCH_3$	9.3	9.4		31.0
$CH_3(CH_2)_{13}COOCH_3$	10.3	10.4		39.2
Dibasic ester				
$H\,COOC(CH_2)_8COOCH_3$		7.5		8.6
$H\,COOC(CH_2)_9COOCH_3$		8.6		10.2
$H\,COOC(CH_2)_5COOCH_3$	9.5	9.5	11.7	11.8
$H_3COOC(CH_2)_6COOCH_3$	10.4	10.3	13.4	13.6
$H\,COOC(CH_2)_7COOCH_3$	11.3	11.3	15.6	15.7
$H\,COOC(CH_2)_8COOCH_3$	12.1	12.1	19.0	19.0
$H_3COOC(CH_2)_9COOCH_3$	13.0	13.0	23.8	23.8
$H_3COOC(CH_2)_{10}COOCH_3$	14.1	14.2	31.0	31.1
$H\,COOC(CH_2)_{11}COOCH_3$	15.5		42.0	
$H_3COOC(CH_2)_{12}COOCH_3$	17.3		56.0	

methyl palmitate, indicating that dehydrogenation was not a required step in the mechanism of autoxidation of methyl palmitate.

Although the location of the oxidative attack along the ester chain could not be determined by NMR, the possibility of a major attack at the *alpha* position or at the terminal methyl group was again ruled out. All indications suggest that the autoxidative attack on saturated fatty esters occurred mainly away from the end of the molecule.

b) Oxidation of the purified hydroxy esters with chromium trioxide in the presence of acetic acid for 1 hr at room temperature resulted in an almost complete degradation of the material. This degradation was shown by Smith and co-workers (19), also by Meakins and Swindels (12), to occur preferentially at the carbon to which the hydroxy group is attached. Two mono- and two dibasic acids are obtained on the oxidation of a molecule containing a single hydroxy group in the middle of the ester. This reaction can be presented in the following general form:

$$\text{R-CH}_2\text{-CHOH-CH}_2\text{-R'-COOH} \xrightarrow{CrO_3,HAc} \text{RCOOH} + \text{RCH}_2\text{COOH} + \text{HOOCCH}_2\text{R'COOH} + \text{HOOCR'COOH}$$

Utilizing such a method, Meakins and Swindels (11) were able to identify an acid obtained from olive leaves as methyl 10, 15 dihydroxyhexadecanoate.

Fig. 1 shows a chromatogram of the products of chromium trioxide oxidation of the purified hydroxy ester. As can readily be seen, the concentrations of the various mono- and dibasic esters were not uniform, nor was there one single major peak. Instead a group of larger peaks of monobasic esters of the fatty acids with long chains of 6-10 carbons appear to predominate in one section of the chromatogram. Similarly dibasic esters with 8-12 carbon-long chains predominate in the other end of the chromatogram. These identifications of the peaks of the oxidized esters were based on comparisons with standard esters on columns, packed with DEGS with 2% phosphoric acid and with Apiezon L, and are shown in Table I. The normalized sizes of the peaks of each series of the esters are represented in Table II. These results also confirm the predominance of esters which were produced by oxidative cleavage near the center of the molecule of the methyl palmitate.

Based on these results, it would appear that the hydroxy groups formed by the reduction

TABLE II

Normalized Concentration of Esters of Mono- and Dibasic Acids Formed by Chromium Trioxide Oxidation of the Methyl Esters of Hydroxy Palmitate

	Normalized Volume, Percentage	
	GS analyses on DEGS w/acid	GC analyses on Apiezon L
Monobasic ester		
$CH_3(CH_2)_5COOCH_3$	3.2	3.3
$CH_3(CH_2)_6COOCH_3$	4.5	2.6
$CH_3(CH_2)_7COOCH_3$	10.9	11.5
$CH_3(CH_2)_8COOCH_3$	11.0	32.6
$CH_3(CH_2)_9COOCH_3$	20.0	17.1
$CH_3(CH_2)_{10}COOCH_3$	18.9	15.4
$CH_3(CH_2)_{11}COOCH_3$	12.8	11.0
$CH_3(CH_2)_{12}COOCH_3$	5.8	4.2
$CH_3(CH_2)_{13}COOCH_3$	1.7	1.5
$CH_3(CH_2)_{14}COOCH_3$	1.8	
$CH_3(CH_2)_{15}COOCH_3$	0.5	
$CH_3(CH_2)_{16}COOCH_3$	Trace	
Dibasic ester		
$H_3COOC(CH_2)_3COOCH_3$		
$H_3COOC(CH_2)_4COOCH_3$		
$H_3COOC(CH_2)_5COOCH_3$	2.2	2.3
$H_3COOC(CH_2)_6COOCH_3$	7.6	9.7
$H_3COOC(CH_2)_7COOCH_3$	18.1	17.7
$H_3COOC(CH_2)_8COOCH_3$	21.2	21.1
$H_3COOC(CH_2)_9COOCH_3$	18.7	19.5
$H_3COOC(CH_2)_{10}COOCH_3$	15.0	16.1
$H_3COOC(CH_2)_{11}COOCH_3$	10.7	7.8
$H_3COOC(CH_2)_{12}COOCH_3$	6.4	5.7

of the peroxides of methyl palmitate were present preferentially towards the middle of the molecule. The indication is that most, if not all, carbon atoms in the chain of the fatty ester are susceptible to oxidation, but those nearer the middle are more readily oxidized than the terminal ones.

In all oxidized samples of fatty esters, a group of four peaks was noted at the end of their chromatograms. Three of the peaks were relatively small, but the fourth peak, which usually appeared second in the group on DEGS and ECNSS-S columns, was of considerable size. Infrared analysis of the hydroxy esters oxidized to keto esters for purposes of the Beckmann transformation revealed that these compounds, or at least the major components of the group, were keto esters of methyl palmitate.

The presence of the large keto peak indicated that the chemical oxidation of the saturated fatty ester or the hydroxy ester was not yet completed. As will be shown later, ketones occur during these oxidations just prior to the split at the carbonyl groups, which yielded a mixture of carboxylic acids. The observed lack of the longer fragments that would have resulted from the chemical oxidation of 1, 2, 3, 4, 14, and 15-hydroxypalmitates would not appear to have been caused by an over-oxidation. Walters (22) reported several studies of oxidation of paraffins which suggested that the primary oxidation tends to occur as far as possible from the terminal group.

Based on the products formed during the oxidation of tricaprin, Crossley et al. (3) suggested that the initial attack of oxygen was primarily at the α-carbon, less at the β-position, and progressively less at subsequent carbons. No direct proof of this hypothesis was offered by these workers. Ramanathan et al. (17) suggested that dehydrogenation followed by the formation of hydroperoxides at carbon 9 was the preferred location of autoxidative attack in methyl stearate. As has been already shown above, no evidence to support this hypothesis could be found during the course of this study.

Endres and his co-workers (4,5) detected during oxidation of tripalmitin at 200C considerable amounts of an hydroxy acid, second only to free palmitic acid in the sample. The origin of this hydroxy acid is apparently the oxidative process although no hydroperoxides could be demonstrated in the triglyceride because of the high temperature of oxidation. After 24 hr of oxidation Enders tentatively identified, in the condensable volatile phase, approximately equal proportions of saturated acids with 9, 10, 11, 12, 13, and 15 carbons with smaller amounts of octanoic and myristic acids. The absence of the shorter-chain fatty acids seems to be in agreement with the results reported above regarding the preferential oxidative attack toward the center of the molecule.

c) Unlike the oxidation with chromium trioxide, only a single mono- and a single dibasic acid are formed by the hydrolysis of an amide which was obtained from an hydroxy ester by the transformation of its oxime. This feature of the Beckmann transformation method suggested that, if the autoxidative attack occurred only at even- or odd-numbered carbon atoms along the chain of the ester, a series of n + 2 carbon mono- and dibasic acids would be observed upon hydrolysis of the amides.

Ross et al. (18) used this method of analysis to confirm the structure of 12-hydroxy stearate, and Christie et al. (2) used it to determine the location of the hydroxy group in butolic acid. Both of these groups studied relatively pure preparations with a single hydroxy group on the molecule under investigation. Under these conditions the presence of smaller amounts of other products of chemical deg-

radation brought about by boiling the ester in concentrated sulfuric acid can readily be ignored. The Beckmann transformation of oximes of the mixture of isomers of hydroxy esters resulted however in the formation of many different mono- and dibasic acids. Appearance of products of the chemical degradation of the residual unoxidized methyl palmitate, as well as the breakdown products of the hydroxy ester, rendered quantitative evaluation of the fatty acids meaningless.

Qualitative examination of the products of hydrolysis revealed that none of the acids formed during the oxidation of the hydroxy esters with chromium trioxide were missing from the chromatograms of hydroxy esters which underwent transformation. This would seem to rule out the possibility of a selective oxidation of only even- or odd-numbered carbons in the chain. The absence of a pair of outstandingly large mono- and dibasic acids would also rule out a highly specific oxidative attack. Autoxidation of methyl palmitate appears to occur nonselectively along the chain of the saturated ester, with a preferential attack toward the center of the molecule.

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Composition of Bile Acids in Ruminants

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ABSTRACT

The bile acids found in sheep bile, beef bile, beef feces, sheep fetus bile, and beef fetus bile have been analyzed by using conventional techniques. Animals maintained on natural and purified diets were used. The bile acids are a complex mixture of isomeric hydroxy- and keto-5β-cholanoic acids which were substituted at one or several of the carbon atoms 3, 7, and 12. Cholic acid is the predominant bile acid found in these species. Deoxycholic acid was the major product formed from cholic acid when the animals were on a natural diet but the concentration of 3α, 12α-dihydroxy-7-keto-5β-cholanoic acid was elevated in the animals that were maintained on a high concentrated purified diet (without roughage). The fetus bile was found to contain nearly all of the bile acids found in the bile of the mature animal but in different concentrations.

INTRODUCTION

FOR MORE THAN A CENTURY there has been a continuous interest in the isolation and determination of bile acids in various animals under different conditions. Although the original isolation and discovery of the bile acids, cholic acid, deoxycholic acid, and chenodeoxycholic acid, was made on human and ox bile, there has been little work published on the complete composition of bile obtained from *Ovis aries* (sheep) and *Bos taurus* (cattle). Haslewood and co-workers have published comprehensive surveys on the bile acids found in different species (1-3), and in recent years much emphasis has been placed on a thorough understanding of the bile acid composition and metabolism of human beings (4-7). The development of new chromatographic techniques has greatly increased the possibilities for isolation and analysis of bile acids, and this has provided the stimulus for much of the research that has been done during the past few years.

The role of steroids in food digestion and absorption in ruminants is unknown. As in other animals, it is thought that the bile acids secreted by the liver of ruminants aid the absorption of fatty acids in the small intestine. Absorption of some fatty acids occurs in the rumen; however it is not known whether bile acids are involved or if the bile acids occur in the rumen. Steroids such as estrone, stilbestrol, and cholesterol have been found to increase cellulose digestion (8). It is not known whether these steroids directly influence the activity of the rumen micro-organisms; however the oral administration of stilbestrol did not have a marked effect on the microbial activity in the rumen of cows (9). The end-products of cellulose digestion are a mixture of volatile fatty acids, especially acetic, propionic, and butyric acids. These acids are thought to be absorbed through the rumen epithelium and partly through the small intestine, and the process may occur with the aid of bile acids. As a prelude to obtaining more information about the possible mechanism that bile acids might have in absorption in ruminants, identification was sought for the bile acids which are found in the bile of sheep and cattle.

Cholic and chenodeoxycholic acids conjugated with either glycine or taurine are the primary bile acids formed from cholesterol in the liver and excreted into the bile. By microbial action in the intestinal tract or the rumen, these two bile acids undergo extensive transformation into a complex mixture or mono-, di-, and tri- substituted 5β-cholanoic acids, which may either be excreted in the feces or be re-absorbed during the enterohepatic circulation and excreted into the bile (5).

Several of the bile acids present in ruminant bile have been identified. Cholic, deoxycholic, and chenodeoxycholic acids were identified in ox bile (3). Wieland and Kishi (10) isolated 3α-hydroxy-12-keto-5β-cholanoic acid, and

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Haslewood (11) described the isolation of 3α, 12α-dihydroxy-7-keto-5β-cholanoic acid and 7α, 12α-dihydroxy-3-keto-5β-cholanoic acids from cow bile. Gordon (12) identified lithocholic, 3β, 12α-dihydroxy-5β-cholanoic, deoxycholic, 3α, 12β-dihydroxy-5β-cholanoic, chenodeoxycholic, and cholic acid in ox bile. Yamasaki (13) reported on the occurrence of cholic, deoxycholic, and chenodeoxycholic acids in sheep bile. This research reports more detailed identification of the mixture of bile acids found in sheep bile and beef bile which was obtained from adults and stillborn animals and in beef feces.

MATERIALS AND METHODS

Extraction and Purification of Bile Acids in Bile and Feces

Bile samples were obtained from the gall bladders of slaughtered sheep and beef animals, that had been fed with either semi-purified or natural rations, and from the gall bladders of the stillborn fetus of the above-mentioned species. Samples of 1-5 ml of bile from each subject were extracted, saponified, and purified as described by Sjövall (14). A fresh fecal sample was collected from a beef animal that was maintained on a natural ration. Twenty-five grams from this sample were extracted and saponified, and the crude lipids were fractionated on a silicic acid (100-mesh) column as described by Eneroth, Gordon, and Sjövall (7).

Preparation of Derivatives for GLC and TLC

The methyl esters (ME) of the bile acids were prepared by dissolving the sample in a small volume of anhydrous methanol and adding a freshly distilled ice-cold diethyl ether solution of diazomethane until a persistent excess of the reagent was obtained. The reaction mixture was allowed to stand at room temperature for 25 min before the excess diazomethane and solvents were evaporated under nitrogen. The methyl esters were taken up in a small volume of acetone. The acetone solution of ME was analyzed by GLC and TLC and was used for the formation of other bile acid derivatives.

Trifluoracetates (TFA) were prepared from the bile acid methyl esters by dissolving the sample in a few drops of dry pyridine and adding an excess of trifluoracetic anhydride. The reaction mixture was heated for 15 min at 35C. The excess reagents were evaporated under nitrogen. The residue was dissolved in acetone, and an aliquot was used for GLC (15,16) and TLC. Partial trimethylsilyl (TMSi) ether derivatives were prepared by adding hexamethyldisilazane (Applied Science Laboratories Inc., State College, Pa.) to the solution of bile acid methyl esters in dimethylformamide, as described by Eneroth, Gordon, Ryhage, and Sjövall (6), and by adding hexamethyldisilazane and trimethylchlorosilane (Applied Science Laboratories) to the solution of bile acid methyl esters in dioxane as described by Briggs and Lipsky (17). The reaction mixture was then directly subjected to analysis by GLC and TLC.

Gas-Liquid Chromatography (GLC)

A modified Barber-Colman gas chromatograph Model 5000 with a hydrogen flame ionization detector was used throughout. The modification consisted of equipping the unit with an oven similar to that used in mass spectrometer-gas chromatography (18,19) so that the columns were interchangeable between the two units. Gas-Chrom P (100-120 mesh, Applied Science Laboratories) was washed, silanized with 5% dimethyldichlorosilane, and coated with 1% QF-1 (methylfluoroalkylsilicone, Wilkens Instrument and Research Inc., Walnut Creek, Calif.) in toluene as described by Horning, VandenHeuvel, and Creech (20). The glass columns (4 ft. × ¼ in. I.D.) were silanized, packed with the support, and conditioned for 72 hr at 250C with a slow helium flow. For analysis of the TFA esters, temperatures of 253, 290, and 290 were used for the column, injection port, and detector respectively. A helium inlet pressure of 50 psi, which provided a flow rate of 65 ml/min, was used. Hydrogen produced by an Elhygen hydrogen generator (Milton Roy Company, St. Petersburg, Fla.) was used at a pressure of 15 psi.

Thin-Layer Chromatography (TLC)

The adsorbent and solvent systems used for the thin-layer plates (20 × 20 × 0.4 cm) were prepared according to Eneroth (21). The detection of the methyl esters (ME), trifluoroacetates (TFA), and partial trimethyl (TMSi) silyl ether derivatives of the bile acids was carried out with the spray reagent used by Kritchevsky, Martak, and Rothblat (22). This reagent is composed of anisaldehyde 0.5 ml, glacial acetic acid 50 ml, and concentrated sulfuric acid 1 ml. After spraying, the plates were heated at 80C to develop colored spots, which were observed in both visible and ultraviolet light (3660Å).

TABLE I
Composition of Rations Fed Sheep and Beef Animals

Purified Diet No. 1		Semipurified Solid Diet No. 2		Semipurified Liquid Diet No. 3.		Natural Diet No. 4	
Ingredient	%	Ingredient	%	Ingredient	%	Ingredient	%
Starch	29.28	Starch	25.84	Molasses	92.98	Ground Milo	63.94
Dextrose	29.38	Dextrose	25.84	Urea (46% N)	4.00	Alfalfa meal	10.00
Cellulose (wood pulp)	30.00	Cellulose (wood pulp)	15.00	Minerals [c]	3.00	Cottonseed meal	10.00
Urea (46% N.)	4.2	Milo	10.00	Vitamins [b] A and D	0.02	Urea (46% N)	1.00
Corn oil [a]	1.00	Polyethylene resin	2.00			Cottonseed hulls	13.50
Polyethylene resin	1.00	Alfalfa meal	10.00			$CaHPO_4$	0.50
Choline chloride	0.10	Urea (46% N)	4.20			$CaCO_3$	0.50
Minerals [c]	4.92	Corn oil [a]	3.00			NaCl	0.50
Vitamins [b] A and D	0.20	Choline chloride	0.10			Vitamins [b] A and D	0.05
		Vitamins [b] A and D	0.02			Santoquin	0.01
		Minerals [c]	4.00				

[a] Santoquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline), added to corn oil at the level of 0.0125% of ration, is an antioxidant.

[b] NOPCO Quadrex contained 20,000 USP units of Vitamin A per gram and 2,500 USP units of Vitamin D per gram.

[c] The mineral composition was: K, 1.25%; Ca, 0.89%; P, 0.30%; Mg, 0.09%; S, 0.10%; Na, 0.33%; Cl, 0.38%; Fe, 156 ppm; Mn, 50 ppm; B, 27 ppm; Zn, 60 ppm; Cu, 11ppm; I, 0.52 ppm; Co, 0.11 ppm; F, 0.97 ppm; Mo, 1.98 ppm; Se, 0.10 ppm; and Cr, 0.11 ppm.

Aluminum Oxide Chromatography

Since the identification of small amounts of many bile acids found in the bile and feces of ruminant animals by conventional methods was very difficult in the presence of major bile acids, the methylated mixtures of bile acids therefore were chromatographed on columns of aluminum oxide (Woelm, grade III) as described by Danielsson, Kallner, and Sjövall (23). The composition of each fraction eluted was assessed by GLC as such and as TFA derivatives and by mass spectrometric analysis of the GLC effluent (GC-MS) as described by Eneroth, Gordon, Ryhage, and Sjövall (6).

Mass Spectrometry-Gas Chromatography

A description of the instrument used has recently been presented (18, 19). The GLC columns were the same as those previously described. The electron energy was 70 ev, the accelerating voltage was 3.5 kv, and the ionizing current 40 to 60 μamp. The temperatures of the column, injection port, separators, and ion source were 250C, 290C, 300C, and 310C, respectively.

Animal Diets

The composition of the four different diets is shown in Table I.

Reference Compounds

a) Authentic bile acids used were donated by Dr. Jan Sjövall: 3-keto-5β-cholanoic acid, 3β-hydroxy-5β-cholanoic acid, 3α-hydroxy-7-keto-5β-cholanoic acid, methyl 3β,12α-dihydroxy-5β-cholanoate, methyl 3α-hydroxy-12-keto-5β-cholanoate, methyl 3α,12α-dihydroxy-7-keto-5β-cholanoate, methyl 3β,7α-dihydroxy-5β-cholanoate, methyl 3α,12β-dihydroxy-5β-cholanoate, methyl 3-keto-12-hydroxy-5β-cholanoate, methyl 3β-hydroxy-12-keto-5β-cholanoate, methyl 3-keto-7α,12α-dihydroxy-5β-cholanoate, and methyl 3α,7α-dihydroxy-12-keto-5β-cholanoate; b) ursodeoxycholic acid was obtained from California Corporation for Biochemical Research, Los Angeles, Calif.; and methyl lithocholate, methyl deoxycholate, methyl cholate, and chenodeoxycholic acid were obtained from Mann Research Laboratories Inc., New York.

RESULTS

The results of GLC, TLC, and MS-GC analyses of bile acids that occur in the bile and feces of ruminant animals are summarized in Table II. The individual bile acids of the complex mixture were characterized by GLC analyses of the partial trimethylsilyl ethers and trifluoroacetyl derivatives and TLC analyses of the partial trimethylsilyl ether, methyl esters, and trifluoroacetyl derivatives. Mass spectrometric analyses of the GLC effluent obtained from the trifluoroacetyl derivatives was performed. In this investigation, comparisons with authentic compounds were carried out so that the identities could be clearly established.

TABLE II

Techniques Used in the Identification of Ruminant Bile Acids[a]

Peak No. (Figure 1 A-F)	GLC			TLC			GC-MS	Identified bile acids
	ME	TMSi	TFA	ME	TMSi	TFA		
	+		+					3β-Hydroxy-5β-cholanoic acid
1	+	+	+	+	+	+	+	3α-Hydroxy-5β-cholanoic acid
2	+		+	+		+		3β, 12α-Dihydroxy-5β-cholanoic acid
	+		+					3β, 7α-Dihydroxy-5β-cholanoic acid
3	+	+	+	+	+	+	+	3α, 12α-Dihydroxy-5β-cholanoic acid
4	+		+					3α, 12β-Dihydroxy-5β-cholanoic acid
5	+	+	+	+	+	+	+	3α, 7α-Dihydroxy-5β-cholanoic acid
6	+	+	+	+	+	+		3α, 7β-Dihydroxy-5β-cholanoic acid
7	+							3-Keto-5β-Cholanoic acid
8	+		+				+	3β-Hydroxy-12-keto-5β-cholanoic acid
9	+	+	+	+	+	+	+	3α, 7α, 12α-Dihydroxy-5β-cholanoic acid
	+		+				+	3-keto-12-Hydroxy-5β-cholanoic acid
10	+		+	+		+	+	3α, Hydroxy-12-keto-5β-cholanoic acid
11	+		+				+	3α, Hydroxy-7-keto-5β-cholanoic acid
12	+	+	+	+	+	+	+	3α, 12α-Dihydroxy-7-keto-5β-cholanoic acid
13	+		+				+	3α, 7α-Dihydroxy-12-keto-5β-cholanoic acid
	+		+				+	3-keto-7α, 12α-Dihydroxy-5β-cholanoic acid

[a]The notation + refers to identification of unknown bile acids by comparing R_t, R_f, and MS with that of reference compounds.

Figure 1 (A-F) shows the gas chromatographic tracings obtained from analyzing the TFA derivatives of the complex mixture of bile acids which were present in mature sheep bile, sheep fetus bile, mature beef bile, beef fetus bile, and mature beef feces.

Monosubstituted Cholanoic Acids

3β-Hydroxy-5-β-cholanoic acid, as it appears from the relative areas of ME peaks, occurs in the bile and feces of investigated ruminant animals at about one-tenth of lithocholic acid concentration. The relative retention time of the TFA derivative of this bile acid is slightly shorter than that of lithocholic acid. Hence it shows up at about the same position of peak 1, Figure 1.

Lithocholic acid (peak 1, Figure 1), which was shown to occur in ox bile (12), is the predominant bile acid in this group and appears to be present in higher concentration in the fetus bile (Figure 1, C and E) than in the bile of mature animals (Figure 1, A, B, and D). Such a high concentration of this compound is probably related to the relatively high concentration of chenodeoxycholic acid (peak 5, Figure 1, C and E) that was shown to be the precursor of lithocholic acid by microbial action in the intestinal tract (5) of rats and human beings. But these results might be better explained by the findings of Mitropoulos and Myant (24) that lithocholic acid is the intermediate in the formation of chenodeoxycholic acid when rat-liver mitochondria were incubated with (4-^{14}C) cholesterol in the presence of a soluble supernatant fraction. The 3-keto-5β-cholanoic acid (peak 7, Figure 1) is not well resolved from the ursodeoxycholic acid (peak 6) and is present in small amounts.

Cholanoic Acids Substituted Both at C_3 and C_7

3β, 7α-Dihydroxy-5β-cholanoic acid, which was shown to be a bacterial artifact product from chenodeoxycholic acid in other species (5), is one of the minor components of ruminant bile acids. Its TFA peak is obscured (masked) by that of deoxycholic acid since it appears at about the same position as peak 3, Figure 1 (A-F). Chenodeoxycholic acid (peak 5, Figure 1), which was reported to be present in the bile of both sheep (13) and beef (3, 12) and to be formed from cholesterol in animal liver (5), is surprisingly low in concentration in the bile of mature ruminant animals (Figure 1, A, B, and D) but very high in the fetus bile of these species (Figure 1, C and E).

Ursodeoxycholic acid (peak 6, Figure 1) and 3α-hydroxy-7-keto-5β-cholanoic acid (peak 11, Figure 1), which were shown to be the products of bacterial transformation of chenodeoxycholic acid in the intestinal tract (5), are present in relatively low concentrations in the bile and feces of cattle and sheep.

Cholanoic Acids Substituted Both at C_3 and C_{12}

3β, 12α-Dihydroxy-5β-cholanoic acid (peak 2, Figure 1), which was identified in ox bile (12) and was shown to be produced in the intestine by action of micro-organisms on cholic acid (5), appears to be produced in relatively small amounts in the ruminants. 3α, 12β-Dihydroxy-5β-cholanoic acid (peak 4,

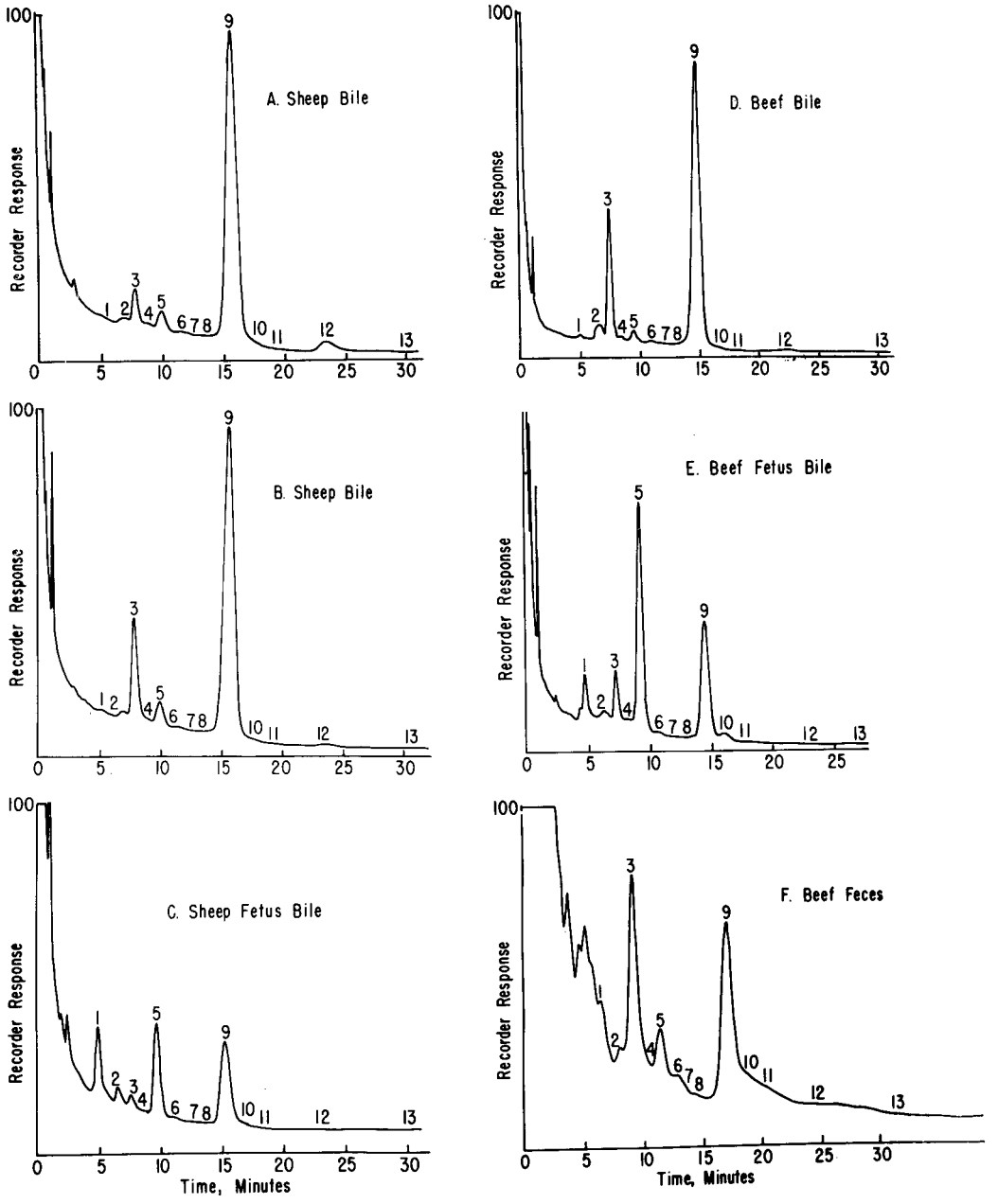

FIG. 1. Gas-chromatographic analysis of TFA derivatives of bile acids obtained from *A*, sheep fed semipurified liquid diet No. 3, Table I; *B*, sheep fed semipurified liquid diet No. 3, supplemented with 1 lb/day of cottonseed hulls, Table I; *C*, sheep fetus; *D*, beef fed natural diet No. 4, Table I; *E*, beef fetus; *F*, feces of beef fed natural diet No. 4, Table I.

Figure 1, A-F) that was shown to occur in ox bile (12) and 3β-hydroxy-12-keto-5β-cholanoic acid (peak 8, Figure 1, A-F) appear, as the present investigations indicate, to be present as minor components in the sheep and bovine bile acids. Both of these bile acids were found to be bacterial products from cholic acid (5).

Deoxycholic acid (peak 3, Figure 1, A-F), which was identified previously (3,12,13), appears to be the major product from the microbial transformation of cholic acid in the in-

testines of mature animals that were fed bulky rations (Figure 1, B, D, and F), but it is relatively low in the bile of animals that were fed high concentrated rations (Figure 1A) and in the bile from fetus (Figure 1, C and E).

3-Keto-12-hydroxy-5β-cholanoic acid, which was shown in other species to be a microbial product from cholic acid (5), is present in small amounts in ruminant bile and feces. Its small TFA peak, though it has a slightly longer relative retention time, is fully eclipsed by that of cholic acid (peak 9, Figure 1, A-F).

3α-Hydroxy-12-keto-5β-cholanoic acid (peak 10, Figure 1, A-F), identified in ox bile (10) and shown to be produced from cholic acid by intestinal microflora (5), occurs in small to moderate concentrations, but its peak was masked to a great extent by the descending side of the cholic acid peak.

Trisubstituted Cholanoic Acids

Cholic acid (peak 9, Figure 1, A-F) was shown to occur in sheep and beef bile (3,12, 13) and to be formed from cholesterol in animal liver (5). It appears to be the predominant bile acid in the bile of mature ruminant animals (Figure 1, A, B, and D) but not in the fetus bile of these species (Figure 1, C and E).

3α, 12α-Dihydroxy-7-keto-5β-cholanoic acid (peak 12, Figure 1, A-F), reported to occur in cow bile (11), is relatively higher in the bile of animals fed high concentrate ration (Figure 1, A) than in the bile and feces of animals when the rations were supplemented with roughage (Figure 1, B, D, and F).

3-Keto-7α, 12α-dihydroxy-5β-cholanoic acid, which was isolated from cow bile (11), and 3α, 7α-dihydroxy-12-keto-5β-cholanoic acid were both shown to be produced from cholic acid by micro-organism action in the intestinal tract of simple stomach animals (5). They occur in relatively small amounts and at about equal concentration (from ME peaks) in the bile and feces of animals investigated by the present studies. The TFA peaks of both these acids appear at about the same position (peak 13, Figure 1, A-F).

DISCUSSION

These studies show that the bile acids found in sheep bile (Figure 1, A and B), beef bile (Figure 1, D), beef feces (Figure 1, F), sheep fetus bile (Figure 1, C), and beef fetus bile (Figure 1, E) are a mixture of a large number of isomeric hydroxy- and keto-5β-cholanoic acids substituted at one or several of the positions 3, 7, and 12.

The qualitative patterns shown in Figure 1, A-F for the occurrence of the bile acids in the bile of mature sheep and beef as well as in the fetus bile of these species and the feces of beef are quite similar. The GLC analyses of the TFA derivatives of bile acids from the sheep bile,[2] on semipurified and purified diets, show about the same quantitative patterns as those obtained in Figure 1, B, which was obtained from the bile of two sheep fed the liquid diet (Diet No. 3, Table I), supplemented with 1 lb of cottonseed hulls daily. Also the beef bile (Figure 1, D) that was obtained from an animal on natural ration (Diet No. 4, Table I) was similar in composition to the various sheep biles assayed. In contrast to this trend, it was of interest to observe that the bile obtained from the two sheep which were fed the liquid diet (Diet No. 3, Table I) only (without cottonseed hulls) was relatively lower in deoxycholic acid but higher in 3α, 12α-dihydroxy-7-keto-5β-cholanoic acid (Figure 1, A) than that of all other treatments (Figure 1, B and D).

On the basis of the scheme proposed by Eneroth, Gordon, and Sjövall (7), which summarized the metabolic interrelations among the various bile acids, and previous observations of the metabolism of labeled bile acids in the liver and intestine of various animals and man (5), a rationalization of the above results can be offered. In ruminant animals fed a natural diet a favorable environment is provided for the microflora inhabiting the digestive tract, and deoxycholic acid is the major product to be formed from the microbial modification of cholic acid (Figure 1, B, D, and F). However, when such conditions no longer exist (Figure 1, A), for example, when the animals are fed very high concentrate rations, the reversible bacterial transformation of cholic acid to 3α, 12α-dihydroxy-7-keto-5β-cholanoic acid becomes an important pathway. This conversion appears to compete effectively with the irreversible pathway that leads to the conversion of cholic acid to deoxycholic acid. The change in microflora found in the host digestive tract is believed to be the corrective factor which is involved.

The high concentration of cholic acid, as compared with the moderately high concentration of deoxycholic acid, and the very low con-

[2] Obtained from four treatments of two sheep each that were on the same semipurified rations (Diet No. 2, Table I) but injected with different levels of urease and from two other treatments, also of two sheep each. In one case the animals were fed purified rations (Diet No. 1, Table I), and in the other they were fed natural rations (Diet No. 4, Table I).

centration of the chenodeoxycholic acid in the bile of mature sheep and beef animals (Figure 1, A, B, and D) raised some question as to whether, in ruminants, cholic and chenodeoxycholic acids are the primary bile acids formed from cholesterol in the liver (5), a transformation known to occur in several other species. Therefore, to help clarify this point, an analysis of the bile of ruminant fetus was conducted. It was found that the bile from the fetus of both sheep (Figure 1, C) and beef (Figure 1, E) provided convincing results that chenodeoxycholic acid is the predominant compound in the fetus bile. Cholic acid occurs in the next highest concentration, and lithocholic acid is higher in concentration than in beef and sheep bile (Figure 1, A, B, and D) and in beef feces (Figure 1, F). The occurrence in the fetus bile (Figure 1, C and E) of almost all the products of the microbial transformation of the primary bile acids found in the mature sheep (Figure 1, A and B) and beef (Figure 1, D) bile as well as in beef feces (Figure 1, F) could indicate that these various products were transferred from the mother's blood to the fetus through the placental membrane.

To establish that a pathway for the elimination of cholesterol from the body of ruminants is made by its conversion into the primary bile acids in the liver (and these, as a consequence of extensive transformation by the microflora of the digestive tract, are excreted as a complex mixture of bile acids in the feces), the study of the beef fecal bile acids was undertaken. The results are shown in Figure 1, F. A comparison of the relative concentrations of cholic and deoxycholic acids in both beef bile (Figure 1, D) and feces (Figure 1, F) indicated the extent of the microbial transformation of cholic acid that occur in the digestive tract. Various bile acids are produced, but primarily the accumulated end-product is deoxycholic acid. The relatively higher concentration of chenodeoxycholic acid in the beef feces (Figure 1, F) than in the beef bile might indicate that this acid is less available for absorption into the enterohepatic circulation than cholic acid.

The composition of the bile acids found in the bile of mature and fetus ruminants and in beef feces has been investigated. The mixture is more complex than has previously been recognized. The evidence points to only small variations in bile acid concentration attributable to different dietary conditions.

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The Fatty Acid and Aldehyde Composition of the Major Phospholipids of Mouse Brain[1]

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ABSTRACT

Phospholipid classes were separated from mouse brain lipid extracts by preparative thin-layer chromatography (TLC). Methyl esters were prepared from the intact phospholipids by direct transesterification at room temperature in the presence of silica gel by using 0.5 M NaOH-methanol in order to prevent interference by aldehydes or derivatives. Dimethyl acetal derivatives of phosphoglyceride alkenyl ethers (alkenyl moiety with a double bond in 1,2 position relative to oxygen linkage) were prepared, using 5% concentrated HCl in methanol, followed by preparative TLC for isolation.

The major phospholipids present were ethanolamine phosphoglycerides (EPG) 39.8%, choline phosphoglycerides (CPG) 39.7%, serine phosphoglycerides (SPG) 15.0%, and sphingomyelin (Sph) 5.4%. One-fifth of the total phospholipids (PL) were in the form of plasmalogens, mainly EPG. Choline and serine plasmalogens were present in trace quantities. The major aldehyde components of the plasmalogens were 16:0, 18:0, and 18:1.

The EPG were rich in long-chain polyunsaturated fatty acids, including 28.8% of 22:6 and 17.0% of 20:4, but contained only 7.2% of 16:0. In contrast, the CPG contained 39.6% of 16:0, and 31.0% of 18:1 with a small content of polyunsaturated fatty acids. The SPG exhibited a still different pattern containing 38.2% of 18:0, 23.2% of 18:1, 24.3% of 22:6, 2.9% of 16:0, and 3.8% of 20:4.

INTRODUCTION

ALTHOUGH THE COMPOSITION of the apolar side-chains influences the properties of biological membranes (1,2) and brain tissue is a rich source of several of the phospholipids, only a small amount of information is available on the composition of apolar side-chains from brain phospholipids. Alkenyl ether concentrations (3) and aldehyde compositions (4) of various species have been reported. O'Brien et al. (5,6) found that fatty acid and aldehyde compositions were specific for each of the phospholipids of human brain. Some data are available for rat brain fatty acids (7-12). The present paper gives the first description of the fatty acid and aldehyde composition of mouse brain. The methods used for derivative formation are selective and convenient for phosphoglycerides which contain alkenyl ether side-chains.

METHODS

Preparation of Lipid Extract

Five male and five female genetically homogenous C57/BL10J mice, 24 months of age, were sacrificed by cervical section. The brains, excluding olfactory lobes, were removed, weighed (4.0 g), and dispersed in 70 ml of chloroform-methanol, 2:1 (v/v) by using a Potter-Elvehjem type homogenizer equipped with a Teflon pestle. The brain extract was filtered, taken to dryness to denature proteolipids, and partitioned with a NaCl solution according to Folch et al. (13). The washed extract was taken to dryness, and the lipids were dissolved in chloroform.

Isolation and Separation of Phospholipids

A 1-cm column containing 10 g of silicic acid (Unisil, Clarkson Chemical Co., Williamsport, Pa.) in chloroform was prepared for the separation of polar lipids from nonpolar lipids. The lipid extract was placed on the column in chloroform solution, nonpolar lipids were eluted with 100 ml of chloroform, and polar lipids were eluted with 200 ml of methanol. The nonpolar lipids were separated for another investigation. Gottfried's method was used for phosphorus determinations (14). The iodine addition method of Gottfried and Rapport (15) without a correction factor was used for alkenyl ether determinations.

Phospholipid classes were separated by preparative TLC by using 0.25-mm layers (Silica Gel G, Brinkmann Instruments, Westbury, N. Y.) impregnated with 0.01 M Na_2CO_3 by

[1]Presented in part at the AOCS Meeting, New Orleans, May 1967.

development with chloroform-methanol-15 M NH_4OH, 65:28:4 (v/v/v) (16). After development, the thin-layer plates were dried briefly with an air gun. Lipid bands were detected under ultraviolet light after spraying with 0.2% 2,7-dichlorofluorescein in ethanol, and the desired phospholipid bands were scraped into tubes for methanolysis.

Preparation of Methyl Esters and Dimethyl Acetals

The methanolysis reaction mixture contained 2-8 μmoles of phosphoglycerides (with or without silica gel) and 3 ml of 0.5 M NaOH-methanol. The mixture was agitated periodically for 30 min at room temperature. After the addition of 3 ml of water, the methyl esters were recovered by extracting three times with 5 ml of hexane. A portion of the lower phase was removed for phosphorus assay. Unreacted lipids from the remaining lower phase were extracted after the addition of 8 ml of chloroform and 1 ml of methanol.

The unreacted lipids were kept at 70C for 2 hr with 5% 12 N HCl in methanol in a culture tube with a Teflon-lined screw cap. After the addition of two volumes of water, the mixture was extracted three times with two volumes of hexane. Dimethyl acetals were separated from the hexane extract by preparative TLC by using 0.25-mm Silica Gel G layers and development with toluene (17). Dimethyl acetals were also prepared directly from the thin-layer fractions by HCl-catalyzed methanolysis, followed by a TLC separation.

In some instances, methyl esters were separated on $AgNO_3$-Silica Gel G TLC plates according to Morris (18).

GLC Analysis of Methyl Ester Mixtures and Dimethyl Acetal Mixtures

Aerograph Model 204-B gas chromatographs (Varian Aerograph, Walnut Creek, Calif.), equipped with hydrogen flame ionization detectors, were used. Stainless steel columns, 6 ft long and ⅛ in. I.D., packed with 10% EGSS-X (an ethylene glycol succinate-silicone copolymer) on 100-120 mesh Gas-Chrom P, were purchased (Applied Science Laboratories, State College, Pa.). Similar columns (Analabs Inc., Hamden, Conn.), packed with 20% C6 DEGS (a stablized diethylene glycol succinate polymer), on 100-110 mesh Anakrom ABS, were made available by Dr. Eric Glende. Flow rates of nitrogen carrier gas and hydrogen were approximately 20 and 27 ml per min respectively. Column temperatures of 186C and 173C were used for the respective columns. Fatty acid methyl esters were also separated on the EGSS-X column by temperature programming from 165C to 200C at 8° per minute after injection of the sample. Temperatures of the injection port and the detector compartment were approximately 290C and 250C respectively.

The methyl esters and dimethyl acetals were identified by plotting the logarithm of the retention times versus chain length (19) and, for the methyl esters, by gas-liquid chromatography (GLC) analysis after separation on $AgNO_3$-impregnated TLC plates and by comparison of retention times with known standards (Applied Science Laboratories; Hormel Institute, Austin, Minn.; National Heart Institute, Bethesda, Md.). Uncorrected peak areas measured by triangulation were used to calculate the composition of mixtures. Quantitative results with Fatty Acid Standards D and F (National Heart Institute) differed from the stated compositions by a relative error of less than 4% for D and 7% for F for major components (>10% of mixture) and less than 6% for D and 15% for F for minor components (<10% mixture).

RESULTS

The total lipid extract from 10 mouse brains contained 233 μmoles of lipid P, which was recovered quantitatively in the polar lipid fraction obtained from the silicic acid column. The polar lipids were separated by TLC. R_f values and the phospholipid composition are given in Table I. The major cerebroside com-

TABLE I

The Distribution of Alkenyl Ethers in Mouse and Rat Brain Phospholipids

		Lipid Phosphorus		Alkenyl Ether		Alkenyl Ether/Lipid P	
	R_f value	Mouse[a]	Rat[b]	Mouse[a]	Rat[b]	Mouse	Rat[b]
EPG	0.58	39.8 ± 1.2	39	92.9 ± 1.4	91.5	46.7	50
CPG	0.50	39.7 ± 1.2	42	4.4 ± 0.6	6.3	2.2	3.3
Sph	0.23	5.4 ± 0.6		ND[c]		ND[c]	
SPG	0.13	15.0 ± 0.6	19	3.5 ± 1.5	2.4	4.6	2.8

[a] Mean ± S.E.M. (n = 4).

[b] From Horrocks and Ansell (20).

[c] Not determined.

TABLE II
The Fatty Acid Composition of Mouse Brain Phosphoglycerides

	Carbon No.	Total PL[a]	EPG[b]	CPG[b]	SPG[b]
		Weight percentage			
16:0	16.0	20.1	7.2 ± 0.37	39.6 ± 1.22	2.9 ± 0.38
16:1	16.5	0.7	0.9 ± 0.06	1.6 ± 0.16	0.3 ± 0.07
18:0	18.0	23.6	16.9 ± 0.84	13.9 ± 0.64	38.2 ± 1.20
18:1	18.6	22.9	21.3 ± 0.28	31.0 ± 0.52	23.2 ± 0.41
18:2	19.2	0.5	0.6 ± 0.09	0.5 ± 0.07	—
20:0	20.0	0.4	—	0.3 ± 0.09	0.6 ± 0.16
20:1	20.5	2.9	3.2 ± 0.31	1.8 ± 0.08	1.9 ± 0.04
20:4	22.3	9.3	17.0 ± 0.57	5.4 ± 0.36	3.8 ± 0.13
22:5ω6[c]	24.2	4.6	4.1 ± 0.23	0.7 ± 0.16	3.0 ± 0.18
22:6	25.7	13.3	28.8 ± 0.93	4.8 ± 0.48	24.3 ± 1.07

[a] Mean of two analyses on a single sample.
[b] Mean ± S.E.M. (two analyses on each of two samples).
[c] Tentative identification.

ponents had R_f values of 0.74 and 0.61. Inositol phosphoglycerides represented less than 1% of the lipid P.

The base-catalyzed methanolysis procedure for preparation of fatty acid methyl esters was quantitative as judged by the absence of fatty acid derivatives in the products which were isolated after subsequent HCl methanolysis. From 85-95% of the lipid phosphorus was recovered after methanolysis. The unreacted lipid fraction which was extracted from the base-catalyzed methanolysis reaction mixture contained 65% of the original alkenyl ethers.

Several fatty acid methyl ester preparations were made by BF_3 and HCl-catalyzed methanolysis. Although the methyl esters were apparently well-separated from the dimethyl acetals by TLC with toluene development, the eluted methyl esters were always contaminated with about 5% by weight of fatty aldehydes as judged by GLC results.

Fatty acid compositions of the total phosphoglycerides and of the separated major phosphoglycerides are given in Table II, and typical gas chromatograms are shown in Fig. 1. The same fatty acid methyl esters were found in the appropriate fractions after TLC on $AgNO_3$-impregnated Silica Gel G. In addition, small amounts of 14:0 and 17:0 were found in the saturated fraction.

The molar ratio of alkenyl ether to lipid P was 0.20. More than 90% of the alkenyl ethers were in the EPG fraction (Table I). The compositions of the dimethyl acetals obtained from the major phosphoglycerides are given in Table III, and a typical gas chromatogram is shown in Fig. 2. No dimethyl acetals with a chain length longer than 18 carbon atoms were found. The EGSS-X column was not used for dimethyl acetal mixtures because the chromatograms showed skewed and extraneous peaks. After two months of use, acceptable chromatograms of dimethyl acetal mixtures were obtained.

DISCUSSION

Mouse brain phosphoglycerides are much like those of the rat. The fatty acid compositions of rat brain phosphoglycerides given by Rathbone (11) are nearly identical with the present data for the mouse. Other reported compositions (7-10) for rat brain phosphoglyceride fatty acids differ in the amount of the

TABLE III
The Aldehyde Composition of Mouse Brain Phosphoglycerides

	Carbon No.	Total PL[a]	EPG[a]	CPG[a]
		Weight percentage		
16:0	16.0	21.4 ± 0.76	25.6 ± 1.70	40.7 ± 1.58
17:br[b]	16.7	0.3 ± 0.09	tr	tr
17:0	17.0	1.6 ± 0.17	tr	tr
18:0	18.0	35.8 ± 0.63	27.8 ± 2.80	29.6 ± 0.94
18:1	18.5	40.4 ± 0.34	46.1 ± 1.43	29.7 ± 0.87
18:2	19.1	1.1 ± 0.09	tr	tr

[a] Mean ± S.E.M. (two analyses on each of two samples).
[b] Tentative identification.

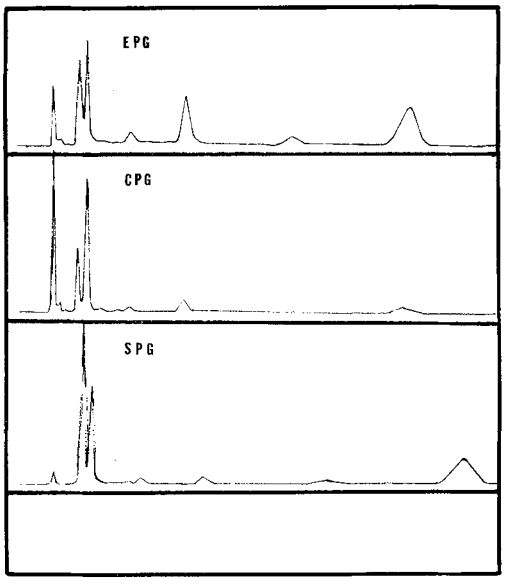

FIG. 1. Gas chromatograms of the fatty acid methyl esters from the three major phosphoglycerides of mouse brain. The marked difference between each of the phosphoglycerides will be noted. The temperature program is described in the text. The retention time for 18:0 was 4.2 min.

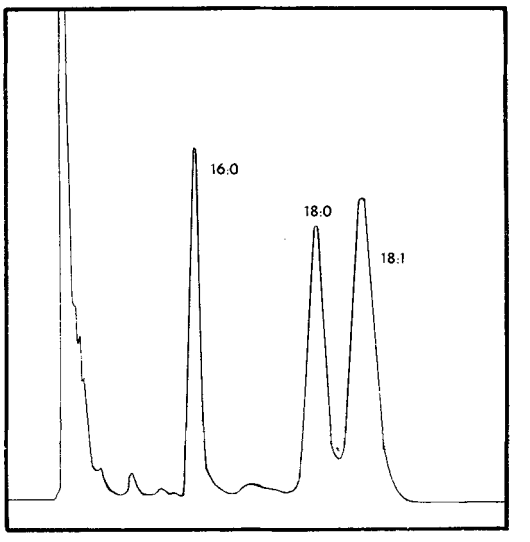

FIG. 2. A gas chromatogram at 173C of the aldehyde dimethyl acetals from the ethanolamine phosphoglycerides. The retention time for 18:0 was 9.6 min.

various 22-carbon polyunsaturated fatty acids. The fatty acid compositions of the EPG, CPG, and SPG are similar to those reported for human gray matter (5,6); the CPG composition of the mouse agrees with the CPG composition of the rat (12). Also the aldehyde composition of the total phospholipids is quite similar to the compositions reported for brains of other species (4-6). The phospholipid composition and the alkenyl ether distribution of the mouse brain and the rat brain are nearly identical (Table I).

Each of the major phosphoglycerides has a characteristic pattern of hydrocarbon substitution, which is summarized in Table IV. Alkyl ethers have not been included in the calculations. The CPG have more saturated and mono-unsaturated apolar side-chains. The EPG are quite rich in polyunsaturated fatty acids, and the SPG are intermediate. The two most characteristic fatty acids for each class are 22:6 and 18:1 for the EPG, 16:0 and 18:1 for the CPG, and 18:0 and 22:6 for the SPG. The relative absence of 18:2 from brain phospholipids has been described previously (6-12). The aldehydes are mainly 18:1, 18:0, and 16:0. The aldehyde patterns for the EPG and the CPG are also rather different.

TABLE IV

A Summary of the Apolar Side-Chains of the Major Phosphoglycerides of Mouse Brain

Type	Double bonds[a]	Total PL	EPG	CPG	SPG
			Mole percentage		
Ester	0	36.0	18.4	53.4	41.1
Alkenyl ether	0	11.8	12.5	0.8	ND[b]
Ester	1	21.6	19.2	33.5	25.4
Alkenyl ether	1	8.1	10.8	0.3	ND
Ester	>1	22.4	39.0	10.8	31.2
Alkenyl ether	>1	tr	—	—	ND

[a] Not including the 1-2 double bond of the alkenyl ethers.
[b] Not determined.

The procedures used in this investigation for the direct preparation of phosphoglyceride apolar side-chain derivatives are selective, convenient, and rapid. The base-catalyzed methanolysis procedure for the preparation of fatty acid methyl esters is quantitative and avoids contamination by aldehydes or derivatives. Use of the unreacted lipids from the step above for the preparation of aldehyde dimethyl acetals avoids most contamination by fatty acid methyl esters. The apparent recovery of alkenyl ethers after base-catalyzed methanolysis was only 65%, probably because of incomplete extraction, but comparison of the EPG composition with the aldehyde composition of the total phospholipids shows that representative samples were obtained. Purification of the acetals by preparative TLC is recommended because of

the possible interference by traces of methyl esters from incomplete extraction, unreacted phosphoglycerides, or from sphingolipids. Evidence of decomposition of dimethyl acetals was observed when a new GLC column was used. Cyclic acetal derivatives (21) of the alkenyl ethers may be better for this analysis. The unreacted lipid residue from the acid-catalyzed methanolysis step may be suitable for the preparation of alkyl ether derivatives (20).

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Effects of Experimental Endogenous Hyperlipemia on Circulating Leukocytes and Erythrocytes

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ABSTRACT

Effects of hyperlipemia on circulating leukocytes and erythrocytes were studied in dogs which were given repeated, intravenous injections of a nonionic detergent, Triton WR-1339.

Erythrocyte lipid concentrations increased from 3.6 ± 0.9 mg/10^{10} cells in control animals to 9.3 ± 1.5 mg in the hyperlipemic group. This increase was accompanied by a shift in the distribution of total fatty acids toward a higher percentage of saturated and monounsaturated acids. In contrast to the changes observed in erythrocytes, the leukocyte lipid content remained unaltered in dogs with serum cholesterol levels ranging from 500 to more than 2,000 mg/100 ml. Leukocyte counts rose whereas hematocrit values, hemoglobin concentrations, and erythrocyte counts decreased. Oxygen utilization studies showed no significant metabolic differences between leukocytes which were isolated from hyperlipemic or control animals. Circulating leukocytes in dogs with an endogenously induced hyperlipemia were shown therefore to maintain normal lipid concentrations and did not participate in lipophage formation, as reported for certain diet-induced lipemias.

INTRODUCTION

BLOOD LEUKOCYTES HAVE been suggested as a source of lipid-laden foam cells in the atherosclerotic arterial intima of man (1,2) and experimental animals including dogs (3,4) and rats (5,6). Studies implicating leukocyte participation in experimental atherogenesis involved diet-induced hyperlipemia, yet few have dealt primarily with the effects of hyperlipemia on the circulating blood cells.

An endogenous type of hyperlipemia was produced in dogs in the present study by chronic intravenous injections of Triton WR-1339 without raising dietary lipids. Possible correlations between plasma lipid concentrations and the metabolism or lipid composition of circulating leukocytes and erythrocytes were sought.

METHODS

Six male and two female mongrel dogs, weighing between 11 and 17 kg, were fed a stock diet containing 61% carbohydrate, 32% protein, and 7% fat for 7 to 9 months. A 20% solution (w/v) of Triton WR-1339 (an oxyethylated tertiary octylphenol formaldehyde polymer, Winthrop Laboratories, New York) was prepared in 0.15 M sodium phosphate buffer at pH 7.2, sterilized in sealed ampoules, and stored at 4C for no longer than 2 weeks. One female and three male dogs were injected intravenously with 100 to 200 mg Triton/kg body weight twice weekly while the remaining four received 0.5 to 1.0 ml buffer/kg.

Venous blood was collected from animals, fasting for at least 12 hr, with either 10 units heparin or 1 mg EDTA/ml. Erythrocytes were sedimented from leukocytes by the following procedure modified from Skoog and Beck (7). Equal volumes of blood and 3% dextran (Nutritional Biochemicals Corporation, Cleveland, Ohio) (mol wt 2-3 x 10^5) in 0.9% NaCl were mixed and allowed to stand 1 hr at 25C. The leukocyte-rich layer was drawn off and centrifuged at 110 x g for 10 min. The loosely packed leukocytes were twice resuspended to 15 times their volume with 0.9% NaCl and centrifuged 5 min at 110 x g. Erythrocytes were washed three times by adding two volumes saline and separated by centrifuging 10 min at 500 x g. The cells were finally suspended in 0.9% NaCl for cell counting and lipid extraction. The ratio of erythrocytes to leukocytes in the leukocyte suspensions varied between 1 and 9 whereas the erythrocyte suspensions were virtually free of leukocytes. All data concerning leukocytes were corrected for contamination by erythrocytes.

Leukocyte oxygen consumption rates were determined manometrically in Warburg type microflasks with a differential respirometer (Model G14, Gilson Medical Electronics, Middleton, Wis.). Between 20 and 50 x 10^6 leukocytes in 2 ml of isotonic Krebs-Ringer phosphate buffer at pH 7.3 were incubated at 37C after the flasks, which contained 0.3 ml 20% KOH in the center well, were gassed

with 5% CO_2 in air. Oxygen uptake was measured, after equilibration, at 15-min intervals for 2 hr.

Lipids were extracted from the leukocytes and erythrocytes with chloroform-methanol (2:1) (8). These extracts as well as lipids from serum samples collected biweekly were analyzed for total cholesterol, free cholesterol, triglycerides, and phospholipids after separation of the extracts into polar and nonpolar lipids by silicic acid chromatography (9). Fatty acid distributions in lipid extracts were obtained by gas chromatography and expressed as weight percentage, calculated from peak areas of unknowns compared with those of internal standards (9). Lactic and pyruvic acids in blood were measured with kits (Boehringer Mannheim Corporation, New York) by an enzymatic method involving an ultraviolet spectrophotometric determination of reduced nicotinamide adenine dinucleotide.

Smears of air-dried peripheral blood leukocytes were examined by light microscopy by using both polarized and standard illumination. May-Grünwald-Giemsa was used for routine morphology, and lipids were stained by oil red O, Sudan Black B, and Nile Blue sulfate. Phospholipids were identified by Baker's method. Cholesterol and its esters were investigated by using Schultz' modification of the Lieberman-Burchard reaction and by osmium tetroxide-alpha naphthylamine for cholesterol esters and triglycerides (10). Differential leukocyte counts were also done after staining with Wright stain.

RESULTS

Serum lipids increased slowly for 4 to 12 weeks in the Triton-treated group until the cholesterol concentration rose to approximately 500 mg/100 ml, after which a rapid rise in all lipids occurred. Variations in response to Triton in 2 dogs are illustrated in Fig. 1 and 2. Dog No. 2 was given alternate 100 mg/kg and 200 mg/kg doses of Triton from the eleventh through the nineteenth week, and the resulting fall in lipids is shown in Fig. 2. The quick return to hyperlipemia was also evident after the twentieth week when the 200 mg/kg injections were resumed. Injections of phosphate buffer without Triton in the control group had no effect on blood lipids. Triton-injected dogs were considered hyperlipemic only when serum total cholesterol exceeded 500 mg/100 ml; control dog values ranged from 140 to 300 mg/100 ml serum. Data pertaining to blood cells isolated from animals which had been maintained under hyperlipemic conditions for 3 to 6 months were grouped since they appeared to be independent of the

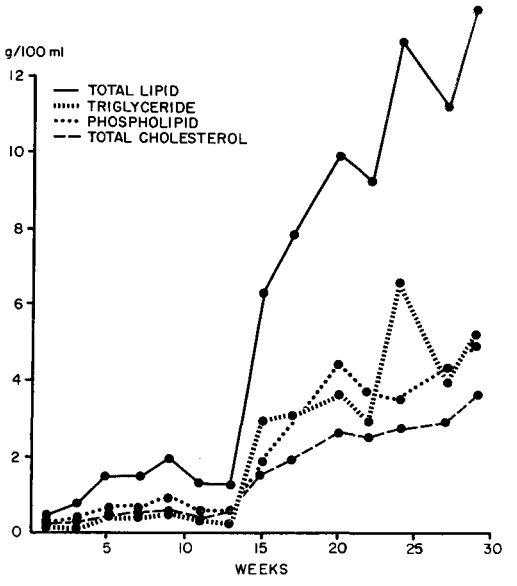

FIG. 1. Variation of serum lipids in Dog No. 1 during the first 30 weeks of Triton injections. The ordinate units in Figures 1 and 2 are given in g/100 ml. Typical control serum cholesterol values approximated 0.2 g/100 ml (200 mg%).

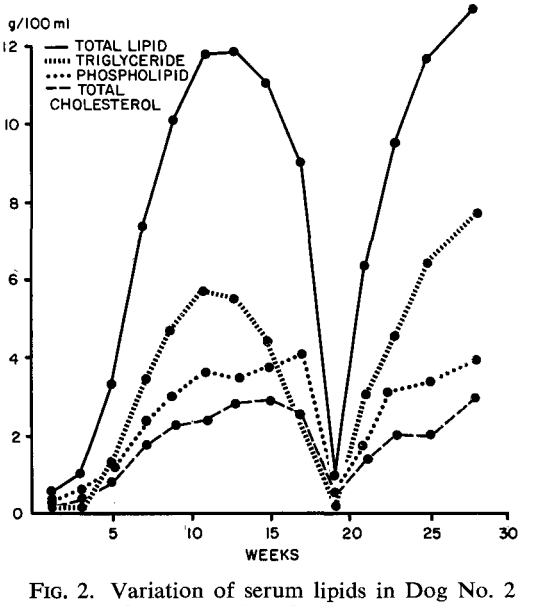

FIG. 2. Variation of serum lipids in Dog No. 2 during the first 30 weeks of Triton injections.

TABLE I
Distribution of Cholesterol, Phospholipid, and Triglyceride in Erythrocytes

	Control %	Triton[a] %	p
Cholesterol	35.1 ± 1.48[b]	35.9 ± 3.86	>0.4
Phospholipid	61.5 ± 1.90	50.5 ± 7.17	<0.001
Triglyceride	3.3 ± 2.01	13.6 ± 9.19	<0.001

[a]Serum cholesterol concentrations at the time of erythrocyte isolation from blood ranged from 810 to 2850 mg/100 ml in hyperlipemic dogs maintained for 3 to 6 months with cholesterol levels exceeding 500 mg/100 ml serum. Results were independent of the degree of hyperlipemia.

[b]Percentage distribution of the 3 lipids by weight ± standard deviation.

degree of hyperlipemia attained at the time of sampling (Table I).

The rate of oxygen utilization by leukocyte suspensions in Krebs-Ringer phosphate buffer was not related to the lipid concentration of the blood from which the leukocytes were isolated. Oxygen uptake by control group leukocytes, expressed as average ± standard deviation, was 70 ± 25 microliters 0_2/hr/10^8 leukocytes and 93 ± 26 ($p>0.1$) with leukocytes from hyperlipemic animals for the first hour of incubation when no substrate was added. Similar results were obtained from limited studies by using 5.5 mM glucose as substrate. In 15 mM succinate the values were 107 ± 33 microliters 0_2/hr/10^8 leukocytes and 123 ± 33 ($p>0.1$) for the control and hyperlipemic groups respectively. Erythrocytes consumed no oxygen even in numbers exceeding 100 times those contained in the leukocyte suspensions. Neither the concentrations nor the molar ratios of lactic and pyruvic acids in plasma changed with increasing blood lipids.

The total lipid content of leukocytes isolated from control dogs was 464 ± 256 mg/10^{10} cells and did not differ significantly from the 403 ± 165 mg/10^{10} cells observed in hyperlipemia. In contrast to the lack of response in leukocytes, the amount of lipid increased from 3.6 ± 0.89 mg/10^{10} erythrocytes to 9.3 ± 1.47 mg ($p<0.001$) in the hyperlipemic group. Comparison of the percentage distribution of total cholesterol, phospholipid, and triglyceride in erythrocytes from both groups (Table I) showed that the cholesterol percentage of the combined lipid remained the same but the proportion of triglyceride increased four-fold in red blood cells of hyperlipemic dogs. The fatty acid distributions in erythrocyte total lipid extracts were also affected by increased lipemia in that saturated and monounsaturated acids were elevated whereas arachidonic was lowered (Table II). Leukocyte fatty acids however did not change from the normal distribution found in isolated total lipid.

TABLE II
Distribution of Fatty Acids in Erythrocytes

Fatty Acid[a]	Control %	Triton %	p
14.0	0.8 ± 0.24[b]	1.3 ± 0.35	<0.005
16:0	19.1 ± 0.87	23.4 ± 0.89	<0.001
16:1	1.6 ± 0.33	2.7 ± 0.63	<0.001
18:0	27.4 ± 2.15	24.0 ± 5.06	>0.1
18:1	14.5 ± 1.47	18.5 ± 2.85	<0.01
18:2	10.7 ± 1.65	13.3 ± 7.06	>0.4
20:4	22.8 ± 3.66	16.0 ± 4.30	<0.01

[a]Number of carbon atoms : number of double bonds.
[b]Percentage distribution ± standard deviation.

Although the lipid content per leukocyte did not change, the white blood cell count rose from 7.2 x 10^6/ml blood to 11.0 x 10^6 ($p<0.02$). The differential leukocyte counts however remained normal in the hyperlipemic animals. Erythrocyte counts exhibited the opposite effect, dropping from 4.8 x 10^9 to 2.8 x 10^9 cells/ml ($p<0.001$). Hemoglobin was also reduced from 15.8 to 11.5 g/100 ml blood ($p<0.001$), and packed cell volume decreased from 46.7 to 33.6 ml/100 ml blood ($p<0.001$). Changes in leukocyte lipid content and vacuole formation were not detected in leukocyte smears by any of the staining procedures listed in Methods, which are being used in present studies to demonstrate increased lipids in leukocytes of patients with familial hyperlipidemias.

DISCUSSION

Leukocytes in rats on an atherogenic diet have been shown to undergo lipid vacuole formation in lymphocytes and particularly in the monocyte fraction where approximately half of the leukocytes are lipophages (11,12). Triton-induced lipemia in the present study with dogs however did not produce increased leukocyte lipid content or a change in the differential leukocyte count although the total white blood cell count was elevated as it was with the rats fed lipid-supplemented diets. The shift toward increased saturation of the fatty acids observed in erythrocytes of the hyperlipemic animals has also been shown recently in the serum and certain tissues of Triton-treated dogs (9,13).

Erythrocyte responses were similar to those

observed with dietary hyperlipemia (12) in that erythrocyte counts decreased together with hematocrit and hemoglobin values. Whether the increase in erythrocyte lipids was caused by actual intracellular incorporation of plasma lipid or an increase in membrane-bound material is not known.

The method employed for leukocyte isolation resulted in a mixed leukocyte population with a distribution of cell types like that in whole blood. Lipid concentration changes occurring in one kind of leukocyte present in small numbers, such as monocytes, could go undetected in a lipid extract of these heterogenous cell suspensions. The cytochemical staining results together with the unaltered fatty acid distribution patterns however support the conclusion that leukocytes are capable of maintaining normal lipid levels even in an extremely lipemic environment produced by a nonionic detergent. Any lipid accumulation in tissues of Triton-treated animals therefore does not come from the penetration of the tissue by lipid-laden peripheral leukocytes or lipophages.

ACKNOWLEDGMENTS

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Autoxidation of Tissue Lipids. II. Monocarbonyl Compounds Formed by the Autoxidation of Methyl Eicosapentaenoate, Methyl Docosahexaenoate, and Cod-liver Oil

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ABSTRACT

Fatty acid analysis of autoxidized cod-liver oil with a peroxide value of 192 showed significant degradation of only eicosapentaenoic, docosapentaenoic, and docosahexaenoic acids of the linolenate family. Purified, mildly autoxidized cod-liver oil with a peroxide value of 28, methyl eicosapentaenoate, and methyl docosahexaenoate produced carbonyl patterns in agreement with the accepted mechanism for olefinic autoxidation. In all cases the major products were propanal and *n*-pent-2-enal as predicted, and *n*-hex-2-enal and *n*-hept-2-enal as reported in the literature for linolenate. In addition, the same cod-liver oil, which had been heated to 188C in vacuum for 1 hr to decompose completely the hydroperoxides before carbonyl analysis, showed the presence of *n*-hepta-2,4-dienal as predicted.

INTRODUCTION

THE FIRST REPORT in this series (1) suggested that a low level of autoxidation occurred in the adipose tissue lipids of vitamin E-deficient rats fed a purified diet containing 5% cod-liver oil. This study was based on carbonyl micro-analysis (2-5), which appears to be one of the most sensitive methods for evaluating the phenomenon.

The monocarbonyl compounds isolated from the tissue lipids of the vitamin E-deficient cod-liver oil-fed rats conformed generally to the pattern predicted by the classical mechanism (6-8) for the autoxidation of olefins of the linolenate type (9-12) with the important exception that *n*-butanal was the principal alkanal, and propanal was not observed. Since the isolated carbonyls apparently resulted from the autoxidation of the linolenic family fatty acids which were incorporated from the cod-liver oil diet, the present study was designed to determine the pattern of carbonyl compounds produced during the low-level autoxidation of cod-liver oil and its constituent polyunsaturated fatty acids. This pattern should also be of interest to those studying autoxidation in food products of aquatic origin.

EXPERIMENTAL

Preparation of Autoxidized Lipids

Cod-liver oil (General Biochemicals Corporation) was dissolved in hexane and rendered carbonyl-free and hydroperoxide-free by passage through a 2,4-dinitrophenylhydrazine/H_3PO_4 reaction column (3), followed by chromatography on activated magnesia (2). When this process was carried out in a nitrogen atmosphere, the resulting oil, after evaporation of the hexane under reduced pressure at 50C, contained no detectable peroxides (13). Methyl eicosapentaenoate and methyl docosahexaenoate (99%) were obtained from the Hormel Foundation and used without further purification.

The purified cod-liver oil was allowed to oxidize at room temperature (25C) in a 100-ml, round-bottom flask for three days with a stream of dry air directed on its surface. The final peroxide value was 28. After autoxidation, one sample of oil was evacuated and purged with nitrogen three times before being heated at 188C (propylene glycol bath) under aspirator vacuum for 1 hr. This procedure has been reported to effect the complete decomposition of hydroperoxides to carbonyl compounds (14). The methyl esters were allowed to oxidize in the same manner for the same period of time as the cod-liver oil. Final peroxide values for these were not determined. A second sample of cod-liver oil was allowed to oxidize for seven weeks to a peroxide value of 192 under the same conditions. This sample was subjected only to fatty acid analysis.

Monocarbonyl Analysis

Carbonyl isolation and analysis were accomplished by the methods of Schwartz et al. (2-5) as in the first study of this series (1). Briefly, the method involves conversion of the carbonyl compounds in the lipid to 2,4-dini-

trophenylhydrazones, separation of the lipid from the derivatives and purification of the derivatives by adsorption chromatography on magnesia and alumina, and fractionation of the individual derivatives by liquid-liquid partition chromatography. The concentrations were determined spectrophotometrically. The results of a single analysis are presented in Table I.

Fatty Acid Analysis of Cod-liver Oil

Methanolysis was accomplished by the AOCS method (15), followed by immediate gas-liquid chromatography on 6-ft × ⅛-in. columns of 12% polydiethylene glycol succinate on 80-100 mesh Gas-Chrom P (Applied Science Laboratories, State College, Pa.). Dual columns in a Perkin-Elmer model 810 chromatograph with a differential hydrogen flame ionization detector were used. Analyses were obtained with a flow rate of 18 ml per minute at STP, injector at 275C, and columns isothermal at 197C and programmed from 155C to 215C at 4° per minute. Samples consisted of 1 μl of a 10% hexane solution. The chromatograms were recorded on a 10-in. chart at 30 in. per hour. Qualitative and quantitative treatment of the chromatograms were performed as previously reported (1). 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%.

TABLE I

Monocarbonyl Compounds Isolated from Autoxidized Cod-liver Oil, Methyl Eicosapentaenoate, and Methyl Docosahexaenoate (moles x 10^8 per g of lipid)

Compound	Cod-liver oil (20.0 g[a])	Cod-liver oil heated[b] (21.6 g[a])	20:5ω3 (2.25 g[a])	22:6ω3 (2.35 g[a])
Alkanals				
C_3	18.6	35.7	31.4	57.8
C_4	2.88	1.90	4.55	6.80
C_5	4.51	3.31	3.66	7.79
C_6	1.08	1.80		3.15
C_7	0.68	2.05		3.83
C_8	0.33	1.50		2.85
C_9	0.47	2.07		1.64
Alk-2-enals				
C_4		0.91		
C_5	2.23	1.96	6.69	11.8
C_6	1.27	3.31	17.8	14.3
C_7		2.74	3.64	9.24
C_8	1.98	0.71	3.78	5.10
Alk-2,4-dienals				
C_7		5.70		
C_8		0.38		
C_9		0.40		
C_{10}		0.59		

[a] Quantity of fat analyzed.

[b] Heated at 188C in vacuum for 1 hr to decompose hydroperoxides.

RESULTS AND DISCUSSION

Fatty Acid Compositions

Table II shows that the untreated, purified, and mildly autoxidized cod-liver oils demonstrated no significant differences in their fatty acid compositions. The composition of the rancid cod-liver oil, which had been allowed to autoxidize to a peroxide value of 192, reveals the autoxidative degradation of almost half of the most highly unsaturated acids. Since these are members of the linolenate family, one would expect the classical carbonyl products characteristic of this family, namely propanal, hepta-2,4-dienal, and pent-2-enal, although the latter compound would have to result from the less stable unconjugated 14-hydroperoxy intermediate.

Monocarbonyl Pattern

In general, the pattern described in Table I is in agreement with expectation. In all cases the major products were propanal and *n*-pent-2-enal as predicted, and *n*-hex-2-enal and *n*-hept-2-enal as reported in the literature for linolenate (11, 12). Dienals, mainly *n*-hepta-2,4-dienal, were obtained only from the heated cod-liver oil, suggesting the stability of the diene hydroperoxides and supporting Schwartz's contention that the reaction column does not convert hydroperoxides to carbonyl compounds (2).

Studies on autoxidized methyl linolenate, the only member of the linolenate family considered previously, have demonstrated that propanal, butanal, the C_4-C_7 2-enals, and hepta-2,4-dienal are the major carbonyl products (11, 12). Frankel et al. (9, 10) have shown that the hydroperoxide concentrate from autoxidized methyl linolenate consists of 9-hydroperoxyoctadeca-10,12,15-trienoate, 12-hydroperoxyoctadeca-9,13,15-trienoate, 13-hydroperoxyoctadeca-9,11,15-trienoate, and 16-hydroperoxyoctadeca-9,12,14-trienoate. The typical secondary products of these hydroperoxides (7) are respectively deca-2,4,7-trienal which would be isolated as a dienal by the Schwartz method since the double bond in the 7 position is not part of the conjugated system, hepta-2,4-dienal, hex-3-enal which would be isolated as hexanal, and propanal.

Theoretical treatment of this phenomenon is complicated by the following factors: a) formation of atypical products from oxygen attack at less reactive α-methylene groups, b)

TABLE II

Fatty Acid Compositions of Cod-liver Oil in Various Stages of Autoxidation (major components[a], wt % total methyl esters ± SE[b])

Fatty acid[c]	Cod-liver Oil			
	Untreated, PV = 25[d]	Purified, PV = 0	Oxidized, PV = 28	Rancid, PV = 192
14:0	4.37 ± 0.16	4.47 ± 0.06	4.43 ± 0.12	4.90 ± 0.08
16:0	10.0 ± 0.22	9.87 ± 0.12	9.61 ± 0.18	11.4 ± 0.26
16:1	8.97 ± 0.07	8.94 ± 0.22	9.03 ± 0.09	10.1 ± 0.26
16:2	1.03 ± 0.06	1.16 ± 0.08	1.03 ± 0.05	1.13 ± 0.06
18:0	2.55 ± 0.05	2.60 ± 0.04	2.74 ± 0.06	2.91 ± 0.06
18:1	21.9 ± 0.16	22.5 ± 0.30	21.8 ± 0.24	24.8 ± 0.28
18:2	1.57 ± 0.07	1.51 ± 0.07	1.71 ± 0.10	1.54 ± 0.14
18:4ω3	2.47 ± 0.13	2.31 ± 0.07	2.21 ± 0.04	1.81 ± 0.08
20:1	13.1 ± 0.24	13.0 ± 0.42	13.2 ± 0.20	14.8 ± 0.12
20:5ω3	9.28 ± 0.11	9.68 ± 0.19	9.16 ± 0.17	5.91 ± 0.05
22:1	7.62 ± 0.20	7.62 ± 0.16	7.95 ± 0.10	8.65 ± 0.10
22:5ω3	1.25 ± 0.05	1.22 ± 0.08	1.27 ± 0.13	0.85 ± 0.06
22:6ω3	9.89 ± 0.10	10.4 ± 0.24	10.0 ± 0.12	5.51 ± 0.30

[a] Major components >1%.

[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] Peroxide value, microequivalents peroxide oxygen per g of lipid.

the presence of unconjugated products, c) autoxidation of the carbonyl products themselves (16), and d) the formation of dihydroperoxides (17) and their decomposition products.

The obvious presence of propanal as the major carbonyl compound formed in these experiments sheds no light on the role of butanal as the major product and the absence of propanal from the adipose tissue lipids of vitamin E-deficient, cod-liver oil-fed rats (1). This phenomenon is still under investigation. The formation of butanal in the present study can be explained by the degradation of a 12, 15-dihydroperoxyoctadeca-9,13-dienoate analog of the 20 and 22-carbon acids. Lillard and Day (16) have postulated the formation of butanal by the autoxidation of hepta-2,4-dienal in the same manner, and the formation of dihydroperoxides of undetermined structure from docosahexaenoate has been reported (17). The autoxidative formation of butanal is probably by the same mechanism as that which produces heptanal from acids of the linoleic family. Yet butanal has been isolated as a major carbonyl component from the Ringer-Locke solution perfusate of isolated rat hearts, in which it was observed to be both plasmalogen-bound and in the free state (18). This, however, could not account for the ten-fold increase in butanal from experimental animals as compared with the controls (1).

ACKNOWLEDGMENTS

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The *trans*-3-Enoic Acids of *Aster alpinus* and *Arctium minus* Seed Oils

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ABSTRACT

The *trans*-3-enoic acids of *Aster alpinus* (dwarf aster, rock aster) and *Arctium minus* (burdock) seed oils have been isolated and characterized. *Arctium* seed oil contains *trans*-3,*cis*-9,*cis*-12-octadecatrienoic acid (9.9%), and *Aster* oil contains *trans*-3-hexadecenoic (7.1%), *trans*-3-octadecenoic (1.9%), *trans*-3,*cis*-9-octadecadienoic (3.0%), and *trans*-3,*cis*-9,*cis*-12-octadecatrienoic (13.7%) acids. *Aster* oil also has an epoxy acid as a minor constituent (ca. 2.0%), which has been identified as *cis*-9,10-epoxy-*cis*-12-octadecenoic acid.

INTRODUCTION

THE FIRST FATTY ACID with *trans*-3-unsaturation to be detected in nature was *trans*-3-hexadecenoic acid, which was isolated from the leaves of spinach and antirrhinum and characterized by Debuch in 1961 (1,2). This acid has since been identified in the photosynthetic tissues of a wide variety of higher plants (3-7) and algae (8-10), and it has been shown to occupy the 2-position of phosphatidyl glycerol almost exclusively (3,10,11). Hopkins and Chisholm (12) first demonstrated the occurrence of this acid in a seed oil, namely, *Helenium bigelowii*, where it was presumably a constituent of triglycerides. At about the same time, two polyunsaturated C_{18} acids with *trans*-3-unsaturation were also characterized as components of seed oils: *trans*-3,*cis*-9,*cis*-12-octadecatrienoic acid from *Calea urticaefolia* (13) and *trans*-3,*cis*-9,*cis*-12,*cis*-15-octadecatetraenoic acid from *Tecoma stans* (14). These three species are all from the family *Compositae*.

Although these acids are obviously related, there was no report of the co-occurrence of several *trans*-3-enoic acids in a seed oil until recently, when this present work was largely completed. Kleiman and coworkers (15) showed that *Grindelia oxylepis* seed oil contained *trans*-3-hexadecenoic and -octadecenoic acids along with minor amounts of homologous *trans*-3-monoenoic acids and of dienoic and trienoic acids containing *trans*-3-unsaturation. They tentatively identified the unusual dienoic acid as *trans*-3,*cis*-12-octadecadienoic acid and the triene as *trans*-3,*cis*-9,*cis*-12-octadecatrienoic acid.

During an examination of a number of *Compositae* seed oils — we noted, on gas-liquid chromatography (GLC) and on thin-layer chromatography (TLC) on silica impregnated with silver nitrate, a number of unusual components of the esters derived from *Aster alpinus* seed oil. One of these behaved exactly like *trans*-3-hexadecenoate in these chromatographic systems and, because of the interest of this laboratory in the occurrence (6-8) and biosynthesis (16) of this acid in photosynthetic systems, it was resolved to characterize all these unusual constituents of *Aster* oil. The screening program of Earle and coworkers (17) had already demonstrated the unusual character of *Aster alpinus* seed oil in that clearly erroneous results were obtained by standard analytical techniques and by the fact that *trans*-ethylenic unsaturation was evident in the infrared spectrum. Among other oils examined by these workers (17), that of *Arctium minus* showed similar discrepancies. As seeds of this species were readily available in the wild, these were investigated also.

As reported in this paper, *Arctium minus* seed oil contained only *trans*-3,*cis*-9,*cis*-12-octadecatrienoic acid as an unusual component in a significant amount, but *Aster alpinus* contained *trans*-3-hexadecenoic, *trans*-3-octadecenoic, *trans*-3,*cis*-9-octadecadienoic, and *trans*-3,*cis*-9,*cis*-12-octadecatrienoic acids along with the normal acids. In addition to these unusual components, *Aster* seed oil also contained a small proportion of an epoxy acid, tentatively identified as *cis*-9,10-epoxy-*cis*-12-octadecenoic acid.

EXPERIMENTAL SECTION AND RESULTS

Extraction of Oil and Preparation of Methyl Esters. Seeds of *Aster alpinus* (dwarf aster, rock aster), obtained from a reputable seedsman, and of *Arctium minus* (burdock), obtained from the wild, were macerated in chloroform-methanol (2:1) with an Ultra-Turrax macerator. After being allowed to stand at room temperature for 2 hr, the seed residues

were filtered off and re-macerated in a further volume of chloroform-methanol. Fatty acid methyl esters were prepared from a portion of each seed oil by transesterification with methanol-benzene-sulphuric acid (20:10:1) under reflux for 1 hr.

Preliminary Chromatographic Studies of Mixed Esters and Isolation of Unusual Components. The mixed esters from *Aster* and *Arctium* seed oils were each analyzed by GLC on a polyethyleneglycol-adipate polyester (PEGA) stationary phase. Besides peaks corresponding to authentic samples of palmitate, stearate, oleate, and linoleate, *Arctium* esters contained a relatively major component (ca. 10%) which emerged after linoleate but earlier than authentic linolenate. *Aster* esters also contained this component (ca. 14%) and, in addition, a minor component (ca. 2%) which emerged between oleate and linoleate and a further major component (ca. 7%) which emerged after palmitate but later than would be expected for palmitoleate. This last component appeared to be identical, on GLC, to methyl *trans*-3-hexadecenoate isolated from photosynthetic tissues (16).

TLC on silica gel impregnated with silver nitrate, with two developments with diethyl ether-light petroleum (1:9), confirmed the presence of unusual unsaturated esters in these two samples. Each separated into spots corresponding to saturated esters, oleate, and linoleate plus an additional spot migrating more slowly than linoleate but faster than a standard sample of linolenate. The *Aster* esters also had a major spot migrating between the saturated esters and oleate, and corresponding exactly to standard *trans*-3-hexadecenoate, plus a minor spot migrating just behind oleate.

Thus the preliminary chromatographic studies indicated that *Arctium minus* contained an unusual trienoic acid and that *Aster alpinus* contained unusual monoenoic, dienoic, and trienoic acids. These components were isolated by preparative argentation-TLC and characterized.

The trans-Δ^3-Monoenes of Aster alpinus. GLC of the unusual monoene fraction, isolated from *Aster* mixed esters, showed the presence of two main components, in an approximate ratio of 4:1. These had carbon numbers (18) of 16.60 and 18.60 respectively on PEGA stationary phase (the carbon numbers of palmitoleate and oleate were 16.30 and 18.30 respectively on PEGA) and of 15.90 and 17.90 on a silicone elastomer (SE-30) stationary phase. The values of 16.60 and 15.90 were identical with the carbon number values of authentic *trans*-3-hexadecenoate on these two phases. There was a small amount (ca. 1% of this fraction or 0.1% of total methyl esters) of a C_{14} homologue with a carbon number of 14.60 on PEGA and 13.90 on SE-30, plus possible traces of odd-chain homologues.

A portion of this fraction (ca. 200 μg) was hydrogenated over Adams catalyst. GLC showed the product to be a 4:1 mixture of palmitate and stearate, thus proving the components to have normal C_{16} and C_{18} chains. A further portion (ca. 400 μg) was oxidized with permanganate-periodate (19) and, after esterification, GLC showed the only products to be tridecanoate and pentadecanoate, again in the approximate relative proportions of 4:1. Thus these unusual monoenoates are 3-hexadecenoate and 3-octadecenoate.

This conclusion was verified by the NMR spectrum of the monoene fraction, which showed peaks at $\Delta = 2.02$ (in parts per million downfield from tetramethylsilane internal standard), corresponding to two protons of a methylene group adjacent to a double bond, and at $\Delta = 2.92$, corresponding to two protons of a methylene group situated between a double bond and an ester group (20,21). There were signals corresponding to two olefinic protons at $\Delta = 5.45$, three carboxymethyl protons at $\Delta = 3.61$, three terminal methyl protons at $\Delta = 0.89$, and to the protons of the chain methylene groups at $\Delta = 1.3$.

The infrared spectrum showed a pronounced band at 960 cm^{-1} corresponding to an isolated *trans* double bond but no trace of a band at 3020 cm^{-1} attributable to *cis* unsaturation. The unusual monoenoic constituents of *Aster* seed oil are therefore conclusively proved to be *trans*-3-hexadecenoic and *trans*-3-octadecenoic acids, probably accompanied by a small amount of *trans*-3-tetradecenoic acid.

The trans-Δ^3-Diene of Aster alpinus. The minor component migrating between oleate and linoleate on argentation-TLC was isolated in only a small amount (< 1 mg). GLC showed a single component with carbon numbers of 18.90 on a PEGA stationary phase, compared with 18.80 for linoleate, and of 17.65 on SE-30. Hydrogenation of a small portion of this sample gave only stearate on GLC. The infrared spectrum showed a pronounced band at 968 cm^{-1} and a weak band at 3020 cm^{-1}, corresponding to isolated *trans*, and *cis*-double bonds respectively.

From the above evidence, this unusual dienoic ester was believed to be *trans*-3, *cis*-9-octadecadienoate. However, as direct oxidative cleavage would not distinguish this structure from a 6,9-octadecadienoate, most of the remainder of this component (ca. 400 μg) was first reduced to the alcohol, with $LiAlH_4$ in ether, before being oxidized with permanganate-periodate. A 6,9-diene would then give 6-hydroxyhexanoic acid whereas a 3,9-diene would give adipic acid. The esterified products of this oxidation comprised methyl nonanoate and a small amount of dimethyl adipate but no trace of 6-hydroxyhexanoate.

Although the structure of this component has not been rigorously proved, it is believed to be *trans*-3,*cis*-9-octadecadienoate. In addition to the evidence described above is the fact that, as a Δ^3-unsaturated analogue of oleate, it co-occurs with the Δ^3-unsaturated analogues of palmitate, stearate, and linoleate and that the increment in carbon number on PEGA from these "parent" esters was in each case similar, 0.6-0.7. Finally it had identical characteristics on argentation-TLC to the more polar of the *trans,cis*-diene isomers derived by partial reduction of *trans*-3,*cis*-9,*cis*-12-octadecatrienoate (see below) under conditions where these isomers are separated and Δ^9-unsaturation is more strongly held than Δ^{12}.

The trans-Δ^3-Triene of Aster alpinus and Arctium minus. The unusual triene fraction isolated from the mixed esters of each of these oils by argentation-TLC appeared as a single component on GLC with a carbon number on PEGA of 19.50. The carbon numbers of linoleate and linolenate were respectively 18.80 and 19.60.

Hydrogenation gave only stearate, and mass spectrometry gave a peak at m/e = 292 for the parent molecular ion, indicating the presence of three double bonds. The NMR spectrum showed two proton signals at Δ = 2.93 and Δ = 2.72, corresponding respectively to a methylene group situated between a double bond and a carboxyl ester group and to a methylene group situated between two double bonds. The infrared spectrum showed bands at 968 cm^{-1} and 3020 cm^{-1} corresponding to isolated *trans*- and *cis*-double bonds.

The position and stereochemistry of each of the double bonds were determined by characterization of the products of partial reduction with hydrazine. Thus the triene ester from *Arctium* (30 mg) was dissolved in ethanol (4 ml), and to the solution was added heptadecanoic acid (30 mg), to provide free carboxylic acid groups the presence of which speeds up the reduction, and hydrazine hydrate (350 μl of 70% solution). The solution was shaken vigorously, to ensure maximum aeration, at 50C for 4 hr. Water was then added, and the product was extracted into ether. The ether extract was washed twice with 10% aqueous KOH to remove the added heptadecanoic acid, then with water to neutrality. The product was separated on argentation-TLC with two developments in ether-petroleum (1:9) into six fractions; the total reduction product (stearate) and unreduced triene and, between these, components corresponding to *trans*-monoene, *cis*-monoene, *trans,cis*-diene, and *cis-cis*-diene.

These were isolated by preparative argentation-TLC. The *cis,cis*-diene corresponded exactly to linoleate on argentation-TLC and on GLC. The *trans*-monoene similarly was identical to *trans*-3-octadecenoate and was proved to be so by $KMnO_4$-KIO_4 oxidation, which gave pentadecanoic acid. The *cis*-monoene fraction gave a double peak on GLC and was clearly separated into two components on a 30% $AgNO_3$-impregnated plate by three developments with toluene at –25C (22). The less mobile component migrated with standard oleate and the other with 12-octadecenoate, and again this was verified by oxidative cleavage to give hexanoic and nonanoic acids and nonandioic and dodecandioic acids. The trienoic acid from *Arctium* seed oil was therefore proved to be *trans*-3,*cis*-9,*cis*-12-octadecatrienoic acid. The trienoic acid from *Aster* oil was similarly characterized.

The *trans,cis*-diene fraction from the partial hydrogenation was separated, like the *cis*-monoene fraction, into two components on a 30% $AgNO_3$-impregnated plate by three developments with toluene at –25C. By analogy with the positionally isomeric *cis*-isomers, the less mobile component is presumed to be *trans*-3,*cis*-9-octadecadienoate, and this isomer was identical to the *trans*-3-dienoate isolated directly from *Aster* mixed esters, which is considered also to be *trans*-3,*cis*-9-octadecadienoate.

The Epoxy Acid of Aster alpinus. On TLC of *Aster* seed oil, it was noticed that there was a minor constituent migrating on TLC more slowly than normal triglycerides but more rapidly than diglycerides, in the position to be expected for a triglyceride containing one epoxy acid or one keto acid. After isolation of this component by preparative TLC, the methyl esters of its constituent fatty acids were prepared by saponification and reaction with

diazomethane. TLC of these esters showed a spot corresponding to normal methyl esters and a more polar component, of similar mobility to an epoxy or keto ester, comprising about one-third of the total mixture. This ester component was isolated, in small amount, by preparative TLC.

That it was not a keto ester was demonstrated when attempted reduction of a portion with sodium borohydride failed to produce a hydroxy ester. To test if an epoxy group were present, a further portion was treated with an anhydrous ethereal solution of HCl, which would cleave any epoxy group to chlorohydrins, and the product was compared on TLC with the original material and with a number of authentic *cis*- and *trans*-epoxy esters and their derived *threo*- and *erythro*-chlorohydrins. The HCl-treated sample showed two spots, more polar than the original ester which was identical to *cis*-9, 10-epoxystearate, corresponding to the standard *threo*-9(10), 10(9)-chlorohydroxystearate isomers. There are sufficient differences in the migration behavior of positional and geometric isomers of epoxy esters and their chlorohydrins on TLC (23) that it appeared reasonably certain from the TLC behavior that this polar ester from *Aster* seed oil was a *cis*-9,10-epoxy acid.

GLC on a PEGA stationary phase showed it to have a carbon number of 23.15, the same as that of authentic *cis*-9,10-epoxy-*cis*-12-octadecenoate, proving that it was a C_{18} epoxy ester and suggesting that it had a double bond. This was verified, and the double bond was shown to be *cis* by argentation-TLC when the compounds migrated more slowly than *cis*-9, 10-epoxystearate but again behaved exactly like authentic *cis*-9, 10-epoxy-*cis*-12-octadecenoate.

The remaining small portion of epoxy ester was converted to the *threo*-dihydoxy ester by acetolysis, hydrolysis, and esterification and was oxidized with permanganate-periodate. On such treatment 9, 10-epoxy-12-octadecenoate would give hexanoate and nonandioate but, because of the small amount of material involved, only dimethyl nonandioate could be detected. However, from the evidence obtained, it was concluded that this polar acid of *Aster alpinus* seed oil was *cis*-9, 10-epoxy-*cis*-12-octadecenoic acid, the acid originally characterized as a constituent of *Chrysanthemum coronarium* seed oil (24).

DISCUSSION

These studies have demonstrated the presence of relatively major amounts of *trans*-3-unsaturated acids in the seed oils of *Arctium minus* and, more particularly, of *Aster alpinus*. Whereas *Arctium* oil contains only the triene (*trans*-3,*cis*-9,*cis*-12-octadecatrienoic acid) in significant quantity, the *Aster* seed oil has a whole family of such acids among its constituents. The structures of these acids have been proved to be *trans*-3-hexadecenoic and -octadecenoic acids, *trans*-3,*cis*-9-octadecadienoic acid and *trans*-3,*cis*-9,*cis*-12-octadecatrienoic acid; *trans*-3-tetradecenoic acid is probably also present in small amount. *Aster alpinus* oil, in addition to these unusual acids, also contains a small proportion of an epoxy acid which is almost certainly identical to coronaric acid (*cis*-9,10-epoxy-*cis*-12-octadecenoic acid). An estimate of the proportions of all of these and of the more common acids present in these seed oils is summarized in Table I.

Table I shows quite clearly that in *Aster* oil there is a *trans*-3-unsaturated analogue of each of the common fatty acids present in significant amount. Moreover, although there is no strictly quantitative correlation, it is also evident that the proportion of each *trans*-Δ^3 acid is at least roughly in accord with the proportion of its "parent" analogue. The biosynthesis of *trans*-3-hexadecenoic acid in the green alga *Chlorella vulgaris* appears to be by direct desaturation of palmitic acid (16). Although it is rather foolhardy to put forward biosynthetic pathways simply on the basis of compositional data, we consider it likely that in *Aster alpinus* seeds these unusual acids arise by the insertion of *trans*-3-unsaturation into preformed palmitic, stearic, oleic, and linoleic acids as a final step. Attempts are being made to define the pathways of biosynthesis of these acids in *Aster* seeds.

The extreme positional specificity of these

TABLE I

Fatty Acid Composition (wt. % as Methyl Esters) of *Aster alpinus* and *Arctium minus* Seed Oils

	14:0	$\Delta^3$14:1	16:0	$\Delta^3$16:1	16:1	18:0	$\Delta^3$18:1	18:1	$\Delta^3$18:2	18:2	$\Delta^3$18:3	18:3	epoxy 18:1
Aster alpinus	tr.	tr.	5.2	7.1	tr.	1.5	1.9	9.1	3.0	56.2	13.7	tr.	2.0
Arctium minus	tr.	....	7.0	....	tr.	1.2	....	7.9	....	74.0	9.9	....	....

trans-Δ^3-acids in the triglycerides of *Aster alpinus* seed oil, but not of *Arctium minus* seed oil, will be described shortly in this journal.

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Rapid Determination of Double-Bond Positions in Monoenoic Fatty Acids by Periodate-Permanganate Oxidation

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ABSTRACT

Tetramethylammonium hydroxide has been used in the extraction and pyrolysis methylation of the carboxylic acids produced by periodate-permanganate oxidation of monounsaturated fatty acid methyl esters. This modification of the von Rudloff procedure allows rapid determination of double-bond positions and analysis of mixtures of positional isomers of monoenoic fatty acids.

INTRODUCTION

DETERMINATION OF THE POSITIONS of double bonds in unsaturated fatty acids by oxidative fission with periodate-permanganate, especially under the conditions developed by von Rudloff (1), is recognized as a reliable, though tedious, procedure. It is achieved with simple apparatus and stable, readily available reagents. The products can be obtained, in virtually quantitative yield, free from contamination by side reactions or over-oxidation. The oxidative splitting of the methyl ester of a monoenoic acid produces a shorter-chain monocarboxylic acid and the half ester of a dicarboxylic acid. In the von Rudloff procedure the half ester is hydrolyzed, and subsequent extraction of the resulting dicarboxylic acid from aqueous solution is the most time-consuming feature of the method. Furthermore, in certain cases, loss of volatile products can occur during recovery and esterification.

These factors have stimulated many recent attempts to devise more rapid and quantitative methods for finding double-bond positions. All of these methods have employed an attack at the double-bond position by ozone (2-7). In the most rapid of these newer techniques Davison and Dutton (3), Nickell and Privett (5) employed pyrolysis of the ozonides to aldehydes in the injection port of the gas chromatograph. In spite of the production of small amounts of by-products, Davison and Dutton were able to obtain an analysis of a complex mixture of C_{18} monoenoic acids comparable with that obtained by the periodate-permanganate method.

The possibility of obtaining equally rapid results with the periodate-permanganate oxidation has recently been provided by development of a technique for the gas chromatographic analysis of aqueous solutions of mono- and dicarboxylic acids by pyrolysis methylation (8). In this technique tetramethylammonium hydroxide (TMAH) is added to the aqueous solution, an aliquot is taken into a probe, dried at 100C, and injected into the gas chromatograph. Pyrolysis of the tetramethylammonium salts to methyl esters, which occurs above 250C, is instantaneous, reproducible, and free from interfering by-products. It seemed likely that use of this procedure with the aqueous solution of products from periodate-permanganate oxidation of unsaturated acids could produce a method for location of double bonds which would require a minimum of time and specialized equipment.

It was hoped initially that the reaction mixture from the oxidation could be treated directly with tetramethylammonium hydroxide, dried in the probe, and injected into the gas chromatograph. Results obtained with this procedure were not sufficiently reliable, principally because of the large proportion of inorganic salts in the reaction mixture after reduction of excess oxidant with metabisulphite.

Also, the low concentration of products necessitated operation of the gas chromatograph at inconveniently high sensitivities.

An alternative method has been developed, which depends upon a simple extraction procedure for concentration of the products and elimination of the inorganic reagents. The procedure depends on the ease with which both the half ester and the monocarboxylic acid oxidation products may be extracted from aqueous solution when appropriate conditions are employed. The products are then concentrated by extraction into a small volume of tetramethylammonium hydroxide solution, an aliquot of which is analyzed by pyrolysis methylation in the gas chromatograph.

The reliability and precision of this tech-

nique was studied in oxidations of pure methyl esters and of mixtures of these standard materials.

EXPERIMENTAL SECTION

Apparatus

An F and M Scientific Corporation Model 400 gas chromatograph with flame ionization detector was used, with a device for probe injection attached to the inlet port (8). The analytical column was a 3-ft × ⅛-in o.d. stainless steel tube packed with 80-100 mesh Diatoport S coated with 10% of its weight of a mixture of nine parts of SE-30 silicone gum and one part of LAC-3-R-728 diethylene glycol succinate (support and stationary phases from Applied Science Laboratories Inc.). This ratio of stationary phases allowed resolution of the methyl esters of the mono- and dicarboxylic acids, without the tailing of peaks which was a feature of the use of separate silicone and polyester columns joined in series (8).

Materials

High-purity methyl esters of monoenoic acids were obtained from the Hormel Institute, Austin, Minn. The oxidant mixture was the same as that used as stock solution by von Rudloff, containing 20.86 g of sodium metaperiodate and 395 mg of potassium permanganate per liter. The *t*-butanol was treated with 1% potassium permanganate solution until a persistent color was obtained and was then distilled. The hexane (Fisher Certified Reagent) was used as received. Tetramethylammonium hydroxide was obtained as a 25% aqueous solution (Mallinckrodt).

PROCEDURE

Reaction Conditions

The investigations of von Rudloff showed that the oxidation of methyl oleate occurred most readily in a reaction mixture containing 30-40% *t*-butanol. In order to simplify pipetting procedures, the present oxidations were carried out with reaction mixtures consisting of 1 ml of stock oxidant solution, 1 ml of potassium carbonate solution (2.5 mg/ml), and 1 ml of *t*-butanol containing 1 mg of the methyl ester or ester mixture to be oxidized. The reaction mixture was shaken in a screw-cap test tube for 1 hr at room temperature. Hexane (3 ml) was then added, followed by one drop of concentrated sulfuric acid, and the mixture was shaken vigorously for 20 sec. The hexane layer was transferred to a second tube, and the remaining reaction mixture was shaken with 3 ml of hexane which had been equilibrated with 33% *t*-butanol. The hexane extracts were combined and then shaken with 0.3 ml of aqueous 1% TMAH, the mixture was centrifuged, and the aqueous layer was transferred to a third tube. A 3 μl aliquot of this solution was taken in the capillary probe, dried in an oven at 100C for 5 min, and then injected into the vaporizer unit of the gas chromatograph. The vaporizer was held at 280C, and during the analysis the column temperature was programmed from 30 to 200C at 3C per min.

Chromatograms were also obtained by pyrolysis methylation of a synthetic mixture of the C_3 to C_9 monocarboxylic acids and the C_4 and C_9 dicarboxylic acids to serve as reference standards for the oxidation products.

Calculations

Peak areas of the gas chromatograms were measured with a planimeter, taking the average of at least four determinations. Relative peak areas were converted into molar ratios by dividing each peak area by the number of carbon atoms in the respective molecule which are capable of giving a response in the flame ionization detector, as described by Ackman (9).

For calculation of the proportions of the positional isomers in the synthetic mixtures of monoenoic acids two alternative procedures were used. In the first the analyses were based on the calculated molar ratios of the monocarboxylic acid fragments alone. The second method was based on the realization that the mono- and dicarboxylic acid fragments from each isomer will contain between them the same number of methyl and methylene carbon atoms, irrespective of the position of the double bond. Thus the sum of the peak areas given by the two fragments from each isomer was taken as being directly proportional to the percentage of that isomer in the mixture.

Rate of Oxidation

To determine the rate at which the oxidations would proceed under the conditions adopted, a series of reaction mixtures was prepared with methyl oleate as the substrate. The mixtures were shaken at room temperature in screw-cap test tubes, and the reactions were terminated at appropriate intervals by shaking with 2 ml of hexane. An aliquot of the hexane layer, which contained any unchanged methyl oleate, was then withdrawn and analyzed by

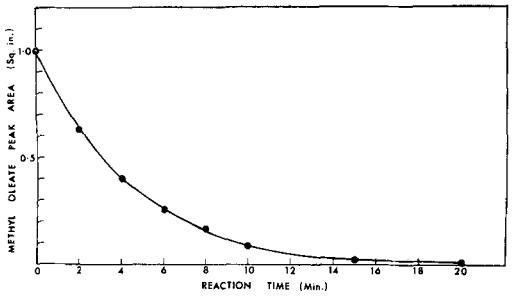

FIG. 1. Rate of oxidation of methyl oleate.

GLC. It was apparent from the results expressed in Fig. 1 that methyl oleate was consumed within 20 min. To show that hydrolysis of methyl oleate was not a factor in its disappearance, a similar experiment was conducted with methyl stearate as substrate. There was no decrease with time in the quantity of this substrate recovered, indicating that the conditions were not sufficiently alkaline to hydrolyze the esters undergoing oxidation.

Extraction Procedure

It was recognized that short-chain fatty acids such as butyric acid, which may be produced by oxidation of some unsaturated acids, are not readily extracted from aqueous solution. It was found, however, that when the solvent mixture employed in the oxidations was acidified and shaken with hexane, the distribution of the short-chain fatty acids was much more in favor of the hexane phase than when the partition was between water and hexane. Ether and chloroform were also investigated as extraction solvents but had the disadvantage of extracting an appreciable proportion of the mineral acid used in the acidification.

To determine the limits of the hexane extraction, several partition experiments were performed. The partition coefficient for butyric acid between water and hexane was determined by shaking 3 ml of water containing 30 mg butyric acid with 3 ml of hexane. The lower layer was removed, treated with 0.1 ml of 25% TMAH, and a 3-μl aliquot was dried and injected into the gas chromatograph for determination of the butyric acid concentration as methyl butyrate. The butyric acid in the hexane upper phase was determined by shaking the hexane with 3 ml of water containing 0.1 ml of 25% TMAH. An aliquot of the extract was analyzed by pyrolysis methylation in the gas chromatograph as before.

The partition coefficient for butyric acid between hexane and water determined in this way was 0.09. The partition coefficient for butyric acid between hexane and 33% *t*-butanol in water was determined in a similar manner and found to be 2.2. Thus a single extraction with hexane served to remove approximately 70% of the butyric acid from an aqueous layer similar to the solvent mixture used in the oxidations. Two extractions of butyric acid from 33% *t*-butanol, first with an equal volume of hexane and then with hexane which had previously been equilibrated with an equal volume of 33% *t*-butanol, left only 12.5% of the butyric acid in the aqueous phase.

The efficiency of extraction of short-chain fatty acids from the oxidation mixture was also checked by preparation of 3 ml of a solution approximating the oxidation solution used and containing 0.5 mg of each of the fatty acids from propionic to pelargonic. The mixture was acidified with one drop of concentrated sulfuric acid and extracted with 3 ml of hexane and then with 3 ml of hexane which had been equilibrated with 33% aqueous *t*-butanol. The combined hexane phases were shaken with 0.3 ml of 5% aqueous TMAH solution, which was then removed and analyzed by pyrolysis methylation in the gas chromatograph. The acids down to C_6 were recovered quantitatively, and the recoveries of valeric, butyric, and propionic acids were 97, 94, and 66% respectively.

RESULTS AND DISCUSSION

The procedure for oxidation of the methyl esters of unsaturated fatty acids and analysis of the products was tested first by application to a range of pure methyl esters.

From the oxidation of each of these compounds only two peaks were obtained in chromatograms of the products, representing the methyl esters of the mono- and dicarboxylic acid fragments (Fig. 2). It was therefore clear that neither the oxidation procedure nor the pyrolysis methylation produced significant amounts of by-products. It was noted previously (8) that, whereas monocarboxylic acids give quantitative yields in the pyrolysis methylation procedure used, the dicarboxylic acids give yields in the region of 90%. The calculated molar ratio of mono- to diester produced from each monoenoic fatty acid was thus always slightly greater than unity. Correction for this effect could be obtained, when desirable, by the use of reference standards.

The same oxidation procedure was used to

determine the proportions of the positional isomers in synthetic mixtures containing methyl petroselinate, methyl oleate, and methyl vaccenate, where the components were present in approximately equal amounts and also where they were present in widely differing amounts.

The analyses obtained for the synthetic mixtures of isomeric C_{18} monoenoic esters (Table I) were within the range of accuracy normally achieved in gas chromatographic procedures (10), irrespective of whether the calculations were based on the molar ratios of the monocarboxylic fragments alone or on the sums of the areas for the mono- and diester products from each isomer. The principal advantage of using the sums-of-areas technique for the calculation of results is in elimination of the need to allow for difference in detector response for each molecular species. Although the yield of the pyrolysis methylation is quantitative for monocarboxylic acids and is about 90% for dicarboxylic acids, it can be calculated that, for the mixtures of isomeric unsaturated fatty acids most commonly encountered, where the positions of unsaturation are near the middle of the chain, this disparity will produce a negligible error of the order of 1%. This can, of course, be avoided by basing the calculations on the monocarboxylic acid fragments alone. This procedure would also be necessary with mixtures in which Δ^2 or Δ^3 unsaturation is suspected since oxalic and malonic acids are completely consumed in the oxidation.

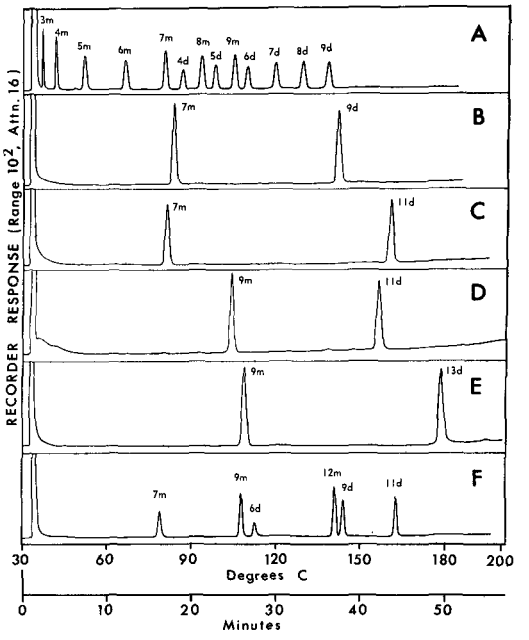

FIG. 2. *A*, Reference mixture (m denotes monocarboxylic ester, d denotes dicarboxylic ester); oxidation products from: *B*, palmitoleate; *C*, vaccenate; *D*, 11-eicosenoate; *E*, erucate; *F*, mixture of petroselinate, oleate, and vaccenate. Column: 3 ft x ⅛ in. packed with Diatoport S coated with 9% SE 30 silicone gum and 1% LAC-3-R-728 diethylene glycol succinate. Programmed temperature 30-200C at 3C per min.

A major advantage of the present technique is that several oxidations and the subsequent extractions can be performed simultaneously. The final solutions of the tetramethylammonium salts of the oxidation products are stable indefinitely at room temperature and can be accumulated for serial gas chromatographic analyses. Only a small proportion of each such solution of products is required for each chromatogram so that many repetitive runs can be performed for each oxidation of 1 mg of unsaturated fatty acid methyl ester. Alternatively the TMAH solution of oxidation products can be concentrated by evaporation so

TABLE I

Determination of Double-Bond Positions in Synthetic Mixtures of Pure Monoenoic Acids

Sample	C18:1 Isomer	Known composition (1%)	Composition calculated from Monoesters[c]	Composition calculated from Sums of areas[c]
1[a]	Δ^6	36.9	37.3 ± 0.34	36.2 ± 0.51
	Δ^9	32.6	31.9 ± 0.16	32.8 ± 0.24
	Δ^{11}	30.4	30.8 ± 0.52	31.0 ± 0.38
2[a]	Δ^6	34.1	32.6 ± 0.32	31.4 ± 0.51
	Δ^9	34.1	35.3 ± 0.70	36.8 ± 0.52
	Δ^{11}	31.8	32.1 ± 0.41	31.8 ± 0.21
3[b]	Δ^6	63.3	62.5 ± 0.42	61.1 ± 0.58
	Δ^9	28.0	27.8 ± 0.45	29.5 ± 0.63
	Δ^{11}	8.7	9.7 ± 0.70	9.2 ± 0.38

[a] Three determinations.

[b] Six determinations.

[c] Mean and standard deviation.

that almost the whole amount of the products may be used for one chromatogram. In this way determinations on as little as 10 μg of unsaturated methyl esters have been achieved, and further reduction to a level of one microgram should be feasible.

ACKNOWLEDGMENTS

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The Distribution of Phospholipids in Some Mammalian Milks

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ABSTRACT

Phospholipids were isolated from cow, sheep, Indian buffalo, camel, ass, pig, and human milks. The distribution of phospholipids was determined by quantitative two-dimensional thin-layer chromatography. The distribution of phospholipids was found to be remarkably constant in the milks of all the species studied, and it is concluded that the phospholipids probably fulfil similar or identical functions in these milks.

INTRODUCTION

THE PHOSPHOLIPIDS OF MILK[1] occur in the milk serum (1,2) and in the membrane which surrounds the milk-fat globule (3-5). Milk phospholipids are of importance in the synthesis of milk lipids (6,7) and in the structure of the fat-globule membrane (3-5). They are also of considerable technological importance in milk-fat products on account of their oxidative instability (8-11).

In recent years there has been much work done on bovine milk phospholipids, and the distribution of the phospholipids and the composition of their fatty acids are now fairly well established (12-14). Until recently there was no comparable information on the phospholipids of other milks, but the fatty acid compositions of the phospholipids from human, sheep, Indian buffalo, pig, ass, and camel milks are now available for comparison (12,15,16). This paper describes a study of the distribution of phospholipids in these milks.

EXPERIMENTAL SECTION

Preparation of the crude milk phospholipids is described elsewhere (12). The crude phospholipids (6-10 μg P) were separated by two-dimensional thin-layer chromatography (TLC) on plates coated with a 250μ layer of silica gel H containing 10% magnesium silicate (17). The plates were developed in chloroform-methanol-28% (w/v) ammonia-water (65:35:5:2.5, v/v) and briefly air-dried. The plates were then left for 10 min on warm wooden boards, cooled, and developed in the second solvent, which was chloroform-methanol-acetic acid-water (65:25:8:4, v/v). The heating of plates after development in the first solvent was essential in this laboratory, otherwise the second solvent swept some of the phospholipids to the solvent front. This heating step has not been found necessary by workers in the United States and Canada, presumably because of differences in ambient temperature and humidity.

Lipids containing phosphorus were detected with modified Zinzadze reagent (18), but equally satisfactory phosphorus analyses were

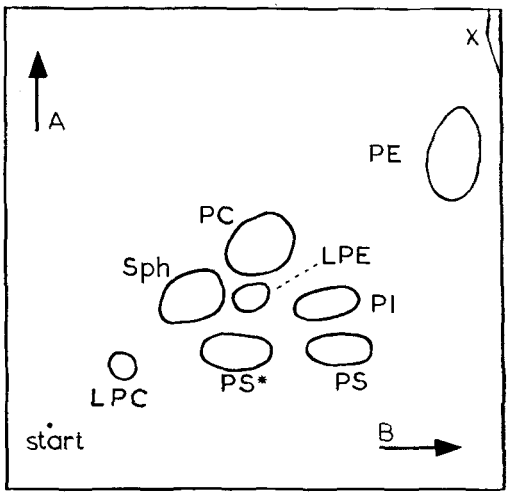

FIG. 1. Separation of milk phospholipids by two-dimensional TLC on plates coated with a 250 μ layer of silica gel H containing 10% by wt. of magnesium silicate. Plates were developed in direction A with chloroform-methanol-28% (w/v) ammonia-water (65:35:5:2.5, v/v), dried, then developed in direction B with chloroform-methanol-acetic acid-water (65:25:8:4, v/v). Lipids were detected with modified Zinzadze reagent. This figure also shows the usual breakdown products found in old or mishandled preparations.

Abbreviations as in text; X = unknown degradation products (of PE?). When silica gel G was used instead of silica gel H + magnesium silicate, phosphatidyl serine was retarded relative to other phospholipids during development in direction B (shown as PS*).

[1] Abbreviations: PE = phosphatidyl ethanolamine, Ceph = cephalin, PC = phosphatidyl choline, PS = phosphatidyl serine, PI = phosphatidyl inositol, LPE = lysophosphatidyl ethanolamine, LPC = lysophosphatidyl choline, Sph = sphingomyelin.

TABLE I
Distribution of Phospholipids in Milk from Several Species, Calculated from the Phosphorus Content of Lipids Separated by Two-Dimensional TLC[a]

	Phospholipid (mole %)							Total choline phospholipids
	PE	PC	PS	PI	Sph	LPE	LPC	
Cow	31.8	34.5	3.1	4.7	25.2	0.8		59.7
Sheep	36.0	29.2	3.1	3.4	28.3			57.5
Indian buffalo	29.6	27.8	3.9	4.2	32.1	1.6	0.8	60.7
Camel	35.9	24.0	4.9	5.9	28.3	1.0		52.3
Ass	32.1	26.3	3.7	3.8	34.1			60.4
Pig	36.8	21.6	3.4	3.3	34.9			56.5
Human	25.9	27.9	5.8	4.2	31.1	3.7	1.4	59.0

[a]Results are the mean of 9 to 12 determinations for each species. Standard deviations for PE, PC, and Sph were 4 to 6% of the actual values, and standard deviations for other phospholipids were 10 to 15% of the actual values.

obtained from spots detected by charring with sulphuric acid/dichromate (19), 50% sulphuric acid, or with α-naphthol reagent for glycolipids (20). A typical separation of phospholipids[1] is shown in Fig. 1.

Phospholipid spots and corresponding blank areas were outlined with a needle and scraped off with a razor blade into graduated 10-ml test tubes. Phosphorus was determined by digestion with 0.3 ml of H_2SO_4, using H_2O_2 as oxidant, and the heteropoly blue color was developed by reduction with ascorbic acid (21). The tubes were then cooled, and the volume was adjusted to 5 or 10 ml. The tubes were then well shaken and centrifuged to remove suspended silica gel. Optical densities were read at 822 mμ (1 μg P gave an optical density of 0.176). Sensitivity was limited by blank values of 0.040-0.080 (depending on spot size), which could not be significantly reduced by prewashing the TLC plates overnight in chloroform-methanol-water (65:35:5, v/v) or by other washing procedures. Phosphorus recoveries were 90-101%.

RESULTS AND DISCUSSION

The distribution of phospholipids in the milks which were studied is given in Table I. In the case of cow and human milks, three or four separate samples were analyzed in triplicate. Only one bulk sample of each of the other milks was available, and three or four separate phospholipid preparations were made from these, and each was analyzed in triplicate.

Some earlier figures for milks (other than bovine) have been published (22,23), but the results are only expressed as PC, Ceph, and Sph. The older results are however in general agreement with the present results. More recently Nagasawa et al. determined the distribution of phospholipids in bovine and human milks after separation by one-dimensional TLC (24). Their results are also in general agreement with those in Table I except that they found large amounts of LPC. Since they extracted the lipids by the Röse-Gottlieb method, which involves the use of ammoniacal solutions, the large amounts of LPC which they found may have been caused by partial hydrolysis of PC. Nagasawa et al. did not however report any lysocephalin fraction, but it seems possible from their published data that lysocephalins might have been included in their PC fraction.

The results in Table I show that PE, PC, PS, PI, and Sph are the normal phospholipids in milks. The small amounts of lysophospholipids in some samples are believed to be breakdown products formed during commercial processing (pasteurization of bovine milk and spray-drying of buffalo milk), but fresh samples of human milk, which were solvent-extracted immediately on receipt, always contained appreciable amounts of LPE. The distribution of milk phospholipids is remarkably regular between the species, and the total content of choline-containing phospholipids is almost constant.

These findings are not in themselves proof, but it seems reasonable to conclude that the phospholipids probably fulfill similar or identical functions in all the milks. Despite interspecies variations in the composition of the milks (15) and their triglyceride fatty acids (25,26), the distribution of phospholipids is comparatively constant. Differences between the phospholipids seem to be almost entirely confined to variations in the fatty acid composition of the phospholipids between species (12,15,16).

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[Received Aug. 9, 1967]

SHORT COMMUNICATIONS

Differentiation of Nitrogenous Phospholipids by Infrared Absorption Between 9 and 11 Microns

THE INFRARED (IR) SPECTRA of phospholipids have many common features (1-3), reflecting the basic structural similarities of this class of compounds. Yet the spectral differences are often adequate to provide a unique identification of a specific phospholipid. Even if it is not possible to determine the exact structure of a compound from its IR spectrum, considerable information about its structure can be obtained by using characteristic absorption bands in various regions of the spectrum. For phospholipid molecules, in particular, the absorption between 9 and 11 microns is exceptionally useful in determining the nature of the nitrogenous constituents of glyceryl or sphingosyl phospholipid. The position and intensity of absorption bands in this region appear to be dependent only on the character of the nitrogenous constituent and are relatively unaffected by the remainder of the molecule. By observing the nature of the IR spectra between 9 and 11μ, one can distinguish between phospholipids with a free amine, a mono- or disubstituted amine, or a quaternary amine.

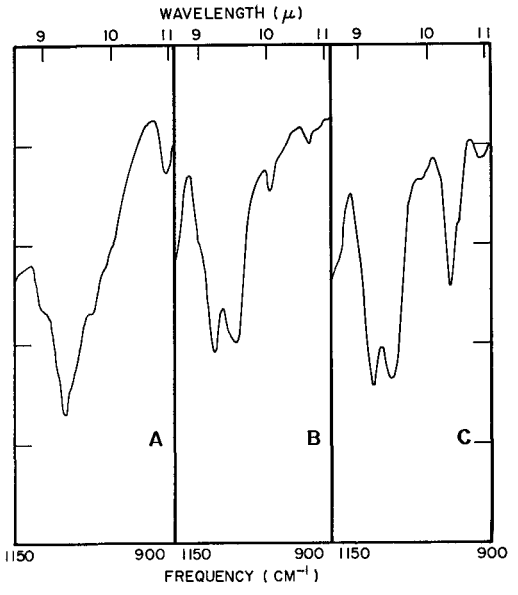

FIG. 1. The infrared spectra of nitrogenous phospholipids between 9 and 11 microns: Curve A, phosphatidyl ethanolamine; Curve B, N-N-dimethyl phosphatidyl ethanolamine; Curve C, phosphatidyl choline. All samples were dissolved in $CHCl_3$ at a concentration of approximately 50 mg/ml. Cells had NaCl windows and 0.1 mm optical path. Spectrophotometer was a Perkin-Elmer Model 521.

Figure 1 shows the IR solution spectra between 9 and 11μ of phosphatidyl ethanolamine, N-N-dimethyl phosphatidyl ethanolamine, and phosphatidyl choline. It is obvious that this region of the spectrum for each phospholipid has a unique character. By using these data and that obtained from the spectra of N-monomethyl phosphatidyl ethanolamine, phosphatidyl serine, and sphingomyelin (spectra not shown), the absorption in this region can be interpreted thus: phospholipids containing a free amine have a single absorption maximum at 9.3μ; those with a monomethyl amine have a strong maximum at 9.5μ and a weak maximum at 9.2μ; the dimethyl amine has a doublet with maximum at 9.2 and 9.5μ; the quaternary amine has a doublet at 9.2 and 9.5μ and a strong singlet at 10.4μ. The strong band at 10.4μ is a unique feature of phospholipids which contain a quaternary amine group. If an ethanolamine analogue of sphingomyelin is ever isolated, one can predict that the IR spectrum in this region will be similar to that of phosphatidyl ethanolamine. The published (1) IR spectrum of ceramide amino ethyl phosphonate, isolated from sea anemone, is similar to phosphatidyl ethanolamine between 9 and 11μ, a finding which lends additional support to this speculation.

Hence the IR spectrum between 9 and 11μ provides a simple and rapid guide with which the nature of the nitrogenous constituent of many phospholipids can be tentatively identified without recourse to more tedious hydrolytic and degradative procedures.

ACKNOWLEDGMENT

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[Received Sept. 19, 1967]

An Inexpensive, Unbreakable Chromatographic Column

FOR THE PAST FEW YEARS we have been using 1- to 4-liter sintered glass funnels packed 10-18 cm deep with silica gel for the isolation and purification of sterols from cacti (1). Recently, when no such funnel was immediately available, we decided to use 1- and 4-liter polyethylene bottles for this purpose. A ¾ to 1¼-in. hole bored into the center of the bottom of the bottle and a ⅜-in. hole in its screw cap were the only alterations required (Fig. 1).

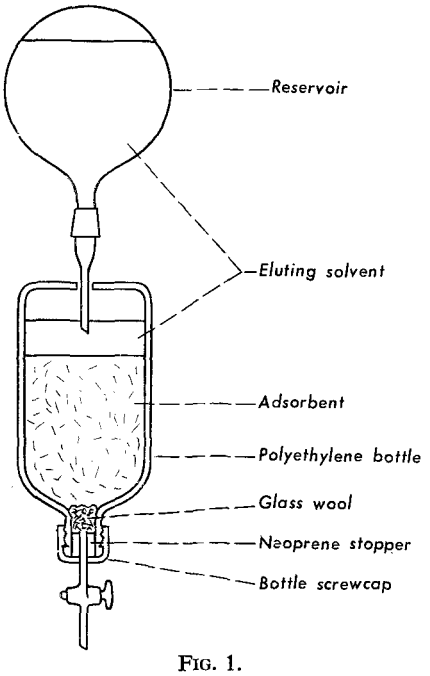

FIG. 1.

A tightly fitting Neoprene stopper, with a stopcock inserted, served to hold the glass wool plug and, above the glass wool, the silica gel. After pouring the slurry of gel in solvent in the usual manner through the hole in the inverted bottle, the gel can be stirred with a glass rod, or a rubber stopper can be placed in the hole and the slurry shaken as in a separatory funnel. A ring stand supports the column, and an additional ring supports the solvent reservoir.

After the slurry has settled, a piece of filter paper pushed through the hole and placed directly beneath it on the surface of the silica gel prevents the surface from being disturbed by addition of the sample and solvent. In a 2-liter volume of silica gel the sterols from 10-15 g of crude nonsaponifiable fractions are readily separated. In our experience, carefully packed large-diameter, low-height columns work as well as narrow-diameter, tall columns. Smaller columns in smaller bottles and columns of silver nitrate-impregnated silica gel can also be used in this fashion.

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[Received June 30, 1967]

Device to Apply Detection Reagents to Microplates

Thin-layer chromatography on microscope slides provides an easy and rapid means for the analysis of lipid mixtures. The simple roller described herein provides a neat, convenient means of applying detection reagents, particularly those which are corrosive, to the slide. It provides a simple alternative to spray techniques.

The roller consists of the bulb section of a 10-ml volumetric pipette, through which a suitable glass rod is placed to serve as a handle. The bulb section is free to rotate on the rod. This gives an all-glass apparatus which permits the use of corrosive reagents, such as concentrated sulfuric acid, etc.

In use the roller is placed on a 10-cm diameter Petri dish with the ends of the pipette part of the roller resting on the edges of the dish. A sufficient quantity of the reagent is then poured into the dish until it contacts the lower surface of the bulb. The roller is rolled back and forth to wet its surface, then immediately picked up and rolled over the slide. With a little practice it is possible to cover the slide quite uniformly. For small amounts of reagent a 10-cm watch glass can be used in place of the Petri dish.

Since the procedure depends to a considerable degree on the surface tension of the liquid, this should be considered in making up detection reagents. Those containing water and/or sulfuric acid spread quite well. Aqueous solutions occasionally will damage parts of the layer. Methanolic solutions require two or

more passes to cover the slide and are not uniform. Grinding the surface of the bulb with 2/0 emery paper will improve its performance with alcoholic solutions. The authors have used the device only on layers bound with $CaSO_4$. It is doubtful if it can be used with unbound layers.

Stable aqueous solutions can be stored in the Petri dishes by using the covers. The seal between the two halves can be improved by grinding the rim of the bottom half with emery paper.

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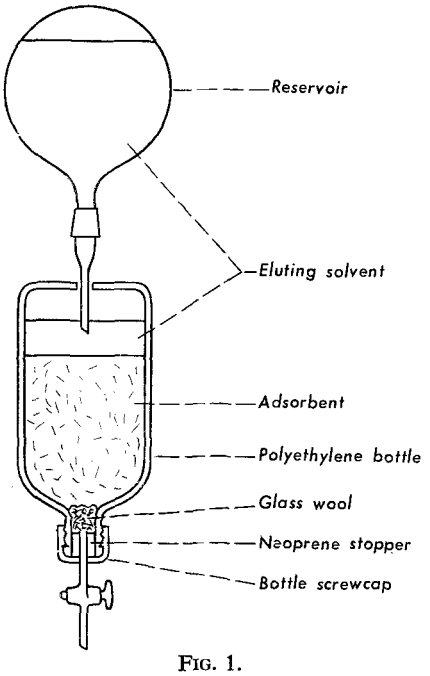

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Fatty Acid Composition of Milk Phospholipids. III. Camel, Ass, and Pig Milks[1]

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ABSTRACT

Phospholipids were isolated from camel, ass, and pig milks, and their fatty acid compositions were determined by gas-liquid chromatography. The specific distributions of fatty acids in phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) were determined. The results are compared with previous results for bovine, sheep, Indian buffalo, and human milks. The milk phospholipids which were studied can be grouped, on the basis of their fatty acid compositions, into those from ruminant herbivores, nonruminant herbivores, and nonherbivores. The phospholipids of camel milk however have features typical of all groups as well as 15% plasmalogen in the PE fraction.

INTRODUCTION

THE FATTY ACID COMPOSITION of the major phospholipids of cow, sheep, Indian buffalo, and human milks have been reported in recent papers (1,2). In the present paper further results are given for camel, ass, and pig milks.

It is well recognized that phospholipids have important functions in milk and milk products (1,3) although they are comparatively minor components (2). It is important therefore to have information on their distribution and structure. Further understanding of their functions may be obtained by making interspecies comparisons. The milk phospholipids studied in the present series of papers were selected in order to complement earlier studies of the milk triglycerides of several species (4). The species represent ruminant herbivores (cow, sheep, Indian buffalo, camel), nonruminant herbivores (ass), and nonherbivores (human, pig), whose milks are (with the exception of pig milk) of dietary significance to man.

EXPERIMENTAL SECTION

Camel and ass milks were obtained through R. Volcani, Faculty of Agriculture, the Hebrew University of Jerusalem, Rehovot, Israel, and were supplied as freeze-dried powders. Pig milk was obtained by courtesy of K. G. Mitchell, National Institute for Research in Dairying, Shinfield, England, and was received as a freeze-dried powder. All milk powders were stored at -20C.

The experimental procedures were basically as described before (1,2). Total lipids were extracted with chloroform-methanol-water, and total phospholipids were isolated by silicic acid column chromatography (1). The phospholipids were then separated on 8-12 preparative thin-layer chromatography (TLC) plates. TLC plates were coated with silica gel H + 10% magnesium trisilicate (5) and developed with chloroform-methanol-ammonia (S.G. 0.88)-water (65:35:5:2.5, v/v). This system separated phosphatidyl serine (PS) from the band containing phosphatidyl inositol (PI) and sphingomyelin (Sph). In previous works PS and PI were not separated by TLC, and results for PS + PI were given (2). The remaining preparative procedures were as previously described (1).

Some samples of phosphatidyl choline (PC) or phosphatidyl ethanolamine (PE) were not readily hydrolyzed by *Crotalus adamanteus* or *Ancistrodon piscivorus piscivorus* snake venoms. *Ophiophagus hannah* venom was therefore used as a source of phospholipase A since the fatty acid composition of the free fatty acids and lysophospholipids after partial hydrolysis (about 70-90% in several cases in the present work) is reported to be the same as after complete hydrolysis (6).

Fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC) on polar and nonpolar liquid phases as before (2) except that 15% EGSS-X on 100-120 mesh Gas-Chrom P (Applied Science Laboratories Inc.) was used instead of diethylene glycol succinate polyester with the same column and operating conditions.

Only one batch of each milk powder was available, and three to five total phospholipid preparations were made from each batch. Triplicate fatty acid analyses were made on all lipid fractions from each total phospho-

[1] For Parts I and II, see References 1 and 2.

TABLE I
Fatty Acid Composition (mole %) of Milk Sphingomyelins

Fatty acid	Camel	Ass	Pig
12:0	0.3		
14:0	3.0	3.9	0.4
14:1	0.3		
15:0	0.8		
15:1	0.3		
16:0	27.7	28.0	15.1
16:1	0.3	3.4	0.3
17:0	1.1		0.3
17:1	0.1		
18:0	5.2	4.7	6.9
18:1	0.8	2.9	0.5
19:0	0.7		0.3
19:1	0.2		
20:0	2.1	7.0	10.5
20:1	0.2	1.3	0.6
21:0	1.3		0.5
22:0	15.9	13.7	17.0
22:1	1.4	0.9	0.7
23:0	10.5	3.5	3.5
23:1	2.1		
24:0	9.8	14.6	20.2
24:1	13.1	16.1	22.0
25:0	1.7		0.8
25:1	1.1		0.4
Total saturated	80.1	75.4	75.5

lipid preparation. Variations on major fatty acids, determined by GLC, were generally ± 5% but, because of the small amount of material available, determinations of minor fatty acids were less reliable.

RESULTS AND DISCUSSION

In this discussion of results, all references to cow, sheep, Indian buffalo, and human milk phospholipids refer to Parts I and II of the present series (1,2) unless other references are given.

The fatty acids of camel, ass, and pig milk Sph (Table I) conform to the normal pattern in that they contain 74-80% saturated acids; the remainder are exclusively monounsaturated. Ruminant milk Sph contains large amounts of 23:0 whereas human milk Sph has a large amount of 24:1. Ass and pig milk Sph belong to the latter group, and camel milk Sph is somewhat intermediate.

In Table II the fatty acid compositions of PS and PI from camel, ass, and pig milks are given. Their degree of unsaturation is between that of PE and PC (Tables III and IV), in agreement with previous results for the other species, and they all contain more 18:2 and 18:3 than was found in the PS + PI of the ruminant milks. Pig milk PI contains comparatively large amounts of 16:1, and this is also in its PE and PC.

The fatty acids of PE (Table III) are more unsaturated than those of the other phospholipids, as was found previously in the other

TABLE II
Fatty Acid Composition (mole %) of Milk Phosphatidyl Serines and Milk Phosphatidyl Inositols

Fatty acids[a]	Phosphatidyl Serines			Phosphatidyl Inositols		
	Camel	Ass	Pig	Camel	Ass	Pig
12:0	0.7		0.4	0.3		0.6
13:0	0.3			0.5		
14:0	2.9	3.8	1.6	1.5	2.2	2.4
14:1				0.3		
i 15:0	0.2			0.1		
ai 15:0	0.3			0.2		
15:0	1.0			0.6		
16:0	14.9	20.0	8.2	9.8	15.3	20.2
16:1	4.4	3.5	3.4	3.3	2.2	8.1
i 17:0	0.2			0.2		
ai 17:0	0.4			0.4		
17:0	0.9			0.9		
17:1	0.9			0.6		
18:0	25.4	23.2	37.5	38.9	23.9	20.5
18:1	18.9	18.3	20.5	19.7	18.8	23.4
18:2	15.6	20.0	19.9	6.7	23.1	14.5
18:3	4.2	10.7	2.0	2.6	11.5	2.0
conj 18:2	2.1			2.1		
conj 18:3	1.1			1.5		
19:0	0.8			1.3		
20:0			0.6	0.2		0.4
20:3	1.3		0.6	2.8	1.2	0.8
20:4	1.6	0.5	1.0	4.6	1.8	4.5
20:5						0.6
22:4						0.6
22:5			2.1			0.6
22:6	1.9		2.2	0.9		0.8
Total saturated	48.0	47.0	48.3	54.9	41.4	44.1

[a] *i* = iso, *ai* = anteiso, *conj* = conjugated.

TABLE III
Specific Distribution of Fatty Acids (mole %) in Milk Phosphatidyl Ethanolamines

	Camel			Ass			Pig		
Fatty acid[a]	α′	β	Total	α′	β	Total	α′	β	Total
12:0	0.3	0.3	0.3						
13:0	0.2	0.1	0.2						
14:0	0.6	0.6	0.6	3.1	2.4	2.7	0.3	0.5	0.4
ald 14:0	0.8		0.4						
ald 14:1	0.8		0.4						
i 15:0	0.1	0.1	0.1						
ai 15:0	0.1	0.1	0.1						
15:0	0.4	0.2	0.3						
ald 15:0	0.5		0.2						
ald 15:1	0.2		0.1						
16:0	14.6	3.0	8.9	19.6	11.3	15.4	12.6	12.2	12.4
ald 16:0	8.7		4.4						
16:1	4.0	6.0	5.0	2.8	3.2	3.0	7.7	7.0	7.3
ald 16:1	1.4		0.7						
i 17:0	0.5	0.5	0.5						
ai 17:0	0.5	0.2	0.4						
17:0	1.5	0.2	0.8						
ald 17:0	0.5		0.2						
17:1	0.8	0.9	0.8						
i 18:0	0.3	0.4	0.4						
18:0	28.7	0.9	14.8	15.7	3.4	9.6	24.2	0.5	12.3
ald 18:0	2.0		1.0						
18:1	26.2	26.1	26.1	54.3	14.4	34.4	47.9	24.6	36.2
ald 18:1	1.6		0.8						
18:2	1.5	34.9	18.1	4.5	41.9	23.2	5.5	30.1	17.8
18:3	0.5	11.3	5.9		21.0	10.5	0.8	3.0	1.9
conj 18:2	1.7	1.1	1.4						
19:0	1.0	0.9	1.0						
20:3		3.0	1.5		1.0	0.5	0.3	1.1	0.7
20:4		6.6	3.3		1.4	0.7	0.7	12.4	6.6
20:5								2.1	1.1
22:5								3.3	1.7
22:6		2.6	1.3					3.2	1.6
Total saturated	61.3	7.5	34.6	38.4	17.1	27.7	37.1	13.2	25.1

[a] *Ald* = aldehydes (tentatively identified by carbon number) from plasmalogens.

milks. Pig milk PE is similar to human milk PE in that it has comparatively large amounts of 20:4 and other long-chain polyunsaturated acids. Ass milk PE, by contrast, has a comparatively large amount of 18:3 but little of the long-chain polyunsaturated acids. The larger 18:3 content is characteristic of nonruminant herbivores, which absorb linolenate from their feed and incorporate it directly into lipids without losses caused by hydrogenation by the rumen micro-flora (7). Camel milk PE resembles ass milk PE in its comparatively large proportion of 18:3, but it has more long-chain polyunsaturated acids. Camel milk PE is unusual in that it contains 15% plasmalogen whereas the largest amount reported in other milk phospholipids is 4% plasmalogen in bovine milk PC (1).

Camel, ass, and pig milk PC (Table IV) are the most saturated of the diacylglycerophospholipids, as was found in the other milks. Camel and ass milk PC contain more 14:0 than do PE, PS, or PI, and ass milk PC has a large amount of 16:0. The relative amounts of 18:3 and long-chain polyunsaturated acids follow the same patterns as in PE, although at lower levels. Camel milk PC contains less than 1% plasmalogen, and the aldehydogenic moeities were not determined by GLC.

From these results it seems that the milk phospholipids which have been studied fit into three groups, based on their fatty acid compositions. The ruminant herbivores have branched-chain fatty acids in all their phospholipids, they have only a few per cent of fatty acids with more than two double bonds, and their Sph contains ca. 30% of 23:0 but little 24:1. The nonruminant herbivore has larger amounts of esterified 18:3, and its Sph has little 23:0 but ca. 16% of 24:1. The nonherbivores are distinguished from the nonruminant herbivore principally by the lesser amounts of 18:3 and greater amounts of long-chain polyunsaturated acids. Camel milk is exceptional because of its PE plasmalogen, also because its phospholipid fatty acids are not entirely characteristic of the ruminant herbivores. Atypical features of the camel milk phospholipid fatty acids are the higher proportions of 18:3 and long-chain polyunsatu-

TABLE IV
Specific Distribution of Fatty Acids (mole %) in Milk Phosphatidyl Cholines

Fatty acid	Camel			Ass			Pig		
	α′	β	Total	α′	β	Total	α′	β	Total
12:0	0.1	0.2	0.2				0.2	0.4	0.3
13:0	0.3	0.1	0.2						
14:0	4.8	5.6	5.3	6.0	6.3	6.1	1.2	2.5	1.8
i 15:0	0.2	0.2	0.2						
ai 15:0	0.2	0.2	0.2						
15:0	2.1	2.1	2.1						
i 16:0	0.4	0.4	0.4						
16:0	41.1	15.4	28.3	63.6	40.7	52.2	34.4	45.4	39.9
16:1	4.0	10.4	7.2	3.3	2.9	3.1	6.1	6.6	6.3
i 17:0	0.4	0.5	0.4						
ai 17:0	0.8	1.2	0.9						
17:0	1.8	0.2	1.0						
17:1	0.6	1.2	0.9						
i 18:0	0.4	0.4	0.4						
18:0	21.6	1.9	11.7	10.0	3.0	6.5	18.9	1.8	10.3
18:1	15.2	20.4	17.8	13.6	8.1	10.9	29.0	14.6	21.8
18:2	3.0	23.0	13.0	3.5	25.7	14.6	8.5	23.1	15.9
18:3	0.7	8.1	4.4		12.0	6.0	1.0	2.1	1.5
conj 18:2	1.6	1.8	1.7						
conj 18:3		1.2	0.6						
19:0	0.7	0.7	0.7						
20:3		1.8	0.9		0.4	0.2	0.2	0.5	0.3
20:4		3.0	1.5		0.8	0.4	0.5	2.2	1.3
22:4								0.3	0.2
22:5								0.3	0.2
22:6								0.2	0.2
Total saturated	74.9	29.1	52.0	79.6	50.0	64.8	54.7	50.1	52.3

rated acids, and the lower 23:0 and higher 24:1 content of the Sph. Camel milk triglyceride fatty acids have however been found to be quite typical of a ruminant (8), and this was confirmed in the sample used in the present work.

The specific distributions of fatty acids between the α- and β- positions generally fit the well-established pattern of increasing preference for the α- position as the chain length of the saturated acids increases, and increasing preference for the β-position as fatty acid unsaturation increases. Exceptions to this are the 16:0 in pig milk PE and PC. Similar exceptions were found previously in human milk PE and PC.

The fatty acids in the β- or 2-position of milk phospholipids differ significantly in composition from those in the 2-position of the corresponding triglycerides (1,2). Present results for pig milk PE and PC likewise differ from those for pig milk triglycerides (9,10) and lend further support to the idea that the biosynthesis of milk triglycerides and phospholipids does not proceed by simple direct routes from a common precursor (1,2). Recent detailed studies by Nutter and Privett (11,12) have led them to similar conclusions (12).

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Phospholipid Reactivation of Plasmalogen Metabolism

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ABSTRACT

This report is concerned mainly with the properties of an enzyme from rat liver microsomes which hydrolyzes the alkenyl ether bond of 1-(1′-alk-1′-enyl)-glycero-3-phosphoryl-choline (alkenyl-GPC hydrolase).

Destruction of the normal environment of the microsomes by treatment with phospholipases A or C caused inactivation of the alkenyl-GPC hydrolase, which was then partially reactivated by the addition of exogenous phospholipids. Both sphingomyelin and diacyl-GPC were efficient in restoring activity; diacyl-GPE was less effective; and monoacyl-GPC and monoacyl-GPE were ineffective. The presence of two long hydrocarbon chains in the lipid activator is apparently required for reactivation, suggesting that interaction of hydrophobic areas of the enzyme with the phospholipid is necessary for maximal activity. High concentrations of sucrose mimicked the effect of phospholipids, and because the sucrose and diacyl-GPC did not show an additive effect, they may reactivate the enzyme in a similar manner.

Disrupting the enzyme's environment by freezing and thawing the preparation also resulted in a loss of enzymatic activity, which was restored by added exogenous phospholipids.

The alkenyl-GPC hydrolase was inhibited by imidazole and some of its derivatives. Histidine and N-acetyl histidine did not inhibit the enzyme, presumably due to the presence of a negative charge on the carboxyl group rather than the steric bulk of that group, since histidine methyl ester did inhibit the enzyme. Kinetic evidence showed imidazole to be a competitive inhibitor. The enzymatic activity of imidazole-treated microsomes also increased following addition of exogenous phospholipids. Imidazole inhibition differed from the phospholipase A-inactivation in that it was partially reversed by KCl, but not by sucrose. Imidazole did not inhibit other microsomal enzymes tested, indicating that it is not a general inhibitor of membrane-associated enzymes.

INTRODUCTION

THE IDEA HAS ARISEN that some enzymes associated with membranes require phospholipids (1-9). The evidence for this is that removal of phospholipids from membranes by some process such as extraction with wet acetone or by treatment with a phospholipase resulted in inactivation of the enzyme. The activity could then be restored to the enzyme by adding exogenous phospholipid to the inactivated enzyme. Thus complexes of phospholipids and proteins occur and are probably very important in determining the properties of a membrane. Studies on the actual mode of interaction of the phospholipids with membrane-associated proteins have shown that both the polar and nonpolar moieties of the phospholipid can interact with proteins (10,11).

Warner and Lands (12) have described an enzyme present in rat liver microsomes which hydrolyzes the alkenyl ether bond of 1-(1′-alk-1′-enyl)-glycero-3-phosphorylcholine. This report presents evidence that this enzyme requires phospholipids. The enzyme is also inhibited by imidazole, and some properties of this inhibition are described.

MATERIALS AND METHODS

Enzyme Assays

The activity of 1-(1′-alk-1′-enyl)-glycero-3-phosphorylcholine alkenyl ether hydrolase (alkenyl GPC-hydrolase) was measured by assaying aliquots from a 1 ml incubation mixture containing 9.9 μmoles of potassium phosphate buffer, pH 7.1, 1.2 μmoles of the substrate, 1-(1′-alk-1′-enyl)-glycero-3-phosphorylcholine, and about 2 mg of microsomal protein. The reaction was started by adding 0.3 ml of a 4×10^{-3} M solution of alkenyl-GPC dissolved in either distilled water or in 0.033 M potassium phosphate buffer, pH 7.1. The aliquots (0.1 ml) were removed at timed intervals and added to a test tube containing 1 ml of chloroform:methanol (2:1, v/v), and the tube was shaken slightly. Then 1 ml of chloroform and 1 ml of chloroform:methanol (2:1) were added successively with shaking. The resulting mixture was filtered through glass wool by passing it through a 9-in. Pasteur pipette containing a small wad of glass wool. The original tube was washed twice with 1 ml portions of

chloroform:methanol (2:1), each of which was transferred to the second tube by passing it through the Pasteur pipette. The resulting mixture was evaporated at 50C on a rotary evaporator, and the residue was taken up in 0.5 ml of methanol. The methanol solution was analyzed for the substrate, 1-(1'-alk-1'-enyl)-glycero-3-phosphorylcholine, by a slight modification of the procedure of Gottfried and Rapport (13). Five-tenths ml of a 2.5×10^{-4}N iodine solution in 3% potassium iodide was added, and the tube was shaken vigorously for about 10 sec and allowed to stand for at least 10 min. Then 4 ml of 95% ethanol were added, the solution was mixed thoroughly, and the absorbance was measured at 355 mμ.

The activity of D-glucose-6-phosphate phosphohydrolase (ED 3.1.3.10) (glucose-6-phosphatase) was assayed by a modified procedure of Harper (14). The reaction was performed in an incubation mixture containing 16.7 mM sodium citrate buffer, pH 6.5, 32 mM glucose-6-phosphate, pH 6.5, and about 1 mg microsomal protein in 1.5 ml. At timed intervals, 0.3 ml aliquots were removed and analyzed for inorganic phosphate. The enzymic assay for acyl-CaA:1-acylglycero-3-phosphorylcholine acyltransferase (acyltransferase) was determined by the spectrophotometric assay described by Lands and Hart (15). The acyl-CoA hydrolase activity was measured by the same method used to measure the acyl-CoA :1-acyl-GPC acyltransferase except that 40-60 mμmoles of the acyl-CoA were used and 1-acyl-GPC was omitted from the reaction mixture.

Preparation of Enzymes

Microsomes were prepared as described by Lands and Hart (15). A partially purified phospholipase A was prepared by dissolving 60 mg of *Crotalus adamenteus* snake venom (Ross Allen's Reptile Institute, Silver Springs, Florida) in 1 ml of 0.05 M Tris-chloride buffer, pH 7.6 and put on a Sephadex G-100 column (2.4 × 36 cm). The phospholipase A was eluted with the same buffer. One-milliliter fractions were collected, and the active fractions were pooled.

Preparation of Phospholipids

The phospholipids were prepared by silicic acid chromatography as described previously (16-18). Sphingomyelin was prepared by dissolving a crude sphingomyelin fraction in a small volume of methanol and then treating it with 1% mercuric chloride in 90% aqueous acetic acid to hydrolyze alkenyl ethers present. Then 2 N methanolic sodium hydroxide was added to adjust the pH to about 9 to hydrolyze any esters present. After the saponification had proceeded 30 minutes at room temperature, the reaction mixture was neutralized by adding ethyl formate, and enough $CHCl_3$ was added to make the chloroform:methanol ratio 2:1. The chloroform layer was washed twice with 0.1 volume of water and then evaporated. The residue was washed 4 times with diethyl ether, and the ether-insoluble sphingomyelin was then purified by silicic acid column chromatography using chloroform-methanol solvent mixtures. The 1-(1'-alk-1'-enyl)-glycero-3-phosphorylcholine was prepared as described before (16) except that twice as much methanolic sodium hydroxide was added, the saponification procedure was allowed to proceed for 30 min at room temperature, and ethyl formate was added to neutralize the saponification mixture.

The sonicated lipids for reactivation studies were made by adding 150 μmoles of a pure phospholipid to a hand homogenizer (Dounce ball-type, Blaessig Glass Co.), and evaporating the solvent. Then 6 ml of cold 0.25 M sucrose, 0.001 M EDTA were added to the homogenizer, and the mixture was homogenized to produce a milky emulsion of the phospholipid. This emulsion was sonicated for 15 min at full power on a Branson model 75SL sonifier using an ice-salt-water bath to maintain the temperature at less than 15C. The sonified mixture was adjusted to a final volume of 15 ml with the sucrose-EDTA. The diacyl-GPC preparations were centrifuged at 100,000 × *g* for 1 hr, and the opalescent solution beneath the floating lipid layer was collected and used.

Treatment of Microsomes with Phospholipases

Microsomes were adjusted to a concentration of 10-15 mg protein per ml with 0.25 M sucrose, 0.001 M EDTA. This suspension was sonicated for 4 min on a Branson model 75SL sonifier at a power of 6 amperes. An ice-salt-water bath was used to keep the temperature between 0C and 10C. The sonicated microsomes were diluted with an equal volume of 0.05 M Tris-chloride buffer, pH 7.4. To the diluted microsomes 60 μl of the partially purified phospholipase A (2 mg protein/ml) and 30 μl of 0.1 M calcium chloride were added for each milliliter of the diluted microsomes, and the solution was rapidly mixed. For phospholipase C treatment, 3 mg of fatty acid-poor bovine serum albumin, 60 μl of a solution of *Clostridium perfringens* phospholipase C (120 mg/ml), and 30 μl of 0.1 M calcium chloride were added for every ml of diluted micro-

somes. When samples were to be used for alkenyl hydrolase assay, 0.4 ml aliquots were removed and added to a test tube containing 0.05 ml of 0.5 M EDTA, pH 7 which stops the phospholipase reaction by complexing the calcium ions required for phospholipase activity.

Phospholipids were extracted from the treated microsomes by adding 0.1 ml aliquots of the microsome-phospholipase mixture to 1 ml of chloroform : methanol (2:1). This mixture was evaporated under nitrogen, and another ml of the chloroform : methanol was added and evaporated. The residue was taken up in 0.1 ml of the chloroform : methanol (2:1).

For thin-layer chromatography, 80 μl of the solution of the phospholipids extracted from the microsomes was spotted on a thin-layer plate spread with Absorbosil No. 2, which had just been heated at 80C-100C for 15 min and cooled to room temperature. Overheating of the plate was avoided because it lowered the R_f of the diacyl-GPE. The separation was accomplished by developing the plate successively in the following three solvent systems:

(1) chloroform : methanol : ethanol : water : acetic acid (100 : 16 : 20 : 4 : 1)

(2) methanol : 95% ethanol (1 : 3)

(3) 95% ethanol : ether : methanol : water (100 : 25 : 25 : 2)

The first solvent was allowed to travel a distance of about 15 cm on the plate. The plate was allowed to dry for about 15 min between the transfers from one solvent to the next. The last two solvents were allowed to travel up to the diacyl-GPE spot which had been detected by rhodamine G. The detection was accomplished by spraying one side of the plate where a standard diacyl-GPE had been put on the plate. The three solvent systems resulted in the separation of the mono- and diacyl derivatives of GPC and GPE from each other. Each compound was identified by comparing its R_f to that of a standard.

After the final solvents had evaporated from the plate, it was sprayed with the molybdate spray described below to detect the phosphate positive areas of the plate. The areas containing the phospholipids were scraped into tubes, and 0.6 ml of 70% perchloric acid was added. This mixture was heated at 185Cfor 2 hr and then cooled to room temperature. Phosphate was measured by the procedure of Bartlett (19).

The molybdate spray was made from 2 solutions. The first solution was prepared by adding 40.11 g MoO_3 to 1 liter of concentrated sulfuric acid and heating gently until the MoO_3 dissolved. The second one was made by dissolving 1.78 g powdered molybdenum in 500 ml of the first solution and heating gently for 15 min. One ml of each solution was added to 3 ml of water to make the spray, which detects phospholipids by producing a blue color.

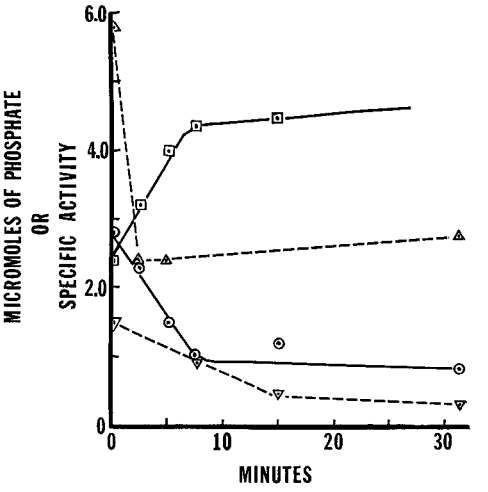

FIG. 1. Inactivation by phospholipase A. Treatment of the microsomes with phospholipase A, the removal of aliquots for alkenyl-GPC hydrolase assay and for phospholipid analysis, the analysis of the phospholipids, and the assay for the alkenyl GPC hydrolase under Methods. The figure shows the amount of diacyl-GPC (O——O), monoacyl-GPC (□——□), and diacyl-GPE (▽— — —▽) present after various amounts of time of phospholipase A treatment of microsomes. The specific activity (in mμmoles/min/mg protein) of the alkenyl-GPC hydrolase (△— —△) is also shown as a function of the time of phospholipase A treatment.

Chemicals

Imidazole was purchased from Eastman Organic Chemicals, 1-methylimidazole from the Aldrich Chemical Co., and the other derivatives of imidazole from Sigma. These compounds were neutralized before use. Urea was obtained from Baker and Adamson, and the alkylated ureas were supplied by George Zografi, Department of Pharmacy, University of Michigan. Glucose-6-phosphate was purchased from Calbiochem.

RESULTS

The Effect of Phospholipase A

When sonicated microsomes were treated with the partially purified phospholipase A, the enzymatic activity of the alkenyl-GPC hydrolase was reduced by 50-70% in 20 min. To show that the phospholipids in the microsomal mem-

TABLE I

Effect of Products of Phospholipase A Reaction on Sonicated Microsomes

Additions	Rate (mμmoles/10 min)
None	375
0.33 μmole each of 16:0, 18:0 and 18:1 fatty acids	375
Santoquin	275
Santoquin + 0.3 μmole 18:3, 0.4 μmole 18:1, and 0.4 μmole 18:2 fatty acids	275
None	350
0.21 μmole monoacyl-GPC	360
0.42 μmole monoacyl-GPC	375
None	325
0.42 μmole monoacyl-GPC	313

The alkenyl-GPC hydrolase activity was determined as described under Methods with 3.9 mg of protein in a 1.1 ml incubation mixture. The incubation mixtures had the amounts of fatty acids or monoacyl-GPC indicated. The enzyme and lipid were allowed to incubate for 5 min before the assay was initiated by addition of the substrate.

Fatty acids were added:

1) from a solution containing 11 μmoles each of 16:0, 18:0, and 18:1 fatty acids and 27 μliters of absolute ethanol per ml of 0.05 M Tris-chloride buffer, pH 7.4. The control had an equal amount of Tris-chloride-absolute ethanol solution containing no fatty acids.

2) from a solution containing 40 μmoles of each 18:1, and 18:2 fatty acids and 30 μmoles of 18:3 fatty acid in 3 ml of 0.05 M Tris-chloride buffer, pH 7.4. A small amount of the antioxidant Santoquin (1,2-dihydro-6-ethoxy-2,2,4-trimethyl quinoline) was present in the polyunsaturated fatty acids, so a solution of Tris and Santoquin containing no fatty acids was added to the control.

Monoacyl-GPC was added:

1) by adding a known amount from a chloroform:methanol (1:1) solution to a test tube and evaporating the solvent under nitrogen. The lipid was dissolved in the incubation medium to which the enzyme and substrate had not yet been added.

2) by adding an aliquot from an aqueous solution containing 4.2 μmoles monoacyl-GPC per ml to the incubation medium (lowest section of the table).

branes were being hydrolyzed by phospholipase A, the lipids were extracted from a microsome-phospholipase incubation mixture at timed intervals and analyzed. The results of such an experiment are shown in Fig. 1. The rate of disappearance of diacyl-GPC was equal to the rate of

TABLE II

Reactivation of Phospholipase A-Treated Enzyme with Diacyl-GPC

Treatment	Rate (mμmoles/10 min)
Sonicated	375
PLA-treated	125
PLA + 0.2 μmole diacyl-GPC	138
PLA + 0.8 μmole diacyl-GPC	263
PLA + 2.0 μmoles diacyl-GPC	338

The microsomes were sonicated and treated with phospholipase A as described under Methods. The alkenyl-GPC hydrolase was assayed as stated under Methods using 3 mg protein. Sonicated diacyl-GPC was added in the amounts indicated.

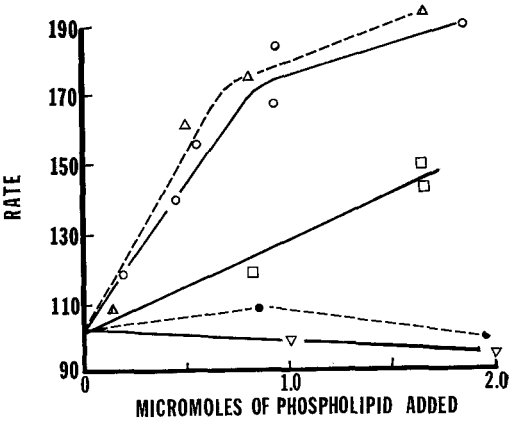

FIG. 2. Reactivation of phospholipase-treated enzyme by phospholipids. The enzyme was sonicated and treated with phospholipase A for 30 minutes as described under Methods. The enzyme was assayed as described in Methods using 3 mg protein. The figure shows the enzymatic rate (expressed as mμmoles/min/mg protein) of the phospholipase-treated enzyme in the presence of different amounts of sonicated diacyl-GPC (O——O), sphingomyelin (△— —△), diacyl-GPE (□——□), monacyl-GPC (●— — —●), and monoacyl-GPE (▽——▽).

appearance of monoacyl-GPC. The rate of gain on monoacyl-GPE (not shown in the figure) was found to be equal to the loss of diacyl-GPE. Thus the rate of loss of the substrates of the phospholipase A reaction was equal to the rate of gain of the products. The data presented in Table I show that the products of the phospholipase A reaction did not inhibit the alkenyl-GPC hydrolase activity. The phospholipase A incubation was done at 20C in the presence of 3×10^{-3} M $CaCl_2$, and the phospholipase A reaction was stopped with 0.06 M EDTA, pH 7.0. Incubation at 20C for 30 min with either of these reagents did not cause inactivation of the alkenyl-GPC hydrolase.

Addition of diacyl-GPC prepared from egg yolks to the phospholipase-inactivated enzyme resulted in a reactivation of the enzyme, and the results presented in Table II show that the amount of reactivation was dependent on the amount of diacyl-GPC added. Other phospholipids were also tested for their ability to restore the inactivated enzyme. The results in Fig. 2 illustrate that both diacyl-GPC and sphingomyelin were good reactivators. Diacyl-GPE could reactivate, but was not nearly as efficient as diacyl-GPC or sphingomyelin. Monoacyl-GPC and monoacyl-GPE were totally ineffective as reactivators.

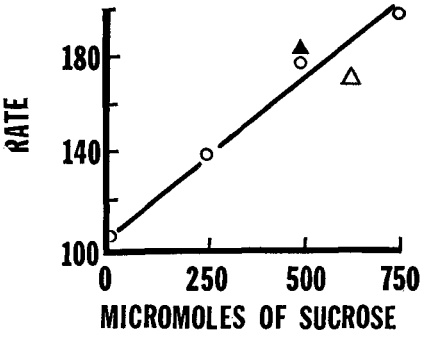

FIG. 3. Reactivation of phospholipase-treated by sucrose. The enzyme was sonicated and treated with phospholipase A for 30 minutes as described under Methods. The alkenyl-GPC hydrolase was assayed as described under Methods using 3 mg of protein. The figure shows the rate (expressed as mμmole/10 min) of the phospholipase-treated enzyme in the presence of different amounts of sucrose (O——O). The rate in the presence of both sucrose and sonicated diacyl-GPC is indicated by the symbols ▲ and △; the amount of sucrose present in the combination experiments is shown on the abscissa, and the amount of diacyl-GPC used was 0.93 μmoles (▲) and 0.47 μmoles (△).

The data in Fig. 3 indicate that high concentrations of sucrose, restored activity to the enzyme. Diacyl-GPC and sucrose did not have an additive effect. Two other polyols, glycerol and mannitol, were tested for the ability to reactivate the enzyme, and neither one restored activity.

The Effect of Phospholipase C

If intact phospholipids are really required by the enzyme, then the enzyme should be inactivated if they are degraded in a different manner. This was tested by treating the microsomes with phospholipase C. Fig. 4 illustrates the phospholipase C degraded both diacyl-GPC and diacyl-GPE in the microsomal preparation. After 40 minutes, the alkylhydrolase was inactivated 50%. When sonicated diacyl-GPC was added to the phospholipase C-inactivated enzyme, activity was restored as indicated in Fig. 4.

TABLE III

Effect of Freezing and Thawing

Conditions	Rate (mμmoles/10 min)
None	
Control	350
Control + 1.9 μmoles diacyl-GPC	363
Frozen and thawed 10 times	257
Frozen and thawed 10 times + 1.9 μmoles diacyl-GPC	363

Microsomal preparations (9 mg protein/ml) were made up in 0.2 M sucrose, 0.001 M EDTA. Each solution was frozen in a freezer maintained at -20C and thawed in a cold water bath kept at 17C or less. Enzymatic activity was determined as described under Methods using 1.8 mg microsomal protein and, where indicated, 1.9 μmoles of sonicated diacyl-GPC.

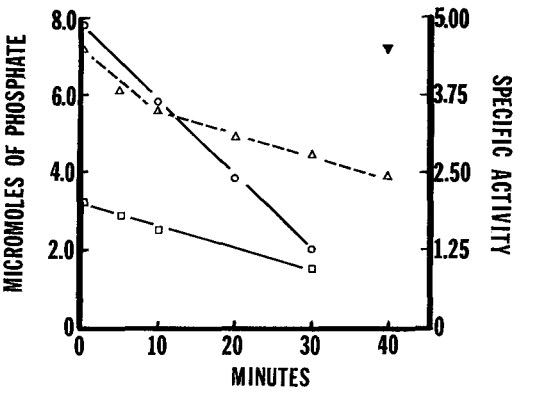

FIG. 4. Inactivation by phospholipase C. The sonication of microsomes, the treatment with phospholipase C, and the analysis of phospholipids are described under Methods. The assay of alkenyl-GPC hydrolase was as described under Methods using 3 mg of protein. Where indicated, 2 μmoles of sonicated diacyl-GPC were added to the assay mixture of the enzyme treated with phospholipase C for 40 min.

The figure shows the amounts of diacyl-GPC (O——O), and diacyl-GPE (□——□) present after different amounts of time of phospholipase treatment. It also shows the specific activity of the alkenyl-GPC hydrolase (△— —△) as a function of the time of treatment with phospholipase C. The specific activity of the enzyme which had been treated with phospholipase for 40 min when assayed in the presence of 2 μmoles of sonicated diacyl-GPC is indicated (▼).

The Effect of Freezing and Thawing

The alkenyl-GPC hydrolase was inactivated by freezing and thawing the microsomes several times. The enzyme activity was about 30-40% of the original value after being frozen and thawed 10 times. The enzyme was reactivated by diacyl-GPC, as shown in Table III. If diacyl-GPC was present during the freezing and thawing process, it failed to prevent the inactivation of the enzyme, and the enzyme no longer showed the ability to be reactivated by phosphatidyl choline. Also large particles settled out of the solution when microsomes were inactivated in the presence of diacyl-GPC.

TABLE IV
Reversal of Imidazole Inhibition

Treatment	Specific activity (mμmoles/min/mg protein)
A — None	14
B — 2.0 μmoles Imidazole/ml	4
A — Centrifuged and resuspended	18
B — Centrifuged and resuspended	19

A preparation of microsomes in 0.25 M sucrose, 0.001 M EDTA was divided into two equal parts. One half was made up to 9 mg protein per ml (total volume = 5 ml) with 0.25 M sucrose, 0.001 M EDTA, and the other half was made up to 9 mg protein per microliter and 2 μmoles of imidazole-chloride, pH 7.0 per microliter. Each diluted preparation was centrifuged for 1 hour at 39,000 rpm in a SW39 swinging bucket rotor. The resulting pellets were resuspended in 5 ml of 0.25 M sucrose, 0.001 M EDTA. The resuspended pellets were then assayed for alkenyl-GPC hydrolase activity as described under Methods.

The Inhibition of Imidazole

Warner (20) found that imidazole inhibited the alkenyl-GPC hydrolase. As seen in Fig. 5, the enzyme was inactivated 50% when imidazole was present in concentrations of 1 μmole per milliliter of reaction mixture. The imidazole inhibition occurred immediately, for the same degree of inhibition occurred whether imidazole was added after the reaction had already begun or was preincubated with the enzyme for 30 min before the reaction was started. The reversibility of the imidazole inhibition of the hydrolase was demonstrated in two ways. Because the enzyme is particulate, it can be centrifuged at 100,000 × *g* for one hour, and the resulting pellet can then be resuspended. When the enzyme was centrifuged from a medium containing 2 μmoles of imidazole per milliliter and resuspended in a medium free of imidazole, the activity returned to control levels (Table IV). Table V reveals that the imidazole inhibition could be reversed by diluting the solution. When the enzyme was assayed with different amounts of substrate in the presence and absence of imidazole and the data were plotted according to the method of Lineweaver and Burk (21), a plot as shown in Fig. 6 was obtained. This graph indicates that imidazole is a competitive inhibitor.

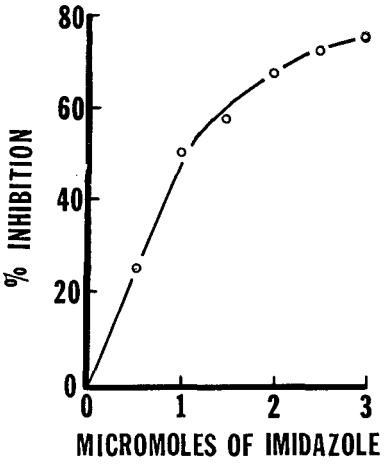

FIG. 5. Inhibition by imidazole. Enzymatic activity was determined as described under Methods using 1.8 mg microsomal protein and a total volume of 1.0 ml. Imidazole was added in the amounts indicated before the reaction was started by adding substrate. The figure shows the percent inhibition of the enzymatic activity obtained with different amounts of imidazole.

Since Warner (20) had shown that histidine did not inhibit alkenyl-GPC hydrolase, the microsomes were treated with other derivatives of imidazole. As shown in Table VI the derivatives containing a negatively charged carboxyl group did not inhibit the enzyme, whereas all other compounds tested inhibited about 75% at a concentration of 0.003 M.

Urea is known to inhibit many enzymes, and as Table VII shows, it inhibits the alkenyl-

TABLE V
Reversal of Imidazole Inhibition by Dilution

Treatment	Rate (mμmoles/10 min)
None	338
+ 2 μmoles Imidazole/ml	75
+ 0.4 μmole Imidazole/ml	300
Imidazole diluted from 2.0 μm/ml to 0.4 μm/ml	294

A microsomal preparation was divided and diluted as described in the legend for Table IV. Then 0.2 ml of each diluted enzyme was assayed as described in Methods. Imidazole hydrochloride, pH 7.0, was added to the diluted microsomes containing no imidazole for the assays containing 2 μmoles imidazole per microliter and 0.4 μmole imidazole per microliter.

TABLE VI
Inhibition by Imidazole and Its Derivatives

Reagent added (3 μmoles/ml)	Rate (mμmoles/10 min)		
None	213	250	263
Imidazole	100	113	
N-Methylimidazole	100		
Histamine		113	
Histidine Methylester		150	75
N-Acetyl-histamine	125	100	150
Histidine	213	275	
N-Acetyl-histidine		263	263

The enzyme was assayed as described under Methods using 1.8 mg of protein. Each reagent was adjusted to pH 7.0 and added 5 min before the substrate solution was added to start the reaction.

TABLE VII
Inhibition by Urea

Additions	Rate (mμmoles/10 min)	Percent inactivation
None	473	—
1.0 M Urea	381	19
3.0 M Urea	184	61
5.0 M Urea	40	92

The enzyme was assayed as described under Methods using 1.3 mg of protein and an incubation volume of 1.1 ml. Where indicated, enough urea was added before the substrate to give the final concentration stated. The reagent and enzyme were allowed to incubate together for 5 min at room temperature before the substrate was added to start the reaction.

GPC hydrolase at high concentrations. The concentration of urea needed to achieve about 60% inhibition was 3.0 M. This is in contrast to imidazole which gave 75% inhibition at 0.003 M. The data in Table VIII also show that the addition of a hydrocarbon side chain to the urea molecule made it a more potent inhibitor of the enzyme. The difference between 1.0 M urea and 0.1 M n-butyl urea demonstrates that the ability of the alkylated ureas to inactivate the enzyme is greater with increasing length of the hydrocarbon chain.

Relief of the Imidazole Inhibition

Since the enzyme preparations inactivated by phospholipases and by freezing and thawing could be reactivated by phospholipids, the effect of these compounds on the imidazole-inactivated enzyme was studied. As Fig. 7 reveals, both sphingomyelin and diacyl-GPC reactivated the imidazole-inhibited enzyme. The amount of reactivation obtained being dependent on the amount of phospholipid added.

Because the phospholipase A-inactivated enzyme showed increased activity in the presence of sucrose, the effect of polyols on the imidazole inhibition was studied. The polyols sucrose, mannitol, and glycerol did not reactivate the imidazole-inhibited enzyme. However, the imidazole inhibition could be relieved by the addition of potassium chloride.

TABLE VIII
Inhibition by Alkylated Ureas

Additions	Rate (mμmoles/10 min)	Percent inactivation
None	330	—
1.0 M Urea	289	12
1.0 M Methyl urea	289	12
1.0 M Ethyl urea	110	67
0.5 M n-Butyl urea	28	92
0.1 M n-Butyl urea	179	46

Conditions were the same as described for Table VII except that 1.0 mg protein was used in an incubation volume of 1.05 ml.

TABLE IX
The Effect of Imidazole on Other Membrane-Associated Enzymes

Enzyme	Specific activity (mμmoles/min/mg) Without Imidazole	3mM Imidazole
Glucose-6-phosphatase	51.5	61.5
Alkenyl-GPC hydrolase	35.0	9.0
Acyl CoA:1-Acyl-GPC-acyltransferase with 20:4 (n-6) acyl-CoA	35.0	29.0
Acyl-CoA hydrolase with 20:4 (n-6) acyl-CoA	22.0	22.0

The enzymes were assayed as described under Methods. When imidazole hydrochloride was included, it was added to a final concentration of 3.0 mM, and it was added before the substrate was added to start the reaction.

Potassium chloride did not reactivate the alkenyl-GPC hydrolase activity in phospholipase A-treated microsomes.

Since phospholipids could bring about a reactivation of the imidazole-treated enzyme, and because phospholipids are known to reactivate certain other membrane-associated enzymes which have been inactivated, the effect of

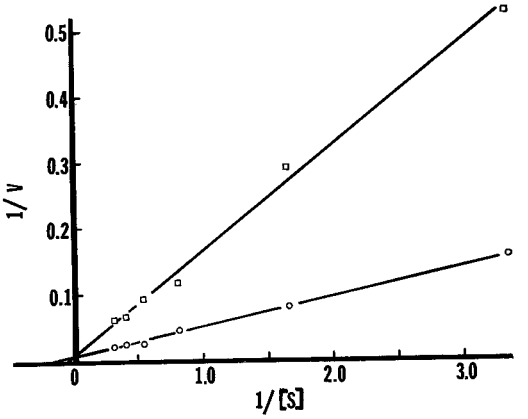

FIG. 6. The effect of substrate concentration on imidazole inhibition. The enzyme was assayed at 25C in the presence of 15 μmoles of potassium phosphate buffer, pH 7.1, 1.8 mg microsomal protein, and the amount of substrate indicated per milliliter of incubation mixture. Aliquots which contained about 0.1 μmole of substrate at zero time were removed and assayed as described under Methods. The reaction was initiated by adding the enzyme. The figure shows the enzyme rate obtained using different amounts of substrate. Where indicated two μmoles of imidazole hydrochloride, pH 7.0, were added before the substrate. The figure shows the plots of reciprocal velocities $(m\mu moles/min)^{-1}$ vs. reciprocal substrate concentrations $(\mu moles)^{-1}$ in the presence (□——□) and absence (O——O) of imidazole.

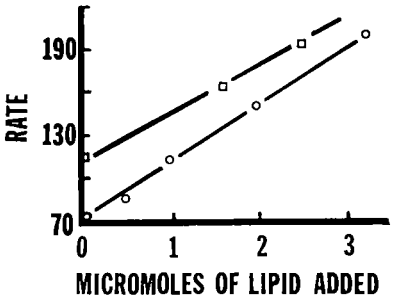

FIG. 7. Relief of imidazole inhibition by phospholipids. The enzyme was assayed as described under Methods using 1.8 mg of protein in the presence of 2 μmoles imidazole hydrochloride, pH 7.0. Sonicated phospholipids were added in the amounts indicated after the addition of the substrate solution. The figure shows the rate of the imidazole-treated microsomes in the presence of different amounts of diacyl-GPC (O——O) and sphingomyelin (□——□).

imidazole on other membrane-associated enzymes was studied. Table IX shows that, of the microsomal enzymes tested, only alkenyl-GPC hydrolase was strongly inactivated by 3.0 mM imidazole.

DISCUSSION

Treatment of the sonicated microsomes with phospholipase A results in the loss of enzymatic activity. This suggests that the loss of activity is due to the destruction of the microsomal phospholipids. The phospholipids were actually degraded by the phospholipase A and the possibility that loss of enzymatic activity was due to the inhibition by the products of the phospholipase reaction was ruled out, as neither fatty acids nor 1-acyl-glycero-3-phosphorylcholine inhibited the enzyme. The fact that polyunsaturated fatty acids did not cause inhibition is especially important, since the fatty acids released from phosphatidyl choline and phosphatidyl ethanolamine by phospholipase A are released from the 2-position of the glycerol moiety (22-24), which contains mainly unsaturated fatty acids in naturally occurring phospholipids (25). Loss of activity was not attributable to any of the reagents used in the phospholipase treatment of the microsomes.

Addition of micellar phospholipids caused reactivation of the phospholipase-treated enzyme, the amount of reactivation being dependent on the amount of lipid added. Thus alkenyl-GPC hydrolase is similar to other membrane associated enzymes which have been treated with phospholipases and reactivated with phospholipids. Reactivation by more than one phospholipid has been demonstrated for other enzymes such as glucose-6-phosphatase from rat liver microsomes (9) and ATPase from sarcosomes (5). Ganoza (9) found that phospholipase A-treated glucose-6-phosphatase was reactivated by both phosphatidyl choline and phosphatidyl ethanolamine, while phosphatidyl ethanolamine was not a very efficient reactivator of alkenyl-GPC hydrolase. Martonosi (5) found that both diacyl-GPC and monoacyl-GPC could reactivate the ATPase and the concomitant Ca^{+2} uptake of the sarcoplasmic reticulum after inactivation with phospholipase C. In fact, monoacyl-GPC was the more efficient reactivator. This is in direct contrast to inactivated alkenyl-GPC hydrolase which could not be reactivated with monoacyl-GPC. Thus microsomal enzymes display some selectivity as to which phospholipids reactivate them, which makes it seem more plausible that phospholipids have specific functions in membranes and that reactivation is more than just an effect of surface-active compounds. This is especially apparent with lipid-deficient mitochondrial ATPase which is reactivated by both detergents and phospholipids, but only naturally occurring phospholipids restore the oligomycin sensitivity characteristic of the untreated enzyme (3). One membrane enzyme has been found which requires a specific phospholipid. This enzyme, β-hydroxybutyric dehydrogenase from mitochondria, shows an absolute requirement for phosphatidyl choline which has at least one unsaturated fatty acid component (4,26).

Since phosphatidyl choline reactivates the alkenyl-GPC hydrolase but phosphatidyl ethanolamine reactivates less, some specificity for the polar group of the phospholipid molecule must exist. That sphingomyelin also reactivates the enzyme supports this conclusion, as it also has phosphorylcholine as a hydrophilic component. However, monoacyl-GPC does not effect reactivation, suggesting that there is also specificity for the apolar part of the phospholipid, two long hydrocarbon chains or else one chain linked to the 2-position apparently being required.

The importance of the hydrophobic areas for this enzyme's activity is also implicated by the fact that alkylated ureas are more potent inhibitors than urea, those with longer side chains being more potent inhibitors. The hydrocarbon side chains presumably interact with hydrophobic areas in the membrane, perhaps disrupting the interaction between the phospholipids and proteins.

The normal membrane environment can

be considered as a phospholipid-protein arrangement, and destruction of the membrane phospholipids by phospholipase presumably destroys the native environment of the enzyme. The effect of adding phospholipid to the inactivated enzyme could be to reconstruct the natural membrane environment.

The phospholipase-treated enzyme was also reactivated by incubating it in high concentrations of sucrose. Because the addition of both phosphatidyl choline and sucrose did not have an additive effect in reactivating the enzyme, it does not seem likely that they acted differently. It is possible that both may cause a conformational change in the enzyme, converting it from an inactive form to an active form. Phospholipids could cause this by direct binding to the enzyme whereas sucrose might induce a similar change through an osmotic effect on the membrane, causing it to contract or become dehydrated. Microsomes are known to behave osmotically (27). If this is the cause of reactivation by sucrose, other polyols might mimic this effect. However, the two that were tested, glycerol and mannitol, did not elicit as large a reactivation as sucrose. Both are much smaller molecules than sucrose, and therefore might penetrate the membrane and not exert an osmotic effect. Sucrose could prove to be a useful tool in understanding how phospholipids reactivate the alkenyl-GPC hydrolase.

Treating sonicated microsomes with phospholipase C results in the inactivation of the enzyme. The phospholipase C reaction did not occur unless albumin was added, which could adsorb any monoacyl-GPC present. This lipid can be a potent inhibitor of phospholipase C (28). Microsomal phospholipids were shown to actually be degraded by this enzyme. The products of the reaction, diglyceride and phosphorylcholine and phosphorylethanolamine, differ from the products of the phospholipase A reaction, providing further evidence that the products of the phospholipase A reaction were not responsible for inactivation of the enzyme. The phospholipase C treated alkenyl-GPC hydrolase was also reactivated by phospholipids. Phospholipase C treatment is a second way to hydrolyze membrane phospholipids and thereby inactivate the alkenylhydrolase, lending support to the idea that it requires intact diacyl phospholipids in its environment for maximal activity.

The process of freezing and thawing microsomes probably inactivates the alkenyl-GPC hydrolase by physical means. The enzyme inactivated by this method was also reactivated with phospholipid. Glucose-6-phosphatase, which also requires phospholipids, is likewise inactivated by freezing and thawing (29). Lusena (30) has shown that the release of β-hydroxybutyric dehydrogenase from mitochondria by freezing and thawing is probably not due to the formation of ice per se, but exposure to transient high concentrations of sucrose and redilution being the main cause of damage. Electron microscopy revealed that under conditions where β-hydroxybutyric dehydrogenase was released, the mitochondria underwent drastic structural changes (31). Freezing and thawing microsomes probably produces similar alterations in their structure, destroying the normal membrane environment and disrupting interactions between the phospholipids and the proteins, thereby inactivating the alkenyl-GPC hydrolase. Adding phospholipid to frozen and thawed microsomes may restore enzyme-phospholipid interactions, and, consequently, enzymic activity.

Imidazole causes immediate inhibition of the alkenyl-GPC hydrolase, indicating that the site of interaction on the enzyme is readily accessible to the imidazole molecule. Urea also inhibits the enzyme, but there is about a 3000-fold difference in the concentrations of urea and imidazole required to achieve the same degree of inhibition. The alkenyl-GPC hydrolase was the only microsomal enzyme of those tested which was inhibited by imidazole, demonstrating it is a specific inhibitor of this enzyme and not a general inhibitor of microsomal enzymes.

Several derivatives of imidazole inhibited the enzyme at the same concentration as imidazole. The two derivatives which did not inhibit the enzyme, histidine and N-acetylhistidine, had a negative carboxyl group present in the molecule. The steric bulk of the carboxyl group does not seem to be the responsible factor, since histidine methyl ester is as potent an inhibitor as imidazole. Thus, the negative charge must prevent histidine and N-acetylhistidine from interacting with the enzyme in such a way as to cause inhibition.

Imidazole is known to bind divalent cations, so it might inhibit the enzyme by removing a tightly bound metal ion. However, the inhibition was reversible by dilution, indicating that imidazole does not remove a metal ion or any other cofactor.

Kinetic studies performed in the presence and absence of imidazole revealed that the inhibition of alkenyl-GPC hydrolase is competi-

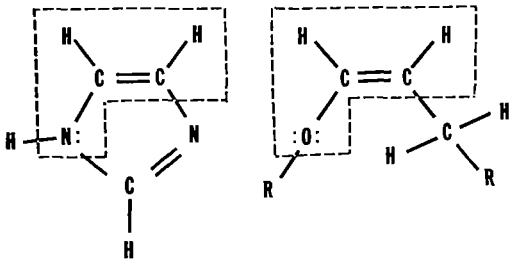

FIG. 8. Structural formulas of imidazole and an alkenyl ether. The dotted line encloses the parts of each compound which are similar in structure.

tive. Reversibility of the inhibition is compatible with this result. Imidazole and the substrate might compete for the same site of the enzyme. Warner (20) has pointed out that there are certain similarities between the structures of the normal substrate and imidazole or its derivatives namely, both have a *cis* double bond with an electron rich center in the vinyl position (Fig. 8). That imidazole inhibition is rather specific for the alkenyl-GPC hydrolase makes the idea that imidazole and the substrate compete for the same site more attractive.

Inhibition by imidazole could be partly reversed by phosphatidyl choline and sphingomyelin, the amount of reactivation being proportional to the amount of lipid added. Thus there are three ways of inactivating the enzyme where addition of phospholipids restores activity; hydrolysis of the lipoprotein phospholipid, freeze-thawing and imidazole addition. The imidazole-inhibited enzyme differs from the phospholipase A-inactivated one in that it is not reactivated by sucrose but is reactivated by potassium chloride. Exactly how the phospholipid reactivates the enzyme is not known for any of the three situations, but further study of the system may provide valuable insight into the role that phospholipids play in the micro-environment of cellular membranes.

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The Effect of the Environmental Temperature on the Fatty Acid Composition and on the *in vivo* Incorporation of 1-^{14}C-Acetate in Goldfish *(Carassius auratus L.)*[1]

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ABSTRACT

Two-year-old goldfish were adapted to 10C and 35C environmental temperatures during a three-week period, and the fatty acids from triglycerides and certain phospholipids were analyzed by gas-liquid chromatography. Over-all unsaturation of the major fatty acids increased with lower temperature in all lipids which were examined although fish maintained at 10C actually had less polyenoic acid in their tissues than did those maintained at 35C.

Fish acclimated to 10C and 30C were injected with 1-^{14}C-acetate, and the activities of the isolated fatty acids were counted. The incorporation of ^{14}C into the fatty acids was much greater at the lower temperature. A comparison of the activities of saturated and unsaturated fatty acids within each temperature group revealed a tendency toward higher incorporation into the unsaturated acids at lower temperature. The possible correlations between accelerated biosynthesis of polyenoic acids and the lower tissue levels of these acids in the cold-adapted fish are discussed.

INTRODUCTION

In the relationships between living organisms and their environment, the temperature is an important factor since it defines the life-sustaining and lethal ranges in both directions. Within this area of existence the organism can adjust itself in individual ways in order to develop and function properly.

Several authors (1-12) have reported an influence of temperature on the fatty acid composition of many different organisms, and explanations for the observed increase of unsaturation at lower temperature and also for its mechanism have been offered (9,10,13).

For homeotherms the metabolic responses to changes of environmental temperatures must be different from those of poikilotherms since the former react to cold exposure by metabolic adjustments tending to counteract the effects of the temperature change. Effects of short-term or prolonged exposure to cold on the metabolism of lipids in homeotherms have been reviewed by Masoro (14).

Among the poikilotherms the aquatic organisms represent a large number in class and species, and their exposure to temperature alterations is direct and intensive as, for example, in fish in which the blood is in continuous close proximity and caloric exchange with the water by its circulation through the gills. The possibilities and the principles for thermal adaptation of fish have been summarized by Brett (15).

An investigation by Lewis (13) on the correlation between temperature and fatty acid pattern showed that certain species of poikilothermic marine animals from Arctic regions have a higher palmitoleic and a lower saturated acid content than do similar species from temperate water. Lewis suggests this to be a possible means of preservation of the "plasmatic viscosity."

According to Farkas and Herodek (16), the melting point of lipids from planktonic fresh water copepods was somewhat lower than the water temperature, following its increase and decline during the entire year. The variations of the melting point were due to changes in the amount of long-chain unsaturated fatty acids, as demonstrated by the iodine numbers of the fat.

In a controlled diet experiment Kayama *et al.* (17) raised guppies *(Lebistes reticulatus)* at 17C and 24C solely on brine shrimp *(Artemia salina)*. The lipids of the warm-water fish contained a higher percentage of palmitic and stearic acids whereas palmitoleic, oleic, and docosahexaenoic acids were decreased.

Holton *et al.* (12) observed an effect of the

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environmental temperature on the fatty acid composition of the blue-green alga *Anacystis nidulans,* in which the major change was reflected in a relative decrease of hexadecenoic acid at a raised temperature.

The sum total of these and many other observations suggest a temperature dependency of fatty acid unsaturation throughout the entire food chain.

In a search for a suitable experimental subject for investigation of these phenomena, the present authors (18) found that muscle and liver lipids from rainbow trout *(Salmo gairdneri)* contain more highly unsaturated fatty acids at lower temperatures and that the specific acids involved were different in the two types of tissues.

Corresponding results were obtained in an investigation (19) on mosquito fish *(Gambusia affinis)* and guppies *(Lebistes reticulatus).* The latter species was also used in a diet-controlled experiment. It was shown that fish on a low fat and those on a complete diet react in quite different ways to temperature changes. In the same study it was found that cold adaptation took place quite rapidly, with few changes occurring, after four weeks. The experiments presented here have two aims: first, to ascertain the distribution of fatty acids in the tissues of fish acclimated to different temperatures and, second, to study the relative rates of formation of saturated and unsaturated fatty acids at the different temperatures. The data describe the effects of temperature extremes on the fatty acid composition of the tissues and on the incorporation of 1-^{14}C-acetate into the tissue fatty acids of goldfish *(Carassius auratus L.).* The experimental results suggest that changes in tissue fatty acids may be a reflection both of an alteration in the relative amounts of triglycerides and phospholipids and of a change in the relative rates of biosynthesis of the fatty acids.

EXPERIMENTAL

The fish, purchased from the Auburndale Company in Chicago, were two years old and 4-5 in. in length with an average weight of 125 g. Three fish each were acclimated to 10C and 35C for the investigation of their fatty acid pattern (experiment 1). For the incorporation of labeled acetate (experiment 2) three fish each were adapted to 10C and 30C. In the following descriptions the cold-water fish in both experiments will be identified as group 1A and 2A respectively, and the warm-water fish as group 1B and 2B respectively.

The acclimation was achieved by gradual immersion of the aquaria in water baths with preset temperatures. Both Groups A did not eat for several days, after which time their food uptake remained somewhat lower than that of both Groups B. All fish appeared to be in good condition throughout the experiments.

It was assumed that the adaptation to the chosen temperatures was completed after three weeks (20,21). In Experiment 1 the fish were killed by freezing on dry ice, and muscle tissue was excised and weighed under nitrogen. (Whenever practicable, the succeeding operations were performed under nitrogen.)

In Experiment 2 the fish were taken out of the water with wet paper towels and injected intraperitoneally with an aqueous solution of 0.1 mc sodium 1-^{14}C-acetate each, with a #25 needle on a tuberculin syringe. These fish were then returned to their aquaria; Group 2B was sacrificed after 4 hr, Group 2A after 7 hr. This time difference was used in an attempt to compensate for the decreased metabolic rate at lower temperature. After decapitation and removal of intestines, organs, and fins, the rest of the fish was weighed and used *in toto.*

The tissues of all groups of fish were cut into small pieces and lyophilized. The lipids were extracted in a Waring Blendor with three 150-ml portions of chloroform-methanol (2:1, v/v). The extracts within each group were combined and filtered, and after the solvent had been removed on a rotary evaporator at 30C, the residues were weighed.

In experiment 2 the fatty acids of the total lipids were investigated; in experiment 1 the neutral lipids and phospholipids were separated, and the fatty acids of each were analyzed separately.

Experiment 1

The total extracted lipid mixtures from the muscles weighed 1.0 g for Group 1A and 0.6 g for Group 1B. For the separation into neutral lipids and phospholipids, 300 mg of each extract were placed on a 250 × 11-mm column of washed activated silicic acid, containing 12% celite (w/w). The lipid fractions were eluted with 150 ml of n-pentane, followed by 300 ml each of 2, 5, 10, and 50% ether in pentane. In order to remove all traces of neutral lipids still on the column, 300 ml of ether was passed through. The phospholipid-containing fractions were then eluted with 300 ml of methanol.

Purity of the eluted neutral lipid fractions was established by thin-layer chromatography (TLC) on Silica Gel G. Solvent systems were

appropriate mixtures of n-pentane and ether. The plates were developed by spraying with chromic acid solution (5g, $K_2Cr_2O_7$ in 100 ml 40% H_2SO_4) and charring. Identification of the lipids was achieved by comparison of R_f's with those of known standards.

In order to isolate the different components of the phospholipid fractions, the mixtures were separated by TLC (22) on Silica Gel G. The first plate of each sample was charred with chromic acid spray and used for densitometric estimation of the amounts of the various components. Two other plates were treated with molybdic acid spray (a solution of MoO_3 and powdered Mo in dilute H_2SO_4) to ascertain the phospholipid nature of the separated fractions. The bulk of the two samples was then chromatographed, sprayed with bromophenol blue indicator (50 mg of bromophenol blue in 100 ml of 0.01 N NH_4OH), and the various phospholipids were scraped off separately. They were recovered from the scrapings by extraction with chloroform-methanol (2:1, v/v), followed by drying over $MgSO_4$, filtration, and removal of the solvent. When the partially purified fractions were rechromatographed in the same manner, no impurities could be detected. They were converted to the methyl esters of their component fatty acids by transesterification with 4% methanolic HCl.

Identification and quantitative determination of the fatty acid methyl esters of all fractions were performed by gas-liquid chromatography (GLC) on a Barber-Colman Model 10 apparatus with a 40 × 0.25-in. column of ethylene glycol succinate, 16.9% on Gas-Chrom P, 80-100 mesh. All calculations of the mass peaks in the chromatograms were carried out by multiplication of peak height by peak width at half-height. The possible presence of hydroxy- and branched-chain acids and of any fatty acids beyond a chain length of 22 carbon atoms was disregarded. The remaining acids were calculated at percentages of the total. For identification, standard samples of known esters were chromatographed under identical conditions.

Experiment 2

Five grams of each total lipid extract (13.4 g in experiment 2A, 16.2 g in experiment 2B) were saponified by boiling under reflux in 3% methanolic KOH for 1 hr, and the unsaponifiable matter was extracted with pentane. From the acidified solutions the free fatty acids were extracted with ether; the combined extracts within each group were washed with water, dried over $MgSO_4$ freed from solvent, and weighed (3.7 g in Group 2A, 4.2 g in Group 2B). After esterification of the acids with an ether solution of diazomethane, both samples were examined by analytical GLC and then separated into fractions of identical chain-lengths on a Wilkens Instrument Company A-100 Aerograph apparatus with a 60 × 0.5 in. column of SE-30 silicone stationary phase, 10% on Chromosorb W support. After rechromatography the fractions were pure as indicated by analytical GLC.

To separate the acids of different degrees of unsaturation within the isolated C_{16} and C_{18} fractions, these mixtures (200 mg for each run) were placed on a column (110 × 14 mm) of silver nitrate-impregnated silicic acid, prepared acording to DeVries (23). The saturated esters were eluted with 10% benzene in pentane, the monounsaturated esters with 2% ether in pentane; the diunsaturated esters were recovered with 3% and 5% ether in pentane. The more highly unsaturated esters were eluted together with ether. These were then (in experiment 2A only) separated by preparative GLC on a Wilkens Instrument Autoprep A-700 with a 10-ft × 3/8-in. column of 30% DEGS on Chromosorb P. The same instrument was also employed for separation of the 20-carbon esters from experiment 2A.

The 22-carbon acids were isolated on a Loenco Model 70 Hi-Flex apparatus with a 4-ft × ¼-in. column of 10% DEGS on chromosorb W by use of a Packard Model 850 fraction collector.

Esters of acids with 14 carbon atoms or less were not separated in either group of experiment 2.

In Group 2B the acids with 20 and 22 carbon atoms were hydrogenated without further separation and were counted together since their activity was too low to give adequate counting rates for individual components.

All fractions were purified by silicic acid chromatography and checked by analytical GLC. The radioactivity of the various fractions was then counted in a Packard Tri-Carb Liquid Scintillation Spectrometer.

RESULTS

In the experiments with cold-water-adapted fish (1A and 2A), the influence of temperature in both groups resulted in an identical total lipid content of the muscle tissue, 3.9% of the wet tissue (Fig. 1). In another experiment 3.3% was found (unpublished results). The lipid content appears to be very sensitive to higher temperatures. In the experiment described, the lipid content was 2.6% at 35C and

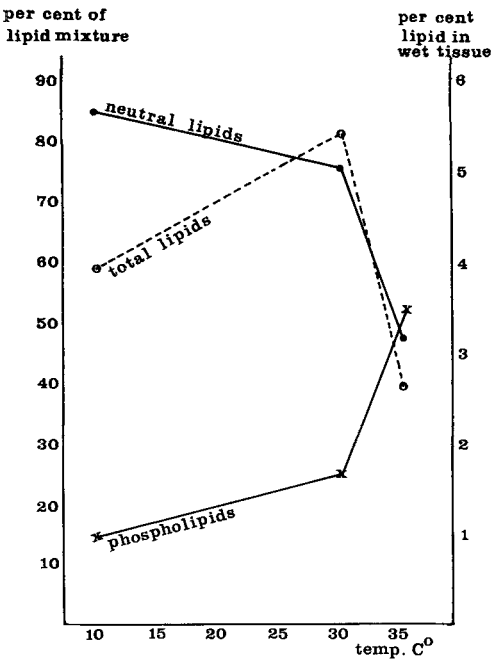

FIG. 1. Changes of the total lipid content (broken line) and of neutral lipids and phospholipids (solid lines) in the muscle tissue of goldfish (*Carassius auratus L.*) with the temperature.

5.4% at 30C (Fig. 1); in an unpublished investigation, which was also carried out at 30C, it was 5.9%.

Within the lipid fractions the ratios of neutral lipids to phospholipids reflect the enhanced deposition of the neutral lipids, mainly triglycerides at lower temperature, and an increase in the proportion of phospholipids at higher temperature (Fig. 1).

Experiment 1

The results of the gas-chromatographic analyses of the methyl esters from muscle lipids from experiment 1 are presented in Table I. The saturated and monounsaturated 16- and 18-carbon acids show the expected increase of unsaturation with declining temperature. However the higher unsaturated acids, mainly linoleic acid, seem to show the opposite effect. The decreased levels of these latter acids at lower temperature occur in both the triglyceride and phospholipid fractions alike.

Estimation of the amounts of the various phospholipids in the mixture by TLC chromatography and charring revealed, for both samples, about 10% sphingomyelin, 40% lecithin, two spots of 15% each for "cephalin," and 10% of an unknown, less polar compound. About 10% of the material remained at the origin. The exact nature of the "cephalin" fractions, whether phosphatidyl ethanolamine or phosphatidyl serine, was not determined.

The major changes of the fatty acids in the various phospholipids involved different acids. For example, the concentration of 16:0 in sphingomyelin was not affected by the temperature changes although the distribution of this same acid into TG and PL, in general, was markedly affected by fluctuations in the environmental temperature.

Palmitoleic acid, present in greater proportion at lower temperature in the total lipid extract and in the major phospholipid fractions, showed the opposite effect in the triglycerides although these changes are of doubtful significance.

The change in the stearic acid of the total lipid mixture seemed to be attributable to the phospholipids, mainly to "cephalin" and the unknown polar compound with smaller changes in sphingomyelin and no change in lecithin, which, as noted above, had the highest increase in palmitic acid. The stearic acid levels in the triglycerides barely changed. The increase in the proportion of oleic acid at lower temperature could be observed in triglycerides and phospholipids. In the latter, the major effect was in the unknown fraction, with a slight change in "cephalin" but essentially no change in lecithin and an opposite effect in sphingomyelin.

Apart from these major fatty acids, the more highly unsaturated long-chain fatty acids were present in smaller proportion. The relative amounts of these acids were increased at higher temperature.

Experiment 2

In the experiments 2A and 2B a comparison of the activities (Table II) demonstrates a strongly enhanced incorporation of 1-^{14}C-acetate into the fatty acids of cold-water-adapted fish. With the exception of stearic acid all components of the mixture showed a profound increase in specific activity, particularly the unsaturated members. Within the fractions of the same chain-lengths, the specific activities tapered off with increase in unsaturation except for the acids with 22 carbon atoms, in which the tri- and pentaenoic acids were several times as active as the rest of the mixture.

A comparison of the total activities of the major fatty acids may reveal more conclusively into which compounds acetate was preferentially incorporated (Table II). Incorporation into palmitic acid of Group A was over twice

TABLE I

Gas-Chromatographic Analysis of Methyl Esters from Lipids of Muscle Tissue of Goldfish Kept at 10C and 35C Water Temperature (Percentage of Total)

	Total lipid		Triglyceride		Total phospholipid		Sphingo-myelin		Lecithin		"Cephalin"		Unknown polar material	
	10C	35C	10C	35C	10C	35C	10C	35C	10C	35C	10C	35C	10C	35C
14:0	1.1	1.1	0.5	1.7	+	+	?	?	+	?	?	?	?	?
14:1	0.1	0.1	+	0.4	+	+	+	?	+	+	0.4	+	?	+
16:0	25.8	27.8	25.2	28.4	25.5	29.8	25.8	25.7	26.8	32.5	9.7	11.9	15.3	21.8
16:1	8.3	7.6	7.7	8.7	5.9	3.1	0.7	+	2.7	+	0.6	+	2.3	3.6
16:2	+	+	+	+	+	+	0.8	+	+	+	+	+	+	+
16:3	+	+	+	+	+	+	+	-	+	+	?	?	+	+
16:4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18:0	5.0	6.2	4.4	4.6	7.8	10.5	23.5	24.4	6.7	6.8	19.6	29.5	7.2	9.2
18:1	45.3	37.4	50.1	43.0	25.6	21.3	20.0	24.5	30.6	31.0	19.8	18.8	31.7	21.8
18:2	7.5	9.6	6.2	8.0	8.5	10.1	5.1	5.2	15.3	12.1	3.9	6.8	28.9	31.4
18:3	+	+	+	+	+	+	+	+	-	-	+	+	+	+
18:4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20:0	0.2	0.1	0.3	0.3	0.7	0.4	0.7	0.8	0.4	0.4	0.8	0.5	+	1.5
20:1	2.1	2.1	2.2	2.5	3.1	2.6	1.5	0.8	1.0	0.6	6.6	1.6	5.7	1.6
20:2	0.6	0.6	0.8	0.5	0.7	+	0.8	+	0.4	+	2.6	+	2.5	0.9
20:3	1.4	1.7	1.1	0.8	5.7	3.8	2.0	1.4	5.3	4.5	6.3	2.9	2.4	1.5
20:4	1.2	2.5	0.4	0.4	5.4	8.1	5.2	6.5	5.5	7.0	12.0	10.3	1.8	2.3
20:5	0.1	0.4	0.5	0.1	2.8	1.0	+	1.4	1.0	1.5	2.1	1.0	0.5	0.8
22:0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22:1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22:2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22:3	+	+	+	0.2	0.5	+	2.6	+	0.8	+	1.5	0.5	+	+
22:4	0.5	0.3	0.6	+	3.0	3.1	7.5	7.7	1.2	2.1	5.7	5.5	1.3	2.1
22:5	+	+	+	+	0.7	+	+	+	0.6	+	1.5	0.9	+	+
22:6	0.8	2.0	+	0.4	4.1	6.2	3.8	1.6	1.7	1.5	6.9	9.8	0.4	1.5

TABLE II

Activities of the Fatty Acids Isolated from Goldfish Kept at 10C and 30C Water Temperature

Identity of fatty acids	10C		30C	
	Spec. activity (CPM/mg x 10^3)	Total activity (CPM x 10^5)	Spec. activity (CPM/mg x 10^3)	Total activity (CPM x 10^5)
14	7.8		2.3	
14:0	16.3	1.8	5.2	0.77
14:1	23.9	0.24	2.3	0.03
16:0	27.2	69.0	8.1	31.0
16:1	25.0	21.0	1.6	1.7
16:2	3.7			
16:3	2.6		Not isolated	
16:4	1.3			
18:0	16.7	8.3	15.0	13.0
18:1	11.9	53.0	2.6	13.0
18:2	1.1	0.84	0.04	0.05
18:3	1.0		Combined	
18:4	0.5		fractions	
20:0	4.9	0.1	0.4	
20:1	15.3	3.2		
20:2	12.3	0.73	Combined	
20:3	4.9	0.69	fractions	
20:4	2.5	0.3	2.7	
20:5	2.2	0.02		
22:0	Not isolated			
22:1	1.1			
22:2	Not isolated		Combined	
22:3	8.2		fractions	
22:4	1.3	6.5	2.4	
22:5	8.4			
22:6	0.17	1.3		

that of Group B, but for palmitoleic acid it was about 12 times as much. This is demonstrated even more clearly by the ratios of the total activities of palmitoleic to palmitic acid within each group (0.3 at 10C and 0.055 at 30C).

The total incorporation of labeled acetate into stearic acid was 1.6 times as high in Group B whereas that of oleic acid, by far the major constituent of the fatty acid mixture, was about four times as great in Group A. The ratios of the total activities of oleic to stearic acid are 6.4 for Group A and 1.0 for Group B.

The octadecadienoic acid in experiment 2A contained a large amount of activity in comparison to experiment 2B. The 1-carbon Schmidt-degradation (24) revealed 80% of the activity to be in the carboxyl group. Gas-chromatographic analysis of the aldehydes and aldehyde esters, derived by ozonolysis and reduction of the ozonides, revealed some heptadecyl aldehyde and octanedioic acid ester semialdehyde, indicating the presence of some 8,11-octadecenoic acid along with the predominant 9,12-(linoleic) acid.

DISCUSSION

In experiment 1 the temperature of 35C was probably rather extreme since it imposes conditions goldfish might never have to endure in their natural habitat. Although it was thought that this temperature might enhance the desired effect on the fatty acid composition, abnormalities of metabolism may have occurred, caused by approaching the upper limits of the temperature tolerance of the fish, and the values in Table I may partially reflect this, especially in the more highly unsaturated long-chain fatty acids. Several authors (15) have reported investigations of lethal temperature limits of poikilotherms. Goldfish seemingly have a wide range of thermal adaptability as compared with other species (25). The major effect of the high temperatures in this type of animal may thus have been largely reflected in an increased metabolic rate.

The increased deposition of triglycerides in the muscle tissue of fish at lower temperature is contrary to findings from studies with homeothermic animals. Thus Patkin and Masoro (26) found adipose tissue from rats to synthesize more fatty acids at lower temperature, but a faster mobilization of these fatty acids in order to meet the higher caloric needs brought the fat content down to the level of the control animals.

In evaluating the changes of the phospholipid fatty acids, it becomes evident that the complex process of temperature adaptation involves quite different reactions of the individual lipids. With alteration of the environmental temperature, a particular fatty acid might

undergo a large change in one type of lipid whereas it would not be affected at all in another, particularly in certain membrane lipids with low turn-over rates.

Although the knowledge of the functions of phospholipids as components of cell walls and their participation in metabolic processes is very limited, it can be concluded nevertheless that both triglycerides and phospholipids are involved in the adaptation to varying environmental temperatures, the latter selectively with fatty acids from the entire spectrum, the former mainly with the 16- and 18-carbon acids.

If, as Lewis (13) has suggested, the preservation of "plasmatic viscosity" is the important result of the changes of the fatty acid pattern, then the triglycerides, as storage material in adipose tissue, would need to be desaturated rather rapidly, after a sudden lowering of the temperature, to avoid solidification. It is also possible that the decreased viscosity is achieved by laying down additional unsaturated fat.

The enzyme systems involved in the changes of mono- and polyunsaturated fatty acids are probably not identical (27). Since the latter acids are largely present in the phospholipids as components of cell membranes and similar structures, they probably will not be affected by temperature changes to the same degree as other fatty acids. Polyenoic fatty acids probably can be expected to change widely only under symptoms of acute starvation after the depot fat is already depleted. The acetate incorporation data however indicate that the newly synthesized polyunsaturated fatty acids will be profoundly affected.

In Experiment 2 the labeled acetate was incorporated to a several-fold larger extent in the cold-water-adapted fish as compared with fish in the warm environment. This effect is obvious in the entire spectrum of the fatty acids, but it holds especially for the unsaturated acids and cannot simply derive from a difference in the incubation time. A metabolic turnover, equaling that for fish in water of 35C during 4 hr, might be achieved after as much as 12 hr in cold-water fish and not after 7 hr, as implied in this experiment.

The difference in the incorporated activities becomes even more apparent, if one considers that, although the total lipid content at 10C is lower, the ratio of neutral lipids to phospholipids is increased (Fig. 1). The greater part of the neutral lipid fraction is triglyceride and, as such, contains a relatively larger proportion of the fatty acids than do the phospholipids.

If it can be assumed that a steady state is reached after the time of incubation, the quotient of the total activities of the unsaturated and saturated fatty acids should be significant for each acid group at each temperature, and it should be largely independent of the incubation time. As long as this quotient from the cold-adapted group, divided by the quotient of the warm-water group, gives a figure greater than 1, the intensified desaturation at lower temperature is evident; the degree is obvious from the magnitude (Table III).

TABLE III

Ratios of Total Activities of Major Unsaturated Fatty Acids and Saturated Fatty Acids

	UF/SF at 10C	UF/SF at 30C	UF/SF at 10C / UF/SF at 30C
16:1/16:0	0.3	0.055	5.5
18:1/18:0	6.4	1.0	6.4
18:2/18:0	0.1	0.004	25.0

To a certain extent the need for synthesis of unsaturated fatty acids includes the higher unsaturated acids of the 18-carbon group, also those with 20 and 22 carbon atoms, in which the saturated components are present only as traces. No explanation can be offered for the finding that some of these long-chain fatty acids of the cold-water-adapted fish are so much more active than others. For example, the high activities in docosatrienoic and pentaenoic acids, which are present only in trace amounts, and the low activity of docosahexaenoic and -tetraenoic acids, both of which represent the major components of this long-chain fraction, may indicate that the former are more active metabolically than the latter.

The strongly active octadecadienoic acid from experiment 2A is probably not entirely linoleic acid since it has been shown that linoleate must be supplied by an external source and cannot be synthesized in the animal organism. The distribution of activity in the carbon chain does not rule out the possibility of a 2-carbon exchange (28) by which labeled acetate could be incorporated into the molecule, but it is a strong indication for its synthesis by chain elongation and desaturation of palmitate via 11-octadecenoic acid (29) or by desaturation of oleic acid in the 6-position.

In conclusion, it should be noted that the apparent discrepancies between the results of experiments 1 and 2 probably result from the contrary operation of two factors. At the higher temperatures, an increased metabolic rate

brings about increased utilization of stored fatty acids, largely the saturated and monounsaturated types. The polyunsaturated fatty acids however, as components of membranes, will not be greatly affected by this depletion and appear to increase proportionately.

In experiment 2, in which only the newly synthesized acids are measured, the effect of lower temperature is to increase relative amounts of the more highly unsaturated acids.

Thus, these two effects appear to give the same results although they take place at opposite extremes of temperature.

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Quantitative Determination of Alk-1-enyl- and Alkyl-Glyceryl Ethers in Neutral Lipids and Phospholipids[1]

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ABSTRACT

A quantitative method for the simultaneous determination of alk-1-enyl- and alkyl-glyceryl ethers is described. Complete hydrogenolysis of carboxylate and phosphate esters of neutral lipids and phospholipids was achieved with lithium aluminum hydride. The hydrogenolysis products of the glyceryl ether containing lipids, alk-1-enyl- and alkyl-glyceryl ethers and alcohols, were identified by thin-layer chromatography (TLC), gas-liquid chromatography (GLC), and infrared spectroscopy. The alk-1-enyl- and alkyl-glyceryl ethers were quantitated by TLC photodensitometry. The specificity of this method can also be extended when used in conjunction with GLC, ion-complexing TLC, zonal scanning, and autoradiography to study composition, isomeric form, and the biosynthesis of glyceryl ethers in neutral and phospholipids.

The percentage of alk-1-enyl- and alkyl-glyceryl ethers in both the neutral lipids and phospholipids of various rat tissues was determined by the described method. Glyceryl ether glycerides represent 0.3-1.2% of the total neutral lipids whereas the glyceryl ether phosphatides of brain, heart, marrow, muscle, and spleen represent 4.5-12.0% of their total phospholipids. Higher concentrations of glyceryl alkyl than of alk-1-enyl ethers were found in the neutral lipids whereas the glyceryl alk-1-enyl ethers were found to predominate in the phospholipids.

INTRODUCTION

CLEAVAGE OF GLYCERYL ALKYL[3] ethers from phosphatides by existing acetolysis procedures (1,2) is not applicable for the release of glyceryl alk-1-enyl[3] ethers from plasmalogens owing to the lability of the alk-1-enyl ether bond (3). Numerous methods based on reactions specific for the alk-1-enyl double bond exist for the indirect analysis of glyceryl alk-1-enyl ethers. After mild acid hydrolysis the products are analyzed by paper chromatography (4-6), by GLC (7-9), and by colorimetry (10-11). The alk-1-enyl content can also be determined by the specific iodination method of Rapport and Franzl (12), modified by Norton (13) and further modified by Williams et al. (14), or by spectrophotometric determination of the dinitrophenylhydrazones (15-17) prepared from the aldehydes released by acid hydrolysis. Recently Owens (18) has described a two-dimensional TLC procedure for the estimation of the plasmalogen phosphatides in each of the phospholipid classes.

The possible instability of alk-1-enyl phosphatides to alkali (19) and the formation of cyclic acetals of alk-1-enyl lysophosphatides subjected to mild acid conditions (19,20) yield results subject to error where these conditions exist. The colorimetric assay based on the Schiff reagent gives high glyceryl alk-1-enyl ether values, while those methods of analysis based on iodination and hydrazone formation yield low values (3). None of the methods mentioned allow the simultaneous determination of alk-1-enyl- and alkyl-glyceryl ethers.

Our method is based on the TLC photodensitometric analysis of the resolved alk-1-enyl- and alkyl-glyceryl ether classes that are formed after lithium aluminum hydride ($LiAlH_4$) hydrogenolysis of phosphate and carboxylate esters. Thompson et al. (21,22) have previously used $LiAlH_4$ for the reduction of larger lipid samples so that the hydrogenolysis products could be separated by silicic acid column chromatography and measured gravimetrically.

This report characterizes the products formed by $LiAlH_4$ hydrogenolysis and demonstrates the applicability of the method for the simultane-

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[3] The term "alkyl" ethers is used throughout the manuscript to denote alkyl- and alkenyl-glyceryl monoethers other than glyceryl alk-1-enyl monoethers, commonly referred to as vinyl ethers. The glyceryl alk-1-enyl ethers can also have additional unsaturation along the hydrocarbon chain.

ous determination of alk-l-enyl- and alkyl-glyceryl ethers quantitatively. The alk-l-enyl- and alkyl-glyceryl ether content of the neutral lipids and phospholipids of a number of normal rat tissues has been determined by this method.

EXPERIMENTAL SECTION

Lipid Isolation and Subfractionation

Lipids were extracted as described previously (23) from the various tissues of young (130-160 g) and adult (> 200 g) female rats (Carworth Farm Nelson's strain) maintained on commercial Dietrich and Gambrill laboratory chow. Forty milligrams or less of total lipids was separated into neutral lipids and phospholipids according to the procedure of Borgström (24) on 0.9 g of silicic acid in a 2-ml fritted glass funnel (0.9-1.4 μ porosity) at room temperature and below 5C. The neutral lipids were quickly eluted with 15 ml of chloroform, and the adsorbed phospholipids were subsequently eluted with 15 ml of methanol.

$LiAlH_4$ Reduction

A known amount (3-5 mg) of neutral lipid or phospholipid was reduced with $LiAlH_4$ by a modification of the procedure by Thompson and Lee (21). Three milliliters of the $LiAlH_4$ reagent (5 mg/ml diethyl ether) was added to each 15-ml conical centrifuge tube (S 14/20) containing the lipid in 0.5 ml of diethyl ether. The centrifuge tubes were immediately connected to water condensers, equipped with a drying tube, and refluxed vigorously for 30 min. The tubes were then removed: 3 ml of distilled water (the first few drops being added cautiously), 3 ml of 4% acetic acid, and 3 ml of diethyl ether were added, and the tubes were stoppered and shaken vigorously for 1 min. Emulsions that usually formed with the phospholipid samples were quickly broken by centrifuging at low speeds for 3-5 min. The diethyl ether phase was then removed with a Pasteur pipette, and the aqueous phase was extracted twice more with 6-ml portions of diethyl ether. The combined diethyl ether extractions were evaporated to dryness under a stream of dry nitrogen, and the last traces of water were removed under reduced pressure. Next, the lipids were transferred quantitatively to a 0.5-dram (2 ml) screw cap vial with Teflon liner, evaporated to dryness, and redissolved with chloroform to a known concentration for TLC analysis.

Thin-Layer Chromatography

Uniform 0.25-mm layers of Silica Gel G were spread on 20 × 20 cm glass plates with a modified Colab applicator (25). The chromatoplates were air-dried, precleaned with diethyl ether-methanol (95:5,v/v), and activated for 20 min at 110C. The chromatoplates were developed in a saturated chamber of either diethyl ether-30% aqueous ammonium hydroxide (100:0.25,v/v) or diethyl ether-water (100:0.5,v/v). The chromatoplates were air-dried, and quantitative densitometric measurements were made according to the procedure of Privett et al. (26). Samples to be recovered from the chromatoplates were visualized by spraying with 0.2% 2',7' dichlorofluorescein in ethanol, and the areas were outlined under UV light. The zones were scraped into a fritted glass funnel and eluted from the adsorbent layer with several volumes of diethyl ether.

Infrared Spectra

The samples were prepared as a thin film on the face of a potassium bromide (KBr) window, and the infrared (IR) spectra were obtained with the use of a Perkin-Elmer Model 337 spectrophotometer. The spectrum of the cyclic acetal in the hydroxyl region was made with an approximately 0.01 molar solution of the acetal in carbon tetrachloride with the aid of a beam condenser and a home-made liquid cell with Mylar windows. The region from 1700 to 2700cm^{-1} of little or no adsorption was not reproduced in the spectra shown.

Materials

The neutral plasmalogen which had been isolated from ratfish liver oil by TLC (27) was a gift from Helmut Mangold and Harold H. O. Schmid, The Hormel Institute, Austin, Minn. The labeled glyceryl ether and the synthetic 1-octadecoxy - 2 - hexadecyl - 3 - phosphoglycerol (glyceryl alkyl ether phosphatidic acid) were made available by Claude Piantadosi and Edward O. Oswald, University of North Carolina, Chapel Hill, N. C.

Other glyceryl alkyl ethers used were synthesized in this laboratory, and their purity and physical and chemical properties have been described (28). Lithium aluminum hydride and silicic acid (325 mesh) were obtained from Metal Hydrides Inc., Beverly, Mass., and Bio-Rad Laboratories, Richmond, Calif. Glass-distilled solvents used in the lipid extractions were obtained from Burdick and Jackson Laboratories Inc. Other reagents and materials were reagent grade and were used without further purification.

RESULTS AND DISCUSSION

Lipid Subfractionation

The reported stability and instability of plas-

TABLE I

Alk-1-enyl- and Alkyl-Glyceryl Ether Concentrations Present in Neutral Lipids and Phospholipids of Various Rat Tissues[a]

Sample No.	Tissue[b]	Lipid type[c]	Percentage neutral lipid and phospholipid	Percentage of N or P Lipid Fractions: Glyceryl alk-1-enyl ether[d]	Glyceryl alkyl ether[e]	Total ether
21	Plasma	N	58.3	T[f]	0.2	0.2
22	Plasma	P	41.7	0.2	0.2	0.4
23	Perirenal	N	99.7	T	0.4	0.4
24	Perirenal	P	0.3	0.9	0.2	1.1
25	Muscle (femoral)	N	52.3	T	0.2	0.2
26	Muscle (femoral)	P	47.7	2.4	0.4	2.8
27	Marrow (femoral)	N	37.2	0.1	0.4	0.5
28	Marrow (femoral)	P	62.8	2.2	2.0	4.2
29	Liver	N	39.5	0.1	0.2	0.3
30	Liver	P	60.5	0.2	0.4	0.6
31	Spleen	N	26.0	0.1	0.3	0.4
32	Spleen	P	74.0	1.6	1.3	2.9
33	Kidney	N	23.0	0.1	0.2	0.3
34	Kidney	P	77.0	0.5	0.1	0.6
35	Heart	N	26.5	0.1	0.3	0.4
36	Heart	P	73.5	1.3	0.3	1.6
37	Brain	N	23.2	0.2	0.1	0.3
38[g]	Brain (5 samples)	P	76.8	2.4 ± .22	0.59 ± .02	3.0
38A[g]	Brain (6 samples)	P	—	2.5 ± .33	0.61 ± .09	3.1

[a] Each value represents the mean of duplicate determinations.

[b] Tissue lipids from Samples No. 21-30 were obtained from 130-160 g of female rats and those from 31-36 were obtained from young adult female rats. Samples No. 37-38A were obtained from old adult male rats.

[c] N denotes neutral lipid, and P denotes phospholipid.

[d] Commonly referred to as vinyl ethers.

[e] Includes glyceryl ethers with unsaturation in the hydrocarbon chain other than the one position.

[f] T represents less than 0.1%.

[g] Brain phospholipids were separated from neutral lipids by silicic acid chromatography at room temperature for Sample No. 38 and below 5C for Sample No. 38A.

malogens subjected to silicic acid chromatography have been reviewed by Rapport and Norton (3). The glyceryl alk-1-enyl ether content of brain phospholipids separated from neutral lipids by silicic acid chromatography at room temperature was not significantly different from those separated at a lower temperature (Table I). The brief period of time (15-20 min) during which the lipids were exposed to the silicic acid probably accounts for the lack of detectable degradation of the alk-1-enyl ethers.

Hydrogenolysis Conditions

The carboxylate and phosphate esters of the neutral plasmalogen and the glyceryl alkyl ether phosphatidic acid were completely cleaved by the method of Thompson and Lee (21). Lower concentrations of the $LiAlH_4$ reagent work equally well and have the advantages of reducing the explosive hazard during the subsequent hydrolysis of excess reagent (20- to 40-fold) and LiAl-alcoholates; also, the amount of precipitate to contend with during extraction is reduced. When tetrahydrofuran was substituted for diethyl ether as a solvent for the reduction, some cleavage of the alk-1-enyl ether linkage occurred (Fig. 1, Lane 3, small spot at the same R_f as the aldehyde in Lane 7). But hydrogenolysis of the neutral plasmalogen in diethyl ether always gave only alcohols and glyceryl alk-1-enyl ethers.

Extraction of Hydrogenolysis Products

The quantitative method necessitates quantitative extraction of the lipid hydrogenolysis products from the reaction mixture. This was determined by the addition of a 1-^{14}C-labeled glyceryl alkyl ether to a series of neutral lipid and phospholipid samples before $LiAlH_4$ reduction. Greater than 95% recovery of the activity was achieved when the aqueous phase was acidified with acetic acid (2%) before extraction, as Thompson (22) suggested. Cleavage of the glyceryl alk-1-enyl ethers did not occur under these conditions or during TLC (Fig. 1, Lane 5).

Identification of Hydrogenolysis Products

Reduction of the glyceryl alkyl ether phosphatidic acid gave rise to only two products (Fig. 1, Lane 1) the R_f's of which corresponded to a glyceryl alkyl ether and a long-chain alcohol. Both the glyceryl alkyl ether and the alcohol identities were confirmed by GLC (29,

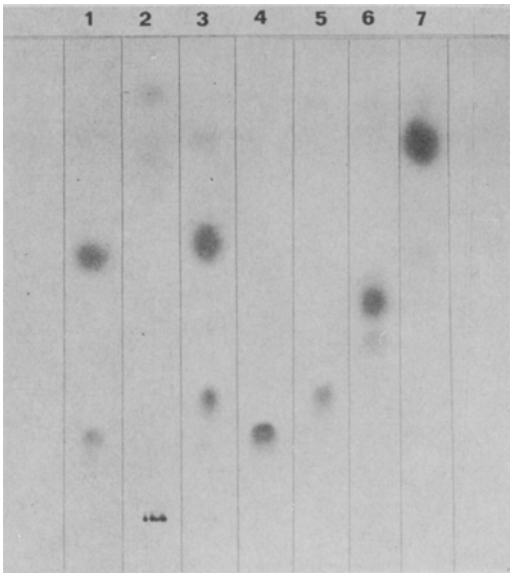

FIG. 1. $LiAlH_4$ hydrogenolysis products of glyceryl alkyl ether phosphatidic acid (Lane 1) and neutral plasmalogens (Lane 3). The chromatoplate also contains glyceryl ether phosphatidic acid (Lane 2), 1-glyceryl *cis*-9-octadecenyl ether (Lane 4), glyceryl alk-1-enyl ether (Lane 5) isolated by TLC after $LiAlH_4$ reduction, cyclic acetal (Lane 6) which was prepared from the glyceryl alk-1-enyl ether shown in Lane 5, and aldehydes (Lane 7) isolated after hydrolysis of glyceryl alk-1-enyl ethers in Lane 5. The solvent system was diethyl ether-30% aqueous ammonium hydroxide (100:0.25, v/v).

30). Reduction of the neutral plasmalogen likewise gave rise to two components: long-chain alcohols, and a component slightly less polar on TLC than a glyceryl alkyl ether (Fig. 1, Lanes 3 and 5). The latter was isolated by preparative TLC (Fig. 1, Lane 5), examined by IR (Fig. 2, top), and converted to the cyclic acetal (19) (Fig. 1, Lane 6).

The IR spectrum of the more polar product formed from reduction of the neutral plasmalogen with $LiAlH_4$ (Fig. 2, top) shows absorption in the region of 3030, 1675, 935, and 740 cm^{-1}, previously shown to be characteristic of glyceryl alk-1-enyl ethers (31,32). Contrary to an earlier report (21), the glyceryl alk-1-enyl ether spectrum is easily distinguishable from that of a glyceryl alkyl ether. The IR spectrum of the cyclic acetal, a compound once confused with the glyceryl alk-1-enyl ether (21), is shown in the lower spectrum of Fig. 2. Four cyclic acetals are possible: *cis* and *trans* 4-hydroxymethyl-2-alkyl-1,3-dioxolane and *cis* and *trans* 5-hydroxy-2-alkyl-1,3-dioxane. Two of the forms were observed on TLC (Fig. 1, Lane 6); however, comparison of the absorption spectra of the hydrogen bonding of the free (3640 cm^{-1}) and intramolecular (3605 cm^{-1}) hydroxyl group (Fig. 2) with those reported by Aksnes et al. (33,34) for the various forms suggests that the *cis* 4-hydroxymethyl-2-alkyl-1,3-dioxolane predominates.

Acid hydrolysis of the suspected glyceryl alk-1-enyl ethers gave rise to aldehydes (Fig 1, Lane 7), and hydrogenation (35) produced glyceryl alkyl ethers. These results demonstrate that the $LiAlH_4$ hydrogenolysis products of a neutral plasmalogen are long-chain alcohols and glyceryl alk-1-enyl ethers. We concluded from the complete phosphate ester hydrogenolysis of the glyceryl alkyl ether phosphatidic acid that plasmalogens would also yield the glyceryl alk-1-enyl ethers. This was confirmed with naturally occurring phospholipids containing plasmalogens.

Thin-Layer Chromatography

The alk-1-enyl- and alkyl-glyceryl ethers are easily separated on adsorbent layers of Silica Gel G, as shown in Fig. 1, 4, and 5. The resolution of these closely related compounds might be attributed to the intramolecular hydrogen bonding of the hydroxyl proton with the electronegative π electron cloud of the alk-1-enyl double bond. However, alk-1-enyl- and alkyl-glyceryl ether diesters have been resolved by TLC (27,35,36) where intramolecular hydrogen bonding is negligible. This suggests that the actual physical shape or conformation of the molecule, as affected by the double bond adjacent to the ether oxygen, also contributes to their resolution.

Although TLC has previously been used to determine the purity of alk-1-enyl- and alkyl-glyceryl ethers isolated by column chromatography (21), the obvious application, the quantitative simultaneous analysis of these two glyceryl ether types by TLC, has not been reported. Because of variation in structure, molecular weight, and degree of unsaturation, conversion of the organic matter to carbon by the charring technique for quantitative photodensitometric TLC analysis requires a linear relationship between peak area and mass over the working range for each compound analyzed (26). A plot of the peak area versus mass, shown in Fig. 3 for the alk-1-enyl-, alkyl- (18:0-1), and alkenyl-glyceryl (18:1-1) ethers, was found to be linear over the range of 3 to 18 μg. Fortunately the curves for each of the glyceryl ethers have the same slopes, and it is possible to use

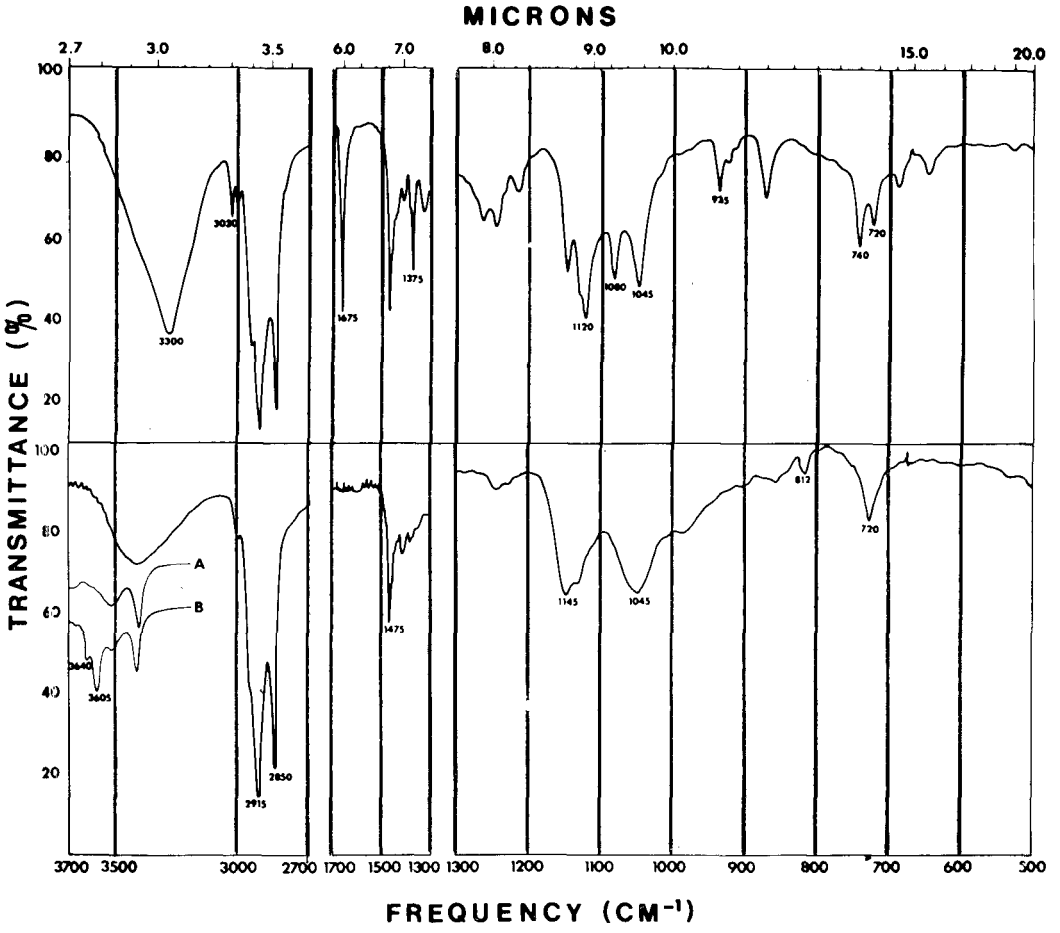

FIG. 2. Infrared spectra of the glyceryl alk-1-enyl ether (top spectrum) isolated by TLC after $LiAlH_4$ reduction of the neutral plasmalogen and the cyclic acetal prepared from the glyceryl alk-1-enyl ethers (bottom spectrum). Absorption of the hydroxyl group hydrogen bonding of the cyclic acetal (0.01 molar) is also shown (B). Absorption of the Mylar window liquid cell, filled with solvent only, is represented by (A).

commercially available glyceryl alkyl ethers as a TLC reference standard.

Glyceryl Ethers in Rat Tissues

Neutral lipids and phospholipids were isolated from various rat tissues and analyzed for alk-l-enyl- and alkyl-glyceryl ether content by the method described in this paper. Chromatoplates depicting the $LiAlH_4$ hydrogenolysis products of the lipids isolated from each of the rat tissues, along with reference compounds, are shown in Fig. 4 and 5. The alk-l-enyl- and alkyl-glyceryl ethers are located on the chromatoplates in the regions d and c, respectively. The hydrogenolysis products of sphingomyelin are among the more polar materials that remain near the origin (Areas a and b). Cholesterol (e) is located immediately below the long-chain alcohols (f). Hydrogenolysis products found in region g of the chromatoplate, usually more prominent in the neutral lipid fraction, have not been identified.

The percentages of alk-l-enyl, alkyl, and total glyceryl ethers of the neutral lipid and phospholipid fractions of the various tissues are given in Table I. All the tissues examined contained both alk-l-enyl- and alkyl-glyceryl ethers; however, the neutral lipids contained less than 0.5% of each. The phospholipids contained more alk-l-enyl than alkyl ethers: more than 1.0% was found in brain, heart, marrow, muscle, and spleen. The greatest concentration of glyceryl alkyl ethers was found in the marrow and spleen phospholipids.

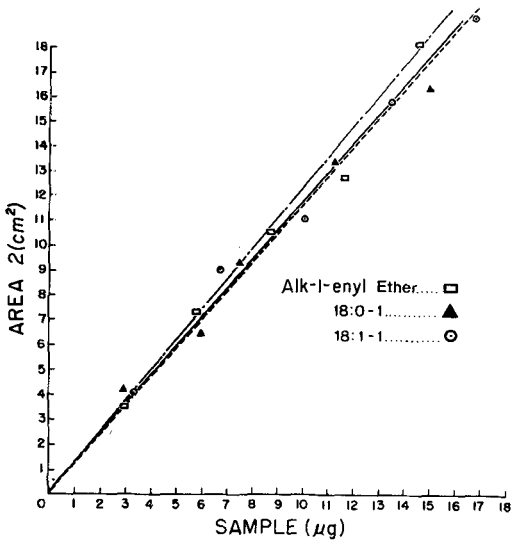

FIG. 3. A plot of peak areas versus mass for alk-1-enyl, alkyl (18:0-1), and alkenyl (18:1-1) glyceryl ethers measured by photodensitometric TLC analysis.

On the basis of the assumption that alk-1-enyl- and alkyl-glyceryl ethers occur mainly as diesters of phosphatides, the percentage of neutral lipids and phospholipids containing an ether bond is approximately three times the values which are shown in Table I. This calculation indicates that tissue glyceryl ether diesters represent 0.3-1.2% of the neutral lipids. These values are subject to some error since it has been shown that glyceryl alk-1-enyl monoether monoesters and the corresponding glyceryl alkyl monoether monoesters also occur in the neutral lipids (37, 38). The 1.3% of total glyceryl ether diesters of the neutral lipid fraction of rat adipose tissue which were reported by Gilbertson and Karnovsky (38) agrees well with the 1.2% we obtained; however, they found the glyceryl alk-1-enyl ethers represented 82% of the total ethers while our results show that the alkyl ether predominates.

In contrast to the low percentages of alk-1-enyl- and alkyl-glyceryl ether glycerides found in the neutral lipids, the glyceryl ether phosphatides of brain, heart, marrow, muscle, and spleen, often regarded as minor components, represent 4.5-12.0% of the total phospholipid fraction. The plasmalogen content of various rat tissues has previously been reported by a number of investigators (14, 17, 39), which is higher in most cases than the values we report. This was expected because previously the neutral plasmalogens of the neutral lipids were analyzed together with the plasmalogens.

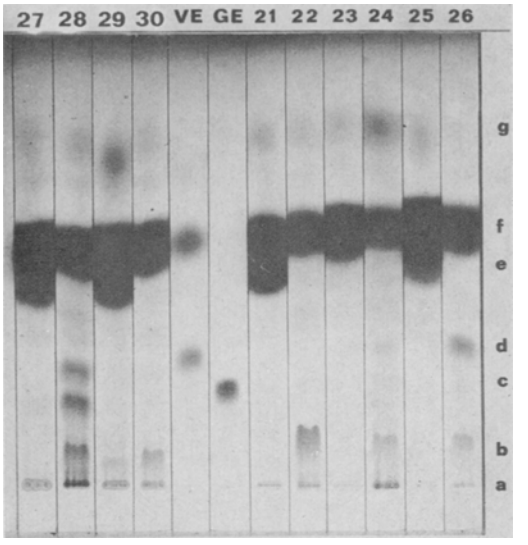

FIG. 4 and 5. Chromatoplates depicting glyceryl alk-1-enyl (Region d) and alkyl-alkenyl (Region c) ethers of several different rat tissues. Odd and consecutive even-numbered lanes represent neutral lipids and phospholipids of plasma 21-22, perirenal 23-24, muscle 25-26, marrow 27-28, liver 29-30, spleen 31-32, kidney 33-34, heart 35-36, and brain 37-38 tissues. Alk-1-enyl- and alkyl-glyceryl ether standards are represented by VE and GE. The solvent system was diethyl ether-30% aqueous ammonium hydroxide (100:0.25, v/v).

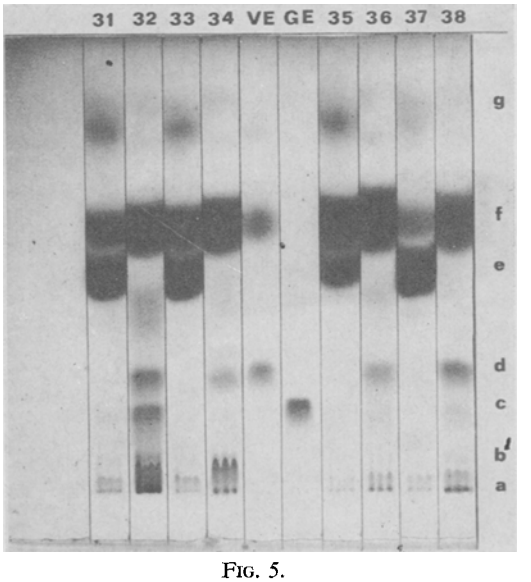

FIG. 5.

Fatty aldehydes shown to exist in the free form (40) would also contribute to the higher plasmalogen values when analyzed by the hydrazone methods. The glyceryl alkyl ether phosphatides, often disregarded, were found to represent 3.1% of the brain phospholipids by

Horrocks and Ansell (41) compared with the 1.8% herein reported. The discrepancies with the literature values can also be attributed to differences in the age, strain, sex, and dietary regime of the rats and method of analysis. The brain alk-1-enyl phosphatide content of female rats (130-160 g) was found to be one-half the value reported for the old adults (> 250 g). The correlation of brain plasmalogen content with age has previously been reported (42).

It would appear that a re-examination of the individual phospholipid classes of these tissues for alk-1-enyl- and alkyl-glyceryl ether phosphatides is necessary and justified.

Precision of the Method

A series of brain phospholipid samples were analyzed for alk-1-enyl- and alkyl-glyceryl ether content to determine the precision of the method. Values for each of the ether types are given in Table I (Samples no. 38 and 38A) with standard deviations. The relative error for the over-all procedure is estimated to be less than 10% for samples containing more than 1 μg of either glyceryl ether type per milligram of total lipid.

Importance of the Method

Our method allows the quantitative determination of both alk-1-enyl- and alkyl-glyceryl ethers simultaneously on relatively small samples of neutral lipids and phospholipids by TLC. Both glyceryl ether types can be analyzed for composition of the hydrocarbon chain by subjecting the mixture (isolated by preparative TLC) to acid hydrolysis, followed by GLC analysis of the glyceryl alkyl ethers (29) and the aldehydes derived from the alk-1-enyl ethers. The alk-1-enyl- and alkyl-glyceryl ethers can be examined for isomeric content by TLC on Silica Gel G impregnated with sodium arsenite or boric acid (28) and alternately by GLC (29). Autoradiography and zonal scanning (43) can also be used in conjunction with this method to study the biosynthesis of these two glyceryl ether classes from various labeled substrates. We are now using the described methods to determine the percentage, composition, isomeric form, and the incorporation of labeled precursors into the alk-1-enyl- and alkyl-glyceryl ethers in neutral lipids and phospholipids of several rat, mouse, and human tumors that we have shown to contain substantial quantities of glyceryl ether diesters (23, 44).

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Cholesterol Metabolism in the Baboon

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ABSTRACT

Studies on the transport of exogenous and endogenous cholesterol in the baboon have been carried out using ^{14}C-acetate, ^{14}C-mevalonic acid, or ^{3}H-cholesterol. The results suggest the following characteristics of cholesterol metabolism in the baboon: the serum α- and β-lipoproteins show no preference in the transport of exogenous or endogenous cholesterol; the half-life ($t_{\frac{1}{2}}$) of disappearance of cholesterol from the whole animal varied between 31 and 50 days; the daily rate of whole body cholesterol biosynthesis in the baboon (47 mg/kg/day) is comparable with that rate observed in the human being (18-41 mg/kg/day); after administration of ^{14}C-acetate, ^{14}C-mevalonic acid, or ^{3}H-cholesterol to baboons, newly synthesized cholesterol esters appear to be incorporated to a greater extent into the serum α-lipoproteins.

INTRODUCTION

In previous studies of the biosynthesis and transport of cholesterol in the baboon, we demonstrated that both exogenous and endogenous cholesterol are transported nonpreferentially by the serum lipoproteins (1,2).

The present investigation was undertaken to elaborate further the manner of transport of free and ester cholesterol (of exogenous or endogenous origin) by the serum lipoproteins, to obtain an estimate of the daily rate of synthesis of cholesterol, and to obtain information on the metabolism of cholesterol esters in the baboon.

MATERIALS AND METHODS

Reagents and solvents of analytical-grade purity were used in these studies. Sodium 1-^{14}C-acetate, or 2-^{14}C-D,L mevalonic acid (MVA), and 7α-^{3}H-cholesterol were purchased from the New England Nuclear Corporation, Boston, Mass. Prior to use, cholesterol purity (>98%) was ascertained by thin-layer chromatography.

The baboons were anesthetized with Sernylan (1-[1-phenylcyclohexyl] piperidine-HCl). Blood samples obtained from the femoral artery or vein were allowed to clot at 4C for 2 to 3 hr, and the serum was then separated.

The labeled compounds were administered by gastric intubation. Cholesterol was dissolved in corn oil; acetate and mevalonate were in an aqueous solution.

Free and total cholesterol were isolated from whole serum and from serum α- and β-lipoproteins by the Sperry and Webb method (3). Serum β-lipoproteins were precipitated by the dextran sulfate method of Castaigne and Amselem (4).

Cholesterol digitonides were solubilized for liquid scintillation counting with methanol, as described previously (5). Aliquots of cholesterol esters were dried in liquid scintillation vials, and 15 ml of scintillation solution were then added. All samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. Samples were counted for a long enough period of time to insure less than 1% counting error ($P < 0.05$).

The cholesterol pool size and the daily rate of cholesterogenesis in the baboon were estimated according to the method of Gidez and Eder (6). These values were derived in the following manner. With the use of a semilogarithmic graph, cholesterol specific activity was plotted on the y-ordinate and time on the x-ordinate (Fig. 5). The linear portion of this curve then was extrapolated to zero time to obtain a value for the "zero time" cholesterol specific activity (DPM/g). The cholesterol pool size, the true pool size, and the daily rate of biosynthesis were calculated according to equations i, ii, and iii, respectively.

$$(P) = (M)/(O) \quad [i]$$
$$(P_T) = (P)\ (R) \quad [ii]$$
$$(S) = (P_T)/(T)\ (W) \quad [iii]$$

where P = cholesterol pool size, g; M = DPM L-MVA administered; O = zero-time cholesterol specific activity, DPM/g; P_T = true cholesterol pool size, g; R = percentage of L-MVA actually converted to cholesterol; S = daily rate of cholesterol biosynthesis, mg/kg/day; T = turn-over time = half-life ($t_{\frac{1}{2}}$) × 1.5; W = weight of baboon, kg.

The baboons used in these studies were ob-

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TABLE I
Dosage Schedule for Administration of Labeled Compounds to Baboons

Baboon No.	Sex	Age	Wt. (kg)	Isotope and form	Total radioactivity administered (mC)	Route of administration
784	M	Adolescent[a]	11.5	1-^{14}C-Acetate	5.2	Oral
808	M	Adolescent	11.6	2-^{14}C-D, L-Mevalonic acid and 7 α-^{3}H-Cholesterol	0.5 5.0	Oral Oral

[a] Approximately 5 to 10 years old.

tained from the Southwest Foundation for Research and Education, San Antonio, Tex., and were maintained on Purina Monkey Chow. A description of these animals and the number and form of isotopes received by each are given in Table I.

RESULTS

Baboon 784 received 5.2 mc of a solution of sodium 1-^{14}C-acetate by stomach tube. Blood samples were obtained at various times up to 104 days, and the free- and ester-cholesterol specific activity of whole serum and of α- and β-lipoproteins was determined. Linear representations of the time-course curves of cholesterol specific activity of these serum fractions from zero to eight days are given in Figs. 1 and 2.

The peak free-cholesterol specific activity was observed between the third and seventh hours. Ester-cholesterol specific activity reached its peak between 1 and 2 days and equilibrated with free-cholesterol specific activity in approximately 24 hr. The specific activity of the ester cholesterol was somewhat higher than that of the free sterol from the second through the eighth day, but the specific activities were approximately equal thereafter.

Patterns of incorporation of acetate into free and ester cholesterol of serum α- and β-lipoproteins during the first 34 hr of this study were found to differ slightly (Fig. 2).

A comparison of the ratio of the cholesterol specific activities in α- and β-lipoproteins is shown in Table II. The ratio of specific activity of α-lipoprotein free cholesterol to that of the β-lipoprotein (α/β) was close to unity at 1 and 3 hr, dropped (0.8-0.9) at 7 and 10 hr, and rose (>1) at 24 and 34 hr. The ratio of ester-cholesterol specific activities in the α- and β-lipoproteins rose to 1.92 at 1 hr, then dropped to 1.08 at 10 hr and 0.9 at 24 and 34 hr. The data suggest a high incorporation of labeled ester cholesterol into the α-lipoprotein, with a rapid equilibration of activity, possibly due to a shift of labeled cholesterol ester from α- into β-lipoprotein.

Essentially similar data were derived from studies with Baboon 808. This animal received simultaneously 5.0 mc 7α-^{3}H-cholesterol and 0.5 mc 2-^{14}C-D, L-MVA by stomach tube. At specified times up to 104 days, blood samples were taken, and the free-, ester-, and total-cholesterol specific activities of whole serum and of α- and β-lipoproteins were determined.

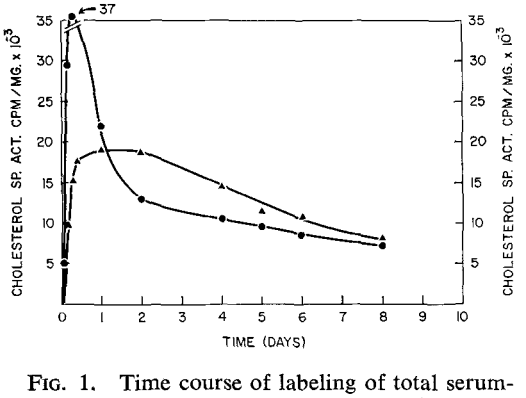

FIG. 1. Time course of labeling of total serum-free (●) and ester (▲) cholesterol. Baboon 784 received 5.2 mc of sodium 1-^{14}C-acetate.

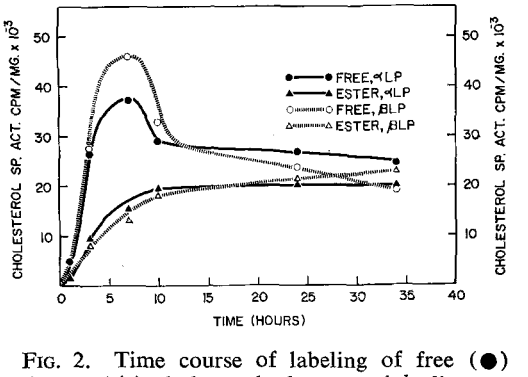

FIG. 2. Time course of labeling of free (●) and ester (▲) cholesterol of serum *alpha*-lipoprotein and free (O) and ester (△) cholesterol of serum *beta*-lipoprotein. Baboon 784 received 5.2 mc of sodium 1-^{14}C-acetate.

TABLE II

Ratio of Cholesterol Specific Activity in *alpha-* and *beta*-Lipoprotein, Derived from Administration of 1-^{14}C-Acetate to Baboon 784

Time (hours)	*alpha-/beta*-Lipoprotein			Ester/Free		
	Free	Ester	Total	*alpha*-LP[a]	*beta*-LP	α/β LP
1	0.925	1.92	1.38	0.356	0.172	2.07
3	1.00	1.18	1.11	0.356	0.300	1.18
7	0.797	1.21	1.01	0.427	0.283	1.51
10	0.865	1.08	0.902	0.675	0.541	1.25
24	1.14	0.897	1.05	0.714	0.910	0.785
34	1.30	0.897	1.05	0.820	1.250	0.656

[a] LP = lipoprotein.

Peak specific activities of endogenous free and ester cholesterol were reached between 3 and 8 hr and between 10 and 24 hr, respectively, as shown in Fig. 3. The serum-free and ester-cholesterol specific activities equilibrated at about 16 hr. After 8 days, the free- and ester-cholesterol specific activities again were equal.

The patterns of radioactivity of free and ester cholesterol derived from 7α-^{3}H-cholesterol are shown in Fig. 4. The peak free- and ester-cholesterol specific activity occurred between 34 and 48 hr. Prior to 24 hr, specific activities of serum-free cholesterol were higher than those of ester cholesterol; at approximately 24 hr, the activities were equal. After 24 hr, ester-cholesterol specific activity again rose and remained slightly higher than the free-sterol specific activity.

Since the pattern of incorporation of ^{3}H or ^{14}C-labeled sterol into the α- and β-lipoprotein fractions was nearly identical to that obtained from whole unfractionated serum, these data have been omitted. Instead, the ratios of specific activity of endogenous (^{14}C-labeled) and exogenous (^{3}H-labeled) free and ester cholesterol in the α- and β-lipoproteins are shown in Table III. As in the data obtained with baboon 784, the incorporation of free cholesterol into α- and β-liproproteins proceeded at an equal rate, but again the incorporation of both endogenous and exogenous cholesterol (as cholesterol ester) into the α-lipoproteins was higher than into the β-lipoproteins, as seen from the high ratios obtained at 1 hr. As observed with baboon 784, both the ^{14}C and ^{3}H ester-cholesterol specific activity in the α- and β-lipoproteins were equal after 8 hr.

The specific activities of the serum-free cholesterol derived from acetate (baboon 784), MVA (baboon 808), or ^{3}H-cholesterol (baboon 808) were plotted graphically in order to evaluate the half-life ($t_{\frac{1}{2}}$) of the disappearance of cholesterol (Fig. 5). The half-life of the slowest components in the decay of cholesterol specific activity, derived from 2-^{14}C-MVA and from preformed ^{3}H-cholesterol, were approximately the same, i.e., 34 and 31 days, respectively.

DISCUSSION

We have calculated the mean values of the serum cholesterol levels (free, ester, α- and β-

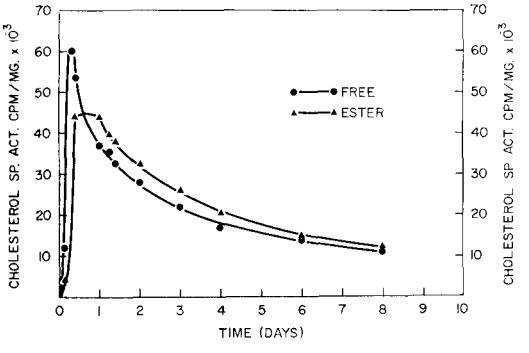

FIG. 3. Time course of labeling of total serum-free (●) and ester (▲) cholesterol. Baboon 808 received 0.5 mc of (2-^{14}C) mevalonic acid via stomach tube.

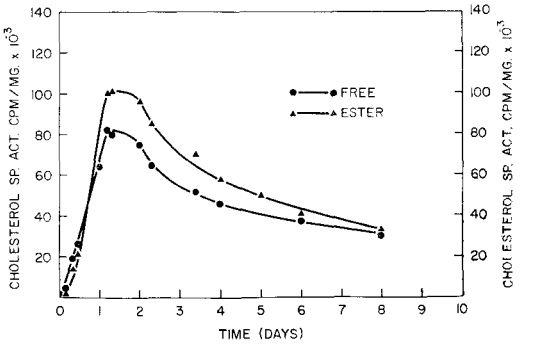

FIG. 4. Time course of labeling of total serum-free (●) and ester (▲) cholesterol. Baboon 808 received 5.0 mc of 7 α-^{3}H-cholesterol via stomach tube.

TABLE III

Ratio of Cholesterol Specific Activity in *alpha-* and *beta*-Lipoprotein, Derived from Administration of Both 2-^{14}C-D, L-MVA and 7α-^{3}H-Cholesterol to Baboon 808

Time (hours)	*alpha-/beta*-Lipoprotein						Ester/Free					
	Free		Ester		Total		*alpha*-LP[a]		*beta*-LP		α/β LP	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
1	0.95	0.93	2.51	1.98	1.44	1.63	0.45	1.08	0.20	0.47	2.25	2.25
8	1.05	1.05	0.98	1.22	0.99	1.09	0.37	0.95	0.39	1.02	0.94	0.93
10	0.82	0.90	0.97	0.97	0.93	0.94	0.78	1.12	0.72	1.03	1.08	1.09
30	1.01	1.00	1.06	1.05	1.00	1.05	1.15	1.03	1.19	1.04	0.97	0.99
72	1.05	1.05	1.00	0.98	1.01	0.99	1.03	1.04	1.09	1.12	0.95	0.93

[a] LP = lipoprotein.

lipoproteins), determined for each animal in the course of this study (Table IV). The fluctuation in total cholesterol levels (in whole serum and in α- and β-lipoprotein) ranged from 10-18% of mean values over a three-month period. The mean values for whole-serum total cholesterol were similar to those reported by other authors (7-9). Values for the free and ester cholesterol present in whole serum and in α- and β-lipoproteins have not been published previously. In this study, the free cholesterol levels (in whole serum and in α- and β-lipoproteins) varied between 20-34% of mean values, while ester cholesterol levels (in whole serum and in α- and β-lipoproteins) varied between 8 and 27% of mean values.

Oral administration of 1-^{14}C-acetate to baboon 784 and of 2-^{14}C-MVA and 7α-^{3}H-cholesterol to baboon 808 gave whole serum-free and ester-cholesterol specific activity patterns similar to those reported in previous studies with baboons (1,2). Peak specific activity of serum-free cholesterol was attained between 3 and 8 hr by using either acetate or MVA as a precursor. Free- and ester-cholesterol specific activity of endogenously derived sterol equilibrated at approximately 24 and 16 hr, respectively. The ester-cholesterol specific activities of α- and β-lipoproteins were approximately equal after eight days.

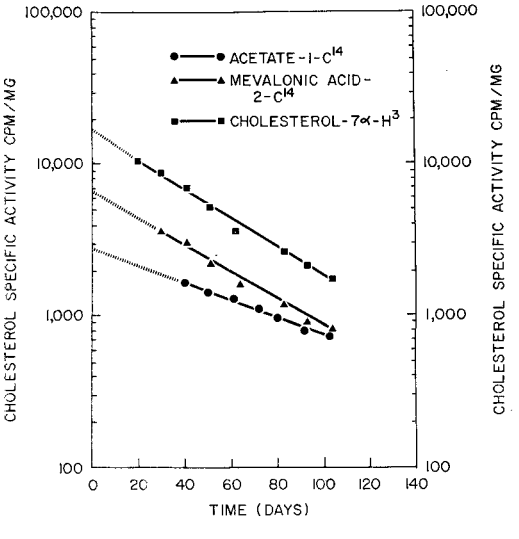

FIG. 5. Serum-free cholesterol specific activity derived from acetate (●), mevalonate (▲), or cholesterol (■) during a 104-day period.

Exogenous serum-free cholesterol reached peak specific activity approximately between 30 and 34 hr; the peak specific activity of ester cholesterol occurred between 34 and 48 hr. Free and ester cholesterol equilibrated at approximately 24 hr. Equilibration of the total cholesterol of α- and β-lipoprotein occurred between 10 to 48 hr whether the sterol source was acetate, MVA, or cholesterol.

However, slight differences in the manner of incorporation of cholesterol (either exogenous or endogenous) into serum α- and β-lipoproteins were noted in the earlier sampling times in these experiments (Tables II and III). After 1 hr the total-cholesterol specific activity was approximately one-and-a-half times higher in the α- than in the β-lipoprotein. This high ratio undoubtedly reflects a proportionately higher incorporation of esterified cholesterol into the α-lipoprotein since at the first hour of sampling the ^{3}H or ^{14}C ester-cholesterol specific activity was usually two to two-and-a-half times higher in the α- than in the β-lipoprotein. On the other hand the ^{3}H or ^{14}C free-cholesterol specific activities were always approximately equal in either lipoprotein fraction. Similar patterns of incorporation of 4-^{14}C-cholesterol into serum α- and β-lipoproteins have been observed in baboons maintained on various nutritional regimens (10). These data suggest that cholesterol esters (the cholesterol moiety of which is derived either from exogenous or endogenous sources) may be incorporated into α-lipoprotein to a greater extent than into β-lipoprotein.

TABLE IV

Mean Serum and Lipoprotein Cholesterol Levels (mg/100 ml) in Baboons[a]

Animal	Serum cholesterol				*alpha*-LP[b]			*beta*-LP			α/β LP		
	Free	Ester	Total	E/F	Free	Ester	Total	Free	Ester	Total	Free	Ester	Tot
B-808 Mean	26.1	93.9	120.4		13.3	42	55.7	18.4	40.3	58.4	0.72	1.04	0.9
±S.D.	±6.3	±10.0	±14.3	3.6	±4.4	±3.2	±7.1	±4.7	±7.8	±9.0	±0.21	±0.27	±0.3
Range	18-42	77-113	99-143		7-25	31-55	41-78	10-30	22-54	40-73	0.32-1.12	0.64-1.76	0.60-
No. Det.	28	28	28		32	32	32	28	28	28	23	23	23
B-784 Mean	30.6	97.1	135.8		10.0	52.9	62.0	15.6	56.8	71.0	0.71	0.92	0.8
±S.D.	±6.2	±23.8	±9.0	3.2	±3.2	±14.2	±11.0	±3.0	±7.1	±11.0	±0.24	±0.20	±0.1
Range	23-47	79-135	113-169		6-13	28-68	38-86	11-20	49-65	50-88	0.65-1.15	0.53-1.10	0.58-
No. Det.	24	24	24		7	7	28	7	7	28	7	7	28

[a] Mean values obtained over entire sampling period.
[b] LP = lipoproteins.

The equilibration between lipoprotein fractions of acetate-derived free and ester cholesterol appeared to be incomplete by 34 hr although equilibration of sterol derived from either MVA or preformed cholesterol apparently had occurred by this time. The observation that the values of the ratios of the α-/β-lipoprotein free- and ester-cholesterol specific activity are similar when exogenous and endogenous sterol data are compared (Table III) suggests similarities in the transport of biosynthesized and preformed cholesterol and is in agreement with our earlier findings (1,2). This conclusion is further supported by the fact that cholesterol derived from MVA disappeared from the vascular compartment at the same rate as did cholesterol of exogenous origin. In contrast with these results, however, are some findings which suggest that subtle differences may exist in the manner of incorporation of exogenous or endogenous cholesterol into various types of cholesterol esters. These findings form the basis of a separate communication (11).

Our results are similar to those of Goodman (12), who found that, after administration of 2-^{14}C-MVA to two human subjects, the free-cholesterol specific activities of very low-density, low-density, and high-density serum lipoproteins were usually equal. Similar results were also reported by Gidez et al. (13). Goodman (12) found that the cholesterol-ester specific activity was markedly different from free-sterol specific activity in each lipoprotein fraction in the early phases (0-48 hr) of his experiments. However, cholesterol-ester specific activity in each fraction seemed to approach an equilibrium value after 48 hr.

Based on the serum-free cholesterol specific activity data derived from 1-^{14}C-acetate, 2-^{14}C-MVA, and 7α-^{3}H-cholesterol, the half-life, the daily synthesis, and the turnover rate of cholesterol were determined. Exogenous and endogenous cholesterol disappeared from the vascular compartment exponentially from approximately 20 to 40 days onward. The half-life values of endogenous sterol, derived from acetate or MVA or from exogenous sources, were 50, 34, and 31 days, respectively. (The difference between the half-life values of acetate- and MVA-derived cholesterol can probably be attributed to differences between baboons.)

Within the limits of error the half-life values of exogenous and endogenous cholesterol of baboon 808 were nearly equal. This finding

TABLE V

Interspecies Comparison of Whole Body Half-Life Values ($t_{1/2}$, Days) for Exogenous or Endogenous Cholesterol

Species	Labeled compound administered	Route of administration	Length of study (days)	Half-life ($t_{1/2}$, days)	Reference
Man	2-^{14}C-D, L-Mevalonic acid	Oral or IV	250	69.6	6
	4-^{14}C-Cholesterol	IV	125-212	71.6	15
	4-^{14}C-Cholesterol	IV	210-300	69-75	14
Baboon	1-^{14}C-Acetate	Oral	104	50	—
	2-^{14}C-D, L-Mevalonic acid	Oral	104	34	—
	7α-^{3}H-Cholesterol	Oral	104	31	—
	4-^{14}C-Cholesterol	IV	120	35-67	17
Dog	4-^{14}C-Cholesterol	IV	60	62	16

suggests that exogenous and endogenous sterols are mixed indistinguishably in the metabolically active cholesterol pool. The identical behavior of these two sterol species was indicated further by the fact that the values of the ^{3}H or ^{14}C free- and ester-cholesterol specific activity in α-/β-lipoprotein were approximately the same at any one time (Table III). Moreover, in several individual studies, after administration of either 2-^{14}C-MVA (14) or 4-^{14}C-cholesterol (15) to human beings, the rate of disappearance of cholesterol was found to be identical (70 and 72 days respectively).

The half-life values of exogenous or endogenous cholesterol of the baboon were found to be lower than those values reported for human subjects (6,14,15) or for dogs (16) (Table V). However, it was calculated that the whole body rate of synthesis of cholesterol was not markedly different from those values reported for human beings.

Based on the endogenous serum-cholesterol specific activity data obtained from baboon 808, the pool size was estimated to be approximately 28 g. The daily rate of synthesis of cholesterol in this animal was calculated to be 47 mg/kg/day. This calculation is based on the assumptions that: a) all administered MVA was absorbed; b) only the L-form was utilized for sterol synthesis; and c) the L-form was not oxidized to $^{14}CO_2$. It has been shown by Gidez and Eder (6) that, in human subjects, only 71-88% of the L-form of MVA is available for cholesterogenesis. The biosynthetic rate calculated for the normal baboon, therefore, must represent a maximum value.

In baboons which were maintained on high-fat, low-cholesterol diets Eggen et al. (17) estimated that the biosynthesis rate was 14-37 mg/kg/day. Gidez and Eder (6) reported that the biosynthesis rate in human beings was 1-4 g/day (corresponding to a cholesterogenesis rate of 18-41 mg/kg/day).

Since 7α-^{3}H-cholesterol was administered orally, it was not possible to determine accurately the daily turnover rate of cholesterol by using the tritium specific activity data. Chobanian et al. (15) found that the daily turnover rate of cholesterol in human subjects on an unrestricted dietary intake was, on the average, 1.68 g/day or 23 mg/kg/day. The data thus derived from studies with baboons and with human beings suggest that a similarity exists between the human subject and the baboon in the daily rate of cholesterol biosynthesis.

These experiments suggest that the cholesterol of the serum lipoproteins is derived non-preferentially from endogenous or exogenous sources. They do not indicate whether equivalent incorporation of free or ester sterol into, or incorporation followed by rapid equilibration between, serum lipoproteins had occurred.

We have recently reported data which suggest that free and ester cholesterol equilibrates rapidly between serum lipoproteins (18). Free cholesterol appeared to exchange between lipoprotein fractions more rapidly than did ester cholesterol. This observation may explain why the ^{3}H or ^{14}C cholesterol-ester specific activity of the α-lipoprotein is higher than the cholesterol-ester specific activity of the β-lipoprotein prior to the eighth hour of the experiment, or it may merely be a reflection of the preferential esterification of free cholesterol of α-lipoprotein. Preferential esterification of the high-density lipoprotein-free cholesterol of baboon serum has been demonstrated in vitro by Glomset et al. (19). Alternately, a combination of both of the above mechanisms may contribute to the observed results.

The information derived to date regarding the biosynthesis and transport of cholesterol in the baboon indicates the following: a) exogenous and endogenous cholesterol are transported generally in a nonpreferential fashion by the serum α- and β-lipoproteins; b) the isotopic data are consistent with the hypothesis that cholesterol esters (the cholesterol portion of exogenous or biosynthetic origin) are incorporated to a greater extent into the α-lipoprotein after the administration of labeled precursors; c) free and ester cholesterol (of exogenous or endogenous origin) probably are incorporated into and exchanged between lipoprotein fractions, both in vivo and in vitro; and d) the whole body rate of biosynthesis of cholesterol in the baboon is comparable with that rate observed for the human subject.

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Studies on the Specificity of a Lipase System from *Geotrichum candidum*[1]

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ABSTRACT

The lipase system from *Geotrichum candidum* preferentially hydrolyzed oleic acid, regardless of position, from the four possible racemic triglycerides containing oleate and palmitate. The rate of hydrolysis of these glycerides was most rapid when the substrate contained two moles of oleate. This acid was also preferentially released from a series of triglycerides containing oleate and two moles of a saturated fatty acid. The chain length of the latter did not alter the specificity for oleate.

Equimolar quantities of oleic and linoleic acids were released when triolein and trilinolein (equimolar mixture) were hydrolyzed by this lipase. No differentiation between oleate and palmitoleate was observed when racemic glyceryl 1-palmitoleate-2,3-dioleate was the substrate. However, only 7.2 M% *cis*-vaccenic acid was released from glyceryl 1-*cis*-vaccenate-2,3-dioleate and 5.4 M% petroselinic acid from glyceryl 1-palmitoleate-2,3-dipetroselinate. It therefore appears that the enzyme may be specific for *cis*-9-unsaturation as well as for *cis*-9, *cis*-12-unsaturation. When specificity was assumed, the fatty acid compositions of the diglycerides obtained from digestions with *G. candidum* were close to theoretical.

INTRODUCTION

THE MICROORGANISM, *Geotrichum candidum*, elaborates an extracellular lipase which preferentially hydrolyzes *cis*-octadecenoic acid from a variety of natural and synthetic fats (1,2). When present, however, varying quantities of linoleic and small amounts of saturated and *trans* unsaturated fatty acids are also hydrolyzed. Nevertheless, the unique specificity of this enzyme would be useful in the study of both synthetic and natural triglyceride structures, particularly with triacid triglycerides containing oleate (3). Application of this lipase to the determination of triglyceride structure has been hampered by lack of information about the properties and specificity of the enzyme. Although the lipase hydrolyzes oleate from synthetic glycerides regardless of position, the effect of position on velocity is not known. Further, the composition of the diglycerides resulting from lipolysis has not always been reported. If *G. candidum* lipase is to be used to produce diglycerides for a stereospecific analysis of triglycerides (3), then the composition of these compounds must be ascertained to assess applicability. Finally, it is not known whether positional isomerism of the double bond in octadecenoates, shortening of the terminal portion of the monoene chain, or the presence of 9, 12-*cis*, *cis* double bond affects the specificity of the lipase.

This paper reports the rates obtained when the four possible triglycerides of oleate and palmitate were hydrolyzed by *G. candidum* lipase and when the pH of the incubation mixture was varied. Also hydrolyzed was a series of mono-oleate-disaturated triglycerides, in which the disaturated portion ranged from caproate to stearate. Finally, the hydrolyses of petroselinate, *cis* vaccenate, palmitoleate, and linoleate were compared with oleate, and these results are included.

EXPERIMENTAL

Substrates

Except for triolein purchased from the Hormel Institute (90.0+%) and purified on a column of alumina (4), the substrates were synthesized essentially as reported by Quinn et al. (5) but on a reduced scale. Purity was ascertained by gas-liquid chromatography (GLC), thin-layer chromatography (TLC), and lipolysis by pancreatic lipase. With the limitations of these methods in mind, purity of the glycerides was estimated to be 99%. The olive oil triglycerides were prepared by eluting commercial olive oil through alumina (4) with petroleum ether-ethyl ether (90:10, v/v). The triglycerides were further purified by crystallization from absolute ethanol at –25C.

Enzyme

The enzyme, a gift from J. A. Alford, was obtained from *G. candidum* as previously de-

[1]Scientific contribution No. 256, Agricultural Experiment Station, University of Connecticut, Storrs.

scribed (6). Several batches of lipase were used, all with similar activity. The enzyme was stored at –7C and, at the time of use, was from one to three years old.

Methods

For each substrate or condition assayed, duplicate samples and a control which contained no enzyme were employed. Unless otherwise indicated, samples were incubated with 5 mg of enzyme and digested for 2 hr. Digestions were terminated with the addition of 1 ml of 20% (v/v) H_2SO_4, and the mixtures were extracted with chloroform-methanol (90:10, v/v) as previously described (7). The microequivalents of fatty acid released were estimated by titrating the extracted samples with 0.05N KOH to the thymol blue end-point. Preparative TLC was accomplished on 20 × 20-cm plates coated to 500 μ with Silica Gel G. Development was in a petroleum ether (bp 35-45C)-ethyl ether-acetic acid (90:30:2, v/v/v) solvent system, and bands were visualized by brief exposure to iodine vapors. GLC was accomplished in Acrograph A-90-C instruments, equipped with hot wire detectors and 10 ft by 0.25 in. columns packed with 18% diethylene glycol succinate (Analabs) on 70-80 mesh Anakrom ABS. Quantitation was facilitated by a Disc integrator attached to the recorder.

Digestions and Analyses

Positional Isomers of Triglycerides Containing Palmitic and Oleic Acid. The numbering system used throughout this paper does not refer to glycerides of optical configuration. Glyceryl-1-oleate-2,3-dipalmitate, glyceryl-1-palmitate-2,3-dioleate, glyceryl-2-oleate-1,3-dipalmitate, and glyceryl-2-palmitate-1,3-dioleate were individually digested at 37C as described (2) except that 100 mg of substrate was employed. The digestion mixtures were extracted; the free fatty acid, residual triglycerides, and partial glycerides were recovered by preparative TLC; and the fatty acid composition was determined as methyl esters by GLC.

α-Oleate-α, β-Disaturated Triglycerides. Glyceryl-1-oleate-2,3-dicaproate, glyceryl-1-oleate-2,3-dicaprylate, glyceryl-1-oleate-2,3-dicaprate, glyceryl-1-oleate-2,3-dilaurate, and glyceryl-1-oleate-2,3-distearate were digested and analyzed as described above except that materials containing short-chain acids were converted to butyl esters and analyzed by temperature-programmed GLC (8).

Factors Affecting Velocity of Lipolysis. The influence of the position of oleic acid within a triglyceride on the extent of digestion was estimated by incubating the glycerides and determining the total fatty acids liberated. The effect of pH on the velocity of lipolysis was observed by incubating triolein in phosphate buffers (ionic strength = 0.1) of the following pH values: 5.8, 6.3, 6.7, 7.3, and 7.8. The values were determined at room temperature, not at the 37C digestion temperature. Digestion conditions were as described (2) except that 15 mg of lipase was added, and a 60-min incubation period was employed. These studies were continued with Tris buffers of the following pH values: 7.0, 7.6, 8.0, 8.1, 8.3, 8.5, 8.7, and 9.0.

Studies on the Geometry of the Substrate. To gain more information relative to the influence of the geometry of the unsaturated acyl chain upon the specificity of the enzyme, the following glycerides were incubated with *G. candidum* lipase: equimolar mixtures of triolein and trilinolein (300 mg substrate/sample); glyceryl-1-palmitoleate-2,3-dioleate (100 mg substrate/sample); glyceryl-1-*cis* vaccenate-2,3-dioleate (150 mg substrate/sample); and glyceryl-1-palmitoleate-2,3-dipetroselinate (150 mg substrate/sample).

Incubation mixtures were prepared with the aid of a Branson Sonifier by emulsifying the substrates in 1% (w/v) gum arabic in Tris buffer (pH 8.2). For each aliquot of substrate, the

TABLE I

Mole Percentage of Oleic Acid Found After Lipolysis of Oleate-Saturated Triglycerides by *Geotrichum candidum* Lipase

Substrate	Residual triglycerides	Free fatty acids	Diglycerides	Monoglycerides[a]
Glyceryl 1-oleate-2,3-dicaproate	31.6	99.0	0.0	0.0
Glyceryl 1-oleate-2,3-dicaprylate	38.1	89.1	4.4	trace
Glyceryl 1-oleate-2,3-dicaprate	31.3	89.8	2.4	trace
Glyceryl 1-oleate-2,3-dilaurate	31.0	86.0	2.0	trace
Glyecryl 1-oleate-2,3-dipalmitate	32.6	89.7	1.2	trace
Glyceryl 1-palmitate-2,3-dioleate	64.4	96.1	48.0	trace
Glyceryl 2-oleate-1,3-dipalmitate	31.4	94.3	trace	trace
Glyceryl 2-palmitate-1.3-dioleate	66.1	97.7	47.7	trace
Glyceryl 1-oleate-2,3-distearate	26.3	82.7	3.2	trace

[a] Very little monoglyceride was formed when the *disaturated triglycerides* were digested.

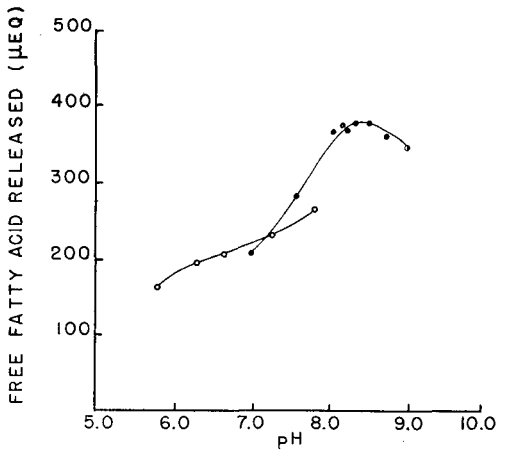

FIG. 1. The average net microequivalents of free fatty acids released when duplicate emulsions containing triolein were digested at several pH levels, first in phosphate buffer (○—○), then in Tris buffer (●—●).

emulsion contained 8 ml of buffer and 0.5 ml of 10% (w/v) $CaCl_2$. For each sample, 15 mg of enzyme was weighed into a digestion beaker and 8 ml of the emulsion added. Samples were digested for 30 min except for glyceryl-1-*cis* vaccenate-2,3-dioleate, where the time was extended to 60 min. The products of lipolysis were recovered, separated, and analyzed except that vaccenate was differentiated from oleate by oxidation (9) and the products of oxidation were identified by GLC with the aid of authentic reference compounds.

RESULTS AND DISCUSSION

Results from the digestion of the synthetic triglycerides containing both oleic and saturated fatty acids are summarized in Table I as the mole percentage of oleic acid observed in each of the products of lipolysis. At least 89% of the free fatty acids was oleic acid when the triglycerides containing palmitic acid were hydrolyzed. Thus the preferential hydrolysis of this acid, regardless of position (1), is confirmed. When triglycerides containing saturated acids other than palmitate were substrates, oleic acid was again hydrolyzed preferentially and the length of the saturated fatty acid did not appreciably alter this specificity.

The fatty acid compositions of the diglyceride and monoglyceride fractions also support the conclusion that *G. candidum* lipase was specific for oleic acid. The diglycerides from the two dioleate triglycerides contained quantities of oleate very close to the theoretical amount of 50%, and the diglycerides from the α-oleate-α, β-disaturated triglycerides contained little or no oleic acid. Thus the lipase produces diglycerides representative of the triglycerides, an important requirement for structural studies employing lipases.

The average net microequivalents of fatty acid, released during the lipolysis of the triglycerides containing palmitic acid, were: glyceryl-1-oleate-2,3-dipalmitate, 86.0; glyceryl-2-oleate-1,3-dipalmitate, 114.7; glyceryl-2-palmitate-1,3-dioleate, 173.3; and glyceryl-1-palmitate-2,3-dioleate, 177.5. Thus, the extent of hydrolysis was approximately proportional to the amount of oleate present in the original triglyceride. The hydrolysis of larger quantities of oleic acid from glyceryl-2-oleate-1,3-dipalmitate than from glyceryl-1-oleate-2,3-dipalmitate was surprising and is a distinct contrast to the specificity of pancreatic lipase for primary positions. Nevertheless, the difference is not so large as to preclude using the enzyme in structural studies of mono-oleate triglycerides.

The results from the oleate triglyceride study suggested that since enzyme activity was low, compared with, for example, pancreatic lipase, an investigation of some factors affecting reaction velocity would be desirable. Studies in

TABLE II

Mole Percentage Oleic Acid or Palmitoleic Acid[a] Found After Lipolysis of Synthetic Triglycerides by *Geotrichum candidum* Lipase

Substrate	Control triglycerides	Residual triglycerides	Free fatty acids	Diglycerides	Monoglycerides
Triolein-trilinolein	58.5	58.4	62.2	56.0	55.0
Glyceryl-1-palmitoleate-2,3-dioleate	66.6	67.4	65.2	71.7	68.9[b]
Glyceryl-1-*cis*-vaccenate-2,3-dioleate[c]	74.6	61.6	92.8	37.6	4.4[b]
Glyceryl-1-palmitoleate-2,3-dipetroselinate[a]	33.6	32.4	94.6	0.9	1.4[b]

[a] Values for glyceryl-1-palmitoleate-2,3-dipetroselinate are palmitoleic; all others are oleic.

[b] Very little monoglyceride was formed.

[c] The oleic acid content was estimated from the methyl nonanoate formed, after methanolysis of the oxidized products. GLC peaks other than methyl nonanoate and dimethyl hendecanedioate were ignored.

which $CaCl_2$ concentration and temperature of incubation were varied indicated that little or no increase in lipolysis of olive oil triglycerides could be obtained by altering these factors. As was expected, increases in time (5-160 min) or enzyme concentration (5-25 mg) gave approximately linear increases in fatty acids released. More important, however, was the finding (Fig. 1) that the optimum pH range for activity was 8.1-8.5, not the pH used in previous studies (1,2). Under the conditions employed, the extent of digestion was almost doubled by increasing the pH from 6.7 to 8.5.

Results from the digestions of the triunsaturated triglycerides are summarized in Table II. Although the enzyme is known to hydrolyze linoleic and palmitoleic acids from a variety of natural fats (2), it was not clear to what extent these acids modified the specificity of *G. candidum* for oleic acid. It is apparent from the results in Table II that the enzyme does not differentiate between oleate and linoleate, since the acid contents of the free fatty acids and partial glycerides was similar to that of the control triglycerides. Similarly, the enzyme did not distinguish between palmitoleic and oleic acids (Table II) as these were released in a 1:2 molar ratio from glyceryl-1-palmitoleate-2,3-dioleate. Thus the presence of *cis* 12 unsaturation or the length of the chain, at least a reduction of two carbons from the terminal portion beyond the double bond, does not influence the specificity of this enzyme for *cis*-9-unsaturation.

To determine whether *cis*-9-unsaturation was a requirement for significant hydrolysis with *G. candidum* lipase, glyceryl-1-*cis*-vaccenate-2,3-dioleate was used as a substrate. Since methyl vaccenate and oleate have the same retention time on the GLC columns employed, it was necessary to oxidize the free fatty acid and glyceride fractions individually before the rates of hydrolysis could be ascertained. Before using the $KMnO_4$-acetic acid procedure (9) on this triglyceride, about 50 oxidations were conducted with triolein, methyl oleate, oleic acid, methyl linoleate, vaccenic acid, and methyl vaccenate to establish the quantitative aspects of the method. In order to obtain sufficient quantities of products for analysis, 20 mg of lipase and a one-hour period of digestion were employed. It is apparent from the results (Table II) that the enzyme preferentially released oleic acid.

The enzyme did not differentiate between oleate and palmitoleate, therefore analysis with the oxidation procedure could be avoided by substituting palmitoleate for oleate when testing possible specificity for isomeric octadecenoates. This was done when testing petroselinate, and the enzyme again preferentially hydrolyzed the *cis*-9-unsaturated fatty acid (Table II).

As was the case in the digestion of the mono- and disaturated triglycerides, the diglyceride compositions obtained in these completely unsaturated substrates are close to theoretical when specificity for *cis*-9-unsaturation is assumed, suggesting that diglycerides produced by this enzyme may be suitable for use in structural studies of triglycerides.

Further testing with the isomers 7-, 8- and 10- is necessary for unequivocal statements; however, the specificity of the enzyme has at least been located between *cis* 6- and 11. It would also be desirable to check, as a substrate, *cis*-9-decenoic acid. It is possible that alterations in the chain beyond *cis*-9 have no influence on the specificity. In any case, the specificity remains an interesting example of active site geometry.

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The Effect of a Polyunsaturated Diet upon Adipose-Tissue Fatty Acids in Young Coronary Males. A Five-Year Cohort Study[1]

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ABSTRACT

The fatty acid composition in the adipose tissue of 38 electrocardiographically confirmed coronary males, mean age 43.7 years, at ideal weight on a 30%-of-calories controlled-fat diet, containing approximately 11.4% of calories as linoleic acid, was studied. The initial linoleic acid concentration in the adipose tissue was approximately 11 mole % of total fatty acids; for approximately the first 12 months it rose slightly and then rapidly increased to about 20% after 24 months. The overall response is sigmoidal in form and fits the equation:

$$\frac{1}{y} = 0.025 + 0.066\ (0.975)^x$$

in which y represents the adipose tissue linoleate as mole percentage of total adipose tissue fatty acids and x is the time in months.

The relative increase in linoleic acid is not attributable to a decrease in any specific fatty acid.

INTRODUCTION

MANY REPORTS have been published in an attempt to correlate the fatty acid composition in human adipose tissue with the fatty acid composition of the diet. Scott et al. (1) noted that the adipose-tissue linoleic acid appeared to reflect the relative percentage of dietary linoleate in human beings. Kingsbury et al. (2) found that the fatty acid composition of the adipose tissue was apparently unrelated to the type of dietary fat and that changes in the lipids of one body compartment are not reliable indications of changes in another over a short period of time. Hirsch et al. (3) noted that the adipose tissue did reflect the diet but postulated that the turnover time of adipose-tissue fatty acids was somewhere between one year and two years. They also postulated the existence of two separate metabolic compartments in the adipose fat: one, a highly labile compartment which is in closer equilibrium with serum fatty acids; and the other, a more stable compartment. None of these works have apparently attempted a direct measurement of the turnover time in human adults. In this report the effect of a polyunsaturated diet upon adipose-tissue fatty acids in young coronary males is presented.

EXPERIMENTAL SECTION

Studies were performed on 38 electrocardiographically confirmed coronary male outpatients, mean age 43.7 years, SD 0.68. These volunteers were obtained from a dietary-managed group which has been described previously (4). The men were placed on a diet designed to approximate 30% of calories from fat and 14.1% from dietary linoleate (Table I) for periods up to five years. All men were within 5% of ideal weight at the start of the study and maintained ideal weight within 5% during the period of the study. Ideal weight was defined as that given in the Metropolitan Life Insurance Tables of desirable weights, which take into account height, age, and body build. Adipose-tissue fatty acids were determined annually by the method of Hirsch et al. (3).

Fatty acids were assayed by vaporphase chromatography on 8 ft × 5-mm I.D. columns containing 16½% diethylene glycol succinate polyester, absorbed on acid and alkali-washed 120-140 mesh Gas-Chrom P. The columns were standardized by using NIH standard mixtures A, B, C, and D. Results of the assays agreed within 2% of the stated concentration in the standard and within 4% for the minor components. The columns had a mean of 1,600 theoretical plates, measured at stearic acid, and a component resolution between stearic acid and oleic acid of 1.18. The preparation of methyl esters has been described previously (5).

Dietary adherence was evaluated qualitatively from the fatty acid composition of the serum-free fatty acids (6) and quantitatively by the serum triglyceride fatty acids (7), determined at 10-week intervals. The degree of adherence was subjectively evaluated by a medical examination and a nutritional interview at 10-week intervals. A detailed food

[1]Presented at the 59th Annual AOCS Meeting, New Orleans, May 1967.

TABLE I
Approximate Composition of Dietary Allowance per Day on Diet (2,000 Calories Average)

	Calories	Protein (g)	Total fat (g)	Saturated fat (g)	Monounsaturated fat (g)	Polyunsaturated fat (g)
Whole egg (none)	(unlimited egg whites permitted)					
Skim milk (1 pint)	180	18.0	0.38	0.22	0.14	0.02
Meat, fish, poultry (8 oz)[a]	402	49.2	22.27	6.21	8.14	7.92
Cottage cheese from skim milk (4 oz)	100	20.0				
Wheat germ (1 tbsp.)	15	1.1	0.37	0.06	0.12	0.19
Bread exchange (4-5)	320	2.1	0.87	0.40	0.20	0.27
Fruit (1 citrus) (3)	250	2.3				
Vegetables (3-4)	100	6.2	1.40			1.40
Potato (1)	100	3.0				
Special margarine (½ oz)	100	0.1	11.00	2.10	5.80	3.10
Corn-safflower oil (1 oz)	250		27.90	3.20	6.30	18.40
Sweets, alcohol, etc.	200					
Total	2017	102.0	64.19	12.19	20.70	31.30

[a]Weekly allowance
lean beef and/or lamb: 1 meal (4 oz)
veal and/or liver: 1 meal (4 oz)
poultry (without skin): 4 meals (16 oz)
fish and seafood (all types): 8 meals (24 oz)

Summary of Composition of Diet

Nutrient	Grams	Gram % of total derived from	% of total calories derived from
Protein	102.0	20.8	20.4
Carbohydrates (by difference)	259.0	52.9	51.8
Fats			
Total	64.2	13.1	28.9
Saturated fatty acids	12.2	2.5	5.5
Monoenoic fatty acids	20.7	4.2	9.3
Linoleate and polyenoic fatty acids	31.3	6.4	14.1

diary for a period of one week was prepared and analyzed on each subject yearly.

RESULTS

The mean dietary linoleate, determined from the serum triglyceride fatty acids, was 11.44% of calories, SD 0.54. The determination of the fatty acids in the serum-free fatty acid fraction, which qualitatively assesses the nature of the dietary fat, confirmed the preponderance of unsaturated fat in the diet. This was further supported by nutritional interviews. The percentage of total calories which were derived from fat was 33.89%, SD 1.46, as determined from data obtained from the food diaries and nutritional interviews.

The effect of the diet upon adipose-tissue linoleic acid as a function of time is given in Table II. These results were obtained by serial determinations over a five-year period on the same men. Because of the different times at which the men were placed on diet, it was not always possible to obtain adipose-tissue samples on the anniversary dates of the men who went on diet. In addition, fatty acid determinations were always performed during the late spring and early summer. Thus, in about half of the men, the second sample was obtained approximately six months after the diet was started. Subsequent samples were obtained at 12-monthly intervals after the second sample.

Linoleate, as mole percentage of total fatty acids in the adipose tissue, increased sigmoidally with time through the period of zero to 60 months. The response approximates the form:

$$\frac{1}{y} = 0.025 + 0.066\,(0.975)^{x}$$

in which y represents the adipose-tissue linoleate as mole percentage of total adipose-tissue fatty acid and x is the time in months. The major increase, approximately 50%, in adipose-tissue linoleate occurred between 12 and 18 months; small increases occurred for the remaining 42 months. The turnover time is of the order of 18 months, which is similar to the turnover time postulated by Hirsch et al. (3). The initial linoleate level of 11%, SD 1.38, is in agreement with the results of Scott et al. (1) for American soldiers and with the

TABLE II

Variation in the Fatty Acid Composition of the Adipose Tissue with Time on an Unsaturated Diet for the 38 Men

	Mole % of Total Fatty Acids				
	Time on diet (months)				
Fatty acid	0	6	12	18	24
12:0	0.94±0.28[a]	2.46±1.48	1.41±0.77	4.73±3.70	2.61±1.02
12:1	0.27±0.13	0.76±0.60	1.94±1.73	0.92±0.42	0.31±0.20
14:0	3.26±0.61	2.33±0.44	1.13±0.42	2.76±0.56	1.67±0.32
14:1	1.42±0.42	7.86±4.38	0.75±0.47	1.35±1.50	0.64±0.25
16:0	21.32±1.20	15.71±3.66	19.88±1.24	16.44±1.13	19.24±0.60
16:1	6.34±0.63	6.65±1.22	5.02±0.91	9.33±1.98	8.68±0.70
16:2	0.33±0.14	1.32±0.65	0.40±0.23	2.11±0.71	0.26±0.37
18:0	5.31±1.07	4.00±1.87	3.14±0.35	3.74±0.60	2.94±0.57
18:1	40.99±2.58	35.90±6.20	49.37±6.00	36.85±3.25	36.45±2.27
18:2	10.92±1.38	11.80±1.90	12.07±2.26	15.24±1.73	19.95±1.40
18.3	1.34±0.26	1.76±0.78	0.82±0.35	0.96±0.19	1.94±0.56
18:4	0.56±0.16	0.16±0.11	—	0.34±0.21	0.75±0.60
20:2	0.52±0.18	0.85±0.44	0.69±0.44	0.34±0.19	1.39±0.86
20:4	1.29±0.71	1.98±1.79	0.57±0.27	0.32±0.24	0.45±0.27
Other saturated	2.41±1.23	1.45±0.58	1.10±0.46	2.85±1.23	1.79±0.73
Other monoenoic	0.20±0.02	—	—	—	0.10±0.10
Other di- and polyenoic	1.94±0.89	6.90±4.65	2.30±1.73	2.74±1.61	1.62±0.61

	Mole % of Total Fatty Acids				
	Time on diet (months)				
30	36	42	48	54	60
1.05±0.84	0.56±0.17	0.56±0.17	0.68±0.28	0.35±0.20	0.45±0.41
0.12±0.09	0.21±0.09	0.37±0.18	0.68±0.25	0.28±0.24	0.05±0.04
2.08±0.32	2.29±0.41	2.58±0.48	2.45±0.39	2.57±0.17	1.65±0.27
0.55±0.20	0.92±0.19	1.20±0.51	0.97±0.32	0.57±0.27	0.88±0.54
19.49±0.33	19.16±1.27	15.61±2.60	18.39±0.94	19.45±1.32	20.69±1.28
7.36±0.91	5.85±0.74	6.24±1.58	7.27±0.91	6.76±1.01	4.06±0.65
0.18±0.09	0.22±0.07	0.57±0.22	0.91±0.08	0.33±0.24	1.08±0.47
3.14±0.35	4.32±0.52	5.18±0.95	4.99±0.62	6.57±2.12	6.53±1.39
43.50±1.96	38.83±2.66	38.87±7.49	33.96±3.06	40.85±4.83	38.51±2.00
17.82±1.80	19.20±1.83	16.84±1.88	20.96±0.98	19.51±1.74	23.68±2.68
1.82±0.37	1.65±0.18	3.08±1.74	1.62±0.29	1.29±1.32	0.87±0.22
0.99±0.07	0.32±0.13	0.81±0.26	0.54±0.21	0.55±0.23	0.22±0.12
0.40±0.31	0.62±0.20	0.29±0.13	1.18±0.47	0.14±0.11	0.13±0.09
0.41±0.31	0.39±0.10	4.22±3.15	0.42±0.15	0.08±0.45	0.07±0.10
0.82±0.34	1.52±0.38	1.20±0.86	1.72±0.41	0.27±0.11	0.78±0.22
—	0.05±0.03	0.30±0.13	0.03±0.02	—	0.04±0.04
0.69±0.10	3.16±0.64	2.37±0.67	2.18±0.51	0.13±0.13	0.06±0.05

[a]Mean ± S. D. of mean.

results of Hirsch et al. (3) for normal adults on a random diet. The upper level approximates the results of Hirsch et al. (3).

DISCUSSION

The fatty acid composition of the adipose tissue as a function of time on diet at ideal weight is given in Table II. Although the relative concentration of linoleic acid increases with time, it does not do so at the expense of any specific fatty acid but rather at the expense of all the remaining fatty acids.

It can be noted that the linoleate level in the adipose tissue after 18 months is significantly higher than the linoleate concentration in the diet. This also parallels the results of Hirsch et al. (3) in a similar subject.

It may also be noted that an apparent lag exists of 6 to 12 months before any appreciable alteration is found in the adipose-tissue linoleate. Hirsch et al. (3) found that, for 20 weeks on a corn oil diet, any change in adipose-tissue fatty acids was imperceptible but after this period the adipose-tissue linoleate rose rapidly. Similar results have been reported in many animal studies. Gordis (8) reported the long-term stability of the triglyceride molecules in the adipose tissue of rats. Privett et al. (9) found that, in the rat, at least 6 months were required for a turnover of lipid in the adipose tissue response to dietary alteration.

By assuming the existence of two pools in the adipose tissue, as suggested by Hirsch et al. (3) and as, in part, supported by Fleisch-

man et al. (6), it would appear that what is being measured is the composition of the more stable pool. It had previously been shown by Fleischman et al. (6) that the labile pool, which is in equilibrium with the serum-free fatty acids, has a turnover rate of approximately three days. This lends support to the idea that the subcutaneous fat measured is in effect part of the more stable pool.

Because of the slow turnover rate of the fatty acids in the adipose tissue, a determination of adipose-tissue fatty acids is valuable in epidemiological studies. It also serves as a monitor of long-term dietary adherence if weight is stable. It suggests further that a nutritional study in which the fatty acid content of the diet is manipulated and which lasts only 18 to 24 months may not be long enough to derive the expected benefit of alteration of the stable fatty acid pool, which is only beginning to change significantly at that time.

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Sphingolipid Metabolism in Leucocytes. I. Incorporation of ^{14}C-Glucose and ^{14}C-Galactose into Glycosphingolipids by Intact Human Leucocytes [1]

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ABSTRACT

Glucosyl ceramide and lactosyl ceramide have been isolated from intact human leucocytes. Incubation of intact white blood cells with either ^{14}C-glucose or ^{14}C-galactose resulted in the incorporation of these tracers into the glycosphingolipids. The products were extracted by conventional procedures and purified by combined silicic acid column and thin-layer chromatography. The bulk of the radioactivity was found in the monohexoside and dihexoside ceramide fractions.

Acid hydrolysis yielded glucose as the principal carbohydrate of the monohexoside ceramide, regardless of the sugar precursor employed. In the dihexoside ceramide fraction, galactose was liberated as the major sugar component. The specific activities of the lactosyl ceramide was found to be greater than that of the corresponding glucosyl ceramide.

INTRODUCTION

THE CONVERSION of labeled percursors, such as ^{14}C-acetate, ^{14}C-glucose, and inorganic ^{32}P-phosphorus into lipid by intact human (1-3) and guinea-pig (4) leucocytes has been described. The bulk of lipid synthesis in whole blood in vitro has been ascribed to the activity of the white blood cell (5,6). Miras et al. have reported the occurrence of galactosyl ceramide and a mixture of lactosyl and digalactosyl ceramide as the principal glycosphingolipids in human leucocytes (7). Interest in the metabolism of these compounds stems from previous observations on the levels of certain sphingolipid hydrolases in white blood cells of patients with several pathological conditions (8,9). The studies demonstrate that leucocytes have the ability to convert both ^{14}C-glucose and ^{14}C-galactose to the glucose moiety of glucosyl ceramide and also the galactose moiety of dihexosides ceramide.

MATERIALS AND METHODS

Whole blood from normal human donors was obtained through the Leukemia Service of the National Cancer Institute, Bethesda, Md., and the white blood cells were prepared in a manner similar to that previously described (8). To 600 ml of the enriched white blood cell suspension, 60 ml of a 6% solution of high molecular weight Dextran ($1.5\text{-}2.0 \times 10^5$) were added. The mixture was stirred, then centrifuged at $500 \times g$ for 5 min. The supernatant solution, which contained the white blood cells, was removed and centrifuged at $2{,}500 \times g$ for 5 min. The pellet was suspended in 50 ml of 0.85% NaCl solution, 150 ml of distilled water were added, and suspension was mixed for 90 sec. Twenty milliliters of 3.6% saline added, and the suspension was thoroughly mixed. The white blood cells were sedimented at $1{,}000 \times g$ for 10 min. ^{14}C-glucose (specific activity; 2×10^8 cpm/μmole) and ^{14}C-galactose (specific activity; 4.7×10^7 cpm/μmole) were purchased from New England Nuclear Corporation, Boston, Mass. Silicic acid was obtained from the Bio-Rad Corporation and heated at 100C overnight before use. Thin-layer plates were prepared by using the Desaga instrument and Silica Gel G (Brinkmann) with or without borate as previously described (10). Chromatograph standards of synthetic glucosyl ceramide, galactosyl ceramide, and lactosyl ceramide were gifts of D. Shapiro, Rehovoth, Israel. Galactosyl galactose was a gift of E. Neufeld, National Institutes of Health.

Each incubation vessel contained from 7.5×10^4 to 1.5×10^5 washed white blood cells, suspended in 0.5 ml of either Tyrodes or Lockes solution. Either 1.8×10^7 cpm of ^{14}C-glucose or 1.2×10^7 cpm of ^{14}C-galactose was added, as noted in the text. The vessels were incubated in air for various periods of

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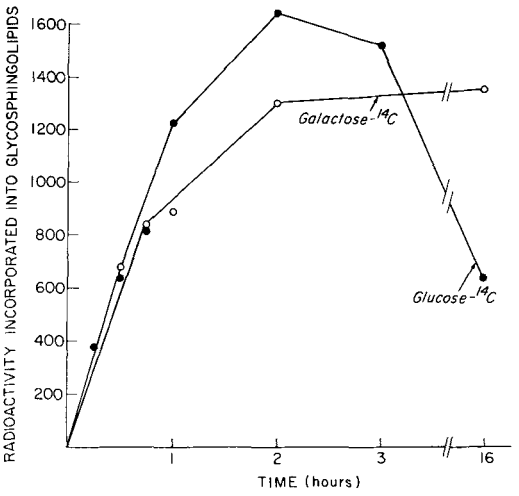

FIG. 1. Time course of incorporation of ^{14}C-glucose and ^{14}C-galactose into lipid by intact leucocytes incubated in Tyrode's solution. Ordinate expressed as total counts incorporated.

time at 37C. The lipids were processed by a modified Folch partitioning procedure (11). The incubation was terminated by the addition of 3.5 ml of methanol, and the vessels were placed in a bath at 55C for 10 min. At this time 7 ml of chloroform was added, and the insoluble material was removed by filtration.

To the chloroform-methanol extract 2 ml of 0.1 M KCl was added, and the mixture was shaken. The upper phase was discarded, and the lower phase was washed by shaking with 4 ml of chloroform-methanol-0.1 M KCl (3:48:47, v/v/v), followed by 4 ml of chloroform-methanol-water (3:48:47, v/v/v). The upper phase from each washing was discarded, and the radioactivity in an aliquot of the final lower phase was determined.

The alkali-labile lipid was removed as follows. The final washed chloroform phase was taken to dryness and saponified by using the method of Marinetti (13). After washing, aliquots of the lower phase were plated, and the amount of radioactivity was determined in a Nuclear-Chicago gas-flow counter. No radioactivity was detected in the final chloroform phase when zero time and boiled enzyme controls were carried through this procedure.

Samples of the chromatographically purified glycosphingolipids were either hydrolyzed with 2N hydrochloric acid for 2 hr at 100C (14) or degraded with periodate-osmium tetroxide (15). The sugars released were chromatographed on Whatman No. 1 paper in ethyl acetate-pyridine-water (1:3.6:1.15) as the solvent system. Standards run in parallel were located with alkaline silver nitrate, and the distribution of radioactivity was determined on a Vanguard strip scanner.

The amount of carbohydrate was quantitatively determined by the phenolsulfuric acid method (16), with standards of glucosyl- and galactosyl ceramide. Thin-layer chromatography on borate-impregnated plates was performed as previously described (10,17). Spots were located by means of iodine vapor. Zones corresponding in R_f to the standards were scraped from the plates and transferred to counting vials, and the radioactivity was determined by liquid scintillation spectroscopy as a thixotropic gel (18).

RESULTS

Initial experiments were designed to determine the optimal media in which to carry out the incorporation experiments. Tyrode's and Locke's solutions (19) were routinely employed since both appeared to be equally satisfactory in preliminary studies.

Time of Sugar Incorporation

Since the purpose of these studies was to establish the nature of the products, it was of interest to determine the period of time required for maximum incorporation. The cells were prepared and finally suspended in five volumes of Tyrode's solution. At the various times indicated in Fig. 1, the reaction was terminated and the samples were processed as described under Methods. Incubation in Tyrode's solution resulted in an effective conversion of ^{14}C-glucose and ^{14}C-galactose into lipid under these conditions.

Lower-Phase Lipids

In order to isolate and identify the glycosphingolipids into which the ^{14}C-glucose and ^{14}C-galactose had been incorporated in a 2-hr time period, the scale of incubation and extraction procedures was increased 20-fold. The lower phases from both the ^{14}C-glucose and ^{14}C-galactose incubation mixtures were evaporated to dryness after saponification and stored in vacuo prior to chromatography. The samples were dissolved in a small volume of chloroform and applied to separate 5-g silicic acid columns (diameter, 1 cm), which had been previously washed with chloroform. The columns were eluted batch-wise with 100-ml volumes of the following solvent mixtures: chloroform; chloroform-methanol (98:2); chloroform-methanol (95:5); chloroform-methanol (9:1); chloroform-methanol (4:1);

chloroform-methanol (2:1); chloroform-methanol (1:1).

The radioactivity was determined in a gas flow counter, and the fractions were concentrated to a small volume so that aliquots could be subjected to TLC. The fractions eluted by the first two solvents contained principally material which migrated to the solvent front. This rapidly moving material, together with a mixture of radioactive monohexosides ceramide, were found in the chloroform-methanol (95:5) fraction. This fraction was evaporated to dryness and rechromatographed on a 5-g silicic acid column by using the first three solvents listed above. The bulk of the material detected in the 95:5 fraction by both iodine vapor visualization and radioactivity was material which migrated with glucosyl ceramide.

The chloroform-methanol (9:1) fraction from the first column was found to contain traces of material which chromatographed both to the solvent front and in the region of monohexoside ceramide, but the major portion of the material migrated with dihexoside ceramide.

Final purification of the products in these two fractions was accomplished by preparative TLC. The samples were applied as a streak on 20 cm × 20 cm Silica Gel G plates together with spots of synthetic standards on either side. After development, the areas of the gel that reacted with iodine vapor and exhibited radioactivity were removed and transferred to small columns. The compounds were eluted from the gel with chloroform-methanol (2:1). The recoveries represented 75-85% of total radioactivity originally applied to the plates. The radioactive homogeneity of these compounds is indicated in Fig. 2.

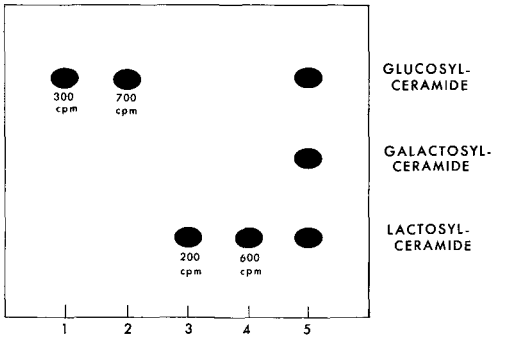

FIG. 2. Diagrammatic representation of the purified glycosphingolipid fractions from human white blood cells. Lane 1. Chloroform-methanol (95:5) from ^{14}C-glucose incubation. 2. Chloroform-methanol (95:5) from ^{14}C-galactose incubation. 3. Chloroform-methanol (9:1) from ^{14}C-glucose incubation. 4. Chloroform-methanol (9:1) from ^{14}C-galactose incubation.

Hydrolysis of Monohexosides Ceramide

Aliquots of the cerebrosides derived from ^{14}C-glucose and ^{14}C-galactose were subjected separately to osmium tetroxide-sodium metaperiodate cleavage, and the sugars released were subjected to paper chromatography. The radioactive products from the hydrolysis procedure were located by use of the Vanguard strip scanner. The radioactivity from the samples derived from incubation with ^{14}C-glucose and ^{14}C-galactose was localized in the area associated with the glucose standard. The radioactive segments of both strips were removed and eluted with water. Carrier nonradioactive glucose was added to both samples that migrated with the glucose standard, and the mixture was oxidized by the hypo-iodide procedure of Moore and Link (20). The potassium gluconate so obtained could be recrystallized to constant specific activity as shown in Table I, Part A. Therefore it can be concluded from evidence of co-chromatography and carrier dilution that both ^{14}C-glucose and ^{14}C-galactose are converted to glucose of the monohexoside ceramide.

Hydrolysis of Dihexosides Ceramide

Aliquots of the dihexoside ceramides derived from the ^{14}C-glucose and ^{14}C-galactose were each hydrolyzed, in order to liberate their intact disaccharides, by the osmium textroxide-sodium periodate degradation procedure. The water-soluble radioactive products obtained in this manner were resolved and identified by

TABLE I
Identification of Hydrolysis Products[a]

Product	Labeled precursors	Specific activity (cpm/mg)
Part A		
Potassium gluconate	^{14}C-Galactose	7.2
Recrystallization I	^{14}C-Galactose	6.3
Recrystallization II	^{14}C-Galactose	6.5
Potassium gluconate	^{14}C-Glucose	16.1
Recrystallization I	^{14}C-Glucose	15.2
Recrystallization II	^{14}C-Glucose	15.5
Part B		
Mucic acid	^{14}C-Galactose	43.6
Recrystallization I	^{14}C-Galactose	39.2
Mucic acid	^{14}C-Glucose	8.5
Recrystallization I	^{14}C-Glucose	7.3

[a]The labeled potassium gluconate and mucic acid were obtained as described in the text.

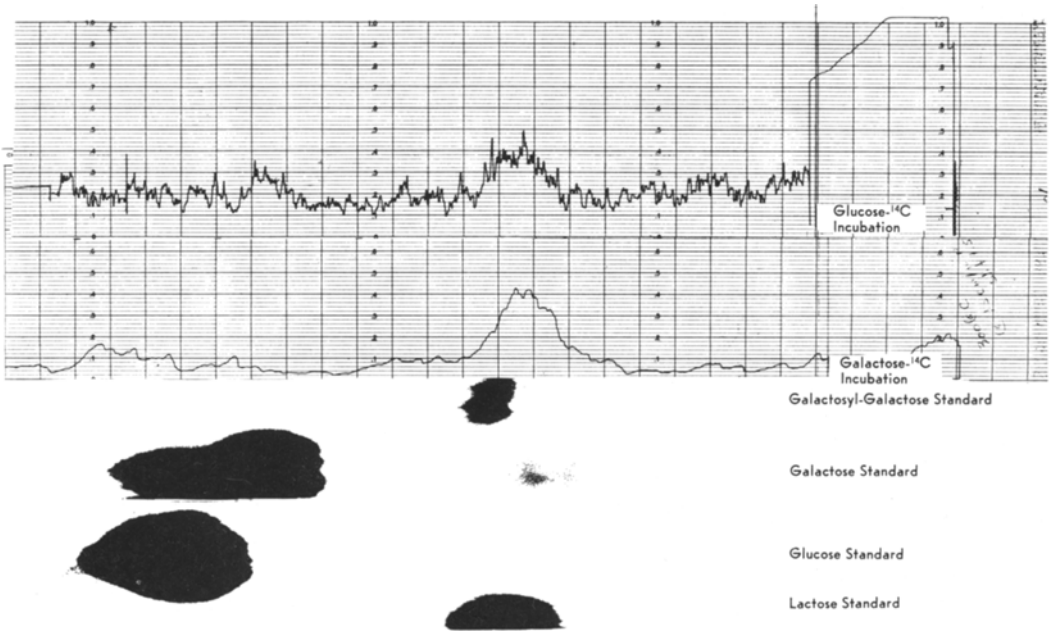

FIG. 3. Tracing of radioactive strips of disaccharides liberated from ceramide dihexosides. Upper tracing from ^{14}C-glucose; lower tracing from ^{14}C-galactose. The upper standard is galactosyl-galactose; the next lower, galactose; the next lower, glucose; and the bottom, lactose.

the same paper chromatographic system and scanning procedures used for the aldohexoses (Fig. 3). It is evident that the labeled material migrated in an area corresponding to disaccharide standards. Little radioactivity was present in the monosaccharide areas, indicating that the original samples were free of monohexoside ceramide contaminants. The areas of paper carrying the radioactive products were removed and extracted with water.

In order to cleave these compounds to their component monosaccharides, they were hydrolyzed with 1 ml of 2 N HCl for 3 hr at 100C. After removal of chloride ion with Dowex-1-acetate resin the samples were lyophilized and chromatographed on paper once again. The sample derived from the ^{14}C-galactose experiment was found to co-chromatogram with the galactose standard; only negligible radioactivity was associated with the glucose standard. Since the hydrolyzed sample derived from the ^{14}C-glucose experiment gave a poor tracing, the areas corresponding to both glucose and galactose standards were eluted and counted. The major portion of the radioactivity (2,200 cpm) was found in the area which migrated with the galactose standard and fewer than 200 cpm were associated with the glucose standard. The radioactivity from the sample derived from the ^{14}C-galactose experiment was also eluted with water.

Carrier non-radioactive galactose was added to each of the eluates, and the mixtures were oxidized with 25% nitric acid to yield mucic acid (21). In both instances, constant specific activity was obtained after repeated recrystallizations (Table I, Part B). When samples of the dihexoside ceramide fractions were subjected directly to more vigorous acid hydrolysis (2 N HCl, 2 hr, 100C) and the liberated hexoses were separated by paper chromatography, galactose was the principal radioactive sugar detected. Therefore it appears that ^{14}C-galactose is the major radioactive sugar in the dihexoside ceramides formed from the incubation of either ^{14}C-glucose or ^{14}C-galactose by intact white blood cells.

Specific Activities of Isolated Fractions

In order to attempt to interrelate the mono- and dihexoside products it was necessary to determine the specific activities of the individual materials. Both total carbohydrate content and radioactivity were determined on aliquots of the purified samples obtained by preparative TLC. These data are presented in Table II.

Identification from Intact Human Leucocytes

Since data concerning the nature of the monohexoside ceramide appeared to be at

TABLE II

Specific Activities of the Monohexoside Ceramide and Dihexoside Ceramide Fractions Obtained from Incubating ^{14}C-Glucose and ^{14}C-Galactose with Intact Human Leucocytes

Product	Labeled precursors	Specific activity (cpm/μmoles sugar)
Monohexoside ceramide	^{14}C-Glucose	21,421
Monohexoside ceramide	^{14}C-Galactose	24,727
Dihexoside ceramide	^{14}C-Glucose	988
Dihexoside ceramide	^{14}C-Galactose	6,660

variance with the observations of Miras et al., who reported this compound to be a galactosyl ceramide (7), it was necessary to establish the nature of this glycosphingolipid of the leucocytes. The lipid was extracted by the Folch procedure (11) from white blood cells which were derived from 40 pints of whole blood. The lower phase, after partitioning, was concentrated to dryness and saponified. After washing, the resultant chloroform phase was dried and applied to a 20–g silicic acid column, and elution was effected with 500 ml of the solvents employed for the product isolation. By essentially identical procedures, described in an earlier section, of rechromatography and preparative TLC homogeneous monohexoside ceramides and dihexoside ceramides were obtained.

The monohexoside ceramide fraction was subjected to TLC on borate plates in solvent systems previously published (10,17). The results are presented in Lanes 1, 2, and 3 of Fig. 4. The monohexoside ceramide migrates in both solvents with authentic standards of glucosyl ceramide and not galactosyl ceramide.

The dihexoside ceramide was subjected to a partial acid hydrolysis (0.3 N HCl, 1 hr, at 60C) in order to remove the terminal hexose. After partitioning of the reaction mixture, the lipid-soluble material was subjected to resolution in the systems previously employed for the monohexoside ceramide. These results are found in Lanes 4, 5, and 6 of Fig. 4. In Lanes 4 and 5 there is some unhydrolyzed starting material, perhaps a trace of a material which migrates with galactosyl ceramide, and the bulk amount of product which migrates as glucosyl ceramide in both solvent systems. Therefore the principal dihexoside ceramide appears to be a lactosyl ceramide. Only a trace of galactosylgalactosyl ceramide seems to be present.

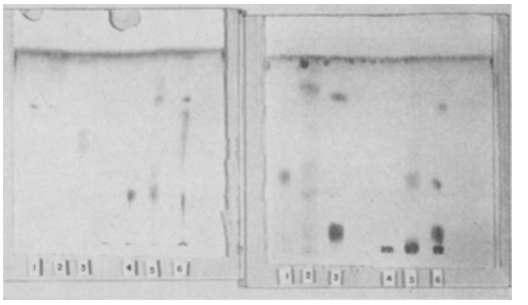

FIG. 4. Thin-layer chromatogram of isolated glycosphingolipids by intact human leucocytes. Left-hand plate run in C-M-H_2O (65:25:4), right-hand plate in C-M-H_2O-NH_4OH (280:70:6:1). Lanes: 1) glucosyl ceramide standard, 2) isolated ceramide monohexoside, 3) galactosyl ceramide standard, 4) lactosyl ceramide standard, 5) partial acid hydrolysis of isolated dihexoside ceramide fraction, 6) mixed standards.

DISCUSSION

The demonstration of glucosyl ceramide as the naturally occurring monohexoside ceramide is in complete agreement with the metabolic studies. This observation is at variance with the finding of Miras et al., (7), who reported the occurrence of a galactosyl ceramide in human leucocytes. However the conclusions of these authors were based on co-chromatography of the isolated material with a galactosyl ceramide standard. Conventional TLC plates are unsuitable for resolving glucosyl ceramide and galactosyl ceramide; however resolution can readily be accomplished by using plates impregnated with Silica Gel G containing sodium tetraborate. Miras et al. have also indicated the occurrence of a mixed dihexoside ceramide which, according to the published data, appears to be comprised of an equal amount of lactosyl ceramide and galactosylgalactosyl ceramide. From the thin-layer chromatogram in Fig. 4 it would appear that the preparation contains principally lactosyl ceramide with only a trace of digalactosyl ceramide.

The metabolic experiments with ^{14}C-glucose and ^{14}C-galactose revealed an incorporation of these sugars into glucosyl ceramide. Paper chromatography and carrier dilution with added glucose of the water-soluble products which were obtained by acid hydrolysis revealed that the bulk of the label was present as glucose. The ceramide released had only negligible radioactivity.

The finding of ^{14}C-galactose as the principal radioactive carbohydrate which was obtained by both total acid hydrolysis of the intact di-

hexoside ceramide fraction and cleavage of the disaccharide liberated from the intact glycosphingolipid was unexpected. The principal dihexoside ceramide isolated from leucocytes is presumably a lactoside. The glucosyl ceramide formed by the acid hydrolysis of the radioactive dihexoside ceramide contained negligible radioactivity. This would indicate that the galactose moiety of the lactosyl ceramide contained the bulk of the label.

The specific activity of the monohexoside ceramide is much greater than that of the dihexoside ceramide irrespective of the labeled hexose precursor employed in these studies (Table II). It is conceivable that the lactosyl ceramide arises by the addition of galactose onto the monohexoside ceramide. However it should be emphasized that glucose was the principal hexose recovered from the labeled monohexoside ceramide and galactose from the labeled dihexoside ceramide. The ratio of the specific activities of the mono- to dihexoside from the ^{14}C-glucose incubation is 20:1, and the ratio from the ^{14}C-galactose incubation is 4:1 (Table II). Thus it may be concluded that galactose is a more efficient precursor of the lactosyl ceramide than is glucose.

The details of the enzymatic reactions responsible for the synthesis of these glycosphingolipids are currently under investigation. It is hoped that these experiments will aid in explaining the observations with intact leucocytes.

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The Maturation of Rat Brain Myelin[1]

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ABSTRACT

Myelin fractions were prepared from brains of 9- to 90-day-old rats by continuous and discontinuous sucrose density gradient procedures. Total protein and lipid content of myelin showed little variation, but lipid composition changed significantly during maturation. Cholesterol, galactolipids, and ethanolamine glycerophosphatide plasmalogen increased whereas choline glycerophosphatide content decreased with increasing age. The changes in lipid composition were more marked in the myelin prepared by the discontinuous than by the continuous gradient technique. The significance of these lipid changes in relation to their organization in the myelin membrane is discussed.

INTRODUCTION

RECENT ADVANCES in subcellular fractionation techniques and lipid determinations have permitted chemical analysis of purified myelin preparations from human subjects and various animals (1-10). Until recently it was thought that myelin had the same composition at all ages (11,12) and that, once formed, it was metabolically inert (13,14). Recent findings have revealed that certain myelin lipids have metabolic activity (15-18). In addition, evidence is becoming available which suggests that the composition of central nervous system myelin changes during maturation. Thus Horrocks et al. (19) reported that the proportion of cholesterol, galactolipids, inositol glycerophosphatide (IGP), and ethanolamine glycerophosphatide (EGP) increased whereas choline glycerophosphatide (CGP) decreased with age in the mouse brain. Desmosterol, a cholesterol precursor, has been found in myelin of young but not adult rats (20,21). The phospholipid composition of white-matter myelin from a 4-year-old human subject was found to be higher in CGP and lower in EGP than in the adult (22). Furthermore, Davison recently has stated that the myelin of young rat brains contains less galactolipid and cholesterol than those of adult animals (23). The present study provides additional evidence that the lipid composition of rat brain myelin does change during maturation.

MATERIALS AND METHODS

Myelin was prepared from pools of six to 24 Sprague-Dawley rat brains by two procedures. The first was that of Laatsch et al. (24); the myelin thus obtained is henceforth referred to as "crude" myelin. The second procedure was carried out as follows. Rat brains were homogenized with 0.29 M sucrose (density, 1.035) in a proportion of 1 g of tissue per 10 ml of sucrose solution for 3 min in a glass homogenizer. The resulting suspension was centrifuged in the cold for 45 min at 15,000 × *g*. The crude microsomal fraction remaining in suspension was discarded, and the pellet containing myelin and other subcellular elements was used to prepare myelin according to Laatsch et al. (24). The resulting myelin was further fractionated by using the continuous sucrose gradient procedure of Autilio et al. (3), and the final preparation is subsequently referred to as "purified" myelin.

The protein content of myelin was determined by the procedure of Lowry et al. (25) with Versatol[3] as a standard. Lipids were extracted according to Folch et al. (26) and separated by two-dimensional thin-layer chromatography (TLC) on Silica Gel G chromatoplates of approximately 400 μ thickness by using chloroform-methanol-7 N ammonia 65:35:4 (v/v/v) in the first dimension, and chloroform-methanol-water 72:28:4.5 (v/v/v) in the second dimension. A typical separation is shown in Fig. 1. The plate was air-dried, and lipids were visualized with iodine vapor. After the cerebrosides, sulfatides, and cholesterol were scraped off along with appropriate

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[3]Versatol, a reconstituted human serum protein mixture, was purchased from General Diagnostics Division, Warner-Chilcott Laboratory Division, Morris Plains, N. J. Using this sample as the standard in the Lowry procedure, the protein content of adult human myelin was found to be in good agreement with analysis by the amino acid procedure (10). Since recent work in this laboratory (30) has shown that myelin protein composition changes with age, data obtained by the Lowry procedure reflects, at best, approximate values of myelin protein content during development.

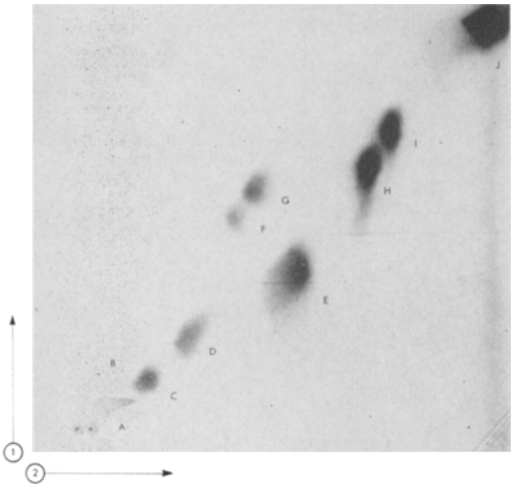

FIG. 1. The separation of myelin lipids by 2-dimensional TLC on Silica Gel G. The plate was developed with chloroform-methanol-7 N ammonium hydroxide (65:35:4, v/v/v) in the first dimension and chloroform-methanol-water (72:28:4.5, v/v/v) in the second. The lipids were visualized by charring with 50% sulfuric acid. Lipid fractions are denoted as follows: A. SGP; B. IGP; C. Sph; D. CGP; E. EGP; F. phrenosine sulfatide; G. cerasine sulfatide; H. phrenosine; I. cerasine; and J. cholesterol.

silicic acid blanks, the plate was sprayed with 50% sulfuric acid and heated at 100C to visualize the phospholipids.

The fractions containing the cerebrosides and sulfatides were immediately placed in separate screw-top tubes, and to each was added 5 ml of a chloroform-methanol-water mixture (65:35:4). After agitation for 2 min in a Vortex mixer, the tubes were centrifuged at 1500 × *g* for 10 min to remove the silicic acid. A 4-ml aliquot of the supernatant was removed and dried at 60C under nitrogen. Galactose standards (5, 10, 25, and 50 μg) were similarly dried. The tubes were cooled in an ice bath, and after 4 ml of cold 0.1% orcinol in 67% sulfuric acid was added to each tube, they were placed in a 79C water bath for 20 min. The tubes were then cooled in an ice bath for 5 min and transferred to a room-temperature bath for another 5 min; optical density of the solutions was read against a reagent blank at 505 mμ. Recovery experiments with cerebrosides, isolated by diethylaminoethyl cellulose column chromatography (27), yielded values between 90 and 100%.

For the microcholesterol determinations 4 ml of chloroform was added to each sample in a Coleman cuvette (19 mm diam.), followed by 2 ml of an acetic anhydride-concentrated sulfuric acid mixture (20:1) in a room-temperature water bath. The tubes were agitated with a Vortex mixer, centrifuged for 5 min at 1500 × *g* to remove the silicic acid, and then placed in the dark. Twenty minutes after the addition of the acetic anhydride-sulfuric acid reagent, optical density of the solutions was read against a reagent blank at 620 mμ. Recovery of cholesterol in these experiments ranged between 95 and 100%.

The various phospholipid fractions and appropriate blanks were individually scraped off and placed in Pyrex test tubes (16 × 150 mm). Phosphorus content was determined by digesting the lipids with 0.9 ml of 70% perchloric acid for 1 hr at 190C in an electric tube heater. After cooling of the reaction mixtures, 7 ml of water, 0.5 ml of 2.5% ammonium molybdate, and 0.2 ml of aminonaphthosulfonic acid reagent (28) were successively added to each tube with mixing between each addition of reagent. The tubes were then heated for 7 min in a boiling water bath, cooled, and centrifuged at 1500 × *g* for 10 min to remove the silicic acid. Absorbance was determined in a Beckman Model DU Spectrophotometer at 830 mμ. The recovery of total phospholipid per plate ranged between 95 and 100%.

The plasmalogen content of the EGP fraction was determined by one-dimensional TLC in the following manner. Four aliquots of myelin lipid (0.3 mg) were applied in 1.5-cm bands. To cleave the plasmalogen into free aldehyde and lysoglycerophosphatide without hydrolyzing the diacyl glycerophosphatides, two of the aliquots were layered with 50 μl of 1.2 N HCl in methanol, and the samples were redried. The plate was developed with chloroform-methanol-7 N ammonia (65:35:4) 15 cm past the origin. The separated lipids were visualized by charring with 50% sulfuric acid and analyzed for total phosphorus. The EGP fraction obtained after acid treatment represents the diacyl and acyl alkyl moieties, and the difference between the acid-treated and the untreated EGP fraction represents the plasmalogen content. This procedure was verified by subjecting EGP fractions of known plasmalogen values, which were isolated from brain lipids by diethylaminoethyl cellulose chromatography, and commercially obtained diacyl EGP and CGP to the acid treatment.

RESULTS

The results of the first series of experiments,

TABLE I

"Crude" Myelin Yield, Protein Content, and Myelin Lipid Composition of Rat Brain[a] During Development

	Days after birth							
	9	13	15	17	19	21	23	90[b]
Myelin protein (% dry wt of myelin)	10.0	10.0	19.0	17.0	17.0	18.0	18.0	22.8
Myelin yield (mg dry wt)	25		34		88		102	225
Cholesterol (mole %)	29.0		35.4		37.8		36.9	40.0
Galactolipids (mole %)	15.1		14.1		17.8		17.0	23.4
Total phospholipids (mole%)	55.9		50.5		44.4		46.1	36.6
SGP and IGP (mole %)	8.2		7.6		7.9		8.2	8.1
Sph (mole %)	2.2		3.0		2.4		3.1	3.1
CGP (mole %)	28.3		21.1		17.1		17.0	9.1
EGP (mole %)	17.0		18.4		17.3		18.0	16.2
EGP-Plasmalogen (mole %)	5.4		9.2		9.3		10.1	12.3
Cholesterol/phospholipid	0.52	0.56	0.70	0.67	0.85	0.76	0.80	1.09
Galactolipid/phospholipid	0.27		0.28		0.40		0.37	0.64
EGP-Plasmalogen/total EGP	0.32		0.50		0.54		0.56	0.76

[a] The eight to 13 brains were used for each experimental day between nine to 23 days after birth, and six brains for 90-day-old animals. Triplicate analyses were performed for each chemical determination. Agreement of replicate analyses of all the individual lipids were 5% or less except for the galactolipids, which were 10% or less.

[b] Average of two preparations.

where "crude" myelin was prepared according to Laatsch et al. (24), are presented in Table I. The content of myelin protein remained relatively constant from 15 to 23 days after birth and was lower than that of adult myelin.

The mole % of cholesterol and galactolipids increased whereas total phospholipids decreased with age. In the phospholipid fraction the mole % of CGP decreased markedly but EGP-plasmalogen increased during development.

When myelin lipid ratios were calculated, the cholesterol to phospholipid ratio increased from 0.52 at nine days to 0.80 at 23 days and finally to 1.09 by 90 days. The galactolipid to phospholipid ratio increased from 0.27 at nine days to approximately 0.4 at 23 days and finally to 0.64 by 90 days. The percentage of plasmalogen in the EGP fraction showed a marked increase from 32% at nine days to about 60% by 23 days; the latter value was still much less than the 76% observed in adult myelin.

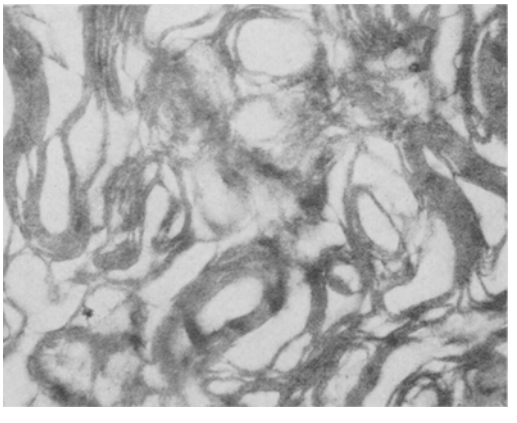

FIG. 2. Electron micrograph of 16-day-old rat-brain myelin (fixed in buffered O_sO_4 and embedded in Araldite 502) Mag. × 108, 800.

The low protein content of myelin prepared by discontinuous gradient suggests that there was no gross contamination since we have found that microsomes contain about 40% protein and mitochondrial fractions contain 60% protein. When these myelin preparations were placed on a continuous gradient and further fractionated, a smaller myelin fraction was obtained, which floated above the main adult band. The less dense material for the 18-day preparation was found to contain more CGP (5.9 mole %) and total phospholipid (7.1 mole %) but less cholesterol (1.8 mole %) and galactolipid (5.1 mole %) than the lipids isolated from the main myelin band. Also, 15% of the dry weight of the less dense fraction was protein as compared with 21% for the purified myelin.

In the second series of experiments all the myelin preparations were fractionated on a continuous sucrose gradient, and only the band corresponding to adult myelin was analyzed. When these preparations were examined by electron microscopy, they showed a high proportion of laminar rings and bands in various stages of disintegration and purity comparable with that seen in adult preparations (Fig. 2). No other subcellular elements were identifiable. The chemical analyses of these "purified" myelin samples are given in Table II. The protein content of myelin obtained from young rats is similar to that of adult animals. An increase in mole % of cholesterol and galactolipids and a decrease in total phospholipids

TABLE II
"Purified" Myelin Yield, Protein Content, and Myelin Lipid Composition of Rat Brain[a] During Development

	Days after birth				
	15	16	18	22	90
Myelin protein (% dry wt of myelin)	20.0	23.2	21.0	22.7	23.7
Myelin yield (mg dry wt)	23	18	26	105	180
Cholesterol (mole %)	36.6	38.0	38.0	37.6	39.5
Galactolipids (mole %)	20.2	19.8	20.1	20.5	24.3
Total phospholipids (mole %)	43.0	42.1	41.9	41.9	36.2
SGP and IGP (mole %)	9.0	7.3	7.5	8.4	7.9
Sph (mole %)	3.6	2.4	2.7	2.3	3.2
CGP (mole %)	15.1	15.3	14.9	14.4	9.4
EGP (mole %)	15.3	17.1	17.0	16.7	15.7
EGP-Plasmalogen (mole %)	8.9	10.5	10.0	10.4	11.8
Cholesterol/phospholipid	0.85	0.90	0.91	0.90	1.09
Galactolipid/phospholipid	0.47	0.47	0.48	0.49	0.67
EGP-Plasmalogen/total EGP	0.58	0.61	0.59	0.62	0.75

[a]The 17-24 brains were used for each experimental day between 15 to 22 days after birth, and six brains for 90-day-old animals. Triplicate analyses were performed for each chemical determination. Agreement of replicate analyses of all the individual lipids were 5% or less except for the galactolipids, which were 10% or less.

were found in animals during maturation. In the phospholipids there was a decrease in the absolute amount of CGP but an increase in EGP plasmalogen. These observed changes in "purified" myelin were less profound than those found in "crude" myelin preparations.

A rise in myelin lipid ratios of cholesterol to phospholipid, galactolipid to phospholipid, and EGP-plasmalogen to total EGP was also observed during development. However this rise in the "purified" myelin fraction was not as large as for the "crude" myelin.

DISCUSSION

It is known that the composition of myelin lipids can vary slightly from species to species (7) and differs within the same species between the central and peripheral nervous system (8, 29). The present report shows that, although the proportion of total protein to total lipid in purified myelin appears to be relatively constant at all ages, the quantity of EGP plasmalogen, sphingolipids, and cholesterol increases whereas CGP decreases during maturation. These findings confirm the preliminary reports of others (19-22) and indicate that the lipid composition of brain myelin changes with maturation. Recent work has shown that the total protein in human myelin remains constant during maturation but the composition of the protein varies. The fraction containing a high proportion of basic amino acids was found to double from infancy to adulthood (30). In young rats, myelin prepared by discontinuous sucrose gradient techniques could be further fractionated on a continuous gradient to give material with a density comparable with adult myelin and a small fraction of lower density which contains less protein, galactolipid, cholesterol, and EGP plasmalogen and more phospholipid than adult myelin. The presence of this fraction in the myelin of young rat brains prepared by the procedure of Laatsch et al. (24) accounts for the more striking changes in the lipid composition observed in the first series of experiments. Removal of this fraction by continuous sucrose gradient technique yielded myelin of similar composition in the 16- to 22-day-old animals. However the latter preparations still had a composition significantly different from that of the adult. The less dense myelin fraction may represent partially disintegrated myelin, microsomes, or mitochondria which result from the method of preparation or may be of the oligodendroglial plasma membrane origin that Davison has postulated as a component of early myelin (22). When myelin of adult rats was prepared either by continuous or discontinuous sucrose gradient techniques, both procedures yielded material of a similar lipid composition.

The significance of the present studies may be viewed from the proposals of Finean (31) and, more recently, Vandenheuvel (32) that the myelin lipid subunit is composed of phospholipid-cholesterol and/or sphingolipid-cholesterol complexes. The molar sum of the noncholesterol lipids exceeds that of cholesterol for all published myelin lipid compositions. Therefore, if all the cholesterol exists in a complexed form with phospholipid or sphingolipid, a portion of the noncholesterol lipid remains uncomplexed. Re-evaluation of the report by Smith and Eng (16) on the metabolic turnover rates of myelin lipids has revealed

TABLE III
Molar Ratios of Brain Myelin Lipids[a] Relative to Cholesterol

	Evans and Finean (8)		Cuzner et al. (7)				Horrocks (34)	(35)
	Guinea pig	Rat	Rat	Rabbit	Ox	Human	Mouse	Monkey
Cholesterol	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
"Stable"	0.96	1.00	0.90	0.90	0.93	1.01	1.03	0.92
"Labile"	0.30	0.45	0.43	0.35	0.38	0.34	0.42	0.28

	Gerstl et al. (10)	O'Brien and Sampson (5)	Norton and Autilio (6)	Norton and Autilio (9)		Eichberg et al. (4)		Eng and Smith (33)
	Human	Human	Ox	Ox	Human	Guinea pig Large	Guinea pig Small	Rat
Cholesterol	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
"Stable"	1.01	0.94	1.00	0.89	0.84	0.95	0.97	1.06
"Labile"	0.42	0.39	0.32	0.31	0.28	0.40	0.65	0.41

[a] The "stable" lipids are EGP, galactolipids, and Sph. The CGP, IGP, and SGP include the "labile" lipids.

that the molar sum of the noncholesterol lipids with the slower turnover rates approximates the molar concentration of cholesterol (33). Table III shows the calculated molar ratios obtained from the brain myelin lipid values of other investigators. The lipids with a slow metabolic turnover rate, that is EGP, sphingomyelin (Sph), galactolipid, and cholesterol are designated as "stable" lipids. CGP, IGP, and serine glycerophosphatide (SGP), lipids with fast turnover rates, are termed "labile" myelin lipids. It is evident that the molar sum of the "stable" lipids corresponds closely to that of cholesterol for the majority of published compositions of myelin whereas the sum of the "labile" lipids shows no correlation with cholesterol. Similar calculations for the myelin samples at various periods of maturation are given in Table IV. It is apparent that the proportion of "stable" lipids to cholesterol is fairly constant at all the ages which were examined.

The present results can be interpreted as further evidence for the possibility that the more "stable" lipids are complexed with cholesterol in the myelin membrane and that these lipids form the backbone of the postulated bimolecular lipid leaflet. Those lipids which have been found to be less "stable" may exist in the membrane in an uncomplexed form and may have nonstructural functions.

ACKNOWLEDGMENT

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TABLE IV
Molar Ratios of Rat-Brain Myelin Lipids Relative to Cholesterol at Various Phases of Development

	Myelin Prepared by Discontinuous Gradient				
	Days after birth				
	9	15	19	23	90
Cholesterol	1.00	1.00	1.00	1.00	1.00
"Stable"	1.18	1.00	0.99	1.03	1.07
"Labile"	1.26	0.81	0.66	0.68	0.43
	Myelin Prepared by Continuous Gradient				
	Days after birth				
	15	16	18	22	90
Cholesterol	1.00	1.00	1.00	1.00	1.00
"Stable"	1.06	1.03	1.05	1.05	1.09
"Labile"	0.66	0.59	0.59	0.61	0.44

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A Study on the Biosynthesis of *cis*-9,10-Epoxyoctadecanoic Acid[1]

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ABSTRACT

Preliminary studies show that red stem, rust-infected wheat plants provide a means for investigating the biosynthesis of epoxy fatty acids. The incorporation of 1-^{14}C-acetate into *cis*-9,10-epoxyoctadecanoic acid occurs at the stage of the infection when sporulation is proceeding, and at the same stage there is at least a fourfold increase in the synthesis of other fatty acids. The epoxy acid appears to be formed by the condensation of acetate units in a process that requires oxygen and is not stimulated appreciably by light.

Labeled stearic and oleic acid are also incorporated into the epoxy acid without undergoing β-oxidation. The rate of conversion of oleic acid is greater than stearic acid, thus indicating that oleic acid is an immediate precursor to 9,10-epoxyoctadecanoic acid.

INTRODUCTION

RECENTLY SEVERAL EPOXY fatty acids or their esters have been isolated from natural sources as discussed in the review of Wolff (1). Epoxy acids such as 9,10-epoxyoctadecanoic acid, 9,10-epoxy-12-octadecenoic, 12,13-epoxy-9-octadecenoic, and 15,16-epoxy-9,12-octadecadienoic acids occur in plant seed oils. Tulloch and Ledingham isolated and observed the presence of *cis*-9,10-epoxyoctadecanoic acid in many fungal spores (2-7). The juvenile hormone, identified as the methyl ester of a branched-chain fatty acid (methyl 10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate), is another example of an epoxy acid or ester present in natural materials (8). It is interesting that all of these sources are tissues in which differentiation may accompany further growth. This suggests that these epoxy acids may be intimately involved in key cellular processes.

The biosynthesis of the epoxy acids has not been seriously considered except in the report by Miwa et al. (9). There is little more known about the formation of epoxide groups in other compounds. Bloom and Shull (10) have observed that certain fungi are capable of introducing epoxide groups at positions of unsaturation in several steroids. Estra-1,3,5(10)-triene-16α,17α-epoxy-3-ol has been shown to be an intermediate in the conversion of estra-1,3,5(10),16-tetraene-3-ol to estra-1,3,5(10)-triene-3,16β,17α-triol by rat liver (11).

With rat (12-14) and swine (15) liver preparations it has recently been shown that the cyclization of squalene is preceded by the formation of 2,3-epoxysqualene.

The possible role of epoxy acids in basic biological processes and the lack of knowledge concerning the formation of the oxirane group in nature prompted the initiation of studies in this laboratory which deal with the biosynthesis and catabolism of epoxy fatty acids. It was the purpose of this work to obtain information on the basic properties of, and the precursors involved in, a suitable system for a detailed study of epoxy acid biosynthesis.

As a source of biological tissue, wheat plants infected with red stem rust appear to be ideal. The *cis*-9,10-epoxyoctadecanoic acid accounts for approximately 19% of the fatty acids in the uredospores of the organism (4). Thus the system is active in the biosynthesis of this epoxy acid. Large percentages (up to 60% of the fatty acids) of other epoxy fatty acids accumulate in some oil-bearing seeds, indicating that maturing seeds of plants such as *Vernonia anthelmintica* would be a suitable system to study (9). However the length of time required to obtain such materials is much greater than the few days required for the production of rust-infected wheat.

MATERIALS AND METHODS

Biological Materials

Wheat plants, variety Little Club, were inoculated, with red stem rust uredospores, *Puccinia graminis* (Pers.) f.sp. *tritici* race 56, according to Daly et al. (16) 7 to 10 days after planting. The infected tissues were used 8 to 12 days after the inoculation.

Incubations

Infected tissue was cut in approximately 2- to 5-mm square sections and collected in ice-

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cold 0.1 M potassium phosphate buffer, pH 4.5. The tissue was washed twice with the same buffer and allowed to drain for a few minutes. Fresh tissue (1.5 g) was placed in a 125-ml Erlenmeyer flask, to which were added the radioactive substrate and enough phosphate buffer to give a final volume of 30 ml. In the acetate incubations the substrate (0.05 μmoles) was added as a solution in one ml of phosphate buffer. The 1-^{14}C-stearic (0.1 μmole) and 1-^{14}C-oleic (0.1 μmole) acids were added in 0.03 ml of 95% ethanol. Incubations, carried out under light conditions, were performed in flasks which were illuminated with fluorescent light of approximately 400 ft-candles. Dark conditions were maintained by using the same conditions except that the flasks were covered with black plastic tape. All incubations were carried out at room temperature on a wrist-action shaker for 4 hr unless otherwise indicated.

Fatty Acid Analysis

The incubations were stopped by filtering the media through 10XX bolting cloth, washing the tissue three times with distilled water, and transferring the tissue to 10 ml of absolute ethanol. The sample was stored at −11C prior to analysis, after which it was homogenized in a glass tissue homogenizer. The homogenate was mixed with two volumes of diethyl ether and centrifuged. The supernatant was transferred to a flask along with two successive diethyl ether extracts of the precipitate. This was further disrupted by grinding the dry material with washed sand or by the use of a Mickel Disintegrator, which was operated for 12 min with 3 g of glass beads and 5 ml of diethyl ether. The disintegrated or ground precipitate was then extracted three times with diethyl ether, and these extracts were combined with the original extracts. The total extract was evaporated to a volume of 10 to 12 min with 3 g of glass beads and 5 ml of and evaporated to dryness. All evaporations in this study were carried out under a stream of nitrogen.

Methyl esters of the fatty acids were prepared by treating the extract with diazomethane, according to the method of Schlenk and Gellerman (17), then transesterifying by refluxing the sample in 0.02 N sodium methoxide for 1 hr as described by Tulloch and Ledingham (2). The methyl esters of the common fatty acids, such as palmitic, stearic, oleic, linoleic, and linolenic, were separated from the ester of *cis*-9,10-epoxyoctadecanoic acid by thin-layer chromatography (TLC). The esters were recovered from the adsorbent by three extractions with ether, and aliquots of the extracted esters were assayed for radioactivity. The remainder were used for degradation or specific activity measurements.

Gas-Liquid Chromatography

Gas-liquid chromatography (GLC) was utilized for the separation and determination of specific activity of the individual fatty acid esters. A 12-ft stainless steel column, 0.25 in. in diameter, was packed with 14.3% diethylene glycol succinate on 90/100 mesh Anakrom AB and operated at 185C with a 90-ml per minute flow-rate of the Argon carrier gas. The Jarrel-Ash Model 700 chromatograph, equipped with an 80:1 effluent splitter and an Argon ionization detector, allowed the trapping of the split effluent vapors at the heated collection port. A temperature of 210C was maintained for the splitter, collection port, and detector; the column injection port was operated at 240C. Specific activity measurements were made by trapping the vapors in 10-mm × 15-mm Pyrex tubes, which were packed loosely with de-fatted cotton, wetted with methanol. With the splitter ratio under consideration, the quantity of ester trapped was determined from the integrated area of the recorder tracing, and the radioactivity was assayed by eluting the trapped material directly into liquid scintillation counting vials with 15 ml of the fluor solution, followed by counting.

The methyl ester of 9,10-epoxyoctadecanoic acid was degraded by a procedure similar to that reported by Tallent et al. (18), in which the methyl ester was first converted to the analogous 9,10-dihydroxy acid by refluxing in acetic acid, saponification, and acidification. The dihydroxy acid was then cleaved with Lemieux's reagent (19); the resulting pelargonic and azelaic acids were extracted and separated by TLC. The separated acids were then decarboxylated by the method of Goldfine and Bloch (20).

Thin-Layer Chromatography

Three TLC systems were used in these studies. A 250μ layer of Silica Gel G was applied to 20-cm × 20-cm plates as a slurry consisting of 2 ml of water for each gram of adsorbent. The plates were air-dried at room temperature, then activated by heating for 1 hr at 100C. The development of plates, in a solvent of ligroin (bp 66-75C, Eastman) and diethylether (70:30), separated carotenes and hydrocarbons (R_f 0.74), the methyl esters of the common fatty acids (R_f 0.62), and the methyl ester of 9,10-epoxyoctadecanoic acid (R_f 0.48).

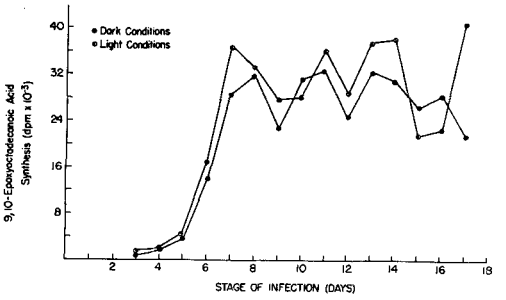

FIG. 1. Incorporation of 1-^{14}C-acetate into *cis*-9,10-epoxyoctadecanoic acid by tissue slices of rust-infected wheat plants.

The chlorophylls and more polar lipids remained at the origin. Dicarboxylic and monocarboxylic acids were separated on plates, prepared in the same manner, with a developing solvent consisting of diethyl ether-heptane-acetic acid (70:30:1). Saturated and unsaturated fatty acid methyl esters were separated on silver nitrate-impregnated TLC plates (21) by using a solvent of heptane-diethyl ether (90:10). To determine the location of the fatty acids or their methyl esters, the thin-layer plates were sprayed lightly with a 2% solution of 2',7'-dichlorofluorescein in 95% ethanol and then observed under an ultraviolet lamp.

Radioactivity Measurements

A Packard Tri-Carb Model 314 EX-2 liquid scintillation spectrometer was used for all counting procedures. The scintillation solution consisted of 15 g of PPO, 150 mg of dimethyl POPOP (Packard Instrument Company), 240 g of reagent grade naphthalene (Baker Chemical Company), 800 ml of reagent grade xylene (Mallinckrodt), and 1,000 ml of spectro-quality *p*-dioxane (Matheson Scientific). The channels ratio method of determining counting efficiency was used for converting cpm to dpm.

Radioactive Materials

All radioactive substrates were obtained from the New England Nuclear Corporation. The purity of the sodium 1-^{14}C-acetate (specific activity of 49.4 μc per μmole) was checked by specific activity determinations of the sodium acetate, isolated from two recrystallizations of a carrier-diluted sample. Decarboxylation of the acetate indicated approximately 95% of the radioactivity in the carboxyl group. The 1-^{14}C-oleic acid (specific activity 8.7 μc/μmole) and 1-^{14}C-stearic acid (specific activity 9.5 μc/μmole) were found to be free of other acids by thin-layer and vapor-phase chromatography of their methyl esters. Decarboxylation of these two acids indicated that the ^{14}C-activity was present in the C-1 position.

Unlabeled fatty acids were purchased from Sigma Chemical Company with the exception of *cis*-9,10-epoxyoctadecanoic acid, which was prepared by epoxidation of oleic acid with peracetic acid (22). The melting point, 59-59.5C, agrees with values in the literature (22,23).

RESULTS

The incorporation of 1-^{14}C-acetate into *cis*-9,10-epoxyoctadecanoic acid was observed five to six days after inoculation of the wheat plants with red stem rust fungus (Fig. 1). After seven days the rate of formation appeared to be at a maximum, and this rate was generally maintained until the plant was killed by the infection. The percentage of acetate in the medium which was incorporated into the epoxy acid in the dark incubation was similar to, although on the average a little lower than, the incubations in the light. The plateau values shown in Fig. 1 represent approximately 0.5% incorporation; however this varied, depending on the severity of the infection of different groups of plants. Incubations with healthy plant tissue or tissue from plants which had been inoculated less than four days prior to the incubation did not indicate any synthesis of the epoxy acid. More than 90% of the labeled epoxy acid which was formed was esterified when extracted.

The synthesis of the other fatty acids also was found to increase five to six days after inoculation of the plants, and this stimulation persisted throughout the life of the plants. For incubations carried out in the light, with tissue from plants inoculated seven to 17 days prior to the incubation, the combined percentages of acetate incorporation into all other fatty acids gave an average value of 10.2% for the 10 incubation trials. The average value for the tissue of noninfected plants of the same ages was 2.6%. Analogous values for the dark incubations were 7.0% and 0.4% for the diseased and healthy tissue respectively. The stage of the infection, where epoxy acid synthesis occurred and common fatty acid synthesis was stimulated, coincided with the uredospore production stage of the infected plant.

The possibility that the 18-carbon chain of 9,10-epoxyoctadecanoic acid is formed by a pathway different from the well-known one that involves the condensations of acetate units

TABLE I

^{14}C-Analysis of *cis*-9,10-Epoxyoctadecanoic Acid from Sodium 1-^{14}C-Acetate Incubations

Experiment	Percentage of ^{14}C-Activity in the Carboxyl Groups of Acids Formed by the Degradation of *cis*-9,10-Epoxyoctadecanoic Acid	
	Pelargonic acid	Azelaic acid
1	1.7	36.7
2	5.9	31.9
3	2.8	33.1
Theoretical	0.0	40.0

was excluded by degradation of the labeled epoxy acid, isolated from incubations in which 1-^{14}C-acetate was used as a substrate. With this substrate, an 18-carbon acid should be produced in which the odd-numbered carbon atoms are labeled. The oxidative cleavage of this acid at the 9-position should yield pelargonic acid, which would not contain any radioactivity in the carboxyl group, and azelaic acid, in which both carboxyl groups would be labeled. The analysis of ^{14}C-activity present in the carboxyl carbon atoms of pelargonic and azelaic acid is presented in Table I. As may be seen, some randomization of the ^{14}C-label does occur. However in complete randomization one would expect 11% of the ^{14}C-activity in the carboxyl group of pelargonic acid and only 22% in the carboxyl groups of azelaic. The 4-hr incubation period would probably allow some randomization to occur.

Oxygen was required in the formation of this acid from acetate (Table II). The type of gas used to flush the incubation vessels did not appear to be important so long as the incubations were carried out in the light. However under dark conditions there was a marked reduction in the synthesis of the epoxy acid when a nitrogen atmosphere was used as compared with an oxygen or air atmosphere. Apparently under light, enough oxygen was liberated by the photosynthetic tissue to allow synthesis to occur at a maximal rate. Also there was a reduction in the amount of unsaturated fatty acids which were formed under

TABLE II

Oxygen Requirements for *cis*-9,10-Epoxyoctadecanoic Acid Synthesis from 1-^{14}C-Acetate

Atmosphere	*cis*-9,10-Epoxyoctadecanoic Acid Synthesis (dpm)	
	Light conditions	Dark conditions
Nitrogen	9,840	948
Air	10,254	10,899
Oxygen	10,793	9,738

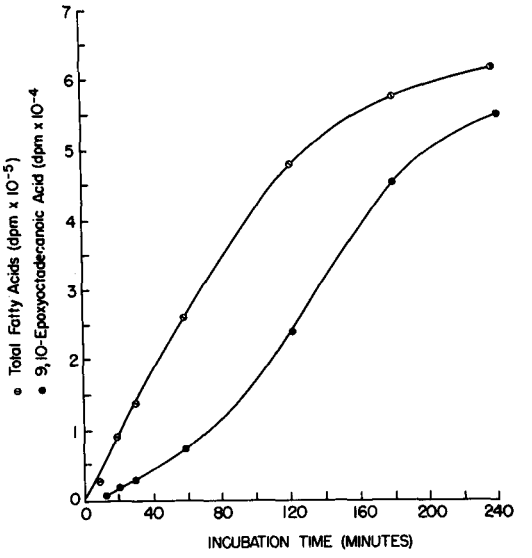

FIG. 2. The 1-^{14}C-acetate incorporation into total fatty acids and into *cis*-9,10-epoxyoctadecanoic acid by tissue slices of rust-infected wheat plants for various incubation periods.

the nitrogen atmosphere when the incubations were carried out in the dark.

Fig. 2 shows the synthesis of the common fatty acids and the epoxy acid as a function of incubation time. Epoxy acid synthesis appeared to lag behind the synthesis of the other acids, indicating that one of the common acids may serve as a precursor for the epoxy acid. The specific activities of the individual acids are shown in Fig. 3. Stearic acid possessed the highest specific activity followed by

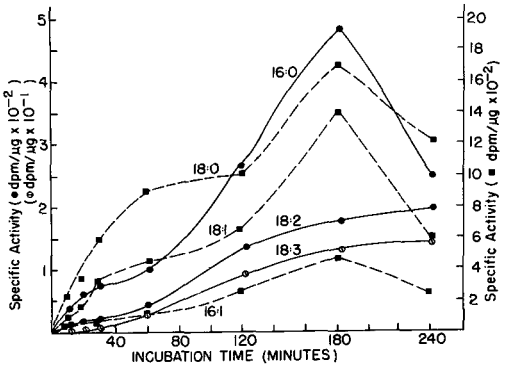

FIG. 3. The specific activity of individual fatty acids isolated from 1-^{14}C-acetate incubations with tissue slices of rust-infected wheat plants. Palmitic = 16:0, palmitoleic = 16:1, stearic = 18:0, oleic = 18:1, linoleic = 18:2, linolenic = 18:3.

oleic acid. Both acids exhibited maximum specific activities at 3 hr. Palmitic acid, which had a lower specific activity than stearic acid, and oleic acid exhibited the same type of curve as did palmitoleic acid. However the specific activity of linoleic, linolenic, and the epoxy acid increased throughout the 4-hr period. The specific activity of the epoxy acid is not shown. However, the curve was identical with the curve for the synthesis of the acid. Little could be gained by a comparison of the specific activities of the epoxy acid with possible precursors because of the pool of fatty acids present in the tissue.

The fatty acid composition of the infected plant tissue, which did not appear to change during the incubation period, is given in Table III along with the common fatty acid compositions of the normal wheat plant and the uredospore.

The high specific activities of stearic and oleic acids in the acetate incubations plus the indication that the epoxy acid carbon chain was formed by the condensation of acetate units led to incubations which used 1-^{14}C-stearic and 1-^{14}C-oleic acids as substrates. Neither of these acids are readily absorbed by the tissue slices. After 4-hr incubations a large portion of the substrate could be recovered from the medium. The percentage of labeled oleic acid found with the tissue after the incubation period was 16.2, 13.4, and 11.7% in three separate experiments. Stearic acid appeared to be taken up at a slower rate as only 11.0, 6.7, and 6.3% of the ^{14}C-acid was detected in the leaf slices. Approximately 84% of the oleic acid and 81% of the stearic acid extracted from the tissue was esterified, indicating that these acids were metabolized, not simply adsorbed in the cuticle of the leaf slices.

Oleic acid was converted to the epoxy acid more readily than stearic acid (Table IV). The ratio of the epoxy acid, which was formed, to the fatty acid, recovered from the tissue, was used to correct for the unequal absorption rates of the labeled substrates. On this basis oleic acid was converted to the epoxy acid twice as rapidly as stearic acid. Without the above corrections the differences were greater. At least 95% of the labeled epoxy acid from either the stearic or oleic acid was isolated in the esterified form. Analysis of the other fatty acids revealed essentially no interconversions except possibly some desaturation of stearic acid.

The epoxy acid isolated from the stearic and oleic acid incubations was degraded as in the acetate incubations. There was essentially no radioactivity in the pelargonic acid isolated from the epoxy acid with either the stearic or oleic acid incubations. In the 1-^{14}C-stearic acid incubations 99.8% and in the 1-^{14}C-oleic acid incubations 96.3% of the radioactivity associated with the azelaic acid was obtained as CO_2 when the dibasic acid samples were decarboxylated. These data show that the synthesis of the epoxy acid resulted from a direct conversion of the substrates rather than β-oxidation of the substrates, followed by resynthesis of the carbon chain.

TABLE III

Fatty Acid Compositions of Normal Wheat Plants, Infected Plants, and Rust Uredospores

Fatty acid	Fatty Acid Composition (Percentage)		
	Normal wheat	Infected wheat	Uredospore
<16	9.6	2.9	2.7
16:0	20.5	17.4	40.8
16:1	7.8	2.6	0.5
18:0	3.6	3.1	4.8
18:1	14.8	10.3	16.7
18:2	20.0	11.7	4.4
18:3	23.3	52.0	10.4
Epoxy	0.0	<0.5	19.7

DISCUSSION

In these experiments both plant and fungal tissues were present in the incubation mixtures. However the formation of the *cis*-9,10-epoxyoctadecanoic acid occurs only in the infected wheat plant. It has been shown conclusively that light stimulates the synthesis of the common fatty acids in photosynthetic tissue and that the enzymes of this system appear to be closely coupled to photophosphorylation (24-26). In the infected wheat plant, common fatty acid synthesis was stimulated by light. However the increase in synthesis owing to the light was comparable with the effect of light on the common fatty acid synthesis of the noninfected plant, thus indicating that the

TABLE IV

Synthesis of *cis*-9,10-Epoxyoctadecanoic Acid from 1-^{14}C-Stearic and 1-^{14}C-Oleic Acids

Experiment[a]	Epoxy Acid Synthesized/Substrate Metabolized (dpm/dpm)	
	Oleic acid	Stearic acid
1	0.192	0.067
2	0.129	0.016
3A	0.061	0.022
3B	0.056	0.023

[a]Different groups of infected plants were used in the experiments of different numbers.

stimulation of common fatty acid synthesis by light was caused by a stimulation of the synthesis in the photosynthetic tissue. The synthesis of the epoxy acid appears to be only slightly stimulated by light. There is a marked increase in common fatty acid synthesis in the infected plant as compared with the noninfected plant. From this evidence it appears that the epoxy acid and a large portion of the common fatty acids are synthesized by fungal enzymes rather than plant enzymes. The effect of light on fatty acid synthesis may be a means of distinguishing between the two types of tissue.

Incubations which utilize acetate as a substrate indicate that the 18-carbon chain of the epoxy acid is formed in the usual manner by the condensation of acetate units. Whether derivatives of malonic or acetic acid are involved cannot be stated from these results.

The observation that stearic and oleic acid are incorporated directly into the epoxy acid indicates that the saturated acid may first be formed, then desaturated. Finally the epoxy group is introduced, as contrasted to an alternative introduction of a functional group at one of the earlier stages of chain elongation. These experiments do not rule out this alternative but do show that the 18-carbon chain can be an active substrate. Enzymatic desaturation of stearate or palmitate has been observed in micro-organisms (27,28), rat liver (29), *Euglena gracilis,* and spinach (26) when suitable forms of the substrates were provided.

In the incubation involving 1-^{14}C-stearic acid little labeled oleic acid was isolated. However it is conceivable that the desaturase and epoxidase activities are located in the same complex hence free oleic is not released; rather it is converted directly to the epoxy acid. Oxygen was shown to be required for the synthesis of 9,10-epoxyoctadecanoic acid from acetate, and it seems quite likely to be involved in several reaction steps. The desaturation of fatty acids requires molecular oxygen in most aerobic organisms (26,29,30), and the atmosphere may be the source of the oxirane oxygen although it could be derived from water. Further study of the role of oxygen in the formation of epoxy acids is definitely needed.

An enzyme catalyzing the hydration of *cis*-9,10-epoxyoctadecanoic acid to form *threo*-9,10-dihydroxyoctadecanoic acid has been isolated from germinating flax rust uredospores (31). Similar enzymatic activities have been observed in wheat rust uredospores (32) and in oil-bearing seeds which contain epoxy-fatty acids (33,34). In these cases the *threo* configuration of the dihydroxy acids was also formed from *cis*-epoxide groups.

It is conceivable that the epoxy acid could be formed by the reverse reaction, i.e., the dehydration of the dihydroxy acid. This suggestion was made by Miwa et al. (9) when they observed that, during the intermediate stage of maturation, *threo*-12,13-dihydroxy-*cis*-9-octadecenoic acid was a major oil component in developing seeds of *Vernonia anthelmintica.* In the mature seed *cis*-12,13-epoxy-*cis*-9-octadecenoic acid accounted for about 68% of the total fatty acids whereas the dihydroxy acid was conspicuously absent. Although hydrolytic reactions frequently favor the hydrated product, the postulate made by Miwa et al. cannot be excluded. A better understanding of this question could be gained by an investigation of the reaction equilibrium with a purified enzyme preparation.

It is interesting to note that Niehaus and Schroepfer have isolated a soluble enzyme from a strain of *Pseudomonas* which catalyzes the sterospecific hydration of oleic acid, yielding 10D-hydroxyoctadecanoic acid (35). This reaction is similar to the over-all reaction observed in the hydration of olefinic linkages in steroids in which an epoxy-steroid is an intermediate. The same enzyme, studied by Niehaus and Schroepfer, also has been shown to catalyze the sterospecific hydration of both the *cis* and *trans* forms of 9,10-epoxyoctadecanoic acid to yield *threo*- and *erythro*-9,10,dihydroxyoctadecanoic acid respectively (36). Perhaps this reaction and another preceding reaction, which converts oleic acid to the epoxy acid, represent intermediate reactions in the formation of 10-hydroxyoctadecanoic acid from oleic acid.

From the evidence it appears that oleic acid or its derivative is an immediate precursor to the *cis*-9,10-epoxyoctadecanoic acid. Similar reactions in steroids, observed in both fungal and mammalian preparations, as well as the position of epoxy groups in other natural epoxy fatty acids support this conclusion.

Attempts to obtain cell-free preparations which were capable of synthesizing *cis*-9,10-epoxyoctadecanoic acid from oleic or acetic acid have not been successful. However, in view of the requirement of acyl derivatives of coenzyme A or acyl carrier protein as substrates in some enzyme preparations that catalyze elongation or desaturation reactions, this is not surprising (26,37). Work is now in progress to obtain cell-free preparations which are active with these acyl derivatives.

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Triglyceride Analysis by Consecutive Liquid-Liquid Partition and Gas-Liquid Chromatography. *Ephedra nevadensis* Seed Fat[1]

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ABSTRACT

The triglyceride composition of *Ephedra nevadensis* seed fat, which contains 16 different fatty acids, has been analyzed by a combination of liquid-liquid partition and gas-liquid chromatography. Triglycerides were first separated by liquid-liquid partition chromatography. The recovered fractions were then analyzed by gas-liquid chromatography to determine the molecular weights of the triglycerides present. Consecutive separation by these two techniques resolved this complex seed fat into 30 different triglyceride groups.

A method for preparative liquid-liquid partition chromatography of triglycerides is described in detail. Highly unsaturated triglyceride mixtures are easily resolved on the basis of "partition number" by using a hexadecane/nitroethane partition system.

INTRODUCTION

THE TRIGLYCERIDE COMPOSITION of natural fats is so complex that no one analytical technique can definite all the components present. Therefore one must employ a consecutive series of separation and characterization techniques to define the triglyceride composition of a fat.

Silver ion adsorption thin-layer chromatography (TLC) has proven to be a rapid and efficient method for the preparative separation of natural fat triglycerides on the basis of unsaturation (1-8). The fractions separated can then be further analyzed by pancreatic lipase hydrolysis (1,6), gas-liquid chromatography (GLC) (2,8), or liquid-liquid partition chromatography (LLC) (5). Silver ion TLC works well with fats containing only saturated, monoene, and diene fatty acids (4-8). However when a triene acid is present, as in linseed oil, only partial resolution of triglyceride bands is possible (3). A useful separation of distinct triglyceride groups cannot be obtained with fats containing four, five, and six double-bond fatty acids. When menhaden or *Ephedra nevadensis* triglycerides are chromatographed on a silver ion TLC plate, for example, one obtains only a streak of varying intensity along the entire length of the plate. Therefore some alternative method must be sought for the preparative separation of highly unsaturated triglycerides prior to further analysis by GLC or lipase hydrolysis.

Vereshchagin and Novitskaya (9,10), Kaufmann et al. (11), and Hirsch (12, 13) have demonstrated that the triglycerides of linseed oil can easily be separated into distinct fractions by using LLC. This indicates that LLC can fractionate highly unsaturated triglycerides better than silver ion adsorption chromatography.

The present work was undertaken to demonstrate how a combination of LLC and GLC can be used to characterize the complex triglyceride composition of *Ephedra nevadensis* seed fat, which contains 17.6% triene and 10.8% tetraene fatty acids. Triglycerides were first separated according to "partition number" by LLC. The recovered fractions were then analyzed by GLC to determine the molecular weights of the triglycerides present.

EXPERIMENTAL PROCEDURES

Materials

Trilaurin, trimyristin, tripalmitin, tristearin, triolein, trilinolein, trilinolenin, and triarachidin of 99% purity were purchased from the Hormel Institute, Austin, Minn., and the Applied Science Laboratories, State College, Pa. *Ephedra nevadensis* seeds were purchased from Harry E. Saier, Dimondale, Mich. All solvents were redistilled prior to use.

Practical grade hexadecane (Matheson, Coleman, and Bell, Norwood, Ohio) was purified to remove all saponifiable impurities. This was necessary to avoid the appearance of extraneous peaks during GLC of the methyl esters from the recovered LLC fractions. Two grams of KOH were dissolved in 4 ml of water; 60 ml of methyl cellosolve, 125 ml of benzene, and 50 ml of hexadecane were added in that order. After refluxing for 2 hr, the product was washed with water and dried over Na_2SO_4; the benzene was evaporated. The residue was taken up in 150 ml of petroleum ether and

[1]Presented at the AOCS Meeting, Chicago, October, 1967.

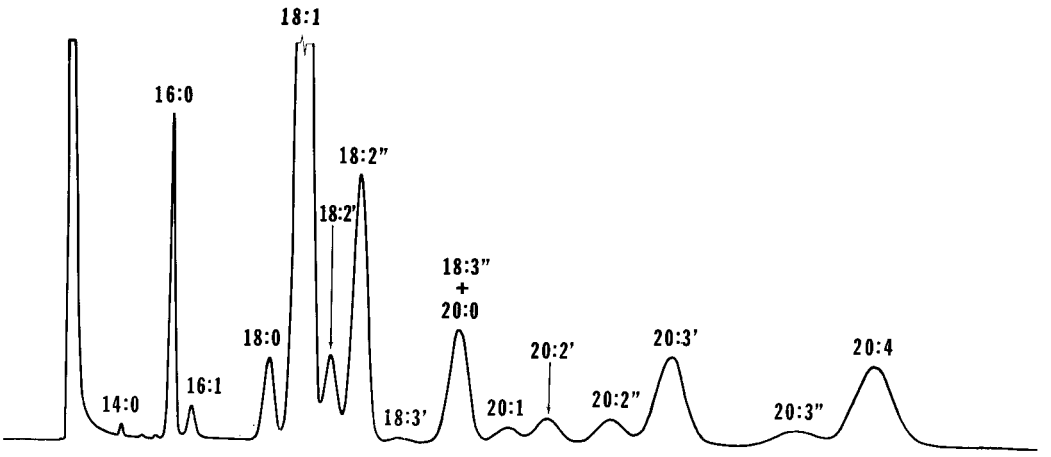

FIG. 1. Gas chromatogram of methyl esters from *Ephedra nevadensis* seed fat triglycerides. Operating conditions: 1.82-m × 2.4-mm I.D. column, packed with 20% diethylene glycol succinate polyester on 60/80 mesh Chromosorb W; column temperature 175C; 30 ml/min N carrier gas; hydrogen flame detector.

passed through a column of 10 g of activated Florisil, followed by 200 ml of petroleum ether. Evaporating the petroleum ether from the eluate yielded a colorless hexadecane free from saponifiable impurities.

Preparation of Triglycerides

The hard shell and testa were removed from each of the *E. nevadensis* seeds. The remaining endosperms were ground in a Waring Blendor, weighed into a tared paper thimble, and extracted with petroleum ether (30-60C) in a Soxhlet apparatus for 4 hr. The oil content of the endosperm was 27.0% on a wet basis. The triglycerides in *E. nevadensis* seed fat were isolated by preparative TLC on 1.0-mm layers of silicic acid, impregnated with Rhodamine 6G. Plates were developed with a mixture of petroleum ether-diethyl ether-acetic acid (79:20:1). 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 chromatoplate indicated that the triglycerides constituted more than 96% of the total fat.

The *E. nevadensis* triglycerides were converted to methyl esters by heating for 60 min at 100C with 5% HCl/CH_3OH in a sealed ampule (14). GLC of the methyl esters showed a series of unusual peaks eluting after 18:3 (Fig. 1). All peaks were identified by the procedure of Privett, Blank, and Romanus (15). The methyl esters were separated into saturated, monoene, diene, triene, and tetraene bands on TLC plates of silicic acid, impregnated with $AgNO_3$. Each band was scraped off the plate, and the methyl esters were recovered. GLC on a 0.53-meter, 3% JXR silicone column identified the fatty acid chain-lengths present in each fraction. GLC on a polyester column identified the number of double bonds for each peak in the original chromatogram. Final peak identifications are given on the chromatogram in Fig. 1, and the fatty acid composition of the total triglycerides is listed in Table II. The exact location of the double bonds in each acid was not determined, but *E. nevadensis* fatty acids apparently resemble those identified in *Ephedra campylopoda* seed oil by Kleiman et al. (21).

LLC of Triglycerides

Triglyceride mixtures were separated by liquid-liquid partition chromatography on TLC plates by using a hexadecane-nitroethane partition system.

TLC plates, impregnated with 8% hexadecane, were prepared by dissolving 4.0 g of purified hexadecane in 85 ml of petroleum ether (30-60C) and adding these to 50 g of silanized silicic acid (Reversil-3, Applied Science Laboratories). After it was shaken vigorously in a stoppered flask, the slurry was poured into a conventional TLC spreader and immediately spread in a 0.25-mm thick layer on 20 × 40-cm glass TLC plates (16). The plates were left overnight in a well-ventilated atmosphere to insure evaporation of all residual petroleum ether. If the plates were used sooner, resolution of triglyceride bands was considerably poorer.

A 4 to 10-mg triglyceride sample was applied across the base of each TLC plate by using a sample streaker (Applied Science Laboratories). The sample was applied to the extreme edge of the silicic acid on each side of the plate. To prevent the silicic acid from falling off the bottom of the TLC plate where it came in contact with the developing solvent, a 1 × 20-cm strip of ether-extracted filter paper was laid across the bottom of the plate, and a similar-size strip of glass was laid on top. Two paper clamps were used to hold this "sandwich" together.

Plates were developed once with nitroethane that had been saturated with purified hexadecane. Band resolution was quite satisfactory after only one development, provided the plates were completely free of the petroleum ether that had been used in their preparation. Ascending development was carried out in a 30 ×30 × 60-cm glass chromatography tank which was lined with nitroethane-saturated blotters and flushed with nitrogen. Development required 4-6 hr, depending on room temperature. After development, the nitroethane was evaporated under a stream of nitrogen.

Triglyceride bands were located by exposing the plate to iodine vapor (5). Fig. 2 shows the wide separation of triolein, trilinolein, and trilinolenin by using this LLC system. For preparative separations, bands were located by exposing only the sides of the plate to iodine vapor since iodine addition to unsaturated fatty acids is known to be irreversible (17). A rectangular Teflon gasket was placed over the center of the TLC plate, and a second piece of glass was placed on top. The resultant "sandwich" was held together with paper clamps while the edges of the plate were exposed to iodine vapor. The center of each triglyceride band, which had not come in contact with the iodine, was scraped off the plate; and the silicic acid was placed in a small chromatography column. The triglycerides (plus hexadecane) were recovered by eluting with 100 ml of diethyl ether. An internal standard of trilaurin was added to the eluate so that the amount of triglyceride in each band could be quantitated during subsequent GLC analysis.

A blank LLC plate was developed in the same manner, and the lipids from a 3 × 10-cm area in the center of the plate were recovered in the usual way. GLC of the recovered material under triglyceride conditions revealed no peaks other than hexadecane. When the recovered material was converted to methyl esters and analyzed on a polyester column, no contaminants were found.

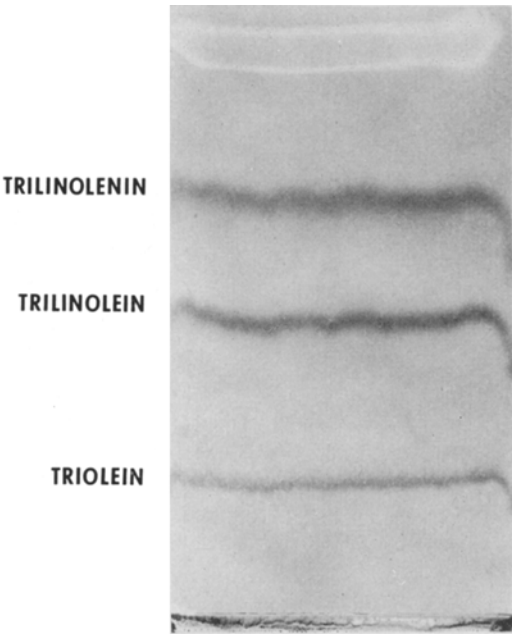

FIG. 2. TLC separation of triolein, trilinolein, and trilinolenin by LLC. Experimental conditions: hexadecane stationary phase supported on silanized silicic acid; one development with nitroethane (saturated with hexadecane) mobile phase; bands located by exposure to iodine vapor.

Partition Number

The separation of triglycerides by LLC has been studied by a number of workers (13, 18, 19) and found to depend on both the molecular weight (carbon number, the number of carbon atoms in the fatty acid moiety of a triglyceride) and the number of double bonds in the triglyceride molecules. This relationship can be defined in terms of a "partition number":[2]

$$\text{Partition number} = \text{Carbon number} - 2\left[\text{Number of double bonds}\right]$$

Molecules of the same partition number travel together in LLC. The triglycerides of a hypo-

[2]Many names have been used to designate the groups of triglycerides which migrate together in LLC. Kaufmann et al. (18) adopted "pc-wertzahl" or "paper-chromatography value." McCarthy and Kuksis (20) used "polarity number"; Hirsch (13) employed "double-bond equivalence." Vereshchagin (19) defined a "K value." The term "partition number" is used in this laboratory. Of all these names "partition number" seems best to describe the process by which the triglycerides are actually separated; and this term has been adopted for the present paper.

TABLE I

Separations Possible by Using Consecutive Liquid-Liquid Partition and Gas-Liquid Chromatography. Hypothetical Fat Containing Only Palmitic, Stearic, Oleic, and Linoleic Acids[a]

Partition number							Carbon number
42	44	46	48	50	52	54	
LLL	OLL	OOL	OOO	SOO	SSO	SSS	54
		SLL	SOL	SSL			
	PLL	POL	PSL	PSO	PSS		52
			POO				
		PPL	PPO	PPS			50
			PPP				48

[a]P = palmitic, S = stearic, O = oleic, L = linoleic, SOO = stearodiolein, OPS = oleopalmitostearin, etc. Triglyceride positional isomers are not distinguished.

thetical fat containing only 16:0, 18:0, 18:1, and 18:2 are grouped according to their partition numbers and carbon numbers in Table I. It is evident that consecutive LLC and GLC could define the triglyceride composition of such a fat in considerable detail.

The partition number of a fatty acid is one-third of the partition number of its corresponding mono-acid triglyceride; oleic acid and triolein have partition numbers of 16 and 48 respectively. To verify that each LLC band contained only triglycerides of a single partition number, an "average fatty acid partition number" was computed from the fatty acid composition of each recovered fraction. Comparison of the experimental average fatty acid partition-number with the theoretical value provided a valuable check on the identity of each LLC fraction.

Preparation of Methyl Esters

Triglyceride fractions, recovered from preparative LLC, contained a large amount of hexadecane, which was removed during methyl ester preparation. The recovered fraction was placed in a stopcock ampule (23), and 0.5 ml of 2% KOH in CH_3OH, 1.0 ml of benzene, and 0.2 ml of methyl cellosolve were added in that order. The ampule was flushed with nitrogen, and the stopcock was closed. After it was shaken, it was placed in a 100C metal block for 30 min, then cooled. The ampule was opened, and 0.8 ml saturated NaCl solution was added. After gentle shaking, the benzene/hexadecane layer was allowed to rise to the top, where the benzene was evaporated under a stream of nitrogen. The remaining hexadecane layer was removed with a capillary pipette and discarded. One extraction with 0.3 ml of petroleum ether reduced hexadecane content to a low level so that it would not interfere with methyl ester GLC. The remaining soap solution was acidulated with four drops of 20% H_2SO_4 and shaken (a K_2SO_4 precipitate often formed). The fatty acids were extracted four times with 0.3 ml of petroleum ether, the extracts were combined in a second stopcock ampule, and the solvent was evaporated. One milliliter of BF_3/CH_3OH solution (24) was added to the fatty acids, and the ampule was closed after flushing with nitrogen. The reaction mixture was heated 4 min in a 100C metal block and cooled. After 1.0 ml of water was added, the methyl esters were extracted four times with 0.3 ml of petroleum ether, then concentrated by evaporation in the sample concentrator described by Archibald and Skipski (23).

Fatty acid compositions were determined by GLC analysis of methyl esters on a 1.82-m and 2.4-mm I.D. column containing 20% diethylene glycol succinate polyester, coated on 60/80 mesh Chromosorb W (7). Peaks were identified by comparison with the elution times of known compounds and by the method of Privett et al. (15).

GLC of Triglycerides

Triglyceride samples were hydrogenated prior to GLC analysis to improve peak resolution and to avoid any thermal decomposition of the highly unsaturated triglycerides. The hydrogenation procedure of Farquhar et al. (25) was employed using freshly distilled dioxane as a solvent in place of ethanol.

GLC analysis of triglycerides was carried out under the conditions described by Litchfield et al. (26). An F&M 400 gas chromatograph, equipped with a hydrogen flame detector and an automatic temperature programmer, was used. The 0.53-m × 2.5-mm I.D. glass column containing 3.0% JXR silicone on 100/120 mesh Gas Chrom Q was heated from 200 to 340C at 4.0C/min with a helium carrier gas flow rate of 100 ml/min. Sample peaks were identified as to carbon number by comparison with the retention times and elution temperatures of known compounds.

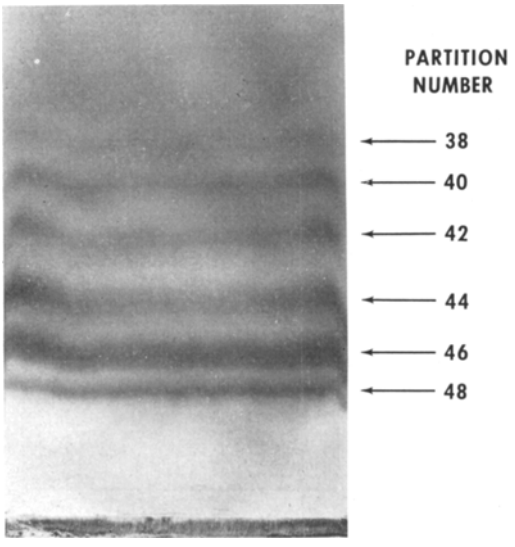

FIG. 3. TLC separation of 10 mg of *Ephedra nevadensis* seed fat triglycerides by LLC. Experimental conditions are the same as for Fig. 2.

Quantitative calibration factors were determined by using a known composition mixture of trilaurin, trimyristin, tripalmitin, tristearin, and triarachidin; all peak areas were corrected accordingly (26). Peak areas were determined by triangulation, and all triglyceride compositions are reported in mole percentage.

RESULTS

Fifty milligrams of *E. nevadensis* triglyceride was applied to five LLC plates, which were

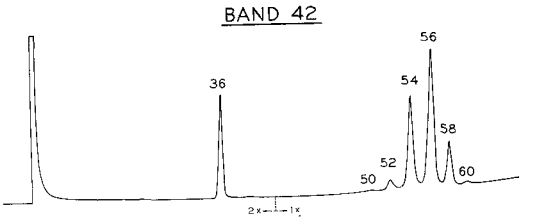

FIG. 4. Gas chromatogram of hydrogenated partition number 42 triglycerides, recovered from preparative LLC of *Ephedra nevadensis* triglycerides. The hexadecane stationary phase elutes in the wide solvent peak; peak 36 is the trilaurin internal standard; and peaks 50 through 60 show the carbon number distribution of Band 42 triglycerides. Operating conditions: F&M 400 gas chromatograph; 0.53-meter × 2.5-mm I.D. glass column, packed with 3.0% JXR silicone on 100/120 mesh Gas Chrom Q; column programmed 200→340C at 4.0°/min; 100 ml/min He carrier gas; flash heater 350C.

then developed in nitroethane. One plate was fully exposed to iodine vapor and photographed to show the clear separation of six triglyceride bands (Fig. 3). The triglyceride bands on the other four plates were located and recovered in the manner previously described. An aliquot of trilaurin solution was added to each fraction as an internal standard.

Part of each recovered fraction was used to prepare methyl esters, which were analyzed by GLC to give the results shown in Table II. The theoretical and experimental average fatty acid partition numbers were calculated for each fraction and are recorded in Table III. The two values showed close agreement in all

TABLE II

Fatty Acid Composition of *Ephedra nevadensis* Triglyceride Fractions Separated by Liquid-Liquid Partition Chromatography

Fatty acid	Total triglycerides	Partition Number					
		38	40	42	44	46	48
				mole %			
14:0	0.2	1.7	1.1	0.9	0.3	0.2	0.2
16:0	6.2	4.7	3.2	5.2	5.6	8.7	9.4
16:1	0.8	3.2	0.4	3.0	1.0	1.2	0.5
18:0	2.8	1.7	1.2	1.1	1.3	2.8	5.3
18:1	40.0	15.0	13.5	19.3	40.1	52.1	65.1
18:2′	3.8	2.7	2.9	5.6	3.4	7.0	2.3
18:2″	12.0	16.8	16.9	20.0	14.2	11.1	3.6
18:3′	0.1	0.5	0.6	—	0.1	—	—
18:3″	7.1	24.0	28.0	18.1	6.6	1.3	1.2
20:0	0.5						
20:1	1.1	0.3	0.6	1.1	0.2	0.5	1.7
20:2′	2.0	1.0	1.0	1.2	1.4	1.2	4.1
20:2″	2.2	0.8	0.6	1.1	1.5	1.7	3.0
20:3′	8.5	4.7	5.3	7.3	8.2	9.5	2.8
20:3″	1.9	1.4	1.0	1.5	1.0	0.8	0.4
20:4	10.8	21.5	23.7	14.6	15.1	1.9	0.4

TABLE III

Average Partition Number of Fatty Acids in *Ephedra nevadensis* Triglyceride Fractions Separated by Liquid-Liquid Partition Chromatography

Triglyceride band	Average Fatty Acid Partition Number	
	Theoretical	Experimental
38	12.67	13.59
40	13.33	13.39
42	14.00	13.97
44	14.67	14.60
46	15.33	15.34
48	16.00	15.98

cases except for Band 38, the smallest of the recovered fractions. The other part of each fraction was hydrogenated and analyzed for triglyceride composition by GLC. A typical chromatogram (Band 42) is shown in Fig. 4. The hexadecane liquid phase from LLC eluted in the wide solvent peak; the trilaurin internal standard gave a carbon number 36 peak; and peaks 50 through 60 showed the carbon number distribution of Band 42 triglycerides. The amount of triglyceride in each of the six LLC fractions was calculated from the trilaurin internal standard peak in each chromatogram (14).

Consecutive LLC and GLC resolved *E. nevadensis* triglycerides into 30 different groups. Quantitative results are listed according to carbon number, number of double bonds (calculated by using formula in Methods section), and partition number in Table IV.

DISCUSSION

Liquid-Liquid Partition Chromatography

LLC is effective for the preparative separation of highly unsaturated triglycerides. Natural fats containing triene (linseed) and tetraene (*Ephedra nevadensis*) acids can be easily separated into discrete triglyceride groups according to partition number. Preliminary experiments with whale oil triglycerides indicate that LLC also shows promise for the preparative separation of marine oil triglycerides containing pentaene and hexaene acids. Triglyceride partition between two immiscible solvents is apparently quite nonselective with regard to fatty acid positional isomers. The *E. nevadensis* triglycerides contain two 18:2, two 20:2, and two 20:3 isomers; but this fact does not impede triglyceride separation by partition number. Neither does the presence of saturated, monoene, diene, triene, and tetraene acids all in the same fat prevent proper separation.

Preparative LLC appears superior to silver ion TLC for the primary separation of highly unsaturated natural fat triglycerides prior to further characterization by secondary techniques. Linseed triglycerides are only partially resolved by unsaturation with the use of silver ion TLC (3), but they are fully resolved by partition number with LLC (9-13). LLC has only 10-30% of the sample capacity per plate that silver ion TLC possesses, but this is easily overcome by using more LLC plates when more sample is desired. Triglyceride and methyl ester GLC require less than 500 μg of material, which is easily obtained from a LLC separation.

Five of the six triglyceride bands separated by LLC show good agreement between their theoretical and experimental average fatty acid

TABLE IV

Component Triglycerides of *Ephedra nevadensis* Seed Fat

Carbon number	Double Bonds										
	1	2	3	4	5	6	7	8	9	10	11
50	0.6	0.3	0.1	tr	—	—	—	—	—	—	—
52	—	3.7	3.0	1.1	0.4	0.1	—	—	—	—	—
54	—	—	12.2	12.6	8.3	3.8	1.0	0.3	—	—	—
56	—	—	—	6.2	12.4	12.7	6.3	3.9	1.7	—	—
58	—	—	—	—	0.9	1.3	1.7	2.0	1.7	1.1	—
60	—	—	—	—	—	—	—	—	0.1	0.2	0.3
Partition number	48	46	44	42	40	38					

partition numbers (Table III). This indicates that errors from oxidation or contamination were not appreciable when more than 0.5 mg of material was recovered from the TLC plates. The smallest, Band 38, however, had an experimental average fatty acid partition number of 13.59 vs. the theoretical value of 12.67. Fatty acids from Band 38 were unexpectedly lower in 18:3 and 20:4 content than Band 40. Appreciable oxidation obviously occurred during separation and manipulation of Band 38, and the fatty acid and triglyceride compositions reported for this band must be somewhat in error. Band 38 contained the least amount of triglyceride (approximately 0.35 mg) of the six bands recovered. Further technique development is evidently needed before recovered samples below 0.5 mg can be handled without oxidation.

A definite band-tailing "edge effect" occurred at both the right and left sides of the LLC plate (Fig. 2 and 3). This could not be prevented by starting the sample application one centimeter away from the side edge of the silicic acid. Therefore the triglyceride sample was applied to the edge of the silicic acid on both sides of the plate so that the edge effect was as far away from the center of the plate as possible. Band tailing at the edge of the silicic acid did not prevent accurate band location since that part of the plate which was exposed to iodine vapor extended well into the area where the triglyceride bands were horizontal on both sides of the plate. This unavoidable edge effect is attributed to two causes: the sharp edge of the silicic acid and the relocation of hexadecane during application of the sample in benzene solution.

Triglyceride Composition

Thirty different groups of triglycerides have been identified and quantitated in *E. nevadensis* seed fat (Table IV). In addition, bands of partition number 36 and 50 were faintly visible when a 30-mg sample (three times normal sample size) was applied to one LLC plate.

In a complex natural fat containing 16 different fatty acids, it is obvious that consecutive LLC and GLC separations will not isolate individual species of triglycerides. Nevertheless certain triglyceride groups can be identified as to fatty acid composition. If the 14:0 content is ignored (0.2% of the fatty acids, hence participating in no more than 0.6% of the triglycerides), then the fatty acid carbon number distribution can be estimated. The C_{50} triglycerides are all $C_{16}C_{16}C_{18}$; the C_{52} molecules are either $C_{16}C_{18}C_{18}$ or $C_{16}C_{16}C_{20}$; and the C_{54} material is either $C_{18}C_{18}C_{18}$ or $C_{16}C_{18}C_{20}$. The C_{56} molecules are either $C_{18}C_{18}C_{20}$ or $C_{16}C_{20}C_{20}$; the C_{58} triglycerides are all $C_{16}C_{20}C_{20}$; and the C_{60} material is all $C_{20}C_{20}C_{20}$.

Certain of the triglyceride groups contain only a single triglyceride or a simple mixture. For example, since all triglycerides of carbon number 60 must contain three C_{20} acids, then the 0.3% C_{60} material with 11 double bonds must be all 20:4/20:4/20:3. Similarly the 0.2% C_{60} triglycerides with 10 double bonds must be a mixture of 20:4/20:3/20:3 and 20:4/20:4/20:2. The 1.1% C_{58} fraction with 10 double bonds contains only 20:4/20:4/18:2 and 20:4/20:3/18:3. And the 0.6% C_{50} material with one double bond must be a combination of 16:0/16:0/18:1 and 16:0/16:1/18:0. Similar calculations can be made for all triglyceride groups, but the remaining 26 groups are more complex mixtures than the four discussed.

The triglyceride data in Table IV cannot be directly compared with any of the numerous fatty acid distribution hypotheses except a 1,2,3-random pattern[3] (22). It is doubtful if any nautral fat follows this distribution pattern exactly, and *Ephedra nevadensis* fat is no exception. A 1,2,3-random distribution of *E. nevadensis* fatty acids would require 14.4% C_{58} triglycerides, but only 8.7% C_{58} material was found.

ACKNOWLEDGMENTS

Advice from Raymond Reiser, H. Wessels, and N. Pelick was greatly appreciated. This investigation was supported in part by grants from the National Institutes of Health (AM-06011) and the Corn Products Institute of Nutrition.

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[3]The confusing nomenclature of fatty acid distribution hypotheses with "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.

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Quantitative Gas Chromatography, Using Retention Times

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ABSTRACT

Diffusion of an injected sample within a gas chromatographic column does not begin from a point source but from a band. Therefore the method of calculating relative areas by using retention time × peak height may require a correction factor to give a more accurate estimate of peak areas. When this correction was applied, the analysis was comparable with that obtained by the more time-consuming triangulation method.

INTRODUCTION

THE RELATIVE PEAK areas obtained in gas chromatography have often been quantitatively determined by triangulation. One modification of the simple one-half (base × peak height) calculation uses the width at half-height × peak height to give values that are less influenced by the difficulty of overlapping at the base of the peaks. Another method of quantitatively determining the relative areas uses the product of retention time × peak height as described by Carroll (1). This method was suggested by Pecksok (2) and used also by others (3,4). In the authors' opinion, this method has advantages over both the triangulation method and the seldom used paper-weighing method.

We have routinely used retention times × peak height in calculating the approximate sample compositions by gas chromatography. As we developed a system for optimum speed and efficiency in analyzing methyl esters of long-chain fatty acids, this method led to low, calculated recoveries of the shorter derivatives. The alternate method of calculation, by triangulation, gave peak areas proportional to the component mass and indicated that the detector response was not appreciably different for the different esters. Thus there was something inadequate in the method that was used for estimating compositions with the aid of retention times. In order to improve the estimated areas and component mass values by the simpler peak-height × retention-time method, we considered corrections which would allow this method to give results comparable with those obtained by triangulation.

EXPERIMENTAL PROCEDURE

Two standard mixtures of methyl esters for gas-liquid chromatography (GLC) were obtained from The Hormel Institute, Austin, Minn., which had the following weight percentage compositions: Standard No. 3—8:0, 20.04; 10:0, 19.99; 12:0, 19.99; 14:0, 19.99; 16:0, 19.99; Standard No. 5—16:0, 24.99; 18:0, 24.98; 20:0, 24.99; 22:0, 25.04. Standard No. 3 (100 mg) was dissolved in 10 ml of carbon disulfide (reagent grade); Standard No. 5 (100 mg) was dissolved in 10 ml of hexane (reagent grade). All solvents gave only the solvent peak when checked by GLC for trace components.

The gas chromatograph was a Barber-Coleman Model 10, equipped with a hydrogen flame detector and operated at 280C. The column was a 6-ft × ¼-in glass column, packed with 10% diethyleneglycol succinate on Chromosorb P (Applied Science Laboratories) and maintained at a constant temperature of 195C. The flash heater was set at 225C. Argon carrier gas was used with a flow-meter value of 60 ml/min (based on a standard calibration of the meter with air). Samples (approx. 1 μl) of each standard were injected onto the column with a 10-μl syringe containing 1.5 μl carbon disulfide as flushing solvent, separated from the plunger and sample by one-μl air spaces. At the time of injection the pen on the recorder was simultaneously moved so

TABLE I
Average Peak Widths at Half-Height and Retention Times[a]

Sample	Peak width	Retention time
Standard No. 3	sec	sec
8:0	3.1 ± 0.1	37.1 ± 0.2
10:0	3.6 ± 0.07	47.4 ± 0.2
12:0	4.6 ± 0.09	64.6 ± 0.5
14:0	6.1 ± 0.08	97.5 ± 0.3
16:0	8.7 ± 0.1	153 ± 0.4
Standard No. 5		
16:0	9.5 ± 0.07	153 ± 0.4
18:0	13.8 ± 0.2	251 ± 2
20:0	21.1 ± 0.1	420 ± 1
22:0	33.8 ± 0.3	717 ± 2

[a]The results for No. 3 are averages of 10 injections and those for No. 5, of six injections (sample size, approximately 10 μg). In each case the measure of variance is the standard deviation. The chart speed of 2 *in./min* gave recorded chromatograms in which 1 mm corresponded to 1.18 sec.

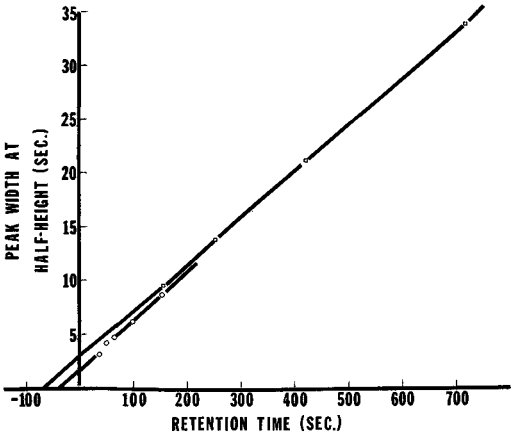

FIG. 1. Peak width at half-height on GLC of methyl esters of long-chain fatty acids vs. the retention time. The □ is standard mixture No. 5; ○ is standard mixture No. 3.

that the injection point was accurately known. Uncorrected retention times were measured from this point of injection.

RESULTS

The uncorrected retention times and peak widths at half-height which were determined are summarized in Table I. The height of the deflection from base line was also determined, but this varied depending upon the amount of standard injected and is not constant from injection to injection. The values for the width of the peaks at half-height for repeated injections of a given sample were easily reproducible and ranged from 3.1 to 33.8 sec with the methyl esters used.

The relationship between peak width at half-height and the retention time for that peak is shown in Fig. 1. The points lie on a straight line that does not go through the origin. In all cases that we have examined, the extrapolated value for the width at half-height at zero time has a finite positive value, ranging from 0.6 to 3.5 sec. Alternatively the line defines an imaginary or virtual injection point which would lie 27 to 72 sec before the real injection point.

Sample calculations of relative peak areas by three methods are shown in Table II. The first method (A) represents the widely used procedure of triangulation and has consistently provided good results with reference standards. The second method (B) uses the product of the retention time and peak amplitude, both of which are conveniently and accurately measured. The third method (C) uses the product of virtual retention time and peak amplitude on the basis that the virtual retention time is directly proportional to the peak width at half-height.

The results from these calculations are shown in Table III. A measure of the error of measurement, indicated by the standard devia-

TABLE III

Comparison of Three Methods of Calculation[a]

Sample	(A)	(B)	(C)
Standard No. 3	%	%	%
8:0	17.7 ± 1.0	13.6 ± 0.2	17.3 ± 0.3
10:0	19.5 ± 0.4	17.5 ± 0.1	19.6 ± 0.1
12:0	20.3 ± 0.4	20.1 ± 0.2	20.7 ± 0.1
14:0	21.3 ± 0.2	23.1 ± 0.3	20.9 ± 0.2
16:0	21.4 ± 0.3	25.8 ± 0.3	21.5 ± 0.3
Standard No. 5			
16:0	25.3 ± 0.2	21.7 ± 0.2	25.3 ± 0.3
18:0	25.5 ± 0.3	24.5 ± 0.2	25.2 ± 0.2
20:0	25.2 ± 0.8	26.6 ± 0.2	25.1 ± 0.1
22:0	24.2 ± 0.2	27.3 ± 0.1	24.3 ± 0.2

[a]The three methods A, B, and C correspond to those described in Table II. The values are averages of six to 10 analyses.

TABLE II

Calculations of Relative Peak Areas

Sample	Peak height (1)	Amp.[a] (2)	Peak width (3)	Retention time observed (4)	Retention time virtual (5)	Calculated areas (A) (1)x(2)x(3)	Per cent	(B) (1)x(2)x(4)	Per cent	(C) (1)x(2)x(5)	Per cent
	cm		mm	cm	cm	cm²		cm²		cm²	
8:0	17.2	1.0	2.8	3.11	5.41	48.1	17.2	53.4	13.8	93.0	17.2
10:0	16.8	1.0	3.2	3.97	6.27	53.7	19.2	66.7	17.2	105.3	19.4
12:0	14.1	1.0	4.1	5.53	7.83	57.8	20.6	77.9	20.1	110.3	20.4
14:0	10.8	1.0	5.5	8.28	10.58	59.5	21.2	89.7	23.1	114.6	21.2
16:0	7.7	1.0	7.9	12.95	15.25	61.2	21.8	100.4	25.9	118.2	21.8
16:0	9.09	1.0	7.9	12.9	18.1	71.4	25.3	116.4	21.6	163.9	25.1
18:0	21.0	0.3	11.1	21.1	26.3	69.9	24.7	132.9	24.7	165.9	25.4
20:0	13.5	0.3	17.8	35.3	40.6	72.1	25.5	143.1	26.6	164.3	25.2
22:0	8.08	0.3	28.6	60.3	65.6	69.3	24.5	146.2	27.2	159.0	24.3

[a]Amp. = amplification setting on the preamplifier of the gas chromatograph. The magnitude of the number indicates the amount of detector signal needed for a full-scale response of the recorder.

tions, was greatest when the peak width at half-height must be measured (A) since even the thickness of the pen tracing affects the small distances that must be measured. The leading edge of the pen tracing was consistently measured for uniform routine determinations. Yet the systematic error in composition with low values for short-chain acids and high values for the larger ones was most evident with method (B), in which only one acid in each series agreed with method (A). Although all three methods gave lower than theoretical values for octanoate, the other acids were adequately determined with methods (A) and (C).

The standards were diluted (1 to 10) in CS_2 and were injected to see if the peak widths at half-height and the virtual retention times could be decreased by this change in solvent and sample size. A small change was noted; the half-height width at zero time for Standard No. 3 changed from 1.3 sec to 0.6 sec, and from 3.5 sec to 2.4 sec for Standard No. 5. These values correspond to shifts in the virtual injection point from –25 sec to –12 sec for Standard No. 3 and from –72 to –52 sec for Standard No. 5. The results in Table IV indicate that these changes lowered the required corrections but were not in themselves sufficient to produce similar values with methods (B) and (C).

DISCUSSION

The hydrogen flame-detector response gives uncorrected peak area percentage values that are approximately equivalent to the weight percentage (5,6) with the series of long-chain fatty acids usually encountered. Additional factors have been proposed to compensate for differences in flame ionization-detector response (7) and for presumed destructive losses in the column (8). Any dimension of the chromatogram that is directly proportional to peak width can give area percentage values that are equal to those from the triangulation method which uses a direct measurement of width. The characteristic property of gas chromatographic peaks of greater width in proportion to the amount of time the sample has been diffusing within the column makes the retention time helpful in calculating relative areas of symmetrical peaks. The results in Fig. 1 however indicate that the application of the sample to the column is not instantaneous but gives a band of material that may be distributed over a range of time from one to three sec (the equivalent peak width at zero retention time). Thus the diffusion within the column does not begin from a point source but rather from a band.

TABLE IV
Effect of Sample Dilution on Calculated Areas[a]

Sample	(A)	(B)	(C)
Standard No. 3	%	%	%
8:0	19.2(18.8)	17.6(17.1)	19.5(18.1)
10:0	19.7(19.2)	18.3(17.8)	19.3(19.1)
12:0	20.3(21.3)	20.0(19.7)	20.0(20.1)
14:0	20.4(20.5)	21.1(21.8)	20.1(20.7)
16:0	20.5(20.2)	23.0(23.6)	21.1(21.9)
Standard No. 5			
16:0	25.0(24.8)	22.1(22.7)	25.3(25.6)
18:0	25.6(25.6)	25.0(24.8)	25.6(25.3)
20:0	25.3(25.4)	26.4(26.2)	25.0(25.0)
22:0	24.1(24.2)	26.6(26.4)	24.1(24.2)

[a]The three methods A, B, and C are indicated in Table II. The results of duplicate analyses are in parentheses.

The width of the sample band in gas chromatography can be expected to depend upon the geometry of the injection chamber, rate of gas flow, flash-heater temperature (which would control the speed of solvent volatilization), and the volatility of the injected sample. Syringe techniques and speed of injection may also contribute one or two tenths of a second to the band width. These variables are all reasonably constant, finite properties of a given gas chromatographic system (including the operator); although their effects can be minimized, they cannot be eliminated.

We believe that one of the principal factors causing a wide initial band in our chromatograph is the rather large volume of the injection chamber of the commercially available columns. This space broadens the bands in much the same way that the "post-column dead volume" does (9). Our studies led to the realization that such factors as solute band-broadening and plate-height abnormalities which are of concern to those who determine the height of the effective theoretical plate (HETP) in columns were indirectly involved in our quantitative estimations of percentage composition by the retention-time × peak-height method. The influence of injection time on the efficiency (or HETP) of GLC columns was indicated by Guiochon (10,11), who noted that this factor "seems to have been completely neglected." He further indicated the magnitude of the contribution of injection time to zone spreading. The problem of initial sample band width was recently commented on by Bartlet and Mason (12), who found that the peak-height × retention-time method gave satisfactory results without corrections.

Our experience suggests that a major dif-

ficulty in satisfactorily applying the convenient retention-time × height method may be due primarily to the injection system used when short retention times are involved. However the approximate band width at zero time can be estimated for a given chromatographic system and used to develop a simple correction factor for quantitative work even when the desire for faster analyses requires short retention times. Samples that are similar in size and composition to the unknowns which are analyzed should be employed in determining this correction factor. An understanding of this correction factor may be useful since it provides results as accurate as those obtained by triangulation and uses measurements that are more conveniently obtained.

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SHORT COMMUNICATIONS

The Lipid Antioxidant Properties of Iodine Compounds

THE RELATIONSHIP BETWEEN THYROXINE and mitochondrial swelling appears to be extremely concentration-dependent. Cash et al. (1) have demonstrated that 1 μM thyroxine exerts a strong lipid antioxidant action while blocking mitochondrial swelling induced by Fe^{++}. This concentration is one-tenth that at which thyroxine itself induces mitochondrial swelling in a similar manner to iodine cyanide (ICN) and I^- (2,3). Thyroxine has been shown by others (4,5) to be a lipid antioxidant, and there is agreement that, at the biological level, it is as effective as α-tocopherol (1,4). The purpose of this study was to investigate the antioxidant properties of compounds related to thyroxine.

Antioxidant activity was determined by measuring oxygen absorption by methyl linolenate (99%, Hormel Foundation) from the air manometrically with a Warburg respirometer at 40C. The control consisted of a buffered suspension of methyl linolenate without antioxidant. The flasks were shaken through an amplitude of 4 cm at 120 strokes per minute. Readings were taken every 10 min for 12 hr.

In addition to the compounds listed in Table I, the iodothyronines, tyrosine, the iodotyrosines, 3,5-dibromotyrosine, iodobenzene, and methyl iodide were tested and found to demonstrate no antioxidant activity. Thyroxine itself appeared to be less potent than has been reported (5). Among the other compounds tabulated, it is evident that the presence of iodine in the position *ortho* to the hydroxyl group of the phenolic compounds reduces antioxidant activity significantly. The similarity between thyroxine and phenol seems to indicate that thyroxine is primarily a phenolic antioxidant. Its activity as an antioxidant is probably independent of its action on mitochondria since the ability of thyroxine to cause mitochondrial swelling is said to be independent of the phenolic hydroxyl group (6). The vast differences in antioxidant activities of thyroxine, ICN, and I^-, all of which have been reported to induce mitochondrial swelling (2, 3), supports this conclusion.

TABLE I
Relative Antioxidant Activities[a]

Compound[b]	Concentration	
	5 x 10^{-6} M	5 x 10^{-9} M
Thyroxine[c]	0.74	0.92
Thyronine[d]	0.66	—
Phenol[e]	0.74	—
2-Iodophenol[f]	1.1	—
2-Naphthol[i]	0.37	—
1-Iodo-2-Naphthol[f]	0.93	—
Hydroquinone[e]	0.54	—
2,6-Diiodohydroquinone[g]	1.2	—
α-Tocopherol[f]	0.45	0.02
Iodine cyanide[c]	0.08	1.1
Iodine[h]	1.3	—
Sodium iodide[e]	1.0	—

[a]Obtained by dividing the μl O_2 absorbed by methyl linolenate (2.00 ml of 2.5 x 10^{-2} M in 0.2 M phosphate buffer of pH 7.2, containing 2% Tween 20) in the presence of the compound in 10 hr by the μl O_2 absorbed by methyl linolenate alone in 10 hr.

[b]Highest purity available from source indicated, selected for their failure to absorb O_2 under the conditions of the experiment in the absence of linolenate.

[c]Nutritional Biochemicals Corporation.

[d]Sigma Chemical Company.

[e]J. T. Baker Chemical Company.

[f]Distillation Products Industries.

[g]K & K Laboratories.

[h]Fisher Scientific Company.

[i]Matheson, Coleman, and Bell.

The observation that α-tocopherol has greater antioxidant potency at 5 $\times$ 10^{-9} M than at 5 $\times$ 10^{-6} M is interesting. The same phenomenon at higher concentrations of α-tocopherol has been both corroborated (8) and contradicted (9).

Probably the most significant result of these experiments is the observation that ICN appears to be the best antioxidant studied at 5 $\times$ 10^{-6} M although it loses its ability to protect methyl linolenate at 5 $\times$ 10^{-9} M. The iodinium ion (I^+), once a popular intermediate in mechanistic speculations, has been shown to have a real existence (7). One is tempted to accept the dissociation of ICN to form I^+ as has been suggested (2). At relatively high concentrations, this ion may act as an antioxidant by reacting with alkoxy radicals:

$$\text{R-}\underset{\underset{\text{O.}}{|}}{\text{CH}}\text{-R} + I^+ \rightarrow \text{R-}\underset{\underset{\text{O}}{||}}{\text{C}}\text{-R} + I^{\cdot} + H^+.$$

Since Michel (3) has suggested a phenyliodonium ion as the active thyroid species, the relationship between the antioxidant activity of ICN and its thyroxine-like action should be clarified.

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[Received Oct. 6, 1967]

Fatty Acid Composition of the Xanthophyll Esters of *Tagetes erecta* Petals[1]

XANTHOPHYLLS FROM PLANT AND ANIMAL sources have been studied extensively by various workers (1,3); however, little work has been done on the fatty acid moiety of xanthophyll ester. Kuhn et al. (4) showed that xanthophylls could occur in nature in the free or esterified form and subsequently isolated lutein dipalmitate from *Helenium autumnale* and predicted that other esters of lutein would be found. Booth (5), in a short communication, outlined a method for extraction of carotene, carotenols, and carotenoid esters from leaves and flowers as a crude mixture.

In earlier studies in this laboratory the composition of the pigment of *Tagetes erecta* petal was investigated (6). Further work indicated that the natural pigments of *Tagetes erecta* show neutral property, whereas the same pigment, when hydrolyzed, showed polar characteristics. This differential behavior was tested by partition distribution using 90% methanol and n-hexane solvent system and also by TLC 20% methylene chloride in ethyl acetate as solvent and Silica Gel G as adsorbent. Furthermore, it was found that the nonpolar characteristic of the natural pigment can be recovered by treating the hydrolyzed pigments with acetic anhydride in pyridine and refluxing the whole solution for 15 min. These findings suggested the existence of ester-type bonds in the natural pigment molecules. This report describes an outline that was adopted using available techniques to analyze the fatty acid moiety of the xanthophyll esters of *Tagetes erecta.*

Tagetes erecta petals, obtained from Special Nutrient, Bay Harbor, Fla., were used for this study. The pigments were exhaustively extracted (five times) from the petals with n-hexane. The combined extracts were washed with distilled water after drying over anhydrous sodium sulfate, filtered, and evaporated to dryness under an atmosphere of nitrogen.

The chromatographic column was a glass tube of 1.5 cm internal diameter and 20 cm length, equipped with a sintered glass filter at one end. Fifteen grams of silicic acid for chromatography (Mallinckrodt, New York) was washed with distilled water and dried at 125C for 3 hr. The silicic acid was then washed three times with 75 ml of chloroform-methanol (10:1) and then with 75 ml of chloroform in a small beaker. The packing of the column was carried out by using the slurry. The extracted pigment was then chromatographed with chloroform as the developing solvent. The neutral pigments along with other neutral lipids were eluted with 150 ml of chloroform and concentrated in vacuo.

Chromatoplates (20 × 20 cm) were coated with a 1-mm layer of Silica Gel G (Merck AG, Darmstadt) in the conventional manner with a commercial spreader. The plates were dried for 1½ hr at 110C and cooled to room temperature before use. The isolated neutral lipids were applied as a thin strip on the chromatoplates and developed with chloroform. The separated bands on the plate were identified by co-chromatography with pure preparations of carotene, cholesterol ester, triglyceride (tripalmitin), di- and monoglyceride. Location of the colorless bands was accomplished by spraying the plates with 2,7-dichlorofluorescein (0.02% solution) and observing under UV lamp. The neutral lipids of *Tagetes erecta* contained only a trace of carotenes and triglycerides and a large amount of xanthophyll esters.

The separated xanthophyll ester band was scraped off the chromatoplate, extracted, and saponified with 30 ml of 20% methanolic KOH. After removal of the unsaponifiable components including the pigment alcohols, the residual solution was acidified, and the fatty acids were extracted with three portions of n-hexane. The n-hexane extracts were combined and

[1]This is a contribution of the Texas Agricultural Experiment Station.

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The chromatographic column was a glass tube of 1.5 cm internal diameter and 20 cm length, equipped with a sintered glass filter at one end. Fifteen grams of silicic acid for chromatography (Mallinckrodt, New York) was washed with distilled water and dried at 125C for 3 hr. The silicic acid was then washed three times with 75 ml of chloroform-methanol (10:1) and then with 75 ml of chloroform in a small beaker. The packing of the column was carried out by using the slurry. The extracted pigment was then chromatographed with chloroform as the developing solvent. The neutral pigments along with other neutral lipids were eluted with 150 ml of chloroform and concentrated in vacuo.

Chromatoplates (20 × 20 cm) were coated with a 1-mm layer of Silica Gel G (Merck AG, Darmstadt) in the conventional manner with a commercial spreader. The plates were dried for 1½ hr at 110C and cooled to room temperature before use. The isolated neutral lipids were applied as a thin strip on the chromatoplates and developed with chloroform. The separated bands on the plate were identified by co-chromatography with pure preparations of carotene, cholesterol ester, triglyceride (tripalmitin), di- and monoglyceride. Location of the colorless bands was accomplished by spraying the plates with 2,7-dichlorofluorescein (0.02% solution) and observing under UV lamp. The neutral lipids of *Tagetes erecta* contained only a trace of carotenes and triglycerides and a large amount of xanthophyll esters.

The separated xanthophyll ester band was scraped off the chromatoplate, extracted, and saponified with 30 ml of 20% methanolic KOH. After removal of the unsaponifiable components including the pigment alcohols, the residual solution was acidified, and the fatty acids were extracted with three portions of n-hexane. The n-hexane extracts were combined and

[1]This is a contribution of the Texas Agricultural Experiment Station.

washed with distilled water. After drying over anhydrous sodium sulfate, the extract was evaporated to dryness.

The methyl esters were prepared by treating the residue with diazomethane in diethyl ether. For the gas-liquid chromatographic analysis, a Research Specialties Model 600 gas chromatograph, equipped with a β-ionization detector, was used. The 6 ft. × 0.25 in. copper column containing 15% (w/w) diethylene glycol succinate on Gas Chrom RZ (Applied Science Laboratories, State College, Pa.) of 60-80 mesh was maintained at 180C with a carrier gas (argon) flow rate of 40 ml/min. Identification of the fatty acids was achieved by comparing the retention times with those of a known standard mixture of high purity. Peak areas were determined by triangulation, and the results were reported as area percentage of fatty acids (Table I). The greatest amount of fatty acid present in xanthophyll esters of *Tagetes erecta* was palmitic acid, followed by myristic acid. Stearic acid was also present in considerable amounts, accounting for about 14.4% of the total. Lauric acid was present only in small amounts. It is quite striking that, except for traces of oleic acid, unsaturated fatty acids were completely absent in xanthophyll esters of *Tagetes erecta.*

TABLE I

Fatty Acid Composition of the Xanthophyll Esters of *Tagetes erecta* and Their Relative Distribution

Fatty acid	Common name	Percentage
12:0	Lauric	2.4
14:0	Myristic	22.6
16:0	Palmitic	60.4
18:0	Stearic	14.4
18:1	Oleic	Trace

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6. Alam, A. U., J. R. Couch, F. M. Farr and C. R. Creger, submitted to Phytochemistry.

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Occurrence of Inosine in Unwashed Lipid Extracts

A CONTAMINANT, later shown to be inosine, was found in chloroform-methanol (2:1, v/v) extracts of human tissues post-mortem. It came to our attention because it was not removed by Sephadex chromatography, following Wells and Dittmer (1), whereas it was readily removed by washing (2) or by dialysis. It was, however, completely removed by the Sephadex column procedure of Siakotos and Rouser (3). The contaminant was found in extracts from brain, retina, iris, cornea, liver, spleen, kidney, lung, pancreas, mucosa of bladder and ileum, leukocytes and platelets, skeletal muscle, myocardium, smooth muscle of uterus, stomach, ileum, and bladder. It was most prominent in the muscle extracts.

Most of the inosine would appear to have been formed post-mortem from adenine nucleotides by well-known enzymatic pathways, and immediate immobilization of tissue metabolism at the time of sampling should reduce inosine contamination. The properties of inosine correspond closely to those of "carnithin", a nitrogenous impurity found in lipid extracts and so named by MacLean in 1918 (4).

The contaminant was detected on silica gel thin layer chromatograms with the aniline-diphenylamine spray reagent (5) as a characteristic, bright-blue spot that was readily distinguished from the dark blue-gray spots given by glycolipids. On chromatograms developed in chloroform-methanol-water (65:25:4,v/v/v) (6), it ran close to choline glycerol phosphatide. In some samples additional bright blue spots, running both in front and behind, were observed.

The major blue-staining substance was isolated and identified as follows. Heart muscle (140g) was homogenized in 7 vol of acetone, and insoluble material was removed by filtration through sintered glass. The solvent was removed from the extract, and the residue was reextracted with chloroform-methanol (2:1,v/v). The amount of 0.2 vol of water was added, the mixture was shaken, and the phases were allowed to separate. The upper phase was taken to dryness, and the resulting residue was extracted with chloroform-methanol (2:1,v/v); then the extract was taken to dryness.

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The dry substance was taken up in chloro-

TABLE I

Ultraviolet Spectral Characteristics of the Blue-Staining Impurity Compared with Observed and Published Values for Pure Inosine

	In Water			In Alkali			In Acid		
pH	5.5		6.0[a]	12.3		11.6[a]	0.3		in 2N-HCl[a]
Sample	Impurity	Inosine	Inosine	Impurity	Inosine	Inosine	Impurity	Inosine	Inosine
Abs. max. (λ)	248.5	248.5	248.5	253	253	253	251	251	251
Abs. min. (λ)	223	223	223	225	225	224.5	220.5	222	221
Abs. 250/Abs. 260	1.63	1.65	1.68	1.075	1.065	1.05	1.20	1.20	1.21
Abs. 280/Abs. 260	0.33	0.30	0.25	0.23	0.22	0.18	0.19	0.16	0.11
Abs. 290/Abs. 260	0.13	0.09	0.03	0.07	0.06	0.01	0.08	0.05	0.0

[a]From Beaven et al. (8).

form-methanol (9:1,v/v) and transferred to a DEAE-cellulose column, which was prepared and eluted according to Rouser et al. (7). The blue-staining material emerged in fractions eluted with chloroform-methanol (7:3 and 1:1, v/v), which were pooled. Solvents were removed, and the dry material was taken up in a small volume of chloroform-methanol (2:1, v/v). After several days at 4C a white precipitate appeared, which was removed by filtration and discarded.

The substance in the major blue-staining spot on thin-layer chromatography was isolated by preparative TLC on Silica Gel G. Its position on the chromatogram was detected in relation to iodine-staining impurities. Methanol was used for elution from the gel from which 30.6 mg of substance was obtained. This was freely soluble in chloroform-methanol and contained less than 0.01% phosphorus. It gave a single blue spot on silica gel thin-layer chromatograms which were developed in chloroform-methanol-water (65:25:4,v/v/v) and water. It co-chromatographed with pure inosine and gave the same hue on staining. The purified material was chromatographed on thin-layer plates, prepared with cellulose (Whatman CC41) and developed in six solvent systems (butan-1-ol-water-conc. NH_4OH [86:14:5,v/v/v]; propan-2-ol-1% aq. $[NH_4]_2SO_4$ [2:1,v/v]; propan-2-ol-conc. HCl-water [68:17:14.4,v/v/v]; propan-2-ol-water-conc. NH_4OH [7:2:1,v/v/v]; ethyl acetate-propan-1-ol-water [4:1:2,v/v/v]; and water). A single ultraviolet-absorbing spot was detected.

In each system this spot had an R_f value identical to pure inosine, and in one or more of the solvent systems the R_f was clearly different from that of adenosine, guanosine, xanthosine, cytidine, and uridine. When added to total lipid extracts, inosine chromatographed exactly with the major unknown blue impurity. When the purified material was heated at 100C in 1N HCl for 75 min and the products were examined by TLC on silica gel plates developed in chloroform-methanol-water (60:40:10,v/v/v), the original blue spot was replaced by a single, slower-running blue band with an R_f identical to *d*-ribose but different from 2-deoxyribose, L-xylulose, glucose, galactose, mannose, and fructose. On cellulose thin-layer chromatograms, developed in water, the original ultraviolet-absorbing spot was absent after acid treatment, and a new absorbing spot was present, which had a lower R_f identical to hypoxanthine.

The ultraviolet spectrum of the purified material showed peaks of maximum absorbance characteristic of inosine. However, absorbance at wavelengths below 240mμ was greater than that given by pure inosine and indicated the presence of other contaminating substances. Calculation of the quantity of inosine in the purified material by use of a molar absorbancy index of 12,250 at 248.5mμ (8) revealed that the preparation was 43% pure. After repeated precipitation from chloroform and preparative TLC on acid-washed cellulose, with water as the developing solvent (9), a substance was obtained which had an ultraviolet absorption spectrum not significantly different from that given by a sample of pure inosine or from published values (Table I). The molar ratio of ribose (10) to inosine (ultraviolet absorption), determined on the pure material, was 1.03.

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Isolation of Tetradecan-1,14-dioic Acid from the Comstock Mealybug, *Pseudococcus comstocki* Kuwana (Homoptera: Pseudococcidae)

THE COMSTOCK MEALYBUG, *Pseudococcus comstocki* Kuwana, is a representative scale insect that attacks various fruit trees in Japan. The body surface is covered with a white wax powder secreted through the cuticle of the scale insect. During this investigation an unidentified acid was found by gas chromatography in the fatty acid fractions of the secreted wax and the body lipids. The relative retention-time of the methyl ester of the unidentified acid compared with that of methyl stearate was 0.75 when a column (1 m × 4 mm) of 10% SE-30 on Celite 545 (60-80 mesh) at 200C was used and 2.68 when a column (2 m × 4 mm) of 15% polyethyleneglycol adipate on Celite 545 (60-80 mesh) at 180C was used. These results suggested that the molecular weight of the unidentified acid was smaller than that of stearic acid and contained an additional polar group as compared with methyl stearate.

Mature adult females (about 10 g) of the Comstock mealybug, reared on pumpkin fruits, were homogenized in an ice-cooled homogenizer. Total lipid (body lipid and secreted wax) was extracted with chloroform-methanol (2:1) (1). Extracted matter was refluxed with 10% ethanolic KOH for 6 hrs. The reaction mixture was diluted with water, acidified with HCl, and then extracted with *n*-hexane. The *n*-hexane extract was concentrated *in vacuo,* and the residue was treated with diazomethane in diethyl ether-methanol (9:1) to methylate fatty acids (2). After the solvent was evaporated, the residue was again dissolved in *n*-hexane and poured onto a column 15 mm in diameter, packed with 20 g of silicic acid.

The fraction eluted with *n*-hexane-diethyl ether (94:6) was concentrated *in vacuo.* Recrystallization from aqueous acetone afforded about 400 mg of colorless plates, mp 41.5-42.0C. Found: C, 67.13; H, 10.51. Calcd. for $C_{16}H_{30}O_4$:C, 67.08; H, 10.58. The methyl ester of the unidentified acid reacted with urea to form a urea-adduct but did not react with 2,4-dinitrophenylhydrazine, acetic anhydride, or $KMnO_4$. The methyl ester was reduced to the hydrocarbon by using lithium aluminum hydride, iodine, and red phosphorus (3). The resultant hydrocarbon was identified as *n*-tetradecane by gas chromatographic analysis.

These results strongly suggested that the unidentified acid was tetradecandioic acid. Methyl tetradecan-1,14-dioate was then synthesized from 1,10-dibromodecane according to the method of Rodinov et al. (4) and was purified by silicic acid chromatography as mentioned before. Recrystallization from aqueous acetone afforded colorless plates, mp 41.5C. The infrared spectrum of the methyl ester of unidentified acid isolated from the scale insect was identical with that of the synthesized methyl tetradecan-1,14-dioate (Figure 1). The gas chromatographic retention-time of the methyl ester of the isolated acid was also the same as that of the synthesized methyl tetradecan-1,14-dioate.

This is the first time that tetradecan-1,14-dioic acid has been isolated from a natural source. The amounts of tetradecan-1,14-dioic acid in the fatty acid fractions of the secreted wax and the body lipid were determined by gas chromatography. The insects were shaken with chloroform to separate the secreted wax from the insect bodies, and the chloroform layer containing the wax material was evaporated to dryness (6).

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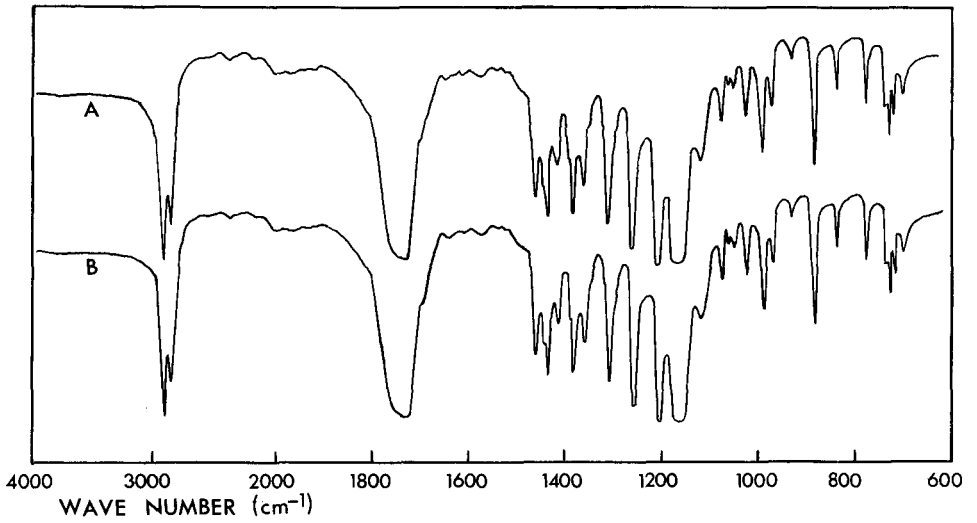

FIG. 1. Infrared absorption spectra of methyl ester of the unidentified acid (A), isolated from the Comstock mealybug and synthesized methyl tetradecan-1,14-dioate (B).

The amounts of tetradecan-1,14-dioic acid in the fatty acid fractions ranged from 8.5 to 17.9% (Table I).

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ACKNOWLEDGMENTS

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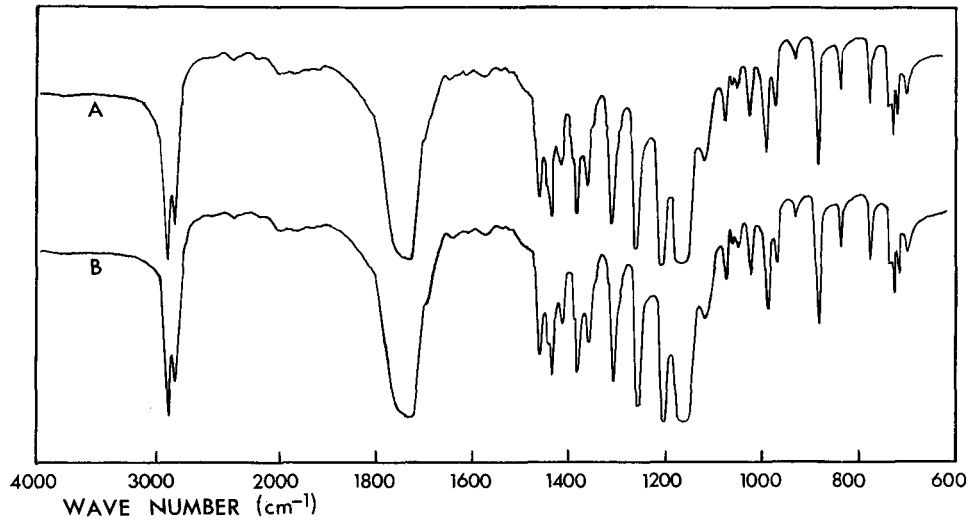
Thin-Layer Chromatography of Phospholipids on Alumina

SILICA GEL G has been used in many studies on the separation of phospholipids by thin-layer chromatography (TLC) even though acidic phospholipids frequently spread or overlap when they are chromatographed on this adsorbent. Separations are improved with acidic or basic additives in the Silica Gel G, acidic, or basic solvent systems, elimination of the calcium sulfate binder, and two-dimensional TLC (1,2). Aluminum oxide has been used extensively for the separation of phospholipids by column chromatography. This adsorbent is seldom used in TLC analysis of phospholipids although Hofmann (3) separated phosphatidyl choline (PC), lysophosphatidyl choline (LPC), phosphatidyl ethanolamine (PE), and sphingomyelin (Sph) on Aluminum Oxide G. In this communication we describe the separation of phospholipids by TLC on basic alumina and the estimation of phospholipid composition by the phosphorus analysis of the TLC spots.

Basic alumina has several advantages. We used the adsorbent to confirm the identity of TLC spots since the phospholipid migration pattern on basic alumina is the reverse of the phospholipid migration pattern on Silica Gel G. Furthermore the acidic phospholipids, phosphatidic acid (PA) and cardiolipin (Cl), are readily separated with this adsorbent. The adsorbent forms water-soluble salts during digestion



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Potato	8.5	10.4

ACKNOWLEDGMENTS

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Thin-Layer Chromatography of Phospholipids on Alumina

SILICA GEL G has been used in many studies on the separation of phospholipids by thin-layer chromatography (TLC) even though acidic phospholipids frequently spread or overlap when they are chromatographed on this adsorbent. Separations are improved with acidic or basic additives in the Silica Gel G, acidic, or basic solvent systems, elimination of the calcium sulfate binder, and two-dimensional TLC (1,2). Aluminum oxide has been used extensively for the separation of phospholipids by column chromatography. This adsorbent is seldom used in TLC analysis of phospholipids although Hofmann (3) separated phosphatidyl choline (PC), lysophosphatidyl choline (LPC), phosphatidyl ethanolamine (PE), and sphingomyelin (Sph) on Aluminum Oxide G. In this communication we describe the separation of phospholipids by TLC on basic alumina and the estimation of phospholipid composition by the phosphorus analysis of the TLC spots.

Basic alumina has several advantages. We used the adsorbent to confirm the identity of TLC spots since the phospholipid migration pattern on basic alumina is the reverse of the phospholipid migration pattern on Silica Gel G. Furthermore the acidic phospholipids, phosphatidic acid (PA) and cardiolipin (Cl), are readily separated with this adsorbent. The adsorbent forms water-soluble salts during digestion

with perchloric acid. Thus phosphorus may be estimated without centrifugation for the purpose of removing insoluble adsorbents such as Silica Gel G.

Alumina (Woelm basic TLC) was obtained from Alupharm Chemicals. Aluminum Oxide G was obtained from Brinkmann Instruments Inc. Aluminum Oxide G purchased before 1964 was used initially in phospholipid separations; however Aluminum Oxide G purchased in 1967 did not separate phospholipids, and this adsorbent was replaced by alumina (Woelm basic TLC) in these studies. Calcium sulfate was obtained from the Baker Chemical Company and passed through a 200-mesh sieve before use. Phosphatidyl serine (PS), phosphatidyl inositol (PI), Sph, and phospholipase D were purchased from General Biochemical Company. Lecithin and cardiolipin (Cl) were purchased from Sylvana Chemical Company. PC and PE were isolated from egg yolk lipids. PA was prepared from PC by hydrolysis with phospholipase D. LPC was supplied by A. F. Robertson, beef heart mitochondria by G. P. Brierley.

An alumina-calcium sulfate mixture (90:10, w/w) was prepared; 40 g of this adsorbent mixture was slurried with 60 ml of water and were used to coat five TLC plates (20 × 20 cm). Plates were air-dried for 15 min and then activated at 150-160C for 1 hr. The tanks, lined with filter paper, were equilibrated for at least 1 hr before developing the chromatogram. Chromatograms were developed with chloroform-methanol-water-pyridine-ammonia (65:27.5:4:2:2, v/v). In two-dimensional chromatography, chloroform - methanol - water (60:40:6) was used as the second developing solvent. All lipid fractions were made visible with iodine vapor, chromic acid charring, or a molybdenum blue reagent for phospholipids. Choline-containing lipids were detected with the Dragendorff reagent, and PS and PE were detected with ninhydrin.

The TLC separations obtained with reference compounds and a total lipid extract of beef heart mitochondria by chromatography in one dimension are shown in Figure 1. The R_f values were PC (0.67), Sph (0.54), LPC (0.43), PE (0.43), and Cl (0.25). PA, PI, and PS remained at the origin. The principal mitochondria lipids, PC, PE, and Cl, were readily separated. Phospholipid fractions showed appreciable streaking when Aluminum Oxide G was used as the adsorbent. The R_f values were decreased, fractions overlapped, and the developing time was increased when alumina was used without the calcium sulfate additive.

Chromatography in a second dimension separated LPC and PE, two lipid fractions which

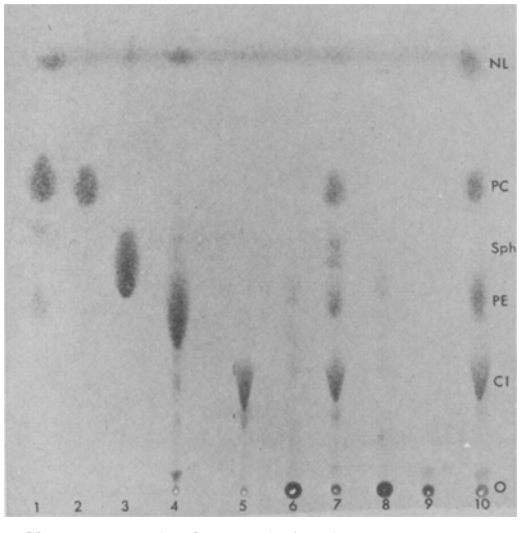

FIG. 1. TLC of phospholipids on an alumina-calcium sulfate adsorbent. The chromatoplate was developed in chloroform-methanol-water-pyridine-ammonia (65:27.5:4:2:2). 1-Lecithin; 2-PC; 3-Sph; 4-PE; 5-Cl; 6-PA; 7-mixture containing PC, Sph, PE, Cl, PA, and PS; 8-PI; 9-PS; 10-mitochondrial lipids. The spots were made visible by charring with chromic acid.

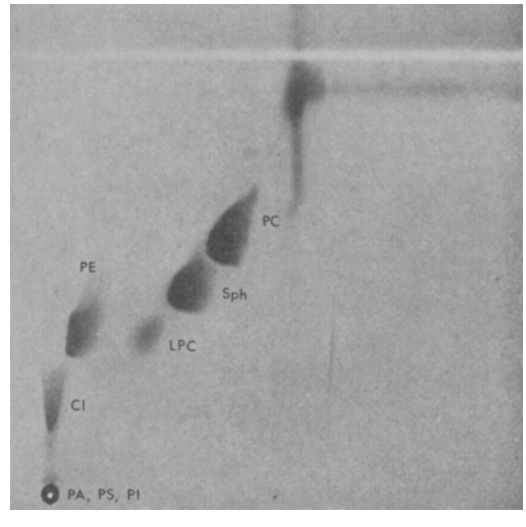

FIG. 2. TLC of phospholipids on an alumina-calcium sulfate adsorbent. The chromatoplate was devleoped in the first dimension with chloroform-methanol-water-pyridine-ammonia (65:27.5:4:2:2) and in the second dimension with chloroform-methanol-water (60:40:6). The phospholipid mixture contained PC, Sph, LPC, CL, PE, PA, PS, and PI. The spots were made visible by charring with chromic acid.

TABLE I
Composition of Phospholipid Mixtures Separated by TLC on Alumina

Component	Mixture A One-dimensional TLC	Mixture B Two-dimensional TLC
	% total phosphorus	
Lecithin	40.0±1.3[a]	14.2±0.4[b]
Sphingomyelin	17.2±1.9	7.1±0.7
Phosphatidyl ethanolamine	15.8±1.4	8.0±1.3
Lysophosphatidyl choline	0	56.0±0.4
Cardiolipin	16.6±1.6	10.6±1.5
Phosphatidyl serine	10.3±1.4	3.9±0.5

[a] The standard deviation was calculated from six TLC plates.
[b] The standard deviation was calculated from four TLC plates.

overlap when they are chromatographed in the first solvent system (Fig. 2). The R_f values in the second dimension were PC (0.61), Sph (0.54), LPC (0.38), and PE (0.09). Cl, PA, and PS remained at the origin. Alumina did not catalyze the hydrolysis of PC since no LPC was detected when chromatographically pure PC was analyzed by two-dimensional chromatography.

Lipid phosphorus was determined by the method of Rouser *et al.* (4) with the following exceptions. The TLC spots were scraped with a spatula onto glass powder paper and were then transferred to the digestion tubes. The sand-bath was maintained at 250C, and samples were heated for 30-40 min. The alumina formed water-soluble salts during digestion with perchloric acid, and the centrifugation step was not required. Reproducibility for one-dimensional and two-dimensional TLC separations was estimated by the analysis of two representative lipid mixtures (Table I). The total phosphorus applied to the plate was estimated by spotting an aliquot in the upper right-hand corner of the plate. From 9-18 μg of phosphorus were applied to the plate in typical separations, and the mean recovery was 99.0%.

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[Received April 3, 1967]

Losses of Fatty Acids During the Saponification Extraction of Small Samples

FISH PROTEIN CONCENTRATE contains 0.2-0.5% residual lipid (1). When 10 mg of the residual lipid was saponified by the method of James (2), modified for semimicro amounts, the amount of fatty acids recovered was only 10-20% of that expected. The sources of the large losses were sought. Saponification conditions were varied by using potassium hydroxide at molarities of 0.1, 0.5, and 1.0*M*, in volumes of 2, 5, and 10 ml and at 1-, 2-, and 4-hr intervals, but the recoveries were not appreciably improved. Experiments were then performed to determine the amounts recovered when pure fatty acids were carried through the procedure.

A known weight of fatty acid (Hormel Institute palmitic, oleic, and arachidonic acids separately or as a mixture) was placed in a 50-ml screw cap vial and freed from solvent with nitrogen. To it were added 5 ml of 0.5M methanolic potassium hydroxide solution and a crystal (approximately 0.1 mg) of hydroquinone. The vial was flushed with nitrogen, capped with polyethylene cone closure (Poly-Seal), heated for 1 hr in a 60-65C water bath, and cooled; to it were added 7.5 ml of distilled water and 1 drop of methyl orange.

The "nonsaponifiable" fraction (Fig. 1) was then removed by 3 extractions with equal volumes of ethyl ether. (All solvents had been redistilled.) The ether extracts were combined, reduced in volume under nitrogen to 5 ml, and washed three times with 5 ml of water (NSF-

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TABLE I
Distribution of Fatty Acids During Extraction

Sample	Weight, mg	% Material in Each Fraction[a] A	B	C	Recovered (A+B+C)
1) Palmitic	10.3	14.6	29.1	56.3	100.0
	10.3	17.5	22.3	60.2	100.0
	98.2	82.5	2.5	15.0	100.0
	99.1	80.7	1.9	16.5	99.1
	100.5	81.4	1.9	15.0	98.3
2) Oleic	9.5	8.4	12.6	81.1	102.1
	9.5	12.6	22.1	67.4	102.1
	9.5	7.4	23.2	69.5	100.1
	9.5	6.3	22.1	71.6	100.0
	114.0	74.6	5.5	18.6	98.7
	114.0	75.3	6.8	18.9	101.0
	114.0	70.7	6.7	19.9	97.3
	114.0	73.7	7.3	18.1	99.1
3) Arachidonic	11.8	14.4	21.2	66.9	102.5
	11.8	14.4	15.3	73.7	103.4
	86.0	65.6	8.7	27.2	101.5
	86.0	73.6	8.0	20.2	101.8
	86.0	67.6	8.6	25.6	101.8
4) Equal-weight mixture of 1-3	13.5	10.4	14.1	75.6	100.1
	13.5	9.6	17.8	72.6	100.0
	94.5	72.1	4.9	24.4	101.4
	94.5	71.4	4.7	25.4	101.5

[a] See Fig. 1 for identification of fractions.

wash, Fig. 1.) The original aqueous fraction contained the "saponified" material. The fractions were then each acidified with 6*N* HCl, and the fatty acids were extracted four times with petroleum ether; the extracts were combined, evaporated, and weighed (Fractions A, B, and C, Fig. 1).

The results (Table I) show the extent to which the fatty acids were distributed into these fractions. The larger samples (100 mg) gave 2-8% losses in the NSF whereas, with the smaller samples (10 mg), these losses amounted to about 20%. The low recoveries of fatty acids in Fraction A and high recoveries in Fraction C indicated that the potassium soaps were appreciably soluble in ether. When fractions from the mixture of fatty acids were analyzed by gas-liquid chromatography for fatty acid composition (Table II), the combined

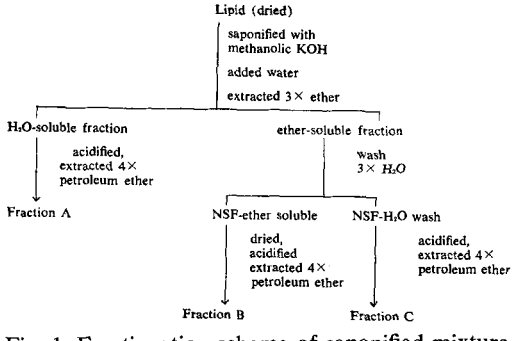

Fig. 1. Fractionation scheme of saponified mixture.

TABLE II
Fatty Acid Composition of Saponification Fractions[a]

Fatty acid	Percentage fatty acid composition of fraction A	B	C	A+C	Mixture composition
C16:0	42;51	25;25	32;31	33;33	31;31
C18:1	30;31	44;45	39;40	38;38	39;39
C20:4	28;28	31;30	29;29	29;29	30;30

[a] Duplicate analyses are shown; sample weights were 14.4 mg. See Fig. 1 for identification of fractions.

aqueous extracts (Fractions A and C) was found to have the same composition as did the original fatty acid mixture. However the NSF (Fraction B) had relatively more oleic acid, indicating that it had a somewhat different partition coefficient than did the others.

Since this fraction represented only 10-20% of the total, the slight increase in oleic acid did not change the analysis appreciably. Because of the larger amounts of lipids commonly used in saponification procedures (2,3), the losses into the NSF would be small, but with decreasing sample size these losses could become large enough to introduce errors of sizable magnitude.

These data show that, with small samples of lipids, appreciable amounts of fatty soaps may be lost when saponified mixtures are extracted with ether. In order to minimize such errors, the ether extracts should be copiously backwashed with water. Even under the washing conditions used in these experiments, 20% of the fatty acids appeared in the NSF when 10 mg-size samples were saponified. If the amount of true NSF in lipid fractions is small, the total lipid fatty acids might better be analyzed by the transmethylation procedures commonly used for purified lipids without prior separation of the nonsaponifiable fraction.

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Individual Molecular Species of Phospholipids. VII. Analysis of Lecithins Containing Ten to Twelve Double Bonds

THE MOST UNSATURATED molecular species of phospholipids so far directly analyzed contain six to seven double bonds (1), but this paper shows that even species of up to 12 olefinic bonds exist and can be handled by the present chromatographic and enzymatic methods.

Lecithin of Baltic herring (*Clupea harengus membras*) served as starting material for the present study. Analysis of this lipid with phospholipase A (EC 3. 1. 1. 4) showed that, of the fatty acids on the C-2 position, 23% were eicosapentaenoic acid (20:5) and 63% docosahexaenoic acid (22:6). The acids at position C-1 consisted mainly of palmitic (61%) and oleic acid (11%), but 17% of docosahexaenoic and some eicosapentaenoic acid were also present. Thus it appeared that the parent lecithin must contain at least a few per cent of molecules with two highly unsaturated fatty acids.

The herring lecithin was converted into diglyceride acetates by hydrolysis with phospholipase C (EC 3. 1. 4. 3) and subsequent acetylation (2). The product moved on silica gel G plates like authentic 1,2-diglyceride acetates. On silver nitrate-containing silica gel G the product was separated into three groups of fractions when chloroform-methanol-water (65:25:4) was used as solvent. The fast fraction (17%) migrated with the solvent front; the middle fraction (70%) traveled in the R_f-range of 0.7, and the slow fraction (13%) lagged behind in the R_f-range of 0.2. After recovery from the plates all these fractions appeared as pure 1,2-diglyceride acetates on ordinary TLC, and argentation TLC showed that they were not contaminated by each other.

Comparison with model compounds on argentation TLC showed that the fast fraction contained zero to three, and the middle fraction five to six double bonds per molecule. The slowest fraction was studied more closely. The molar ratio of long-chain ester, acetyl ester, and glycerol in this fraction was 2.07:0.89:1.0 (Theory 2.0:1.0:1.0). Analysis of the long-chain fatty acids revealed 23% eicosapentaenoic (20:5) and 72% docosahexaenoic acid (22:6). GLC of the slow fraction on SE-30 (3) revealed one small and two large peaks (Fig 1). Their carbon numbers, based on saturated 1,2-diglyceride acetates, were 38.7, 40.4, and 42.1 respectively when only the carbon atoms in the two long chains were considered. The peaks likely represented the molecular species 20:5-20:5-2:0, 22:6-20:5-2:0, and 22:6-22:6-2:0. In conformity with this the material revealed after hydrogenation one small and two large peaks of carbon numbers 40.0, 42.0, and 43.9.

These conclusions were confirmed and extended by converting another sample of the herring lecithin into dimethyl phosphatidates by hydrolysis with phospholipase D (EC 3. 1. 4. 4) and subsequent methylation with diazomethane (1,4). These derivatives were fractionated like the diglyceride acetates by argen-

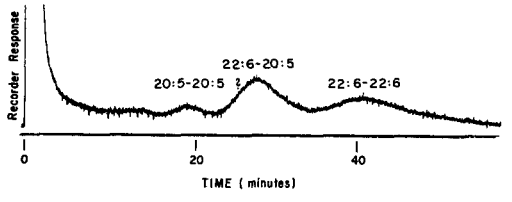

FIG. 1. GLC of highly unsaturated diglyceride acetates (the slow fraction), derived from Baltic herring lecithin.

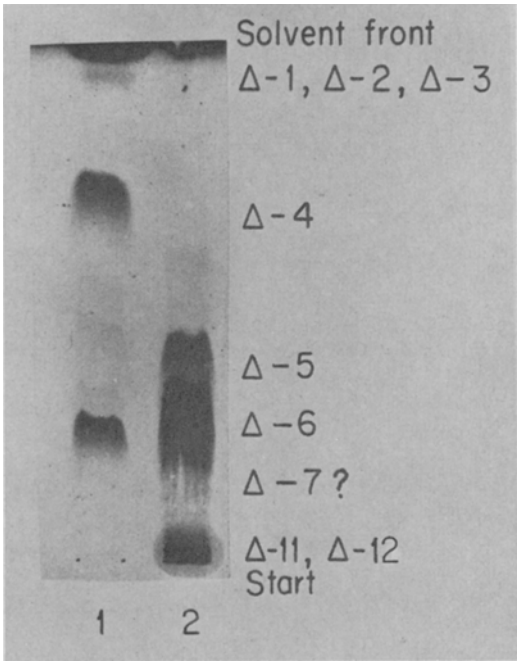

FIG. 2. Argentation TLC of dimethyl phosphatidates with chloroform-methanol-water (90:10:1). 1. Derivatives of egg lecithin. 2. Derivatives of Baltic herring lecithin.

tation TLC (Fig. 2). The most unsaturated fraction was isolated and partially hydrolyzed with pancreatic lipase (EC 3. 1. 1. 3). The reaction proceeded in the same manner as that of authentic dimethyl phosphatidate; free fatty acids and lysophosphatidic acid dimethyl esters as well as intact starting material were found in both reaction mixtures. The lysolipid formed from the herring derivative contained about equal amounts of 20:5 and 22:6; these are C-2 fatty acids according to the model experiment. The free fatty acid fraction of the hydrolysate was almost pure 22:6 with traces of 20:5. This fraction represents C-1 acids according to the model experiment. Thus the principal molecular species of the slow fraction must have been dimethyl phosphatidates of the structure 22:6-20:5 and 22:6-22:6.

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Lipid Contaminants: Polypropylene Apparatus and Vacuum Pumps

SEVERAL REPORTS have been issued on lipid contaminants from various laboratory sources, such as vacuum pump exhausts, cigarette smoke, hand cream, rubber tubing, and others (1); plastic bags, probably vinyl (2); and polyethylene bottles (3). It has been shown that dry adsorbent powders can take up lipids from plastic containers without the intervention of a solvent. (It appears that adsorbent manufacturers are still disregarding this source of contamination.) Tygon tubing and related brands of vinyl tubing have been known to contribute their plasticizers to solvents.

To this list must be added polypropylene. This plastic is often described as resistant to organic solvents, but we have noted that several lipids are rapidly extracted from graduated polypropylene cylinders when they are used to measure out methanol, chloroform, or hexane. The lipids are readily detected on evaporating the solvents to dryness. Thin-layer chromatography on Silica Gel G with hexane-ether (85/15) disclosed seven spots, detected with alkaline bromothymol blue. The R_f values (multiplied by 100) were *0*, 3, *20*, 23, 28, *75*, and 80. The numbers in italics refer to the more intense spots. The contaminants have not been identified, but we know from the fabricator of the cylinders that antioxidants are added to the plastic.

We thought that prolonged soaking might leach out the contaminants, thus making the cylinders usable in lipid work. Two four-day periods of standing with chloroform-methanol (1:1) yielded much solid matter in both extracts. By this time the cylinders were no longer straight, and the attempt was discontinued. It is possible that apparatus of less sensitive shape could be cleaned up adequately by sufficient soaking.

The problem of vacuum pump oil contamination of laboratory atmospheres can be eliminated quite nicely by attaching filters to the pump exhausts. These filters are available from the Arthur H. Thomas Company. They are excellent also for decreasing the noise of vacuum pumps.

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Lipid Contaminants: Polypropylene Apparatus and Vacuum Pumps

SEVERAL REPORTS have been issued on lipid contaminants from various laboratory sources, such as vacuum pump exhausts, cigarette smoke, hand cream, rubber tubing, and others (1); plastic bags, probably vinyl (2); and polyethylene bottles (3). It has been shown that dry adsorbent powders can take up lipids from plastic containers without the intervention of a solvent. (It appears that adsorbent manufacturers are still disregarding this source of contamination.) Tygon tubing and related brands of vinyl tubing have been known to contribute their plasticizers to solvents.

To this list must be added polypropylene. This plastic is often described as resistant to organic solvents, but we have noted that several lipids are rapidly extracted from graduated polypropylene cylinders when they are used to measure out methanol, chloroform, or hexane. The lipids are readily detected on evaporating the solvents to dryness. Thin-layer chromatography on Silica Gel G with hexane-ether (85/15) disclosed seven spots, detected with alkaline bromothymol blue. The R_f values (multiplied by 100) were *0*, 3, *20*, 23, 28, *75*, and 80. The numbers in italics refer to the more intense spots. The contaminants have not been identified, but we know from the fabricator of the cylinders that antioxidants are added to the plastic.

We thought that prolonged soaking might leach out the contaminants, thus making the cylinders usable in lipid work. Two four-day periods of standing with chloroform-methanol (1:1) yielded much solid matter in both extracts. By this time the cylinders were no longer straight, and the attempt was discontinued. It is possible that apparatus of less sensitive shape could be cleaned up adequately by sufficient soaking.

The problem of vacuum pump oil contamination of laboratory atmospheres can be eliminated quite nicely by attaching filters to the pump exhausts. These filters are available from the Arthur H. Thomas Company. They are excellent also for decreasing the noise of vacuum pumps.

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Calculation of the NMR Spectrum of Double-Bond Protons in Aliphatic Systems[1]

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ABSTRACT

A simplified subspectral method is presented to determine coupling constants and chemical shifts for the group $-CH_2-CH=CH-CH_2-$ in aliphatic compounds. Results are given for methyl esters of oleic, elaidic, erucic, and linoleic acid. These results are in agreement with more elaborate calculations.

INTRODUCTION

VALUES OF CHEMICAL SHIFTS and coupling constants obtained by detailed analysis of NMR spectra are commonly used as a source of structural information. In problems involving the configuration at a carbon-carbon double bond a knowledge of the coupling constants often distinguishes clearly between *cis* and *trans* isomers. A type of double bond frequently met in aliphatic compounds is characterized by the group $-CH_2-CH=CH-CH_2-$. Analysis of the spectrum of the protons at the double bond is, in principle, straightforward and gives the coupling constants necessary for a structural assignment. In the analysis of actual spectra, complications arise owing to the large number of lines produced by coupling with the CH_2 groups. It is shown in the following sections that the total spectrum can be considered as consisting of several partly overlapping subspectra. Classification of the subspectra and formulae for line positions and intensities are given, and the method is applied to some fatty acid esters.

THEORY

It is possible in certain limiting cases to introduce an effective chemical shift in the analysis of NMR spectra (1). The effective chemical shift δ_A of a nucleus A is defined as the chemical shift in the external field δ_0, corrected by the contributions from spin-spin coupling with nuclei N, for which Eq. (1) is valid.

$$J_{AN}/(\delta_A-\delta_N)<<1 \qquad (1)$$

Since the spin orientations of the nuclei N can combine in several ways, there are a number of effective chemical shifts corresponding to A.

The chemical shifts of $-CH=$ and $-CH_2-$ are in the present case approximately 5 ppm and 2 ppm, measured downfield from TMS. The coupling constants J_2 and J_1 (Fig. 1) are of the order of 10 Hz. At a resonance frequency of 60 MHz, Eq. (1) is accordingly fulfilled, and we can assign effective chemical shifts to H_A and H_B by including the spin contributions from H_1, H_2, H_3, H_4. Where $\delta_A = \delta_B$ we obtain a single line spectrum, as usual for two equivalent protons, while $\delta_A \neq \delta_B$ gives an AB pattern of four lines. It is possible in this way to subdivide the spectrum into a number of single lines and AB patterns, which might be partly overlapping.

To proceed further, we shall distinguish between two main cases on the basis of the symmetry properties of the surroundings of the double bond. The symmetric case is symbolized in Fig. 1 and the asymmetric case in Fig. 4.

The Symmetric Case

The simplified system in Fig. 1 will be used to calculate the line pattern for H_a and H_b. Application of the model implies that the double bond is in symmetrical surroundings. This is a good approximation in aliphatic chains when two or more CH_2 groups are linked to each side of the double bond. Fig. 1 implies further, that the coupling between a β-CH_2 and a double-bond proton tends to zero. As the results will show, this assumption is largely justified.

Since there is essentially free rotation about the C-C single bonds, protons in a CH_2 group will be equivalent. So long as we regard the long-range coupling between the two $-CH_2$

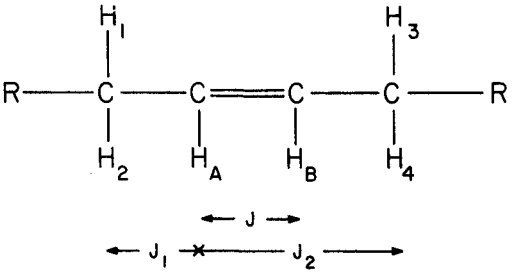

FIG. 1. Simplified model for double bond in symmetric surroundings.

[1]Issued as NRC of Canada Publication No. 10033.

TABLE I

Description of the Possible Subspectra Arising from Different Combinations of Spin in Two $-CH_2-$ Groups

Spin orientation of protons in CH_2 groups		Symmetric case	Asymmetric case
H_1H_2	H_3H_4	Species	Species
s_1	s_1	1	1
s_1	s_0		2
		2	
s_0	s_1		3
s_1	a_0		4
		3	
a_0	s_1		5
s_1	s_{-1}		6
		4	
s_{-1}	s_1		7
s_0	s_0	5	8
s_0	a_0		9
		6	
a_0	s_0		10
s_0	s_{-1}		11
		7	
s_{-1}	s_0		12
a_0	a_0	8	13
a_0	s_{-1}		14
		9	
s_{-1}	a_0		15
s_{-1}	s_{-1}	10	16

groups as infinitely small, each of them will be described adequately by the four stationary spin functions for a two-spin system (2): s_1 s_0 a_0 s_{-1}.[1] Combinations of these four possibilities for each of the CH_2 groups gives a total of 16 species, all of which can be taken as part of the effective chemical shift for H_A and H_B. The 16 systems (species) all occur with the same statistical weight. Owing to the symmetry of the system, only 10 different spin combinations are found (Table I), and the total spectrum can be regarded as the sum of 10 subspectra.

For coupling between groups with widely separated chemical shifts like $-CH=$ and $-CH_2-$, only the component of the spin in the direction of the magnetic field is of importance (2). The effective chemical shift of H_a will correspondingly differ from δ_0 by an amount $\pm J_1$ or 0, arising from the coupling to H_1 and H_2. The effect from both CH_2 groups is additive. In the following section the subspectra that make up the total spectrum are discussed in some detail.

Subspectra for the Symmetric Case. Species 5, 6, 8. When the two protons in a CH_2 group have a vanishing spin along the magnetic field, their contribution to the local field at the site of a double-bond proton is zero. The double-bond protons in this species will therefore have the same chemical shift δ_0. Two protons with identical chemical shifts, which still have the same resonance field after inclusion of spin coupling to all other spins, give no observable spin-spin splitting. We shall, therefore, expect only one line with an intensity ¼ of the total spectrum at the true chemical shift δ_0.

Species 1. In this system we have a net influence on the effective chemical shift coming from the parallel spins in the CH_2 groups. Using the notation indicated in Fig. 1, H_1 and H_2 will together contribute $+J_1$ to the shift of H_a and $+J_2$ to the shift of H_b. In the same way H_3 and H_4 contribute $+J_2$ and $+J_1$ at H_A and H_B. The result is that both protons show an increased effective chemical shift $\delta_A = \delta_B = \delta_0 + J_1 + J_2$. With the same argument as in the former case one expects a one-line spectrum of intensity 1/16 at the position $\delta_0 + (J_1 + J_2)$.

Species 10. All the arguments mentioned for species 1 apply to this case except for the sign of the local contributions to the effective chemical shift. The resultant spectrum is a single line of intensity 1/16 at $\delta_0 - (J_1 + J_2)$.

Species 2, 3. In this case the local fields at the two double-bond protons will differ, i.e., $\delta_A \neq \delta_B$. One proton will have the effective chemical shift $\delta_0 + J_1$, the other $\delta_0 + J_2$. The spectrum will be a four-line AB type of pattern with a separation J between inner and outer lines, centered at $\delta_0 + (J_1 + J_2)/2$. The summed intensity of the four lines is ¼. Line positions and intensities for the separate lines are given in Table II.

Species 7,9. As a mirror image of Species 2,3 the spectrum is found to be an AB type of pattern centered at $\delta_0 - (J_1 + J_2)/2$.

Species 4. The effective chemical shifts of the two double-bond protons are $\delta_0 + (J_1 - J_2)$ and $\delta_0 - (J_1 - J_2)$. The AB spectrum will be centered at the undisturbed chemical shift δ_0 with a summed intensity of ⅛.

In Table II line positions and intensities are given for all 15 lines as a function of J, J_1, and J_2. It will be noted that an important feature in the spectrum will be a triplet, with splitting $(J_1 + J_2)$ and intensity ratio 1:4:1, which accounts for ⅜ of the total intensity.

Procedures and Results for Monoenes. The data in Table II were derived without indication of the possible *cis-trans* configuration at the double bond, and the considerations apply equally well for both types.

To find the actual values of the coupling

[1] s_1 : $\alpha\alpha$
s_0 : $(\alpha\beta+\beta\alpha)/\sqrt{2}$
a_0 : $(\alpha\beta-\beta\alpha)/\sqrt{2}$
s_{-1} : $\beta\beta$

TABLE II

Line Positions and Intensities for $-CH_2-CH=CH-CH_2$ in Symmetric Surroundings

$M_2 = (J_1+J_2)/2$
$Q_1 = 4(J_1-J_2)^2 + J^2$
$Q_2 = (J_1+J_2)^2 + J^2$
$F_1 = (Q_1+J)/(Q_1-J)$
$F_2 = (Q_2+J)/(Q_2-J)$

Species	Line No.	Line position	Line intensity × 16
1	1	$+J_1+J_2$	1
2, 3	2	$M_2+(Q_2+J)/2$	$2/(F_2+1)$
	3	$M_2+(Q_2-J)/2$	$2F_2/(F_2+1)$
	4	$M_2-(Q_2-J)/2$	$2F_2/(F_2+1)$
	5	$M_2-(Q_2+J)/2$	$2/(F_2+1)$
4	6	$(Q_1+J)/2$	$1/(F_1+1)$
	7	$(Q_1-J)/2$	$F_1/(F_1+1)$
	8	$-(Q_1-J)/2$	$F_1/(F_1+1)$
	9	$-(Q_1+J)/2$	$1/(F_1+1)$
5, 6, 8	10	0	4
7, 9	11	$-M_2+(Q_2+J)/2$	$2/(F_2+1)$
	12	$-M_2+(Q_2-J)/2$	$2F_2/(F_2+1)$
	13	$-M_2-(Q_2-J)/2$	$2F_2/(F_2+1)$
	14	$-M_2-(Q_2+J)/2$	$2/(F_2+1)$
	15	$-J_1-J_2$	1

constants, certain limiting values can be imposed by inspection of the experimental spectrum. The final determination is made by trial and error, with a synthesis of the total spectrum for different values of the parameters. When theoretical line spectra show that a close fit has been obtained, further refinement can be obtained by computing and plotting the summed total spectrum (vide infra). By this method, values given in Table III for different monoenes are found. The calculated values all lie within the empirical limits given in most textbooks (2). The identical value of J found for the two *cis* monoenes justifies the assumption of spectral independence of differences in chain structure which are remote from the double bond.

The values of J_1 are seen to change by 0.5 Hz from the *cis* to the *trans* configuration. Changes of this magnitude would be expected by comparison with the results for 1,3-dichloro propene (3). Likewise the small negative value of J_2 is close to the values given for a series of unsaturated molecules (4).

The chemical shifts of the double-bond protons in *cis* and *trans* compounds have been measured in CCl_4 solutions (ca. 1 mole/liter). Oleic and erucic acid esters gave a shift of 5.27 ppm and elaidic acid ester 5.33 ppm, downfield from internal TMS.

The calculated spectrum for oleic acid ester is reproduced in Fig. 2, and a comparison with the experimental spectrum (5) shows that it is possible to account for all line positions and intensities except for a slight asymmetry in intensity towards higher field. Fig. 3 shows the calculated spectrum of elaidic acid ester. It is interesting to compare the spectra in Fig. 2 and 3. With $J_{trans} > J_{cis}$ the width of the spectrum of elaidic acid should be bigger than for oleic acid. Even though this is true, the intensity distribution in the spectrum is seen to make oleic acid appear to have the wider spectrum.

The Asymmetric Case

In the preceding sections it was assumed that the surroundings of the double bond were symmetric, giving rise to a spectrum with three coupling constants and one chemical shift. This approximation is valid if the double bond is surrounded by several CH_2 groups on both

TABLE III

Coupling Constants in Some Unsaturated Fatty Acid Esters

Compound	J_1	J_2	J_3	J_4	J
Oleic acid methyl ester	7.0	−1.6	7.0	−1.6	10.6
Elaidic acid methyl ester	6.5	−1.4	6.5	−1.4	15.2
Erucic acid methyl ester	7.0	−1.6	7.0	−1.6	10.6
Linoleic acid methyl ester	8.2	−1.2	6.8	−2.2	10.7

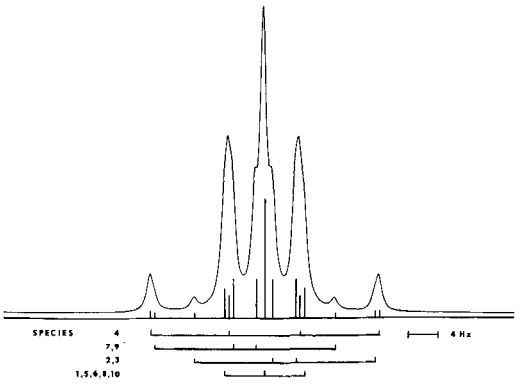

FIG. 2. Theoretical spectrum of double-bond protons in oleic acid. At the bottom line, positions are given for the individual subspectra. Above this the total line spectrum is given, and at the top, the contour of the summed spectrum.

sides. If this is not the case, it is still possible to divide the spectrum into a number of subspectra originating with different species, as described for the symmetric case, although the number of subspectra now increases.

Asymmetric surroundings can give rise to three types of spectra: I. five different coupling constants and two different chemical shifts for the double-bond protons; II. three different coupling constants and two different chemical shifts for the double-bond protons; and III. five different coupling constants and one chemical shift common to both double-bond protons.

In the following sections the subspectra for type III will be developed and applied to the determination of the coupling constants in linoleic acid ester.

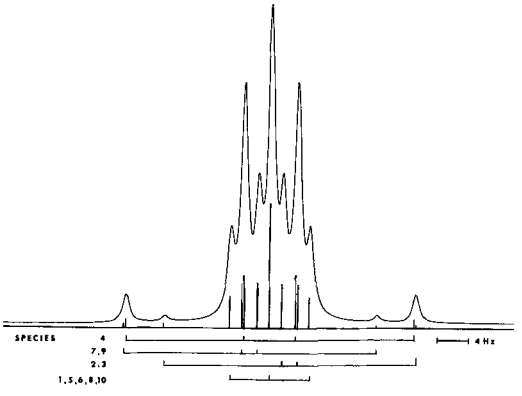

FIG. 3. Theoretical spectrum of double-bond protons in elaidic acid. At the bottom line, positions are given for the individual subspectra. Above, the total line spectrum is given, and at the top, the contour of the summed spectrum.

Subspectra for Asymmetric Case III. In Table I the possible spin combinations for neighboring CH_2 groups are given. In this asymmetric case there are 16 different species, which give rise to nine subspectra (Table IV).

Species 8,9,10,13. The same arguments apply as in the symmetric case. No influence on the chemical shift of H_a and H_b can be expected when the total spin of each CH_2 group in the direction of the field is zero. The result is a single line representing 1/4 of the total intensity, located at the true chemical shift δ_o of the double-bond protons.

Species 1. Although this spin combination in the symmetric case resulted in the same effective chemical shifts for H_a and H_b, one now gets two different shifts. Using the notation defined in Fig. 4, the effective shifts become: $\delta_a = \delta_o + J_1 + J_4$; $\delta_B = \delta_o + J_2 + J_3$. Spin coupling between H_a and H_b gives rise to an AB type of pattern centered at $\delta_o + (J_1 + J_2 + J_3 + J_4)/2$, corresponding to 1/16 of the total intensity.

Species 16. The sign of the local contributions are reversed as compared with species 1. The effective chemical shifts are given by $\delta_A = \delta_o - (J_1 + J_4)$; $\delta_B = \delta_o - (J_2 + J_3)$ and lead to an AB type of spectrum similar to species 1 but centered at $\delta_o - (J_1 + J_2 + J_3 + J_4)/2$.

Species 3,5. In these the contributions to the effective chemical shifts from H_1 and H_2 cancel with the effects of H_3 and H_4 and lead to effective chemical shifts $\delta_o + J_3$ and $\delta_o + J_4$ for H_A and H_B respectively. The resulting AB type of spectrum is centered at $\delta_o + (J_3 + J_4)/2$ and has a summed intensity of 1/8.

Species 11,14. The spectrum is a mirror image of the one calculated for Species 3,5 with respect to the true chemical shift δ_o.

Species 2,4; 12,15. From the arguments for Species 3,5 are obtained two AB type of spectra located symmetrically around δ_o. One corresponds to Species 2 and 4, with its center at $\delta_o + (J_1 + J_2)/2$; the other corresponds to Species 12 and 15, centered at $\delta_o - (J_1 + J_2)/2$. Each of the AB patterns has a summed intensity of 1/8.

Species 6. H_1 and H_2 contribute to the effective chemical shifts of H_A by $+J_1$ and of H_B by $+J_2$. At the same time H_3 and H_4 give contributions of $-J_4$ and $-J_3$ to H_A and H_B respectively. The final effective shifts of the two protons are $\delta_A = \delta_o + J_1 - J_4$; $\delta_B = \delta_o + J_2 - J_3$. As a result, one obtains an AB type of spectrum located at the position $\delta_o + (J_1 + J_2 - J_3 - J_4)/2$ with a summed intensity of 1/16.

TABLE IV

Line Positions and Intensities for $-CH_2-CH=CH-CH_2-$ in Asymmetric Surroundings

$M_1 : (J_1+J_2+J_3+J_4)/2$
$M_2 : (J_3+J_4)/2$
$M_3 : (J_1+J_2)/2$
$M_4 : (J_1+J_2-J_3-J_4)/2$
$Q_1 : (J_1-J_2-J_3+J_4)^2+J^2$
$Q_2 : (J_3-J_4)^2+J^2$
$Q_3 : (J_1-J_2)^2+J^2$
$Q_4 : (J_1-J_2+J_3-J_4)^2+J^2$
$F_1 : (Q_1+J)/(Q_1-J)$
$F_2 : (Q_2+J)/(Q_2-J)$
$F_3 : (Q_3+J)/(Q_3-J)$
$F_4 : (Q_4+J)/(Q_4-J)$

Species	Line No.	Line position	Line intensity × 32
1	1	$M_1+(Q_1+J)/2$	$1/(F_1+1)$
	2	$M_1+(Q_1-J)/2$	$F_1/(F_1+1)$
	3	$M_1-(Q_1-J)/2$	$F_1/(F_1+1)$
	4	$M_1-(Q_1+J)/2$	$1/(F_1+1)$
2, 4	5	$M_3+(Q_3+J)/2$	$2/(F_3+1)$
	6	$M_3+(Q_3-J)/2$	$2F_3/(F_3+1)$
	7	$M_3-(Q_3-J)/2$	$2F_3/(F_3+1)$
	8	$M_3-(Q_3+J)/2$	$2/(F_3+1)$
3, 5	9	$M_2+(Q_2+J)/2$	$2/(F_2+1)$
	10	$M_2+(Q_2-J)/2$	$2F_2/(F_2+1)$
	11	$M_2-(Q_2-J)/2$	$2F_2/(F_2+1)$
	12	$M_2-(Q_2+J)/2$	$2/(F_2+1)$
6	13	$M_4+(Q_4+J)/2$	$1/(F_4+1)$
	14	$M_4+(Q_4-J)/2$	$F_4/(F_4+1)$
	15	$M_4-(Q_4-J)/2$	$F_4/(F_4+1)$
	16	$M_4-(Q_4+J)/2$	$1/(F_4+1)$
7	17	$-M_4+(Q_4+J)/2$	$1/(F_4+1)$
	18	$-M_4+(Q_4-J)/2$	$F_4/(F_4+1)$
	19	$-M_4-(Q_4-J)/2$	$F_4/(F_4+1)$
	20	$-M_4-(Q_4+J)/2$	$1/(F_4+1)$
8, 9, 10, 13	21	0	8
11, 14	22	$-M_2+(Q_2+J)/2$	$2/(F_2+1)$
	23	$-M_2+(Q_2-J)/2$	$2F_2/(F_2+1)$
	24	$-M_2-(Q_2-J)/2$	$2F_2/(F_2+1)$
	25	$-M_2-(Q_2+J)/2$	$2/(F_2+1)$
12, 15	26	$-M_3+(Q_3+J)/2$	$2/(F_3+1)$
	27	$-M_3+(Q_3-J)/2$	$2F_3/(F_3+1)$
	28	$-M_3-(Q_3-J)/2$	$2F_3/(F_3+1)$
	29	$-M_3-(Q_3+J)/2$	$2/(F_3+1)$
16	30	$-M_1+(Q_1+J)/2$	$1/(F_1+1)$
	31	$-M_1+(Q_1-J)/2$	$F_1/(F_1+1)$
	32	$-M_1-(Q_1-J)/2$	$F_1/(F_1+1)$
	33	$-M_1-(Q_1+J)/2$	$1/(F_1+1)$

Species 7. This is related to Species 6 by a change of sign for the local contributions to the effective chemical shifts. An AB type of pattern corresponding to $\delta_A = \delta_o-(J_1-J_4)$; $\delta_B = \delta_o-(J_2-J_3)$ will result. The spectrum is centered at $\delta_o-(J_1+J_2-J_3-J_4/2$, with a total intensity of 1/16.

In Table IV line positions and intensities are listed as functions of the five parameters involved.

Experimental Results for Linoleic Acid. According to the theoretical predictions, 33 lines are expected in the spectrum of linoleic acid.

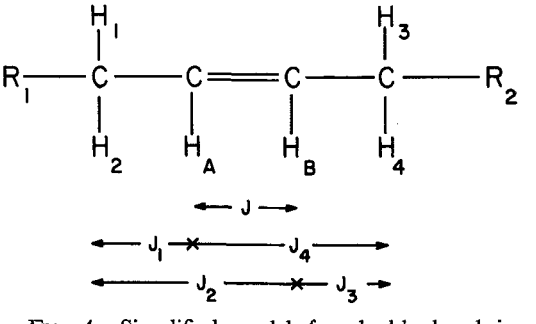

FIG. 4. Simplified model for double bond in asymmetric surroundings.

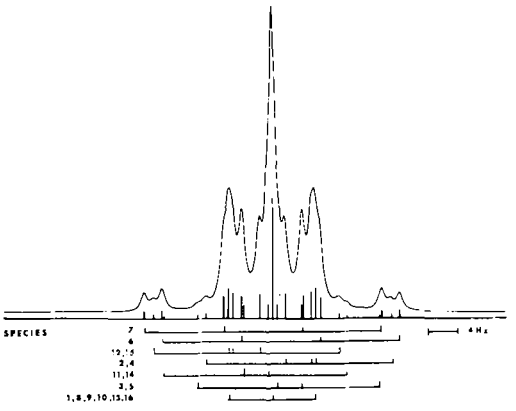

FIG. 5. Theoretical spectrum of double-bond protons in linoleic acid. At the bottom line, positions are given for the individual subspectra. Above, the total line spectrum is given, and at the top, the contour of the summed spectrum. In the example, calculated quartets corresponding to Species 1 and 16 have degenerated to a single line each.

The number of lines appearing in the experimental spectrum is far less than this, which makes an unambiguous interpretation impossible. By introducing the reasonable assumption that the coupling constants are not drastically different from those found in oleic acid, it is possible to find only one combination that fits the observed spectrum. Comparison of the data given in Table III shows that the double-bond coupling constants are the same for the *cis* isomers studied. The coupling constants to the CH_2 groups differ slightly from those observed for oleic acid. This might be attributable to minor changes in electron distribution but is more likely an effect of a change in statistical weight of the rotational isomers about the =C–C– bond. The latter interpretation is supported by the value of the chemical shift 5.28 ppm, the same as for the *cis* monoenes.

DISCUSSION

In applying the subspectra method, two cycles of adjustments are generally needed. On the basis of empirical information from related molecules, a theoretical spectrum can be calculated by using Table I or II, and comparison with the experimental spectrum allows a rough assignment of subspectra. By means of the explicit relations between line positions and spectral parameters, further adjustment can be made until the calculated line spectrum agrees with the experimental one. To obtain a more sensitive check on the validity of the derived parameters, an envelope of the theoretical spectrum is calculated using a Lorentzian line shape and a common half-line width for all lines. Small adjustments in the parameters will then bring the envelope into close agreement with that of the experimental spectrum. Our experience indicates the uncertainty in the final parameters to be less than 0.2 Hz. As a consequence of the approximations involved in the use of effective chemical shifts, it will not be possible to match the experimental spectrum completely. The difference occurs in the intensity distribution. Although the experimental spectrum is slightly asymmetric, the calculated spectrum is symmetric. Where the ratio in Eq. (1) is ~ 0.05, asymmetry in the intensity distribution only amounts to a few per cent.

To assume the validity of the final parameters, a digital calculation using the LAOCOON IV program (6) was performed. The spectra thus obtained agreed with those obtained by the subspectral method within 0.1 Hz.

The subspectral method is seen to have two general applications.

In spectra exhibiting a number of separate sharp peaks, an easy way of obtaining a reliable set of starting parameters for digital computation and iteration (6) is provided by the subspectral method.

Spectra consisting mainly of a strongly overlapping pattern are not compatible with the iterative procedures in digital computation, and the analysis has to be done by trial and error. Examples of this type are analyzed more efficiently by use of the subspectral method. In problems where the ratio in Eq. (1) is considerably bigger than in the present case, a complete solution cannot be expected by use of subspectra. In such cases, results of a subspectral analysis still provide a valuable basis for further digital refinement.

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Influence of Sex and Gonadal Hormones on Lipid Metabolism in Essential Fatty Acid-Deficient Rats[1]

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ABSTRACT

Previous work has shown differences between male and female rats in their ability a) to mobilize linoleic acid from adipose tissue when the supply is limited; b) to maintain higher levels of circulating and liver arachidonic acid when dietary linoleic acid is limited; c) to prevent accumulation of cholesteryl ester (CE) in the liver; and d) to increase the proportions of polyunsaturated fatty acids (PUFA) in their plasma lecithins.

Recent studies are reviewed which show that a) essential fatty acid (EFA)-deficient rats exhibit the same kinds of sex differences as do rats on complete diets; b) these differences are mediated or at least influenced by estrogen; c) some of the differences may be attributed to differences in body size which result in less need for PUFA in structural phospholipid (PL); d) the rate of conversion of linoleic to arachidonic acid may differ under certain experimental conditions; and e) female rats have higher proportions of stearic and arachidonic acids in their liver lecithins than do males, which may relate to their higher rate of lecithin synthesis via methylation of phosphatidylethanolamine.

INTRODUCTION

OBSERVATIONS THAT SEX DIFFERENCES exist in the metabolism of fat extend back to the early studies of Burr and Burr (1), who showed that female rats which were made deficient in essential fatty acids (EFA) stored more fat than did comparable males. This effect was more pronounced when the rats were fed a diet rich in saturated fat than when they received a fat-free diet (2). These authors suggested that the female rat had a more efficient system for utilizing its EFA than did the male and conversely that the male rat was more sensitive to an EFA deficiency than was the female, especially when the bulk of the calories was derived from fat.

Subsequently it was demonstrated that the requirement for linoleic acid in female rats was about one-third that of the males (3) and that males showed greater depression of growth and more severe dermal symptoms when fed an EFA-deficient, fat-free diet.

Since that time there has followed a series of papers from a number of laboratories which have suggested possible sex differences in the intermediary metabolism and utilization of the EFA. Coleman et al. (4) demonstrated sex differences in the requirement for EFA as regulators of cholesterol metabolism. Gonadectomy of EFA-deficient rats, fed a fat-free diet, appeared to favor esterification of liver cholesterol with saturated fatty acids and a reversal of sex differences in lipid metabolism, particularly in the males. Morton and Horner (5) showed that EFA deficiency in the absence of fat led to a greater accumulation of liver triglyceride (TG) and cholesteryl ester (CE) in male rats than in females and to greater changes in the fatty acid pattern of liver lipids. The increase of trienoic and decrease of dienoic acids in the liver lipid classes were much more pronounced in males than in females. Cholesterol supplementation, which tends to increase the severity of the effect of an EFA deficiency (6), decreased the concentration of dienoic and tetraenoic acids in the (PL) of livers of male rats appreciably more than in those of females. Thus female rats appeared to utilize available linoleic acid more efficiently than did males.

In the course of studies concerning the effects of dietary cholesterol, Lis and Okey (7) and Okey et al. (8) observed sex differences in the levels and in the composition of both liver and plasma CE. They indicated differences in the metabolism of PUFA when rats were fed a diet containing either cottonseed or coconut oil, i.e., a diet rich or poor in linoleic acid. Experiments designed to measure retention of linoleic acid in adipose tissue when a dietary fat, high in linoleic acid, was changed to a low one showed that female animals were better able to mobilize linoleic acid from adipose tissue than were comparable males (9,10).

A further indication of differences between

[1]Presented at the symposium on essential fatty acids in honor of George O. Burr, AOCS Meeting, Los Angeles, April 1966.

males and females in intermediary metabolism of EFA was the finding that cholesterol-fed female rats maintained higher proportions of arachidonic acid in PL of plasma and liver, as well as in plasma CE, than did males. The ratio of stearic : palmitic acids in the PL of both liver and plasma was higher in females than in males, indicating a possible difference in the distribution of PL subclasses (11). Monsen et al. (10) subsequently showed that neither the presence nor absence of cholesterol or widely varying amounts of dietary linoleic acid influenced these sex differences in plasma PL fatty acid composition.

In order to determine whether sex hormones would produce differences in lipid composition similar to those observed in intact male and female rats, Lyman et al. (13) studied the effects of small doses of testosterone and estradiol on the lipids of liver and plasma in castrated male rats. The results demonstrated that estradiol-injected, castrated males had levels of plasma cholesteryl arachidonate similar to the high levels in females whereas castrated controls had lower levels, resembling those of testosterone-injected castrates and intact males. Furthermore, the ratios of stearic : palmitic acids in the plasma PL were about twice as high in the intact female and estradiol-treated rats as in the males or androgen-treated animals.

Fractionation of the plasma PL into subclasses and determination of the fatty acid composition in each class demonstrated that differences in the ratios of fatty acid resulted not from a change in the relative proportions of the subclasses as first hypothesized but were attributable principally to a higher level of stearic and arachidonic acids in the lecithin fraction of female or estrogen-injected rats as compared with intact male and testosterone-treated animals. These effects seemed strongly influenced by estradiol because the absence of testosterone in the castrated controls did not change the "male" pattern. Analysis of liver lipids of these animals showed similar sex differences in their fatty acid patterns (Lyman et al., unpublished).

Thus available information up to the time of these studies indicated differences between male and female rats in their ability a) to mobilize linoleic acid from adipose tissue when the supply is limited; b) to maintain levels of circulating cholesteryl arachidonate and of PL-arachidonate in both liver and plasma when dietary linoleic acid is low; and c) to prevent CE accumulation in the liver under certain dietary conditions. Furthermore, the sex differences seemed to be mediated or at least strongly influenced by estradiol. This led to the question whether the decrease of susceptibility of female rats to the effects of an EFA deficiency could be demonstrated and explained by sex differences in the utilization or metabolism of EFA. We therefore studied sex differences and hormone influences in the mobilization, distribution, and metabolism of EFA during the onset of an EFA deficiency.

MOBILIZATION OF LINOLEIC ACID

Differences in the utilization of EFA would be expected to be more evident when the supply of EFA is limited, particularly during the early stages of a deficiency, and before the concentration of EFA reached similarly low levels in both sexes. In order to evaluate the sequence of events during the development of an EFA deficiency, we attempted to obtain data from nearly all major lipid compartments of the body. Therefore the lipids of plasma, liver, and carcass of groups of rats were determined at intervals during the development of an EFA deficiency (14,15). In addition to intact males and females, groups of castrated male rats were included, injected with either estradiol benzoate, 30 μg/week (E group), testosterone propionate, 2 mg/week (T group), or hydrogenated coconut oil which served as carrier of the hormones (O group). These groups were intended to show whether differences between males and females were caused primarily by the effects of the estrogen or by those of the androgen. The diets were of a semipurified nature and contained adequate levels of protein (casein and albumin), vitamins, minerals, choline, and methionine. The source of fat was 10% of hydrogenated coconut oil ($<0.01\%$ linoleic acid by analysis). Five or more rats of each group were killed for the lipid analyses of each individual rat before the diet was fed and at 2, 3, 4, 6, and 9 weeks after the diet had been fed. The animals grew normally for about 4-6 weeks, after which time their weight gains began to level off. As expected, the males (and castrated males injected with testosterone or coconut oil) grew more than did the females (and castrated males injected with estradiol) and were about 30 g heavier after 9 weeks on the deficient diet.

Carcass Composition

Carcass fat increased in all groups during the first 4 weeks of the deficiency, then remained quite constant at about 10% of carcass weight. Fig. 1 shows that, although the percentage of linoleic acid in the carcass fat of

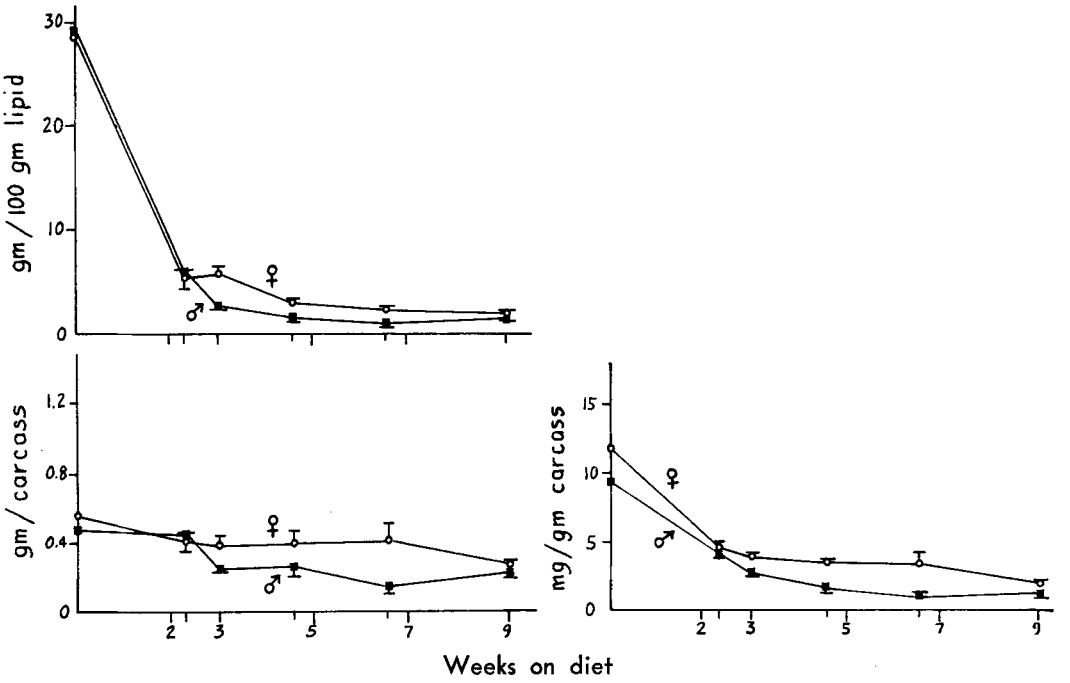

FIG. 1. Linoleic acid content of carcass fat of rats during the development of an EFA deficiency. ♂ = males, ♀ = females. Vertical lines on the figures are half SE values of the means from five or more rats.

both males and females decreased abruptly from 30 to about 5 in the first 2 weeks, total carcass linoleic acid decreased only slowly throughout the deficiency period (from about 500 mg to 300 mg in all groups). Because of the smaller body size of females, the concentrations of linoleic acid in their carcasses (mg/g carcass) was significantly greater than in those of males during the earlier stages of the deficiency. By 9 weeks however differences no longer were evident. It appeared therefore that mobilization of linoleic acid from carcass depot did not differ appreciably between males and females. Females, because of their smaller size, would have had more linoleic acid available, per unit of body weight, to meet EFA requirements of other tissues. These results appear to be contrary to those of the depletion study (9). It has been shown however (10) that the course of depletion of linoleic acid in mice is diphasic. Thus the rate of loss is much faster initially when the concentration of linoleic acid is relatively high (the case existing in the previous experiment) than when it is below a critical level. Since in the EFA-deficiency study all measurements were made after the time of rapid depletion of linoleic acid, it is possible that sex differences during this time were missed. Also, a recent publication (16) has shown that only about half of the carcass 18:2 acid of rats fed hydrogenated coconut oil for 95 days was the 9,12-isomer, linoleic acid. The remainder consisted of the 8,11 and 6,9-isomers. Since the isomeric 18:2 acids were not separately determined in the present experiment, it is impossible to state definitely whether males differed from females in their proportions of the three isomers. So, although it appears from the results presented in Fig. 1 that there are no significant sex differences in the total 18:2 acid removed from the lipid depot during EFA depletion, a final decision of the relative amounts of the possible isomers in both sexes must await a more complete analysis.

Liver Composition

Fig. 2 shows liver weights for the five groups of animals. As expected, males had larger livers than did females. Unexpectedly the E group (estradiol-injected) had livers as large as those of the males and the T group (testosterone-injected). Because of their smaller body weight, the estradiol-treated rats therefore had larger livers, per body weight, than

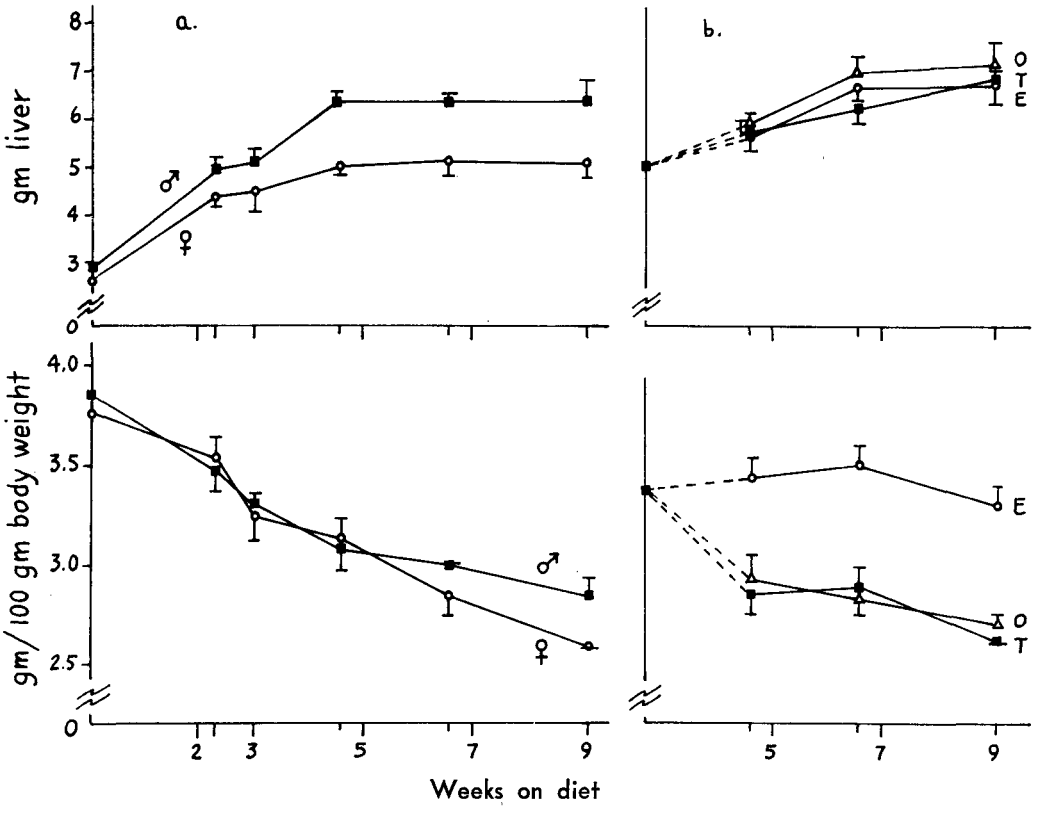

FIG. 2. Liver weights of rats during the development of an EFA deficiency: a) intact males (♂) and females (♀); b) castrated males treated with estradiol (E), testosterone (T), or the vehicle oil (O). Castration was performed, and hormone injections were begun 2 wk after the animals had been put on EFA-deficient diet. The first group of these animals was analyzed 1 wk later, i.e., after 3 wk on the diet.

those of any of the other groups. The reasons for this finding are not known. Possibly EFA-deficient rats have an increased sensitivity to the effects of estrogen because the same doses (30 μg/week), administered to animals which were fed a normal diet, did not produce such a response (13). Others have recently shown that relatively high doses of estradiol, administered to rats which were fed high-fat diets, greatly increased both α- and β-lipoproteins in the serum (17). A similar effect in the EFA-deficient rats, in response to a small dose of estrogen, might also explain the rise noted in plasma TG (Fig. 9).

The fat content of livers increased in all groups as the deficiency progressed (Fig. 3). Since the amount of liver lipids was proportional to liver weight, the intact males had higher lipid levels than did females, and the E group (estradiol-injected) had levels more closely resembling those of males than of females. Phospholipids comprised about 80% of the total liver lipids. The amount of this lipid at each of the intervals reflected closely the growth or weight of the liver (Fig. 2). Consequently females consistently had less liver PL than did males whereas the E group, with the enlarged livers, had PL levels similar to those of males and the T group (testosterone-injected).

Cholesteryl ester represented a minor portion of liver total lipid, equivalent to about 2% in males and only 0.6% in females. The amount remained unchanged in males during the course of the deficiency but was significantly decreased in females (Fig. 4). Castrated rats injected with estradiol showed a similar reduction in total CE in spite of the fact that their livers were as large as those of the male rats. Thus it appears that estradiol removed or prevented excessive accumulation of CE in the liver and that the effect was independent of liver size. Unesterified cholesterol (Fig. 4) and triglycerides (not shown) represented a small part

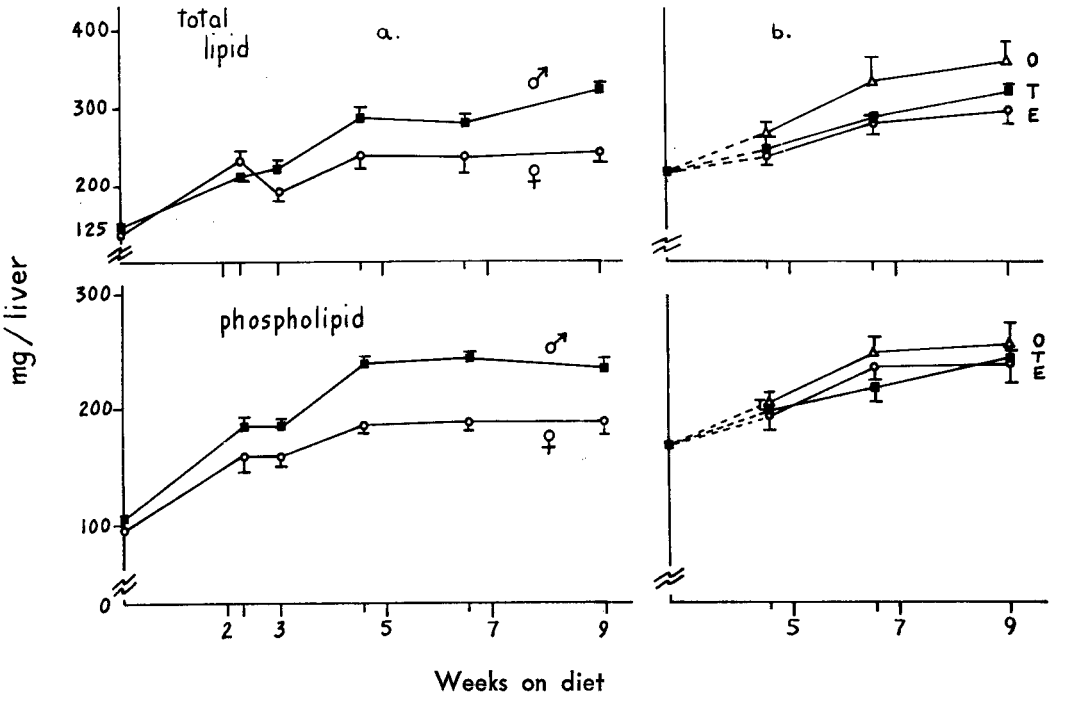

FIG. 3. Liver total lipid and phospholipid content of rats during the development of an EFA deficiency. (For symbols see Fig. 2.)

of the total liver lipid. No unusual differences were observed in these lipid fractions and generally their levels paralleled closely the size of the liver.

Fig. 5 shows the effect of the EFA deficiency on the proportions and amounts of liver cholesteryl linoleate and arachidonate. The proportion of cholesteryl linoleate fell to 3% or 4% in both males and females within the first 2 weeks of the deficiency and remained at that level. The proportion of cholesteryl arachidonate decreased promptly in males whereas females maintained the percentage of arachidonic acid initially present until late in the development of the deficiency. However, when the cholesteryl linoleate or arachidonate was calculated in terms of amount per liver, it is evident that males had quantities of both fatty acids similar to those of the females in spite of the greater percentage of CE in the male livers. It was the esters of oleic and palmitic acids and, to a certain extent, of palmitoleic and eicosatrienoic acids that contributed to the higher amounts of liver CE in the male rat. Aftergood and Alfin-Slater (18), on the basis of results with rats fed a fat-free diet, have suggested that estradiol may enhance esterification of cholesterol with the more unsaturated fatty acids and thus promote removal of cholesterol from the liver. Our results, obtained with rats fed hydrogenated coconut oil and given smaller doses of estradiol, are in agreement with this concept.

Liver PL accounted for more than 95% of the total liver arachidonate. Whereas the proportion of liver PL linoleate (not shown) decreased from about 13% to 5% after 2 weeks of the deficiency, the decrease in the proportion of arachidonate was much slower (Fig. 6). It was compensated by nearly equivalent increases in the proportion of an eicosatrienoic acid (20:3), identified as principally the 5,8,11-isomer derived from oleic acid. Females lost arachidonic acid and accumulated eicosatrienoic acid at a slower rate than did males. After 9 weeks of the deficient diet however, these differences had mostly disappeared.

Although there were significant sex differences in the proportions of arachidonic acid, no differences in the total amount of liver PL arachidonate or linoleate (Fig. 7) were apparent even though males had about 60-70 mg more PL in their livers than did females (Fig. 3). During the first 2 weeks of the deficiency, during a period of rapid liver growth (Fig. 2), the PL arachidonate actually increased. Since little or no change in the amount of liver linoleate occurred, all available lin-

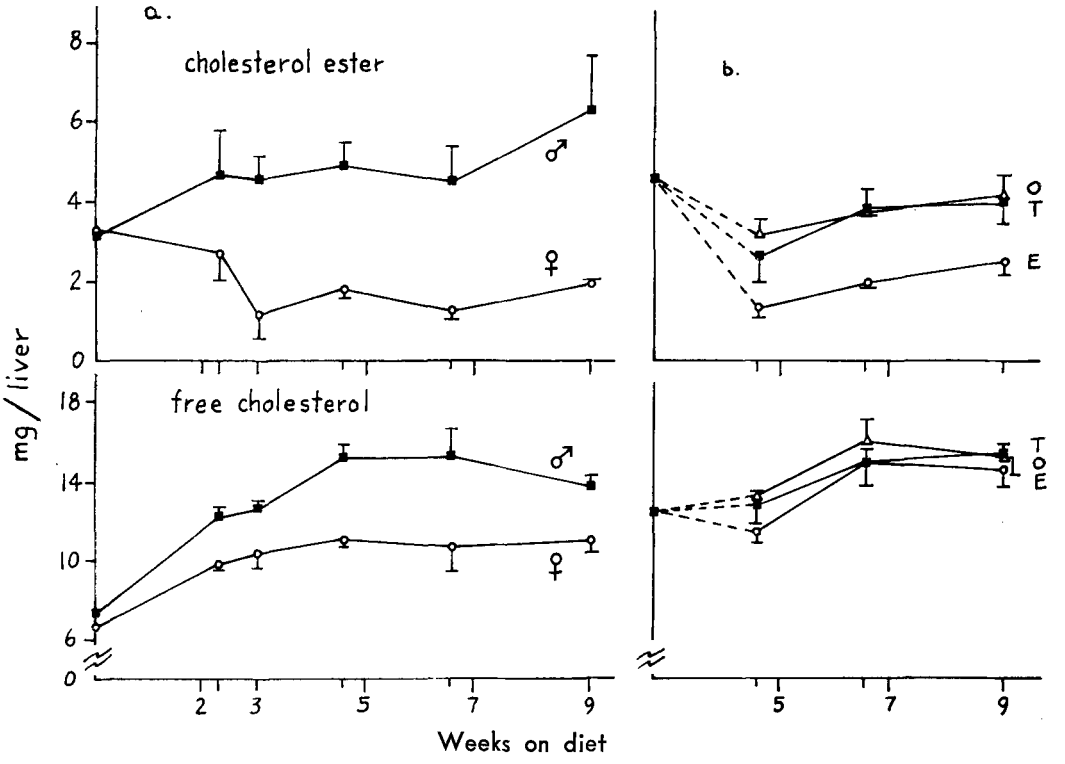

FIG. 4. Liver cholesteryl ester and free cholesterol content of rats during the development of an EFA deficiency. (For symbols see Fig. 2.)

oleic acid must have been rapidly converted into arachidonic acid, and the amount converted would appear to represent the maximum available in both sexes from the limited supplies of the precursor.

The much larger amounts of 20:3 and palmitic acids in the male liver PL (Fig. 8) suggests that, when the supply of arachidonic acid is limited, growth continues for a time by substituting the 20:3 acid for the arachidonic acid in the newly synthesized PL. The faster growth and concomitant greater need for PL synthesis result in the accumulation by males of PL, rich in 20:3 acid, in their tissues and organs more rapidly than do females. The earlier formation of relatively large amounts of such abnormal PL in tissues of male rats, with subsequent impairment of normal cell membrane function, may provide an explanation for the relative resistance of female rats to the symptoms of EFA deficiency. It also may explain why EFA-deficiency symptoms are relatively easy to produce in young animals whereas adult animals are quite refractory. Additional evidence that growth is the critical factor in the amount of 20:3 acid incorporated into PL, rather than a direct effect of estrogen, is provided by the hormone-treated groups (Fig. 8). All the animals in these groups, regardless of treatment, had large livers with about the same amount of liver PL as had the males (Figs. 2 and 3). All groups also had levels of 20:3 and palmitic acids in their PL comparable with those in males.

Plasma Composition

Phospholipid concentration (Fig. 9) in males and in the testosterone-injected group decreased slightly wheras that of females remained essentially unchanged after the 3-week period. Estradiol administration however caused a sharp increase in plasma PL and also in plasma TG. This may be a reflection of a greater responsiveness of the EFA-deficient animals to the estradiol treatment.

The concentration of either the free or esterified cholesterol (not shown) did not change significantly in the course of the deficiency in any of the groups. The concentrations of linoleic, arachidonic, and 20:3 acids in plasma CE are shown in Fig. 10. Linoleic acid concentration decreased to the same

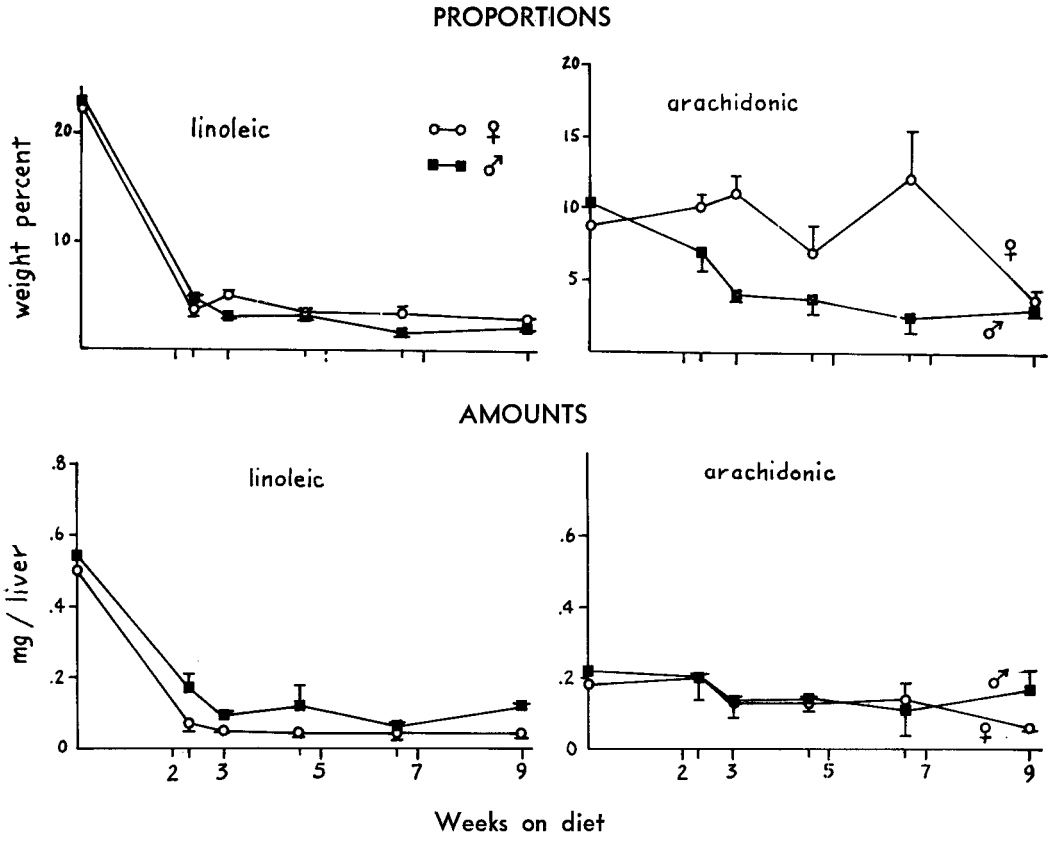

FIG. 5. Linoleic and arachidonic acid content of liver cholesteryl ester from rats during the development of an EFA deficiency. (For symbols see Fig. 2).

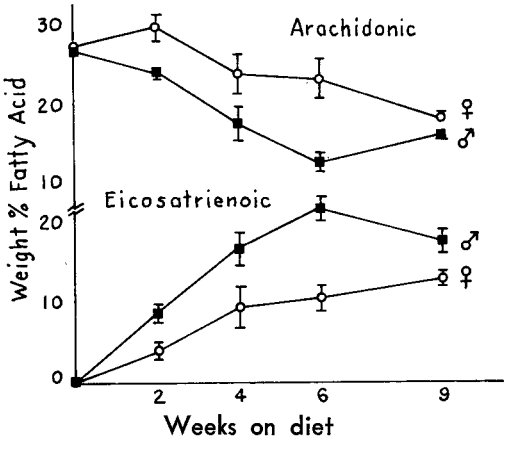

FIG. 6. Proportions of arachidonic and eicosatrienoic acids in liver phospholipids from rats during the development of an EFA deficiency. (For symbols see Fig. 2.)

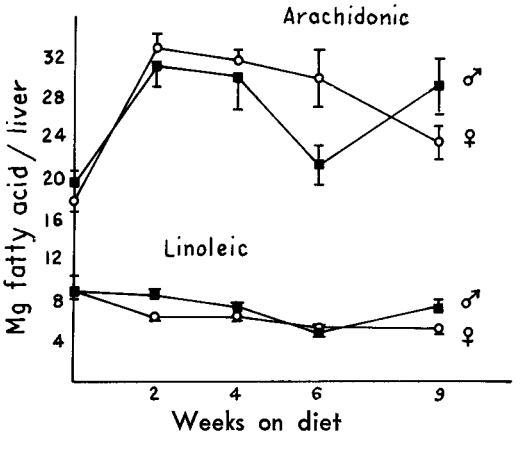

FIG. 7. Amounts of arachidonic and linoleic acids in liver phospholipids from rats during the development of an EFA deficiency. (For symbols see Fig. 2.)

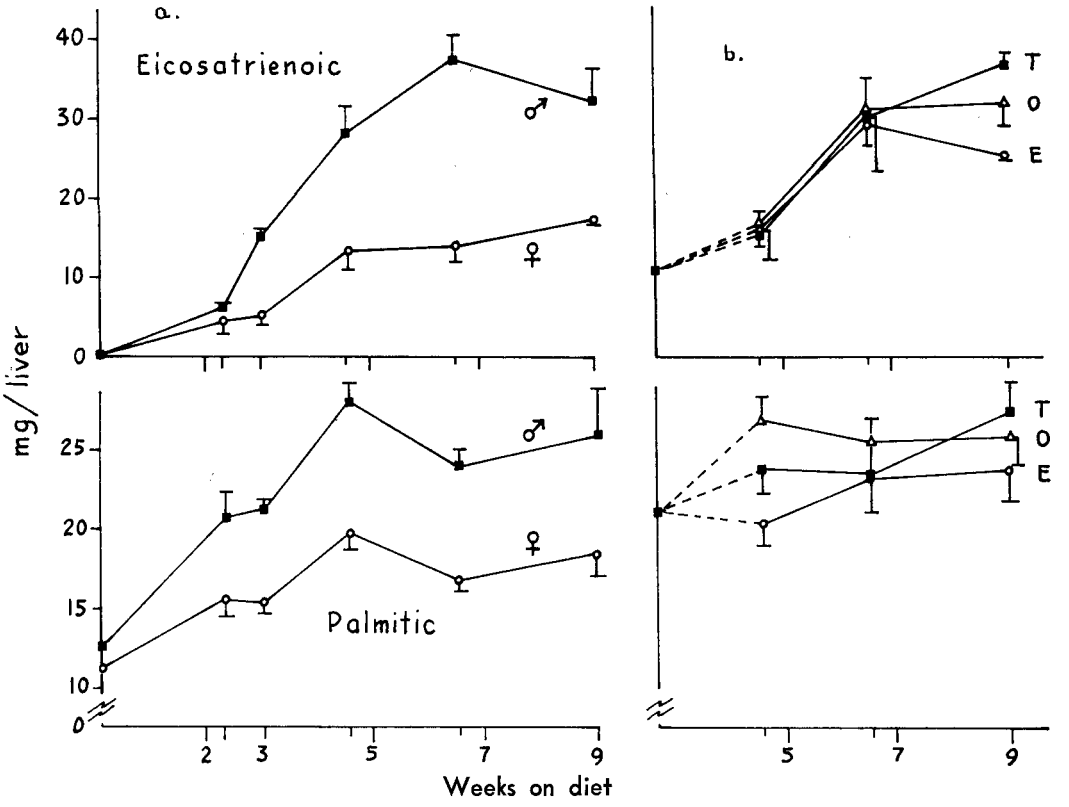

FIG. 8. Amounts of eicosatrienoic and palmitic acids in liver phospholipids from rats during the development of an EFA deficiency. (For symbols see Fig. 2.)

low levels in both the males and females during the first 2 weeks, similar to the effects seen in the livers (Fig. 5). The major fatty acid in this plasma lipid fraction was arachidonic acid, which decreased only slightly in females as the deficiency progressed. Thus a higher concentration of phospholipid arachidonate in females compared with males was observed in these EFA-deficient rats than had been observed in normal animals (13). The greater decrease in cholesteryl arachidonate in males was accompanied by a larger increase in the 20:3 acid. The concentration of this acid in all three castrated groups was similar to that of intact males (Fig. 10).

From Fig. 11 it can be seen that the pattern of the concentrations of arachidonic and 20:3 acids in the plasma PL were strikingly similar to those of the plasma CE (Fig. 10). It is well known that rat liver CE and plasma CE differ greatly in their fatty acid composition; the latter has significantly more arachidonic acid and the former contains more palmitic and oleic acids. Sperry (19) and, more recently, Glomset (20) have investigated the problem and provided evidence that the high proportion of PUFA in plasma CE arises from a plasma transesterification reaction between the β-unsaturated fatty acids of the plasma lecithin and free cholesterol. Although Sugano and Portman (21) also consider that plasma transesterification reactions play a major part in determining the fatty acid composition of the plasma CE, Roheim et al. (22), Goodman and Shiratori (23,24), and others (25) believe that the liver is the major source of the plasma CE and their composition in the plasma is determined by a process of preferential secretion of cholesteryl esters rich in PUFA.

In our experiments the parallelism between the concentrations of arachidonic and 20:3 acids in plasma PL and CE (a phenomenon not exhibited by the other fatty acids) in both sexes throughout the deficiency period indicates that these lipid fractions may be in equilibrium with each other, possibly in the same lipoprotein (22,26). Therefore the data support the postulation that the transesterification of free cholesterol with the β-unsaturated fatty acids of PL could have occurred in the plasma.

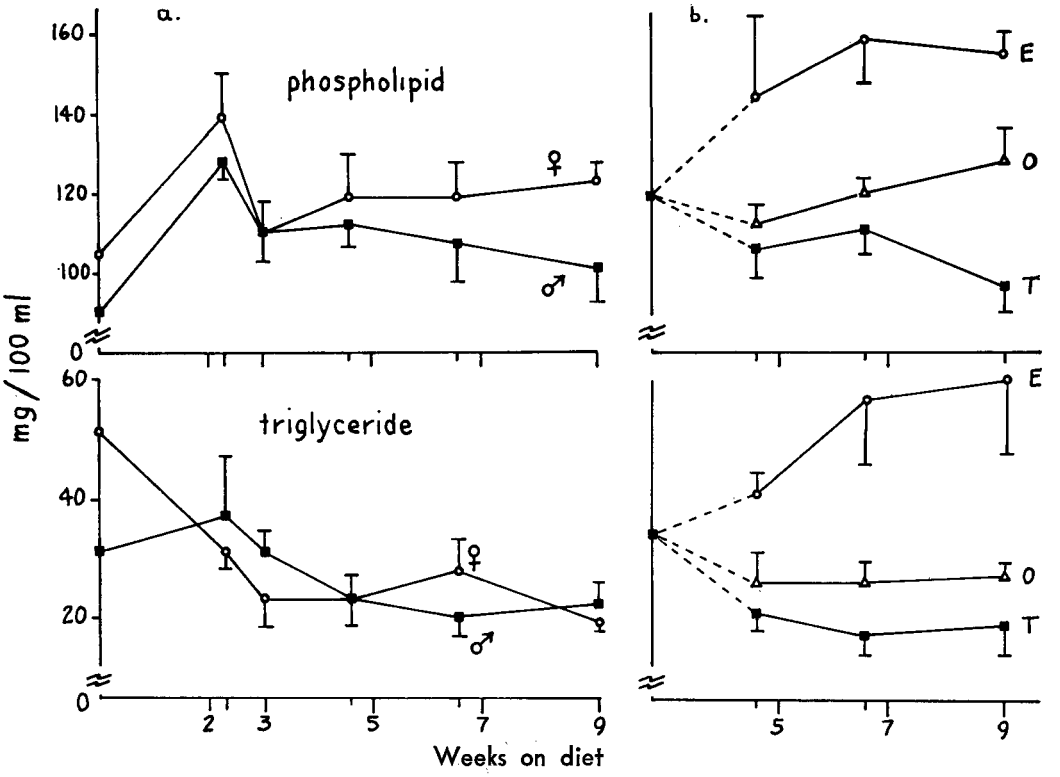

FIG. 9. Plasma phospholipids and triglycerides in rats during the development of an EFA deficiency. (For symbols see Fig. 2.)

Yet, a selective secretion of esterified cholesterol from the liver cannot be ruled out, especially since the patterns of the liver PL arachidonic and 20:3 acids resemble so closely those of the plasma (Fig. 6).

CONVERSION TO ARACHIDONIC ACID

We next considered the possibility that gonadal hormones may influence the conversion of linoleic acid to arachidonic acid (27). The experimental design was to measure the incorporation of ^{14}C from 1-^{14}C-linoleic into arachidonic acid of the major lipid fractions of liver and plasma of castrated, EFA-deficient rats that had been injected with either estradiol or testosterone. Animals were killed 6 hr after administration of an oral dose of 1-^{14}C-linoleic in safflower oil. Table I shows that, by 6 hr after feeding of the labeled linoleic acid, most of the radioactivity in the liver lipids was concentrated in the PL and TG fractions. Plasma CE however had a much higher proportion of the label than did the liver CE. In the liver, the estradiol-injected group had a higher proportion of activity in the PL and a lower proportion in the TG than did the testosterone-injected group. Specific activities (SA) of the fractions show that the CE had the highest SA in the plasma whereas the TG had the highest SA in the liver.

Table II shows the SA of linoleic and arachidonic acids for the various lipid fractions. It is evident that plasma cholesteryl esters were more heavily labeled with both fatty acids than were those of the liver. The results for the PL were equivocal however; only the testosterone-injected group showed significant differences between liver and plasma.

These results indicate that, under the conditions imposed, plasma cholesterol is esterified with linoleic and arachidonic acids more rapidly than is liver cholesterol, thus providing further evidence that esterification of cholesterol in the plasma may be a major metabolic reaction in the rat.

Table III shows the specific activities of linoleic and arachidonic acids in the liver PL after they had been subfractionated into a mixture of phosphatidylethanolamine (PE) and phosphatidylserine (PS), into a fast-moving lecithin fraction (F-PC) characteristically rich

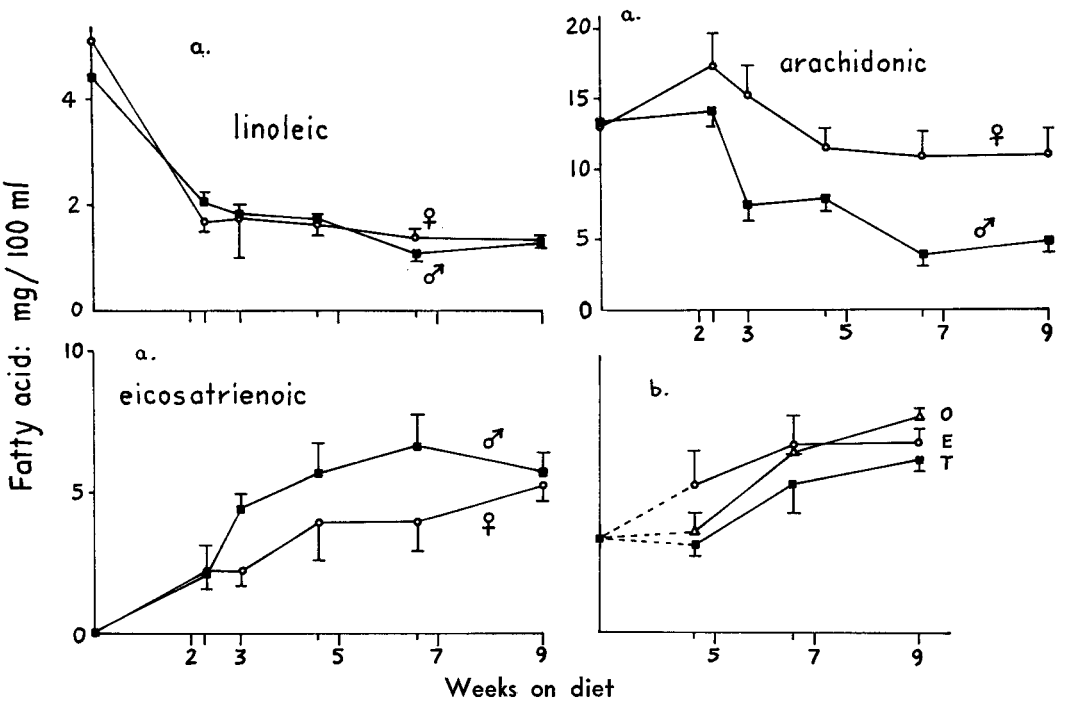

FIG. 10. Concentrations of linoleic, arachidonic, and eicosatrienoic acids in plasma cholesteryl esters from rats during the development of an EFA deficiency. The linoleic and arachidonic acid concentrations in the castrate groups were similar to those of their intact counterparts; the O group was between the E and T groups. (For symbols see Fig. 2.)

TABLE I
Distribution of Radioactivity and Specific Activity in the Lipid Classes of Liver and Plasma[a]

Group[b]	Distribution[c] (% of total) in lipid fraction of:		Specific activity[d] of fraction (cpm/μM x 10^{-3})	
	Liver	Plasma	Liver	Plasma
		Cholesteryl ester		
E	0.99±0.10	26.7±4.2	1.72±0.23	7.82±1.2
T	0.53±0.16	25.9±4.5	0.84±0.25	7.72±0.7
		Triglycerides		
E	30.9±3.6	29.3±3.0	18.0±1.7	3.37±1.3
T	47.3±3.8	34.2±2.2	23.4±3.0	6.38±0.9
		Phospholipids		
E	61.2±2.6	25.8±2.0	1.47±0.06	2.04±0.08
T	47.0±3.5	22.1±1.5	1.41±0.06	2.21±0.08

[a]Groups of six EFA-deficient, hormone-treated rats, 6 hr after intubation with 1-^{14}C-linoleic acid (means ± SEM).

[b]E = estradiol-injected castrated male rats (30 μg/week) subcutaneously. T = testosterone-injected castrated male rats (2.0 mg/week) subcutaneously.

[c]Percentage of activity recovered in plasma and liver respectively, as cholesteryl ester, triglyceride, and phospholipid. In liver about 5% and in plasma about 15% of the total activity was in a fraction containing mono- and diglycerides and unesterified fatty acids.

[d]Calculated from count/min/100 ml plasma (or 100 g liver) and μmole of cholesteryl ester (or triglyceride or phospholipid)/100 ml plasma (or 100 g liver), calculated as cholesteryl-oleate, triolein, and distearoyl-phosphatidylcholine respectively.

TABLE II
Specific Activity of Linoleic and Arachidonic Acids of Plasma and Liver Lipids[a]

Group[d]	(count/min/micromole fatty acid) x 10^{-3}			
	18:2[b]		20:4[c]	
	Liver	Plasma	Liver	Plasma
		Cholesteryl ester		
E	3.73[e]	15.2	0.46	2.17
T	2.01	11.6	0.35	2.35
		Triglycerides[f]		
E	15.2	1.89		
T	23.1	3.50		
		Phospholipids		
E	3.19	3.93	0.66	0.96
T	3.26	4.40	0.62	2.61

[a]Groups of six EFA-deficient, hormone-treated rats, 6 hr after intubation with 1-^{14}C-linoleic acid (means).

[b]Linoleic acid.

[c]Arachidonic acid.

[d]E = estradiol-injected castrated male rats (30 μg/week) subcutaneously. T = testosterone-injected castrated male rats (2.0 mg/week) subcutaneously.

[e]SEM were 10-20% of the means and have been omitted for the sake of clarity.

[f]Triglyceride fractions contain only negligible amounts of arachidonic acid.

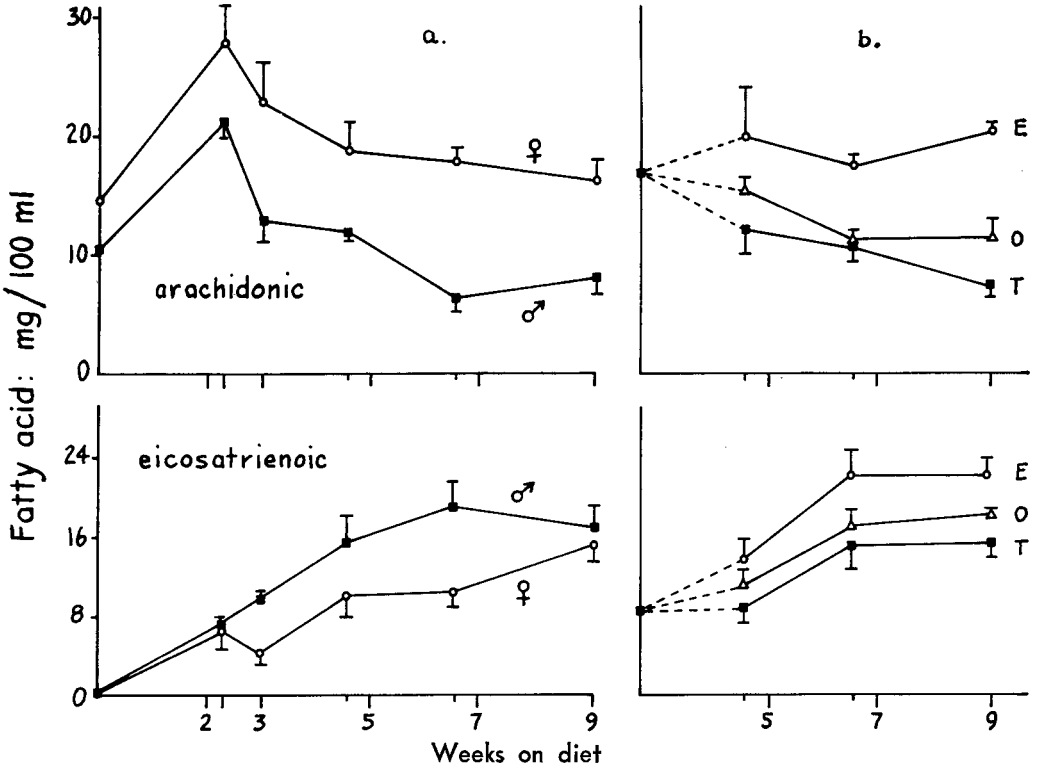

FIG. 11. Concentrations of arachidonic and eicosatrienoic acids in plasma phospholipids from rats during the development of an EFA deficiency. (For symbols see Fig. 2.)

TABLE III
Specific Activity of Linoleic and Arachidonic Acids in Liver Phospholipid Classes[a]

Group[b]	PL class[c]	Specific activity in (count/min/micromole fatty acid) x 10^{-3} 18:2[d]	20:4[d]	18:2[e]/20:4
	Phosphatidylethanolamine and phosphatidylserine (PE and PS)			
E	1.78	3.5	0.60	5.8
T	1.29	3.1	0.50	6.3
	Fast-moving phosphatidylcholine (F-PC)			
E	2.83	4.4	1.09	4.1
T	2.17	3.9	0.38	10.3
	Slow-moving phosphatidylcholine (S-PC)			
E	3.05	3.9	1.05	3.7
T	2.31	3.3	1.13	2.9

[a]Groups of six EFA-deficient, hormone-treated rats, 6 hr after intubation with 1-^{14}C-linoleic acid (means).

[b]E = estradiol-injected castrated male rats (30 μg/week) subcutaneously. T = testosterone-injected castrated male rats (2.0 mg/week) subcutaneously.

[c]Calculated from count/min in phospholipid class/100 g liver and μmole of phospholipid class/100 g liver.

[d]Calculated from count/min/100 g liver in linoleic or arachidonic acid respectively of the phospholipid class and μmole of linoleic or arachidonic acid in phospholipid class/100 g liver.

[e]Ratio of count/min/μmole of linoleic to arachidonic acid.

in stearic and arachidonic acids, and into a slower-moving lecithin fraction (S-PC) with a preponderance of palmitic and linoleic acids (28).

There were no differences in the SA of the cephalins (PE and PS) between the estradiol- and testosterone-injected groups, but in the F-PC fraction of the estradiol-injected group the SA of arachidonic acid was nearly three times that of the testosterone-injected group. The high ratio of SA of linoleic acid to that of arachidonic acid in the testosterone-injected group indicated that little conversion of linoleic acid to arachidonic took place whereas the smaller ratio in the group treated with estradiol suggests that the female hormone may have facilitated a more rapid conversion or an accelerated transfer of labeled arachidonic acid into this lecithin fraction.

ACKNOWLEDGMENTS

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Relative Incorporation of Acetate and Glucose into Glycerides of *Glomerella cingulata*

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ABSTRACT

Cultures of the fungus *Glomerella cingulata* were pulse-labeled for 30 min with 1-^{14}C-acetate at ages from 2 to 12 days old. The greatest incorporation into triglycerides and phosphoglycerides occurred in the youngest cultures, but more of the radioactive acetate was diverted to the phosphoglycerides than to the triglycerides in these cultures.

In another series of experiments the incorporation of 1-^{14}C-acetate or 2-^{14}C-glucose into the triglycerides and phosphoglycerides of the fungus was investigated at 15-minute intervals for 1 hr. Hydrolysis of the two classes of glycerides revealed some labeling of glycerol from acetate; but, not surprisingly, acetate was incorporated into the fatty acid moieties to a greater extent than into the glycerol moieties, and there was relatively greater incorporation of 2-^{14}C-glucose into glycerol than into fatty acids. Some relationships of these results to the growth and development of *G. cingulata* are suggested, and implications relative to control mechanisms are pointed out.

INTRODUCTION

In animal tissues the synthesis of triglycerides and phosphoglycerides proceeds for several steps over a common pathway; the pathway then diverges at L-1,2-diglyceride (1). The available knowledge suggests that a similar situation exists in higher plants and in fungi (2-4). However the extent to which triglyceride and phosphoglyceride synthesis occur relative to each other under differing physiological conditions is not known, and the mechanism by which such synthesis is regulated after the diversion at L-1,2-diglyceride is poorly understood. Since fungi produce triglycerides and phosphoglycerides as major classes of lipids (5), they should be convenient organisms for studying the regulation of glyceride metabolism. Moreover, during the logarithmic stages of fungal growth, many membranous organelles are formed, and presumably such formation depends, at least in part, upon rapid synthesis of phosphoglycerides. There is also some evidence that triglycerides are accumulated in older fungal cells (6,7). Therefore the regulation of glyceride synthesis in fungi may be closely related to their growth and development. Accordingly this study was undertaken to investigate the relative incorporation of selected precursors into triglycerides and phosphoglycerides as a function of age and to determine the relative incorporation of precursors of both fatty acid and glycerol into the fatty acid and glycerol moieties of the two classes of glycerides. Two readily permeable and utilizable compounds, 1-^{14}C-acetate and 2-^{14}C-glucose, were used as precursors.

MATERIALS AND METHODS

Growth and Labeling of Fungus

The culture of *G. cingulata* was obtained from Jack Ziffer, Pabst Laboratories, Milwaukee, Wis., and was maintained on potato dextrose agar slants. The composition of the nutrient solution in g/liter was: glucose (10); asparagine (4); KH_2PO_4 (1.0); $MgSO_4 \cdot 7H_2O$ (0.5); $CaCl_2$ (0.1); biotin (0.005). One milliliter of a trace element solution was added to each liter of the nutrient solution. The composition of the trace element solution in milligrams per liter was: $Na_2B_4O_7 \cdot 10H_2O$ (88); $(NH_4)_6Mo_7O_{24}$ (64); $FeCl_3 \cdot 6H_2O$ (960); $ZnSO_4 \cdot 7H_2O$ (8,800); $CuCl_2$ (270); $MnCl_2 \cdot 4H_2O$ (72). Each flask was inoculated with 1 to 2 mg conidia per 100 ml of nutrient medium. In order to label the glycerides, 1-^{14}C-acetate was added to the cultures at a concentration of 10 μc/100 ml of medium, and 2-^{14}C-glucose was added at 20 μc/100 ml of medium. Specific activities of these labeled compounds were 1-^{14}C-acetate 2.0 mc/mm and 2-^{14}C-glucose 1.96 mc/mm. Both labeled compounds were obtained from New England Nuclear Corporation.

When the incorporation of precursors into the glycerides was studied as a function of age, mycelial pellets of 2 to 12 days of age, in 250-ml Erlenmeyer flasks, were incubated with the labeled precursors for 30 min. However, when the incorporation of precursors into the glycerides was studied for 1 hr, 50-ml samples of

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TABLE I

Incorporation of 1-^{14}C-Acetate into Triglycerides and Phosphoglycerides of *G. cingulata* as a Function of Age with Cultures Pulse-Labeled for 30 Minutes at Indicated Ages

Age (days)	Radioactivity in cpm per mg Total Lipid	
	Triglycerides	Phosphoglycerides
2	66,918	192,243
3	8,556	21,118
4	364	1,189
5	893	2,062
6	1,616	6,137
7	733	2,047
10	101	196
12	127	194

two-and-a-half to 3-day-old mycelial pellets were taken at 15-minute intervals from a labeled culture in a single one-liter Erlenmeyer flask by using 50-ml B-D Plastipak syringes, modified as follows: the tips of the syringes were drilled out to about 1.5 mm (I.D.), and 2.5-cm lengths of 2.5-mm (I.D.) surgical tubing were then affixed to the syringe tips. To complete the modification, 15-cm lengths of 2-mm (I.D.) glass tubing were inserted into the open end of the surgical tubing to serve as the syringe needle.

Treatment of Lipids

After incubation of the fungus cultures with the radioactive precursors, labeled samples of the cultures were rapidly filtered or centrifuged, and the mycelium was immediately extracted at room temperature for 3 hr with $CHCl_3$/MeOH (2:1 v/v). Extraction was repeated twice for 3 hr each time. Between the first and second extractions, the mycelium was macerated in a Waring Blendor in $CHCl_3$/MeOH (2:1 v/v), for 2 min to facilitate the subsequent extractions.

The extracted lipids were separated from nonlipid labeled and unlabeled compounds on Sephadex G-25 by using the method of Siakotos and Rouser (8). The purified lipids so obtained were then separated into sterol ester, triglyceride, sterol, and phosphoglyceride fractions by using a scaled-down version of the procedure previously described (9). The purity of the fractions obtained by column chromatography was monitored by thin-layer chromatography and autoradiography of the thin-layer chromatograms.

Alkaline hydrolysis of purified total lipids and of triglyceride and phosphoglyceride fractions was carried out with 0.1N methanolic KOH, and the completeness of hydrolysis also was monitored by thin-layer chromatography and autoradiography of the thin-layer plates. Contamination of triglyceride and phosphoglyceride glycerol in the hydrolyzates by water-soluble labeled compounds was avoided by removing such compounds prior to silicic acid chromatography and alkaline hydrolysis by using the procedure of Siakotos and Rouser (8). Contamination of the glycerol by fatty acids was avoided by making four consecutive extractions of the acidified hydrolyzates (5.0 ml). Twenty milliliters of redistilled petroleum ether (40C to 60C) was used for each extraction. The combined petroleum ether extracts were reduced in volume and made up to the same volume as the methanolic fraction for assay of radioactivity.

Assay of Radioactivity

Free fatty acids were recovered from hydrolyzates in petroleum ether and were assayed for radioactivity in this solvent. However the purified total lipids and all of the lipid fractions obtained by column chromatography were taken to dryness in vacuo and redissolved in $CHCl_3$/MeOH (2:1 v/v) so that problems arising from differential quenching might be minimized. Determinations of radioactivity were carried out with an ANSitron liquid scintillation spectrometer by using standard toluene and dioxane scintillation solutions of 2,5-diphenyloxazole and 1,4-bis-2-(phenyloxazolyl)-benzene (10,11). Determinations of radioactivity were made by using 0.2-ml aliquots of the various samples in 15 ml of the appropriate scintillation system. Under these conditions quenching was low.

RESULTS AND DISCUSSION

The data reported in Table I are from an experiment in which cultures of *G. cingulata* were pulse-labeled for 30 min with 1-^{14}C-acetate as a function of age. Three phases of incorporation into the glycerides are evident from this table: Phase I, a period of great activity when the fungus was two to three days old; Phase II, a period of low activity when the fungus was more than seven days old; and Phase III, a period of intermediate activity encompassing the time span four to seven days old.

In Phases I and III the amounts of radioactivity incorporated into the phosphoglycerides were two-and-a-half to four times greater than the amounts incorporated into the triglycerides; in Phase II the amounts of radioactivity incorporated into the phosphoglycerides were about one-and-a-half to two times greater than the amounts incorporated into the triglycerides. Although the total amounts of precursor incorporated into the triglycerides and

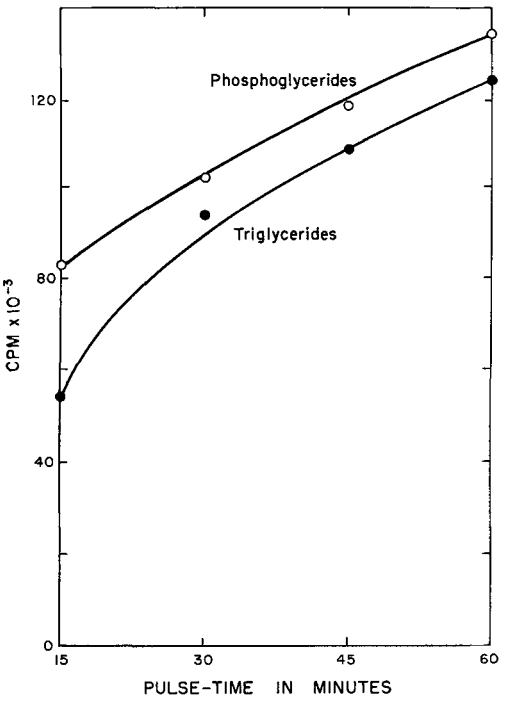

FIG. 1. Incorporation of 1-^{14}C-acetate into glycerides of late log phase *G. cingulata* mycelium.

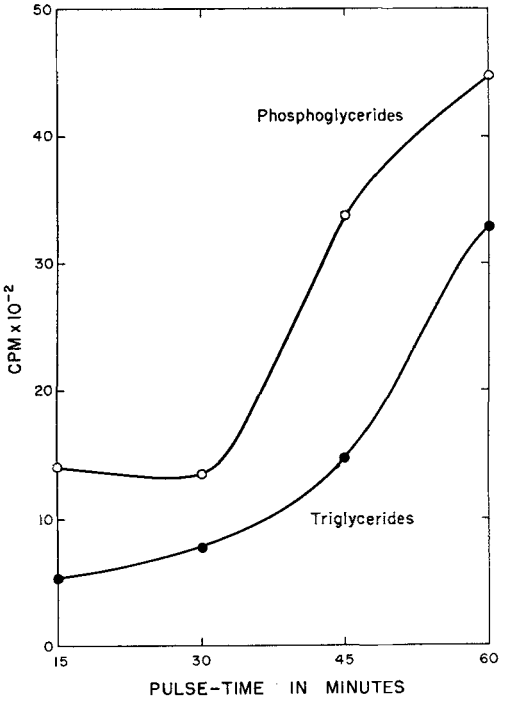

FIG. 2. Incorporation of 2-^{14}C-glucose into glycerides of late log phase *G. cingulata* mycelium.

phosphoglycerides during growth varied from experiment to experiment (as would be expected of total values of other physiological indices, such as soluble protein, nitrogen, dry weight of mycelium, or amount of lipid formed), nevertheless the three phases of incorporation and the second peak shown in Table I, the peak between four and seven days old, were reproducible in three separate experiments which were carried out at three different times.

Figures 1 and 2 are representative of time-course experiments that were carried out under somewhat different conditions from the experiments represented by Table I. Late log phase cultures (two-and-a-half to three days old), in one-liter flasks, were labeled with 1-^{14}C-acetate or 2-^{14}C-glucose and then sampled at 15-minute intervals for 1 hr. In this type of experiment the differences in radioactivity between the triglycerides and phosphoglycerides were relatively small but consistently indicative of greater incorporation into the phosphoglycerides than into the triglycerides. Thus, in three separate experiments, the radioactivity of the phosphoglycerides was in all cases at least 10% greater than the radioactivity of the triglycerides. This slightly greater radioactivity of the phosphoglycerides may be indicative of at least the following two possibilities: either a) there was more rapid exchange between fatty acids of the phosphoglycerides and newly synthesized fatty acids of lysophosphoglycerides than between fatty acids of species of triglycerides or b) phosphoglyceride synthesis

TABLE II

Radioactivities (cpm) of Fatty Acids and Glycerol from Triglycerides and Phosphoglycerides of Late Log Phase *G. cingulata*. Precursor: 1-^{14}C-Acetate

Pulse-time (minute)	Triglycerides		Phosphoglycerides	
	Fatty acids	Glycerol	Fatty acids	Glycerol
15	5,984	33	7,554	354
30	9,937	81	10,956	505
45	11,650	147	11,848	507
60	13,411	73	14,459	771

TABLE III

Radioactivities (cpm) of Fatty Acids and Glycerol from Triglycerides and Phosphoglycerides of Late Log Phase *G. cingulata*. Precursor: 2-^{14}C-Glucose

Pulse-time (minute)	Triglycerides		Phosphoglycerides	
	Fatty acids	Glycerol	Fatty acids	Glycerol
15	60	14	138	86
30	61	6	107	75
45	151	11	332	110
60	352	28	350	209

was favored over triglycerides synthesis.

Results on the labeling of the fatty acid and glycerol moieties of the triglycerides and phosphoglycerides in late log phase cultures are reported in Tables II and III. It appears, superficially, that these tables show that incorporation into the fatty acid moieties was favored regardless of whether 1-^{14}C-acetate or 2-^{14}C-glucose was used as precursor. However, assuming that one mole of glycerol is formed from one-half mole of glucose and that three or four moles of glucose yield one mole of fatty acid, then it becomes apparent that the fatty acid moieties were not more heavily labeled when 2-^{14}C-glucose was the precursor. In the case of acetate as precursor, the relative labeling of fatty acid and gycerol can be explained by recalling that this precursor is incorporated directly into fatty acids but indirectly (via oxaloacetate) into glycerol. Further examination of Tables II and III shows two other points of interest. These are the generally low incorporation of 2-^{14}C-glucose into the two classes of glycerides and the greater radioactivity of phosphoglyceride glycerol than triglyceride glycerol. The low incorporation of 2-^{14}C-glucose may be caused in part by dilution of the radioactive glucose by glucose in the medium.

The main points emerging from the experiments on incorporation during growth (represented by Table I) are that, in actively growing cultures of *G. cingulata,* labeled precursors are incorporated into phosphoglycerides to a larger extent than into triglycerides, that increased or decreased incorporation into the phosphoglycerides, is paralleled by increased or decreased incorporation into the triglycerides, and that, although the total incorporation of precursors into these two classes of glycerides changed markedly during active growth, the ratio of phosphoglyceride to triglyceride incorporation, by comparison, changed relatively little. One of several reasons for this unequal distribution of radioactivity between the glycerides could be that during rapid cell division, meeting the requirements for phosphoglycerides (such as membrane formation) is more important to the cell than synthesizing a carbon source (e.g., triglyceride) for future use.

Although the experiments reported in this paper were not designed to investigate the mechanism by which triglyceride and phosphoglyceride synthesis is regulated in *G. cingulata,* the fact that decreased or increased incorporation into the phosphoglycerides invariably was accompanied by similar effects with the triglycerides does suggest one of two possibilities: either a) *G. cingulata* contains a single metabolic device for controlling phosphoglyceride and triglyceride synthesis, or b) it contains more than one such regulatory device and these devices are simultaneously and precisely regulated in parallel to meet changing physiological conditions.

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Glyceride Structure of *Cardamine impatiens* L. Seed Oil

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ABSTRACT

A group of unusual triglycerides, in which one of the acyl groups is a vicinal dihydroxy acid with one of the hydroxyl groups acetylated, has been isolated from *Cardamine impatiens* L. (Cruciferae) seed oil. Hydrolysis of these triglycerides with castor bean lipase facilitated isolation and identification of a mixture of C_{18}, C_{20}, C_{22}, and C_{24} hydroxy acetoxy fatty acids. Pancreatic lipase hydrolysis data revealed that these monoacetylated dihydroxy acid residues are esterified exclusively with one of the α-positions of the glycerol moiety. The remaining acyl groups are comprised of ordinary C_{18} unsaturated acids (which occupy 98% of the β-position), palmitic acid, and C_{20}, C_{22}, and C_{24} monoenoic fatty acids.

INTRODUCTION

SEED OIL OF *Cardamine impatiens* L. has recently been reported to contain 25% of a mixture of saturated, vicinal, dihydroxy acids with chain lengths of 18, 20, 22, and 24 carbon atoms. Essentially all the diols have the *erythro* configuration (1). In a preliminary communication (2), we concluded that these dihydroxy acids, together with ordinary long-chain acids and acetic acid, were constituents of unusual triglyceride components which comprised 40% of *Cardamine* oil; however the exact nature of these components remained unclear.

This paper presents results which show that these unusual triglycerides have one or both of the structures shown in Fig. 1. These substances, which will be referred to as hydroxy triglycerides, differ from one another only in chain length and degree of unsaturation of the acyl groups; they migrate as a single spot in thin-layer chromatography (TLC) on Silica Gel G and have been treated as an entity throughout this investigation.

EXPERIMENTAL PROCEDURES AND RESULTS

General

Infrared (IR) analyses were done on 1% solutions in carbon disulfide, chloroform, or carbon tetrachloride (depending on the solubility of the sample) with a Perkin-Elmer Model 137 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained with a Varian A-60 spectrometer on deuteriochloroform solutions containing tetramethylsilane as the internal reference. Mass spectral data were determined with a Nuclide 1290-G spectrometer. TLC analyses were done on plates spread with 250 μ layers of Silica Gel G. *Cardamine impatiens* seed oil was obtained and analyzed as described previously (1).

Isolation and Analysis of Hydroxy Triglycerides

Hydroxy triglycerides were separated from normal triglycerides of *Cardamine* oil with a 2.3 × 30-cm column of 100–140 mesh Adsorbosil CAB (Applied Science Laboratories Inc.). These separations were done in batches of 1.5 g of oil with the solvent system ethyl ether-petroleum ether, 70:30 (v/v). Fractions showing hydroxyl absorption and acetate absorption in their IR spectra were combined (40% of whole oil) to give the hydroxy triglyceride fraction. This fraction was demonstrated to be a glycerol-based lipid by the method of Holla et al. (3), in which the glycerol moiety is converted to triacetin and identified by gas-liquid chromatography (GLC). NMR indicated that glycerol α-carbon (multiplet centered at 5.76τ), olefinic (4.63τ), acetate (7.93τ), and C-methyl protons (9.12τ) were present. The fraction did not react with periodate, was optically active $[\alpha]_D^{27.5C}$ + 1.4° (c = 10, $CHCl_3$, visual polarimeter), and contained 13.5% oxygen by direct measurement. Migration characteristics of this fraction on TLC can be seen in Fig. 2, Sample 4. Its position on the plate demonstrates that it is made up of triglycerides since it migrates slightly faster than distearin. If it were composed of diglycerides containing a dihydroxy fatty acid moiety with one hydroxyl group free, it would be considerably more polar than normal diglycerides.

Chemical Hydrolysis of Hydroxy Triglycerides

Hydrolysis of the hydroxy triglycerides by refluxing 2 hr with 1% H_2SO_4 in methanol yielded a mixture of ordinary fatty acid methyl esters (excluding acetic acid) and dihydroxy acid methyl esters in a 2:1 molar ratio. This ratio was determined by separating the mixture

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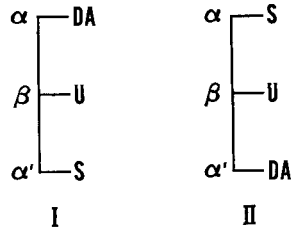

FIG. 1. Schematic representation of *Cardamine impatiens* triglyceride structures; D = dihydroxy acyl moiety, A = acetyl moiety, U = unsaturated C_{18} acyl moiety, and S = saturated or long chain ($>C_{18}$) acyl moiety.

into the two ester types on a 1.4 × 25-cm column of Adsorbosil CAB and weighing each fraction. Ordinary esters were eluted with chloroform and the dihydroxy esters with methanol-chloroform (5:95). TLC indicated that hydrolysis was complete. Alkaline hydrolysis in aqueous alcohols (e.g., 5% KOH in 80% aqueous methanol), followed by esterification with diazomethane, gave the same result. However, when the hydroxy triglycerides are refluxed 3 hr or more with alkali in absolute alcohol (e.g., 5% KOH in absolute methanol), the esterified products contain a small amount (less than 10%) of an artifact. This artifact was isolated by preparative TLC and yielded both ordinary and dihydroxy acids upon hydrolysis with acidic methanol, but its exact structure is not known.

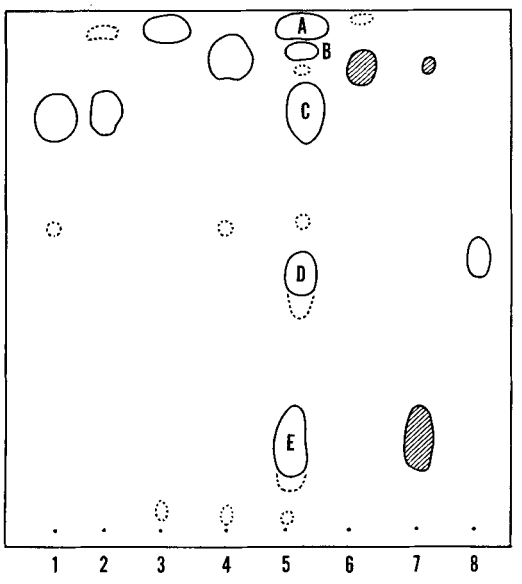

FIG. 2. Tracing of thin-layer chromatogram on Silica Gel G developed with methanol-chloroform (2:98). Spots were visualized with dichlorofluorescein and H_2SO_4 charring. The shaded spots did not char. Samples are: *1*, methyl esters of fraction DA; *2*, methyl 13(14)- and 14(13)-hydroxyacetoxydocosanoate; *3*, methyl oleate; *4*, *Cardamine* hydroxy triglycerides; *5*, methyl esters of pancreatic lipase hydrolysis products of *Cardamine* hydroxy triglycerides; *6*, distearin; *7*, monostearin; and *8*, methyl esters of *Cardamine* dihydroxy acids.

Enzymic Hydrolysis of Hydroxy Triglycerides

Castor bean lipase was isolated and purified according to the methods of Ory et al. (4,5). Under the selected conditions the enzyme did not hydrolyze methyl oleate or 9(10)-hydroxy-10(9)-propionoxystearic acid to any measurable extent. About 0.300 g of sample to be hydrolyzed was weighed into a 25 × 70-mm screwcap vial, and 0.020 g of purified enzyme, 0.5 ml of 0.1M Tris buffer (pH 8.0), and 1.5 ml of water were added. This mixture was acidified with 0.1M acetic acid to pH 4.3, and the vial was flushed with nitrogen and capped tightly. The contents were stirred vigorously for 3-5 hr at 25C. Hydrolysis of glyceryl ester linkages was complete after 3 hr; this point was established by the absence of monoglycerides and starting triglycerides from the products as indicated by TLC. These hydrolysis products, recovered by ether extraction, were converted to methyl esters (by treatment with diazomethane) to facilitate separation of the mixture of acetylated dihydroxy acid fragments (designated DA, Fig. 1) from the unsubstituted acyl fragments (S and U, Fig. 1).

Isolation and Analysis of Fraction DA

Fraction DA was separated from other fragments by chromatographing the methyl ester mixture on a 1.4 × 30-cm column of 100–140 mesh Adsorbosil CAB. At the start of the run the solvent was 20% ethyl ether in petroleum ether (bp 30-60C). The ethyl ether content was increased in increments of 10% until the most polar materials were finally eluted with 50% ethyl ether in petroleum ether. Normal fatty acid methyl esters recovered by this procedure totaled 64 mole %, and the acetylated dihydroxy acid (DA) methyl ester fraction accounted for the remaining 36 mole % [calculated from the weight of each fraction isolated and the known composition reported previously (1)]. Unacetylated dihydroxy acid esters were not observed. Each ester fraction gave only one spot on TLC. The hydroxyl and acetate absorption observed in the IR spectrum of the hydroxy glycerides was retained in esterified fraction DA.

The NMR spectrum of fraction DA methyl esters revealed equivalent numbers of methoxyl

(6.33τ), acetate (7.93τ), and C-methyl (9.12τ) protons. No olefinic proton signal was observed. Elemental analyses indicated that esterified DA contained 18.4% oxygen (by difference). The calculated oxygen content is 18.5% by assuming a mixture of hydroxy acetates with the amounts of the various chain-length homologs found previously (1).

An additional sample of fraction DA was isolated (yield, 30% of theoretical) as the mixed free acids by column chromatography on Adsorbosil CAB. Ethyl ether-petroleum ether was the eluting solvent, and the ethyl ether content was increased gradually in increments of 5%. Excessive tailing caused a low yield, but a sample of fraction DA containing ca. 90% of the four homologs was obtained and was shown to have a neutral equivalent of 475, indicating monomeric substances.

Chemical Hydrolysis of Fraction DA

Refluxing 0.073 g of the methyl esters of DA with 25 ml of 2% sulfuric acid in methanol for 3 hr under nitrogen yielded 0.065 g of dihydroxy acid methyl esters. Analysis of the product by GLC under conditions described previously (1) revealed that only a trace of ordinary fatty acid esters was present (methyl acetate was volatilized in the work-up process). A parallel experiment in which DA was refluxed with 5% potassium hydroxide in 80% aqueous methanol for 2 hr and the resulting products were esterified with diazomethane, produced the same results. The absence of ordinary esters was confirmed by TLC. By this method it was also demonstrated that both hydrolyses were complete.

Synthesis of Methyl 13(14)-Acetoxy-14(13)-Hydroxydocosanoate

Erucic (*cis*-13-docosenoic) acid (0.410 g) in 3 ml of chloroform was treated at room temperature dropwise with a solution of 0.420 g of *m*-chloroperbenzoic acid (6) in 5 ml of chloroform. After the mixture was stirred magnetically for 1.25 hr, excess peracid was destroyed by addition of a 5% solution of sodium sulfite. The chloroform layer was withdrawn and washed successively with sodium bicarbonate solution and water. Removal of the chloroform and crystallization of the crude product from warm petroleum ether yielded 0.330 g of *cis*-13,14-epoxydocosanoic acid, mp 62-63C [lit. value, 63.5C (7)]. The epoxide was converted to methyl 13(14)-acetoxy-14(13)-hydroxydocosanoate by refluxing for 2 hr with glacial acetic acid, followed by treatment with diazomethane.

Identification of Fraction DA by GLC, TLC, and IR

Analysis of the synthetic methyl acetoxyhydroxydocosanoate and of the methyl esters of fraction DA by GLC was carried out on a 60 × 0.3-cm O.D. column packed with 3% OV-1[2] on 60-80 mesh Gas-Chrom Q (Applied Science Laboratories Inc.). The column temperature was programmed at 4C/min from the starting temperature of 145C; helium was the carrier gas. An F&M Model 810 chromatograph with a hydrogen flame detector was used. Methyl acetoxyhydroxydocosanoate emerging from the column at 197C. Fraction DA methyl esters gave four peaks, the major one emerged also at 197C. The next largest peak emerged at 208C and was attributed to the C_{24} hydroxyacetate. Two minor peaks emerging at 173C and 185C were attributed to the C_{18} and C_{20} hydroxyacetates respectively. On TLC, fraction DA methyl esters and synthetic methyl acetoxyhydroxydocosanoate each gave a major spot of R_f 0.81 as indicated in Samples 1 and 2 respectively (Fig. 2). In addition, the IR spectrum of the synthetic material was identical to that of fraction DA methyl esters.

Conversion of Fraction DA to α-Ketols

A 0.116-g sample of fraction DA methyl esters in 5 ml of dry acetone was treated for 5 min at room temperature with 0.4 ml of Jones reagent (8). The reaction mixture was diluted with water; the oxidized products were recovered by ether extraction and were transesterified by refluxing with 2% sulfuric acid in methanol. IR analysis of the resultant α-hydroxy ketones (1%, CCl_4) indicated hydroxyl (3450 cm^{-1}), ester carbonyl (1725 cm^{-1}), and ketone absorption (1700 cm^{-1}). The intensity of the ketone band nearly equalled that of the ester carbonyl band. Synthetic hydroxyacetoxydocosanoate was similarly converted to the corresponding α-ketol.

We had no success in cleaning these α-ketols with periodate although other workers have done this with C_{18} fatty acid derivatives (9). A similar cleavage reaction utilizing lead tetraacetate (10) was carried out on the α-ketols. Although cleavage was complete, over-oxidation led to the isolation of carboxylic acids as the only products.

Mass Spectra of Fraction DA and Hydroxyacetoxydocosanoate

The revealing portions of the fragmentation pattern for methyl hydroxyacetoxydocosanoate

[2]OV-1 is a silicone stationary phase with a high percentage of phenyl groups. It is similar to SE-30 in separating characteristics but superior to SE-30 in thermal stability.

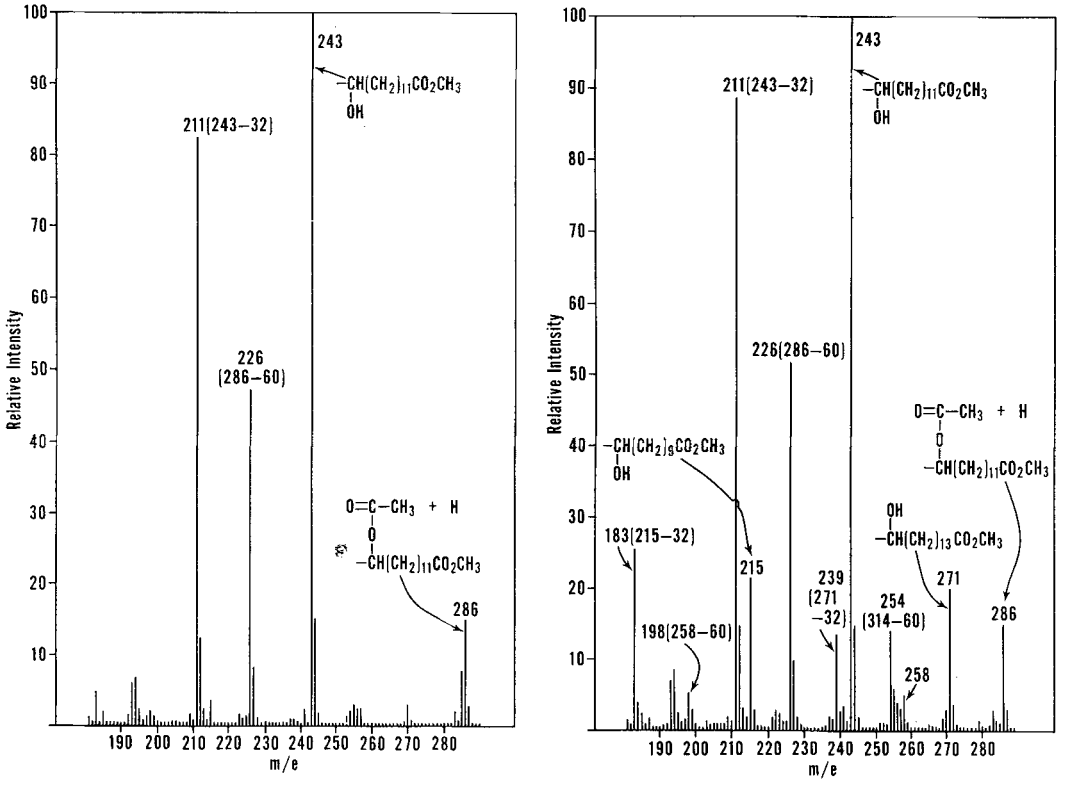

FIG. 3. Portions of mass spectra of methyl 13(14)- and 14(13)-dihydroxyacetoxydocosanoate (left), and methyl esters of *Cardamine* fraction DA (right).

and for the methyl esters of fraction DA are depicted in Fig. 3. The pattern shown in Fig. 3A for the synthetic methyl hydroxyacetoxydocosanoate is characterized by peaks due to both isomers (i.e., 13-hydroxy-14-acetoxy- and 14-hydroxy-13-acetoxydocosanoate) since both should be present in equal amounts. Cleavage between carbons 13 and 14 of the 13-hydroxy isomer gives the base peak at m/e = 243. Further fragmentation (loss of CH_3OH) of the 243 peak and subsequent rearrangement, probably to the ketene (11), gives the peak observed at m/e = 211. Similarly cleavage of the 13-acetoxy isomer yields two principal fragments, m/e = 286 and m/e =226. Probably the 226 peak arises by loss of acetic acid from the m/e = 286 peak. It could also be derived from the fragment appearing at m/e = 243 by loss of –OH, but this cleavage does not seem to occur appreciably in hydroxy acids (11).

The hydroxyacetoxy methyl esters from *Cardamine* oil give much the same fragmentation pattern (Fig. 3B). The four peaks are present in about the same proportion and are attributable to the C_{22} hydroxyacetate, the major constituent of the mixture. Fragmentation of the C_{24} isomer is consistent with that of the C_{22} and gives peaks at m/e = 314 (not shown), 271, 254, and 239. The 314 peak is caused by a fragment comparable with that causing the 286 peak but with two additional methylene groups. A similar pattern results from the C_{20} isomer, with peaks at m/e = 258, 215, 198, and 183. These data indicate that the acetate functions in this glyceride are approximately equally distributed between the vicinal hydroxyl groups of each dihydroxy acid.

Pancreatic Lipase Hydrolysis of Hydroxy Triglycerides

This reaction procedure was based on the semimicro method developed by Luddy et al. (12). To a 0.200-g sample of the *Cardamine* hydroxy triglycerides in a 25 × 70-mm vial was added 0.060 g of pig pancreatic lipase, EC 3.1.1.3, (Nutritional Biochemicals Corporation, Cleveland, O.), 3 ml of 1M Tris buffer (at pH 8.0), 0.3 ml of 22% calcium chloride solution, and 1 ml of 0.1% sodium cholate solution. A Teflon-coated magnetic stirring bar was placed in the vial, the contents were flushed thoroughly with nitrogen, and the vial was tightly

capped. After the vial was placed in a water bath maintained at 35-40C, the contents were agitated vigorously by a magnetic stirrer for 15 min. The hydrolysis products were isolated by ether extraction of the acidified reaction mixture; the ether extract was washed with water, concentrated to 5 ml, and reacted with diazomethane. TLC of the esterified mixture on Silica Gel G gave the chromatogram reproduced in Fig. 2, Sample 5.

Pure fractions of the hydrolyzed normal acids (as methyl esters) and of the monoglycerides, spots A and E respectively (Fig. 2, Sample 5), were obtained by column chromatography on Adsorbosil CAB. The ester mixture in petroleum ether (soluble with warming) was applied to the column, and the solvent was changed to chloroform after all the normal esters had been eluted with petroleum ether. Chloroform eluted the material contained in spot C (methyl esters of DA), but early fractions were contaminated with residual hydroxy triglycerides (spot B), and the final fractions contained some material from spot D (diglycerides containing one normal acid and one acetylated dihydroxy acid).

GLC analysis of the trimethylsilyl ethers of the materials in spot D demonstrated that unacylated dihydroxy acid methyl esters were not present. Most of the monoglyceride portion (spot E) was obtained pure with 2% methanol in chloroform although it was eluted from the column slowly. Methyl esters of the monoglyceride acyl groups were prepared by acid-catalyzed transesterification. Analysis of these esters by IR and TLC established that neither hydroxylated nor acetylated material was present.

Methyl esters of normal acids from the pancreatic lipase hydrolysis mixture were analyzed by GLC as described previously (1), and the resulting area percentages were: $C_{14:0}$, 0.3%; $C_{16:0}$, 9.7%; $C_{18:0}$, 1.8%; $C_{18:1}$, 4.4%; $C_{18:2}$, 5.2%; $C_{18:3}$, 2.0%; $C_{20:0}$, 2.1%; $C_{20:1}$, 18.3%; $C_{20:2}$, 1.8%; $C_{22:0}$, 1.6%; $C_{22:1}$, 45.2%; $C_{22:2}$, 1.0%; $C_{24:0}$, trace; $C_{24:1}$, 6.0%; and unidentified, 0.6%. The esters derived from the monoglyceride fraction were: $C_{16:0}$, 0.6%; $C_{18:1}$, 24.0%; $C_{18:2}$, 62.2%; $C_{18:3}$, 12.0%; and unidentified, 1.2%.

DISCUSSION

The experimental work shows conclusively that the unusual hydroxy triglycerides of *Cardamine impatiens* oil have acetylated dihydroxy acids attached to one of the α-positions of glycerol; saturated and long-chain ($>C_{18}$) ordinary acids attached to the other α-position; and oleic, linoleic, and linolenic acids esterified at the β-position. As far as we know, kamala seed oil (13), ergot lipids (14), and *Sapium sebiferum* seed oil (15) are the only previous examples of glycerides containing acylated hydroxy acid moieties. Estolides or polyestolides, in which a chain of two or more hydroxy acids is built up (13), were not observed in *Cardamine* triglycerides as shown by TLC data, and this conclusion is supported by the 2:1 molar ratio of ordinary acids to dihydroxy acids.

Other workers (14,16) have encountered difficulty in hydrolyzing esters of secondary hydroxyl groups by standard methods. However, we experienced no difficulty in removing the acetate group described in this work by either acid or base hydrolysis. Its removal is probably facilitated considerably by the neighboring group effect of the hydroxyl group on the adjacent carbon atom (17).

Preparation of α-ketols from the corresponding hydroxy acetoxy compounds involves an hydrolysis step (to remove acetate) after the hydroxyl group has been oxidized to a keto group. This hydrolysis with either acid or base may cause equilibration (18,19) between the two isomeric forms of the resulting α-hydroxy ketones. Therefore the use of these α-ketols in cleavage reactions designed to pinpoint the original location of the acetate function may lead to erroneous results. In any event, the failure of periodate to cleave the α-ketols described here is surprising since Cramp et al. (9) used this method to cleave a C_{18} α-ketol. The C_{22} and C_{24} chain-lengths of our compounds cause a solubility problem, and the nature of the fat globule surface may be such that periodate is unable to effect cleavage.

A brief consideration of the question of possible acyl migration in the acetylated dihydroxy acids is pertinent to this work. Acyl migration in glycerides was first observed by Fischer (20) and since has been shown to be a common occurrence in partial glycerides (21) and in acetylated carbohydrates (22). The generally accepted mechanism for these migrations involves a cyclic intermediate (20,23,24). Mass spectral data for these hydroxy acetates from *Cardamine* oil lead to the conclusion that the acetate group is distributed approximately equally between the vicinal hydroxyl groups. This conclusion is disturbing because natural biosynthetic processes tend to be selective, and one might expect to find preferential acetylation. This preferential acylation may actually exist but equilibration by heat or electron im-

pact, or both, may have occurred and thus given the observed spectra.

Little is known concerning the preference of hydroxy acids for the various glycerol positions. However saturated acids and acids longer than C_{18} are usually found on the glycerol α-positions in plant lipids where they have a clear-cut choice of position. Therefore the marked preference of the acetylated dihydroxy acids for the glycerol α-positions is not surprising since the dihydroxy acid residues are saturated and almost all have more than 18 carbon atoms. In contrast, estolide moieties in ergot lipids (14), although not directly comparable to our acetylated acids, seem to exhibit no clear-cut preference for either the α- or β-positions of glycerol.

Recent work has shown that linoleic acid is the direct precursor of ricinoleic acid in ergot oil (25). The speculation is made (14) that the biosynthesis of ricinoleic acid and its acylation (in ergot lipids) may occur directly from linoleic acid which is already in triglyceride form. Such a mechanism could possibly be operative in *Cardamine* triglycerides as well. Cruciferae seed oils generally contain erucic (*cis*-13-docosenoic) acid, and many have small amounts of nervonic (*cis*-15-tetracosenoic) acid as well (26,27). Since these long-chain monoenes would normally be attached to the α-position of glycerol, it is conceivable that the dihydroxy acids could be derived by hydroxylation of the corresponding monoenes after the triglyceride structure has been elaborated. Epoxy acids might serve as immediate precursors of the dihydroxy acids, or they might be subject to ring opening by acetate, which would give the hydroxy acetoxy acids directly. This last choice may be even more attractive when one considers that no diacetoxy derivatives were observed and that the acetylation step appears analogous to the chemical acetolysis of an epoxy group in that both hydroxy acetates are formed in equal amounts.

Tentative conclusions given in our preliminary report (2) that a dicarboxylic acid and an alkali-stable linkage were present in *Cardamine* hydroxy glycerides have now been shown to be erroneous. Interpretation of the original NMR data for fraction DA was based on the assumption that ordinary fatty acids were an integral part of DA when, in fact, they were impurities. An unidentified component (V) found earlier (2) was probably not a material containing alkali-stable linkages but more likely was an artifact formed by base in nonaqueous solvents in what appears to be a transesterification reaction.

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Retinol Inhibition of Some Proteolytic Enzymes

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ABSTRACT

Automated analyses were used to determine the effect of retinol on the activity of the following proteolytic enzymes: ficin (EC 3.4.4.12), bromelain (EC 3.4.4.24), trypsin (EC 3.4.4.4.), chymotrypsin A (EC 3.4.4.5), papain (EC 3.4.4.10), clostridiopeptidase A (EC 3.4.4.19), pepsin (EC 3.4.4.1), cathepsin D (EC 3.4.4.23) from rat-liver and rat-kidney lysosomes and the nonspecific proteolytic enzyme, pronase. Of these proteolytic enzymes only ficin, bromelain, and rat-kidney lysosomal cathepsin D were inhibited significantly by 1 x 10^{-4} M retinol.

Some nonproteolytic enzymes not inhibited by retinol were acid phosphatase (EC 3.1.3.2), β-acetylglucosaminidase (EC 3.2.1.30), arylsulfatase (EC 3.1.6.1), and pyruvate kinase (EC 2.7.1.40). The inhibition of cathepsin D varied with the substrate used, being greater with hemoglobin than with ovalbumin or bovine serum albumin. Carotene and retinol inhibited ficin and cathepsin D to similar extents. Retinol inhibition of ficin was partially reversible. These studies of proteolytic enzyme inhibition by retinol serve as a simple model for studying retinol-protein interactions in vitro.

INTRODUCTION

HIGH CONCENTRATIONS of retinol are known to inhibit β-glucuronidase (EC 3.2.1.31). A large number of lipids were tested, and inhibition by retinol was relatively specific. The β-glucuronidase was inhibited 70% by 1 × 10^{-4} M retinol and by 8 × 10^{-5} M cholesterol (1,2). Glutamic dehydrogenase (EC 1.4.1.3) is also sensitive to similar lipid inhibitors and is inhibited 64% at 1 × 10^{-4} M retinol. In vivo, retinol interacts specifically with opsin to produce rhodopsin in the visual cycle (3) and binds to a specific protein carrier in blood plasma during transport (4). The inhibition of enzyme activity by retinol has provided a simple model for studying in vitro the binding of retinol to specific proteins. This paper describes retinol inhibition of ficin (EC 3.4.4.12), bromelain (EC 3.4.4.24), and kidney lysosomal cathepsin D (EC 3.4.4.23).

MATERIALS AND METHODS

Lysosome Preparations

Rat-liver lysosomes were prepared by the method previously described (5). Rat-kidney lysosomes were prepared by a procedure similar to that previously described (6). The animals used were male Sprague-Dawley rats (200-250 g). The lysosome-rich fraction from liver was suspended in 0.45 M sucrose, which contained 0.4 mg glycogen per milliter; that fraction from kidney was suspended in 0.6 M sucrose. The lysosome preparations were carried out at 2-4C. The resulting lysosome-rich fractions were frozen and thawed before carrying out cathepsin reactions.

Fluorometric Enzyme Analysis

The Technicon Auto-Analyzer (Technicon Instruments Corporation, Chauncey, N. Y.) was employed for automated analyses of several proteolytic enzymes. Tryptophan released from hemoglobin, ovalbumin, bovine serum albumin, or casein was measured fluorometrically with an Aminco-Bowman spectrophotofluorometer (American Instruments Company, Inc., Silver Spring, Md.) with an excitation wavelength of 275 mμ and fluorescence at 348 mμ. The flow diagram used has been described previously (7).

For measurement of cathepsin D activity, the reagents pumped into the reaction coils were: hemoglobin, bovine serum albumin, or ovalbumin at a concentration of 10 mg/ml of 0.1 M sodium acetate buffer (pH 5.0), which contained 0.1% Triton X-100. Automated analyses require a detergent to obtain optimum flow patterns and to minimize protein adhesion in the tubing. Triton X-100 is compatible with many hydrolytic enzymes (8) and does not affect enzyme kinetic parameters (9). Ethanolic solutions of inhibitor compounds were pre-mixed with the enzyme in the sample cups just prior to assay, with a final ethanol concentration of 5%. The values for percentage of inhibition were based upon control of enzyme activity in the presence of 5% ethanol.

Trypsin (EC 3.4.4.4), 2 × cryst. (Mann Research Laboratories), chymotrypsin A (EC 3.4.4.5), 3 × cryst. (Mann Research Laboratories), and pronase from Nutritional Biochemicals Corporation were assayed with hemoglobin as substrate. The hemoglobin concentra-

TABLE I
Comparison of Retinol Inhibition of Several Proteolytic Enzymes[a]

Enzyme	Retinol in Reaction Mixture 10^{-5} M	10^{-4} M	10^{-3} M
	% Inhibition		
Ficin	27	44	57
Bromelain	29	45	56
Papain	0	0	2
Clostridiopeptidase A	4	7	14
Pronase	0	0	28
Trypsin	0	0	10
Chymotrypsin A	0	0	13
Cathepsin D			
Liver lysosomes	0	4	19
Kidney lysosomes	17	62	70

[a] Incubations were for 20 min at 37C. Substrate for cathepsin D assays was hemoglobin, 10 mg/ml.

tion was 10 mg/ml in 0.1 M Tris (Tris [hydroxymethyl] amino methane) buffer (pH 8.0).

The activities of ficin, 2 × cryst., (Nutritional Biochemical Corporation), bromelain (Pineapple Research Institute of Hawaii), clostridiopeptidase A (EC 3.4.4.19) (Calbiochem), and papain (EC 3.4.4.10), 2 × cryst. (Nutritional Biochemical Corporation), were measured by using as substrate Hammersten quality casein, 10 mg/ml, which was prepared by the method of Whitaker (10). This substrate was pumped into the Auto-Analyzer at a casein concentration of 10 mg/ml in 0.1 M Tris buffer (pH 8.0), which contained 0.1% Triton X-100. When papain and ficin activities were measured, 1.25×10^{-2} M cysteine was included in the substrate. All of the enzymes were diluted sufficiently in 0.25 M sucrose to give similar product absorbancies upon hydrolysis of the various substrates.

In studies where high levels of retinol could possibly have interfered with tryptophan fluorescence, results were confirmed by measuring the reactions by spectral absorbance at 280 mμ.

Measurements of inhibition were made under similar reaction conditions for each enzyme and substrate. Inhibition was measured during initial enzyme reactions. The fundamental knowledge available on these enzymes and the manual methods for measuring the effect of inhibitors on enzymes applies directly to these automated methods. A detailed study of automated enzyme kinetic parameters, especially substrate concentration and inhibition by retinol, has been made with β-glucuronidase (2), and this study has shown the validity of using automated techniques for measuring enzymic reactions.

TABLE II
Retinol Inhibition of the Hydrolysis of Hemoglobin Bovine Serum Albumin and Ovalbumin by Rat-Kidney Lysosomes

Retinol, final concentration	Hemoglobin	Bovine serum albumin	Ovalbumin
		% Inhibition	
1×10^{-5} M	0	33	0
1×10^{-4} M	50	33	6
1×10^{-3} M	72	56	30

EXPERIMENTAL RESULTS

Retinol Effect on Proteolytic Enzymes

A comparison of the effect of retinol on several proteolytic enzymes is given in Table I. At 1×10^{-4} M retinol, only ficin, bromelain, and kidney lysosomal cathepsin D were significantly inhibited. Crystallized pepsin (EC 3.4.4.1), pre-incubated with 7×10^{-4} M retinol for 45 min, was not inhibited.

Effect of Retinol on Other Enzymes

Several nonproteolytic enzymes were tested for inhibition by retinol. Acid phosphatase, β-acetylglucosaminidase, and arylsulfatase, three lysosomal enzymes, and pyruvate kinase were not inhibited when assayed in the presence of retinol at levels similar to those which resulted in cathepsin D, ficin, and bromelain inhibition.

Arylsulfatase from limpet, rat-liver, and rat-kidney lysosomes and from rat colon mucosa were all tested. Retinol at 6×10^{-4} M had no effect on the rat-liver and limpet arylsulfatase. As reported previously (11), activation was obtained with this enzyme from rat colon and rat kidney. In this study 24% activation was found.

Retinol Inhibition of Rat-Kidney Lysosomal Cathepsin with Various Substrates

Autoanalysis with fluorescence read-out was used to obtain the data presented in Table II. Inhibition of cathepsin from rat-kidney lysosomes was variable, depending upon the substrate used. Hydrolysis of hemoglobin was inhibited 50% when retinol was pre-mixed immediately before assay with 1 ml of suitably diluted kidney lysosomes so that the retinol concentration of the final reaction mixture was 1×10^{-4} M. Hydrolysis of bovine serum albumin and ovalbumin was inhibited 33% and 6% respectively.

Inhibition of Rat-Kidney Lysosomal Cathepsin D and Ficin by Various Lipids

In Table III it is shown that, of the lipids tested for inhibition, retinol is relatively specific for the inhibition of rat-kidney lysosomal ca

TABLE III
Effect of Lipids on Cathepsin D from Rat-Kidney Lysosomes

	Lipid[a] added and % inhibition or activation
Inhibition	Carotene, 42; cholesterol, 30; retinol acetate, 51; retinol, 50
Slight inhibition or none	Lecithin, 7; testosterone, 10; estradiol, 0; squalene, 0; vitamin D_3, 10
Activation	Cholic acid, 10; coenzyme Q_{10}, 16; deoxycholic acid, 5; phytol, 22; progesterone, 3; α-tocopherol, 5

[a]Lipids were added to the enzyme at concentrations of 100 μg/ml enzyme. Incubations were for 20 min at 37C with hemoglobin substrate.

thepsin D. Only cholesterol and carotene, of the many other lipids tested, resulted in inhibition of catheptic activity.

Reversibility of Ficin Inhibition

Table IV shows that the degree of inhibition of ficin by retinol and carotene is similar to that for cathepsin D. The automated fluorescence technique was used to determine whether or not ficin inhibition was reversible. Table V shows the details of the experiment, which indicate that the inhibition was partially reversible upon dilution.

DISCUSSION

Protein-lipid complexes occur in all tissues and cells. Westphal (12,13) has discussed the significance of protein binding to lipids and steroid hormone interaction with proteins. Retinol is known to bind to a specific protein carrier in blood plasma (4) and to interact specifically with opsin in the visual cycle (3). These specialized cases of retinol binding with protein are well known, but few studies of retinol-protein binding in vitro are reported in the literature. The relatively specific inhibition of the lysosomal enzyme β-glucuronidase by retinol and cholesterol has recently been reported

TABLE IV
Effect of Lipids on Ficin Activity

	Inhibitor[a] and % inhibition
Inhibition	Carotene, 16; cholic acid, 7; coenzyme Q_{10}, 16; α-tocopherol, 5; retinol acetate, 23; retinol acid, 30; retinol, 44
No inhibition	Arachidonic acid, cholesterol, estradiol, linoleic acid, linseed alcohol, oleic acid, phytol, soybean alcohol, squalene

[a]Lipids were added to the enzyme at concentrations of 100 μg/ml enzyme. Incubations were 20 min at 37C.

(1,2). This present study of the effect of retinol on proteolytic enzymes provides an additional model for studying in vitro a retinol-protein interaction as well as serving to extend the information on the effect of retinol on the activities of enzymes. The main advantages of studying enzyme inhibition are that all of the techniques of enzyme kinetic analysis can be used and that the more detailed knowledge of the enzyme substrate specificity, active sites, etc., is available.

Survey of the effect of retinol on some lysosomal enzymes has shown that β-glucuronidase (1,2) and cathepsin D are significantly inhibited at 1×10^{-4} M retinol and that acid phosphatase, β-acetylglucosaminidase, and arylsulfatase are not inhibited. Since proteolysis by rat-kidney cathepsin D was found to be inhibited, several other well-known proteolytic enzymes were also studied to determine whether or not inhibition was general for this group of enzymes. Ficin and bromelain were found to be inhibited, but papain, clostridiopeptidase A, pronase, trypsin, chymotrypsin A, and pepsin were not.

The differences in the levels of inhibition of cathepsin D with the three substrates (Table II) may be related to their different protein characteristics and presentation as proteolytic substrates. Also, the binding of retinol by these

TABLE V
Reversibility of Retinol Inhibition of Ficin upon Dilution

Experiment No.[a]	Condition Prior to Enzyme Analysis		Condition of Enzyme Analysis		Florescence Units	Inhibition
	Ficin	Retinol	Ficin	Retinol		
	mg/ml	μg/ml ficin	mg/ml	μg/ml ficin		%
1	0.2	0	0.2	0	6.0	
2	0.2	100	0.2	100	5.5	8.3
3[b]	0.8	400	0.2	100	5.0	16.7
4	0.8	0	0.8	0	23.8	
5	0.8	400	0.8	400	15.6	34.4

[a]Experiment 1 is control for Experiments 2 and 3; Experiment 4 is control for Experiment 5.
[b]Enzyme and retinol were diluted 1:3 before determining activity against the casein substrate (pH 7.0).

substrates may be important. Bovine serum albumin and ovalbumin strongly bind retinol (14). This problem was not studied in further detail.

With the main emphasis on retinol and cholesterol, the specificity of lipid inhibition of ficin and rat-kidney cathepsin D was found to be similar to that for β-glucuronidase; cholesterol (in the case of β-glucuronidase and cathepsin D), carotene, and retinol inhibited the three enzymes to the greatest extent. Retinol, retinyl acetate, and carotene were moderate inhibitors of cathepsin D and ficin as they also were of β-glucuronidase. The major difference in specificity of inhibition was that cholesterol inhibited cathepsin D 30% but did not inhibit ficin. Another interesting set of differences between cathepsin D and ficin is that cholic acid, coenzyme Q, and α-tocopherol all gave small inhibitions of cathepsin D and small activations of ficin. In the context of this study these small divergent effects were not studied further. As was found with β-glucuronidase (2), inhibition of ficin by retinol could be partially overcome by dilution of the enzyme-inhibitor mixture before addition of substrate.

Retinol inhibition of β-glucuronidase and glutamic dehydrogenase is probably a result of disaggregation of these enzyme molecules into inactive units (2). There are no known common properties of ficin, bromelain, or cathepsin D which allow explanation of their inhibition by retinol. In the only previous studies of lipid inhibition of any of these three enzymes, sorbate ion was found to be a powerful inhibitor of ficin. However the mechanism of sorbate inhibition involves an irreversible reaction with the essential sulfhydryl group of ficin (15). In all of these studies of retinol inhibition of ficin, excess cysteine was present and retinol inhibition was reversible upon dilution.

It is probable that a common characteristic of the enzymes sensitive to retinol is their general hydrophobic nature. As indicated by their binding of nonpolar compounds, β-glucuronidase and glutamic dehydrogenase have many hydrophobic sites. Also, since the retinol and the other lipid compounds were used as micelle dispersions, the sensitive enzymes could be inhibited by binding through many general hydrophobic sites; certainly they are not specific for retinol as is the protein opsin.

In the search for functions of retinol in the animal body, considerable attention is directed to its membrane-active reactions. Dingle and Lucy (16) have reviewed retinol interaction within lipoprotein membranes. Excess retinol penetrates lysosomal membranes with release of lysosomal enzymes (17), it penetrates and expands red blood-cell membranes and causes lysis (18), and it causes considerable membrane damage to fibroblasts (19). With increasing knowledge of retinol interaction with proteins and lipoproteins, this information on retinol inhibition of ficin, bromelain, and kidney lysosomal cathepsin D is of biochemical interest.

ACKNOWLEDGMENTS

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Tritium and ^{14}C Counting in Tissue Samples by Using Liquid Scintillation Method

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ABSTRACT

The combustion method has been modified to increase the recovery of tritiated water after combustion of a tritium-labeled tissue sample. This was accomplished by cooling the bottom of the combustion flask in a dry ice-acetone bath while irradiating the top with an infrared lamp. The procedure resulted in at least 92% to 102% recovery of the tritiated water. The NCS solubilizer was found to be superior to hyamine for solubilizing ^{14}C labeled tissue samples. The samples yielded light yellow-colored solutions when incubated for 15 hr at 50-55C. The counting efficiency of this solution was 75% or higher.

INTRODUCTION

LOW SPECIFIC ACTIVITIES of tritium in tissue samples are difficult to determine directly by solubilizing the tissue samples in hyamine or NCS base (Nuclear Chicago Corporation, Ill.). Even though the base is miscible in the scintillation fluid, the counting efficiency is quite low owing to severe quenching. To overcome the problem, the labeled tissue samples are combusted, and the activity of the resulting tritiated water is determined. The combustion method of Kelly (6) has been partially modified by Oliverio et al. (8), but the results obtained in our laboratories were not consistent. Therefore this method was modified to obtain higher recoveries of tritiated water after combustion, and the equipment was simplified for easier assembly.

The ^{14}C labeled tissues, or organic compounds, have been solubilized in many different systems. Passman et al. (10), Frederickson and Ono (5), Bruno and Christian (2) used hyamine; Petroff et al. (9) used methanolic KOH; Mahin and Lofberg (7) used perchloric acid with H_2O_2; and Eldefrawi (4) used HNO_3 acid oxidation. These methods were evaluated, and though hyamine was satisfactory, it gave low counting efficiency. Later another quaternary ammonium base which was soluble in toluene, the NCS solubilizer, became available and was compared with hyamine and the other methods for solubilizing tissue samples or organic compounds.

One milliliter of NCS can dissolve tissue samples weighing 50-100 mg when left for seven days or more at room temperature. Hyamine dissolved little of the tissue under similar conditions. Complete solubilization of tissue samples was obtained at 50-55C with both hyamine and NCS solubilizer. Some flocculation was visible when tissue samples were dissolved in perchloric acid – H_2O_2, methanolic KOH, HNO_3, or hyamine. The counting efficiencies were low when samples were dissolved in hyamine, perchloric acid, or HNO_3 acid but where higher in NCS and methanolic KOH. In the case of methanolic KOH, if the samples were not counted the same day, the efficiency dropped to less than 30%, and the background counts were three times the normal.

Taking into consideration the tissue-solubilizing properties and the counting efficiency of the scintillation fluid containing a base, the NCS solubilizer was found quite satisfactory.

METHODS AND RESULTS

Tritium Counting in Tissue Samples

Combustion of the tritiated tissue sample was carried out in a two-liter heavy wall flask. Instead of the usual platinum wire, 20-gauge Chromel wire was used.

It was wound in a tight spiral (Fig. 1), with the coils in the form of a conical basket to hold the sample. The wire from the basket was fused into a 20-mm × 8-mm glass rod. The rod was inserted into a No. 9 rubber stopper. When the stopper was inserted tightly into the flask, the basket hung freely one-third above the bottom of the flask. To prevent any loss of tritiated water which was produced during combustion of the tissue sample, a copper band, 1/2 in. wide with half of a worm-drive hose clamp screwed at each end, was used. Tightening of the copper band in place prevented any loss of moisture during combustion.

Dialysis tubing, made of cellulose, was used for holding tissue samples. The dialysis tubing was cut into 1 to 1½-in. lengths, cut open on one side, and converted to a cone with the aid of Duco cement. The pointed end of the cone was pressed to make a flat base for holding the tissue samples. Two holes were punch-

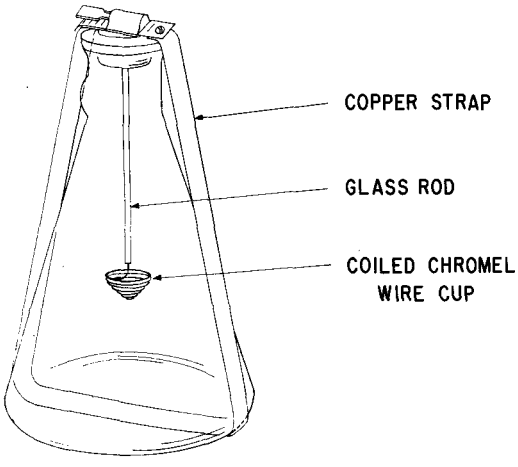

FIG. 1. Heavy wall flask for combustion of the tritiated tissue sample.

ed on the sides of the cone, through which a wire was passed. The wire kept the cone hanging freely in an upright position. Wet tissue samples, e.g., liver, muscle, duodenum, kidney, skin, etc., weighing up to 50-100 mg, or measured volumes of 0.1 to 0.2 ml of urine or blood, were transferred to these cones hanging on the wire. The samples were allowed to dry overnight under an infrared lamp.

To determine percentage recovery, known amounts of tritiated sodium acetate standard in alcohol were pipetted onto the dried tissue samples. The alcohol was allowed to evaporate, and a strip of black paper 3/16 in. × 5/8 in. was attached to the cone containing the sample to assist in ignition. The Chromel wire basket containing the sample was transferred to the flask, and the flask was purged with pure oxygen for a period of 2 to 3 min. The stopper was clamped tightly with the help of the copper band. The flask was then transferred to a Thomas Ogg safety chamber, equipped with an infrared (IR) lamp. The sample ignited easily when the IR light was focused on the black strip of paper. The combustion was complete within a few seconds.

The flask was transferred to a dry ice-acetone bath; taking care that only the bottom of the flask was immersed. After 5 min the top of the flask was irradiated with an IR lamp for 30 min from a distance of 12 in. The bottom of the flask remained in the dry ice-acetone bath. After irradiation the top of the flask was allowed to cool while the bottom still remained in the dry ice-acetone bath. The stopper was removed, and 20 or 40 ml of the scintillation fluid [4 g of 2,5-diphenyloxazole (PPO), 100 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl-POPOP) per liter of toluene] containing 20% alcohol was added. The stopper was reinserted, the solution was swirled, and the flask was immersed in a crushed ice bath. After equilibration to bath temperature for approximately 10 min, 10 or 20 ml of the counting solution were withdrawn and counted in a liquid scintillation counter.

TABLE I

Recovery of the Tritiated Water after Combustion of the Dried Tissue Samples

	Before modification of the method	After modification of the method
CPM recovered	2916-4861	4471-4957[a]
Percentage recovery	60-100	92-102

[a]Sodium acetate – H^3 (32,400 dpm/10μl) was used as standard. Efficiency of counting $\cong$ 15% in both cases.

The percentage recovery of tritiated water (Table I) was in the range of 60-100% by using the method of Kelly whereas the modified method consistently gave recoveries of 92-102%.

In the case of tritium counting, oxygen quenching has been reported by Conway and Grace (3) and Baggett et al. (1). Leaving the samples in the dark for more than 4 hr however gave reliable and reproducible cpm (counts per minute). There were no changes in count rate even after four days when the samples were equilibrated with air, as suggested by Conway and Grace (3).

Efficiency of counting for each sample was determined by the internal standard method, which corrected for any quenching because of water, alcohol, and oxygen.

^{14}C Counting in Tissue Samples

To study the incorporation of ^{14}C-glycine in the skin, liver, and muscle after intraperitoneal injection in a rat, the tissue samples were removed, chopped with a scalpel, weighed, and transferred to liquid scintillation counting vials containing 1 ml of NCS solubilizer. Vial caps with tin foil linings were used. After transfer the vials were allowed to stand in an incubator at 50-55C overnight. Use of higher temperatures solubilized the tissue samples within 4 to 6 hr, but the intense yellow color, so produced, caused severe quenching problems. The vials were removed from the oven and allowed to cool to room temperature. Fifteen milliliters of toluene counting solution without alcohol was added to each vial. The samples were transferred to the liquid scintillation

TABLE II

Comparison Between NCS Solubilizer and Hyamine 10x

Tissue[a]	NCS Mean DPM/100 mg of tissue ± standard deviation	Hyamine Mean DPM/100 mg of tissue ± standard deviation
Skin	3699 ± 387	4331 ± 1117
Liver	20215 ± 113	19887 ± 1808
Muscle	2968 ± 946	4151 ± 670
Range of Efficiency (%)	65 - 75	49 - 58

[a]Ten samples of each tissue, weighing up to 50-100 mg, were incubated in one ml of either base at 50C for 15 hr. Efficiency for each sample was determined by internal standard method.

counter and dark-adapted for 4 hr or more before counting.

The present method eliminates the pipetting error after digestion of the tissue samples, as is the case in solubilization with methanolic KOH. There is no loss of volatile end-products as occurs in the nitric acid oxidation method. The counting solutions were always less yellow when the tissue samples were solubilized with NCS than with hyamine. In addition, the counting efficiencies of the tissue samples dissolved in NCS were always higher than with hyamine (Table II). Quenching owing to the dissolved tissue sample in NCS was initially corrected by the use of an internal standard, but with the availability of an external *gamma* source it has become easier to ascertain the efficiency of each vial in a short interval of time. The efficiency obtained from the external standardization source was checked several times with an internal standard method, and the two methods agreed within a range of ± 0.1%.

To correct for the drift in the instrument, a set of quenched standards was always counted with the unknown quenched samples.

An experiment was carried out to determine whether differences in the volume of scintillation fluid affected the counting and the efficiency of each vial. The variations in volume had little effect on CPM or on the efficiency of counting each vial (Table III).

Tissue fluorescence did not interfere in counting ^{14}C when NCS was used for solubilizing tissue samples. The CPM were consistent

TABLE III

Effect of Various Volumes of Scintillation Fluid on CPM and Efficiency

(Standard Used: Toluene ^{14}C-4370 dpm/10μl)

Volume of scintillation fluid used in ml	CPM	Efficiency
1	3216	73.3
2	3238	73.8
4	3295	75.1
6	3220	73.4
8	3168	72.3
10	3295	75.1
12	3287	74.9
14	3221	73.4
16	3220	73.4
18	3154	71.8
20	3060	69.7

if the samples were allowed to remain in the dark for more than 4 hr. When methanolic KOH was used for tissue digestion, tissue fluorescence affected the cpm.

These methods yielded higher recovery of tritiated water after combustion. The NCS solubilizer was quite satisfactory in solubilizing ^{14}C-labeled tissue samples. The efficiency of counting was 75% or higher.

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The Total Synthesis of Phosphatidyl(Dioleoyl)Hydroxy-L-Proline and Its Activity in Blood-Clotting Systems

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ABSTRACT

The phthalimidomethyl ester of *N*-anisyloxycarbonyl-hydroxy-L-proline was combined with phosphorus oxychloride and *rac*-1,2-diolein. The diolein was made by large-scale preparative application of the method of Krabisch and Borgström (1). The protected phosphatide, obtained by the phosphorylation reaction, was stripped of its protective groups under mild conditions. The phosphatidyl(dioleoyl)-hydroxy-L-proline was purified by TEAE cellulose (acetate) chromatography, as developed by Rouser (6), also by silicic acid chromatography. Aqueous dispersions of the material were tested for anticoagulant activity in the antithromboplastin test and the Hicks-Pitney test. The new phosphatide had about one-tenth of the activity of beef brain phosphatidylserine.

INTRODUCTION

THE TOTAL SYNTHESIS of phosphatidyl(distearoyl) hydroxy-L-proline from an optically active 1,2-distearin was achieved by Baer and Zschocke (2). However, because saturated phosphatides are not dispersible in water, it is not possible to test the phosphatide of Baer and Zschocke in biological tests. From experience with phosphatidylserine and phosphatidylethanolamine it can be expected that unsaturated phosphatides would be sufficiently dispersible in water to be tested. In order to synthesize unsaturated phosphatides of this type, it is necessary to use protecting groups that can be removed without altering the double bonds. In a synthesis of unsaturated phosphatidylserines (3,4) we employed the phthalimidomethyl group to protect the carboxyl of serine, and the anisyloxycarbonyl group to protect the amino group. This technique has now been used with hydroxy-L-proline (Fig. 1).

Although phosphatidylhydroxy-L-proline has not been found in nature, it has sufficient similarity to phosphatidylserine to make its possible biological activity a matter of great interest. The relation between structure and activity might be clarified by the synthesis of a variety of phosphatidyl amino acids. This is particularly emphasized by the recent report that a phosphatide containing an unidentified hydroxy-amino acid (not serine) is a renin activator (5).

PROCEDURES AND RESULTS

Materials and Methods

Hydroxy-L-proline (M. A. grade) was obtained from Mann Laboratories, New York, and oleic acid (Purum) for the 1,2-diolein from Fluka A.G., Buchs, Switzerland. The TEAE cellulose (selectacel, No. 83, standard grade) was obtained from Carl Schleicher and Schuell Inc., Keene, N. H. A portion of 100 g was put into a 4-liter beaker, filled with distilled water. After most of the TEAE cellulose had settled, the water was decanted. This process was repeated 10 times to remove fines. The TEAE cellulose was then washed with 0.1N sodium hydroxide, washed with water until neutral, cycled through the chloride form, and finally restored to the washed OH form. It was next washed with methanol, dried in a high vacuum, and converted to the acetate form with glacial acetic acid according to the earlier methods of Rouser (6,7).

Magnesium oxide ("light" grade) was obtained from British Drug Houses, Poole, England. Anisyl azidoformate was made as described before (4). Peroxide-containing solvents, like tetrahydrofuran, were freed of peroxides by distilling over triphenyl phosphine. Solvents were dried, where necessary, over molecular sieves (4). Hydrazine (95% grade) was from Eastman Kodak. Silicic acid was Biosil of Bio-Rad Laboratories, Richmond, Calif.

All operations were conducted under nitrogen when unsaturated fatty acids or unsaturated phosphatides were treated. Transfers were made in the glove box of I2R Inc., Cheltenham, Pa. Infrared spectra were determined on Nujol mulls by using the Infracord (Perkin-Elmer Inc., Norwalk, Conn.). Melting points were determined on the Kofler hot bench.

rac-1,2-Diolein. The *rac*-1-(2′-tetrahydropyranyl)-glycerol was made according to Barry and Craig (8), and it was acylated with oleoyl chloride to form *rac*-1,2-dioleoyl-3-(2′-tetrahydropyranyl)-glycerol. The tetrahydropyranyl group was removed from this in 10-g batches, according to Krabisch and Borgström (1). The

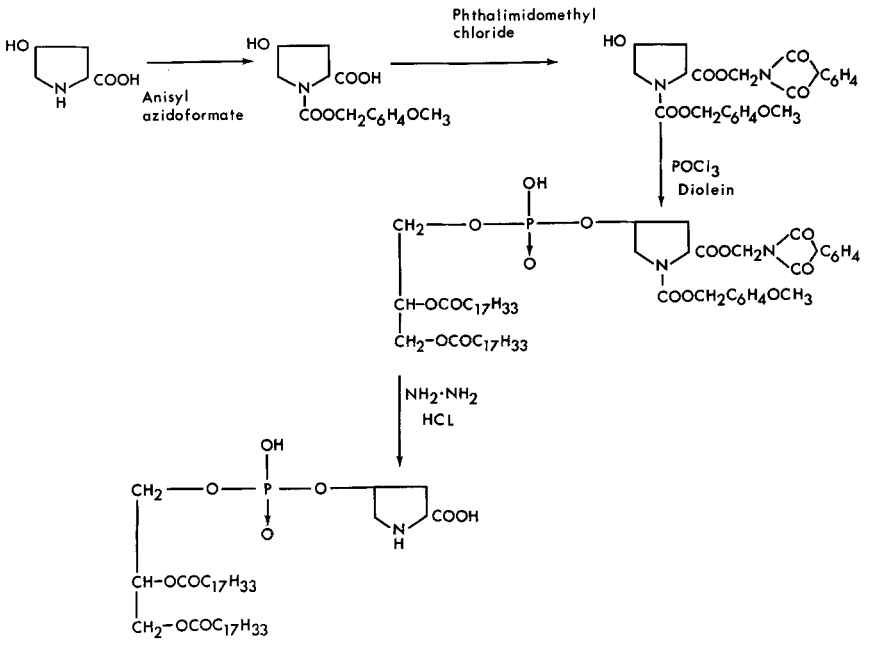

FIG. 1. Scheme for the synthesis of phosphatidyl(dioleoyl)hydroxy-L-proline.

removal required 13.9 ml of concentrated hydrochloric acid, added to a solution of the tetrahydropyranylglyceride in 280 ml of U.S.P. ether and 280 ml of methanol. After 10 min at room temperature the mixture was diluted with water and extracted with heptane. The diglyceride was purified according to the methods of Crossley et al. (9,10) and was examined by TLC with the use of Silica Gel H treated with boric acid (11) and also silica gel treated with silver nitrate (with the solvent isopropyl alcohol-chloroform 1.5:98.5) (12) by comparison with authentic 1,2-diolein and 1,3-diolein. From this TLC investigation it appeared that the material was 1,2-diolein with traces of impurities.

p-Methoxybenzyloxycarbonyl-hydroxy-L-proline. The protected amino acid was made by the general procedure of Weygand and Hunger (13) by the action of anisyl azidodoformate on the amino acid in the presence of magnesium oxide. The product was an oil obtained in 73% yield. Its infrared spectrum was consistent with the structure expected, 5.65 μ(carboxy) 5.90 μ(urethane carbonyl), 2.9 μ and 9.2 μ(secondary OH).

The substance was characterized as a dicyclohexylammonium salt, obtained by adding dicyclohexylamine to a solution of the acid in dimethyl formamide and then precipitating the product with acetone and ether. This gave a gelatinous solid which was recrystallized from ethanol-ether, mp 165C (dec.). The carbonyl bands in this material were now shifted to 5.87 μ and 6.08 μ.

Anal. Calcd. for $C_{26}H_{40}N_2O_6$: C, 65.52; H, 8.46. Found: C, 65.53; H, 8.41.

Phthalimidomethyl Ester of p-Methoxybenzyloxycarbonyl-hydroxy-L-proline. The preceding dicyclohexylammonium salt (26 g) was put into 200 ml of dry dimethylformamide, and the mixture was heated to 70C. An equimolar amount of phthalimidomethyl chloride was dissolved in the clear solution. The mixture was stored in a stoppered flask at 40C over-night. The addition of water precipitated an oil, which was rubbed to induce crystallization. The crystals were filtered and washed thoroughly with water. They were dried and recrystallized from ethanol. The yield was 85%, mp 147C $[\alpha]_D = -20.1$ (c=1%, $CHCl_3$).

The infrared spectrum showed the typical spectrum of this type of compound (3) with four bands in the carbonyl region (5.39; 5.70; 5.80; 5.89 μ).

Anal. Calcd. for $C_{..}H_{..}N_2O_8$: C, 60.79; H, 4.88; N, 6.17. Found: C, 60.76; H, 4.80; N, 6.10.

Dioleoylglycerophosphoryl-N-anisyloxycarbonyl-hydroxy-L-proline Phthalimidomethyl Ester. A solution of 7.85 g (17.3 mM) of the phthalimidomethyl ester of anisyloxycarbonyl-hydroxy-L-proline and 2.6 ml (19 mM) of triethylamine in 100 ml of dry tetrahydrofurane

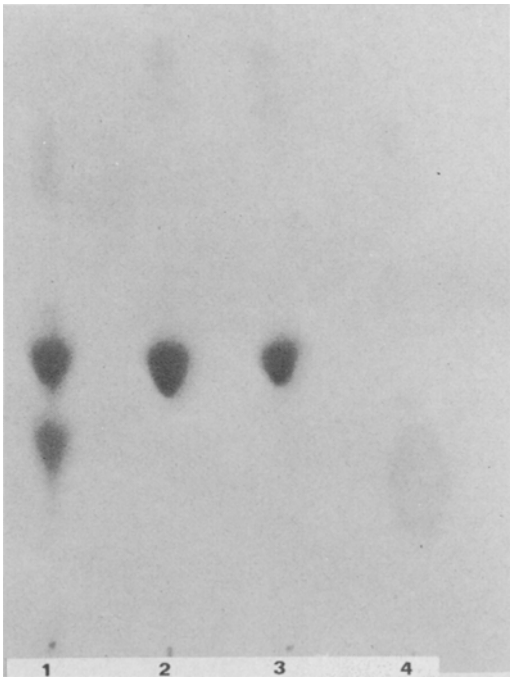

FIG. 2. TLC of phosphatidyl(dioleoyl)hydroxy-L-proline eluates from TEAE cellulose column. Channels 2 and 3—material eluted with glacial acetic acid; Channel 1—fraction eluted with methanol after the acetic acid eluate; Channel 4—last eluate (not phosphorus compounds), obtained with NH_4OH in $CHCl_3$-MeOH.

All spots are revealed with stain for phosphorus of Long et al. (17). Ninhydrin, followed by the Ehrlich reagent, revealed only single spot in Channels 2 and 3 and two spots in Channel 1. The slow-moving material in Channel 1 is probably lysophosphatide.

was cooled in ice water with stirring. To this was added 7.9 ml (86.5 mM) of phosphorus oxychloride. After 30 min at room temperature the triethylammonium chloride was removed by centrifuging, and the precipitate was washed twice with dry tetrahydrofurane. The combined tetrahydrofurane was evaporated in vacuo, first with a water aspirator and finally in the vacuum of an oil pump. The oily residue was dissolved in 100 ml of dry benzene. Then 2.6 ml (19 mM) of triethylamine and a solution of 10 g (16 mM) of *rac*-1,2-diolein in 100 ml of dry benzene was added. After standing over-night at room temperature, the mixture was centrifuged, and the solution was evaporated in vacuo. The residue was dissolved mostly in ether, and the ethereal solution was decanted from some insoluble gum. The ether was washed with ice-cold 0.5N sulfuric acid and water and dried over sodium sulfate. Evaporation of the ether gave an oil weighing 17.5 g. TLC of this material was carried out on silica gel H (E. Merck, Darmstadt) by using the solvent system of Grisdale and Okany (14), which has given good results with phosphatidylserine preparations.

This solvent system is a mixture of seven volumes of solvent No. 1, consisting of chloroform-methanol-acetic acid-water (65:25:8:4), with three volumes of the upper layer of solvent No. 2, consisting of *n*-butyl alcohol-acetic acid-water (4:1:5). The TLC plates were stained with dichloro-fluorescein, iodine, and the chlorine-tolidine procedure of Pataki (15) for carbobenzoxyamino acids. Only the major spot reacted with all three reagents although there were three spots shown. One reacted only with iodine (recovered diglyceride) while the other contaminating material was shown by the dichlorofluorescein. This was taken to indicate that the material was pure enough for removal of the protecting groups. Chromatographic purification was avoided in order to avoid oxidation.

Phosphatidyl(dioleoyl)hydroxy-L-proline. The preceding material was freed of its protecting groups in portions adapted to rapid column chromatography without overloading the columns as follows. To a solution of 2.4 g of the product in 50 ml of ethanol cooled to −15C was added a solution of 0.71 ml of 3.09 M hydrazine in ethanol. After 45 min at room temperature a second portion of 1.07 ml of 3.09 M hydrazine was added. The mixture was stored at 38C for two days. It was then evaporated, and the residue was dried thoroughly in a high vacuum. The residue was dissolved in 20 ml of dry chloroform, and the solution was cooled to 0C. To the solution was added a solution of 39 ml of 0.38N dry hydrogen chloride in dry chloroform that had been precooled to 0C. The mixture was kept at 0C for 45 min. The time selected was judged to give the maximum yield of phospatide as determined by TLC of the reaction mixture periodically. At the end of this period the solvents were removed at 0C by using a Langdon pump as recommended by Rouser et al. (6). Chloroform was added, and the distillation was repeated to remove hydrogen chloride.

A column of 35 g of TEAE cellulose (acetate form) was prepared, and the preceding crude phosphatide was separated on this column by elution (6) with chloroform-methanol 7:3, chloroform-methanol 1:1, pure methanol, and acetic acid chloroform 1:3. Finally the

pure phosphatide was removed from the column with glacial acetic acid, and this was freeze-dried to yield 174 mg of light powder.

The appearance of TLC of some of the fractions from the column is shown in Fig. 2. The solvent system of Grisdale and Okany (14) was used with Silica Gel H. The plates were first stained with ninhydrin, giving a yellow spot with phosphatidylhydroxy-L-proline. This yellow spot was converted to a red spot by spraying with Ehrlich reagent (16). Finally, after it was sprayed with the phosphate reagent of Long et al. (17), the spot turned blue. At the same time the phosphate reagent revealed the presence of traces of impurities (Fig. 2) near the solvent front. These impurities were removed by chromatography of the material on silicic acid columns in the usual manner (3,4). The progress of the separation was followed by TLC and the ninhydrin test. The pure material was eluted with 15% methanol in chloroform when Bio-sil was employed. The recovery of material showing only a single spot by TLC (Fig. 3) was 60% of the material applied to the silicic acid column. The phosphatide was also homogeneous when 300 μg were applied to paper impregnated with silicic acid according to the methods of Marinetti (18). On such paper it moves slightly behind phosphatidylserine if the di-isobutyl ketone-acetic acid-water solvent is used.

The fatty acids, obtained from the phosphatides, were converted to methyl esters and determined by GLC with the Barber-Colman Model 20 as tritium detector and an 8 ft. ⅛ in. stainless steel column, containing a packing of 20% DEGS on 60/80 mesh Chromosorb W. The mixture was separated isothermally with a column temperature of 185C. The fatty acids were 95% oleic acid with traces of palmitic, palmitoleic, heptadecanoic, stearic, and linoleic acids. This composition essentially duplicated the composition of the Fluka Purum fatty acid which was used as starting material. The phosphatidyl(dioleoyl)hydroxy-L-proline had $[\alpha]_D$ −5.17 (1% $CHCl_3$).

Anal. Calcd. for $C_{44}H_{80}NO_{10}P$ (814.1): C, 64.91; H, 9.91; N, 1.72, P, 3.80. Found: C, 64.40; 65.01; H, 10.08, 10.13; N, 1.91, 1.51; P, 4.04, 3.92.

Activity of Phosphatidyl(dioleoyl)hydroxy-L-proline (PHP) as an Inhibitor of Plasma Thromboplastin Formation and Brain Thromboplastin Activity. The effects of solubilized phosphatidyl(dioleoyl)hydroxy-L-proline in blood-clotting tests were compared with those of solubilized phosphatidylserine.

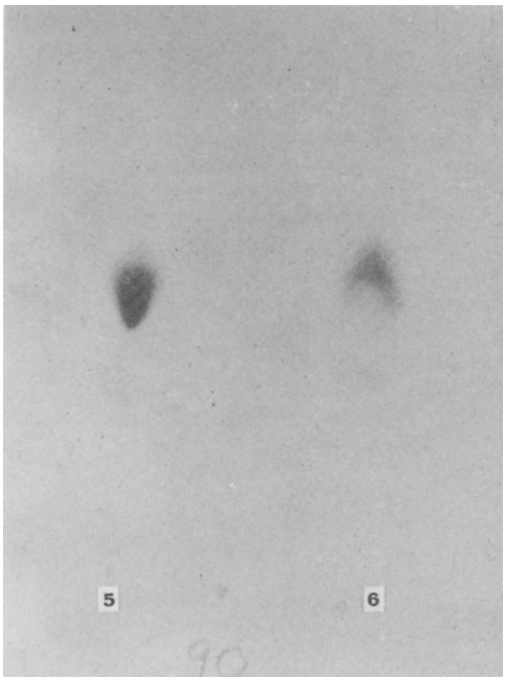

FIG. 3. TLC of phosphatidyl(dioleoyl)hydroxy-L-proline eluate from silicic acid column. Channel 5—pure phosphatidylhydroxy-L-proline; Channel 6—lecithin and lysolecithin from egg (The stain employed was that of Long et al. for phosphorus compounds (17).).

Attempts were made to solubilize the free acid form of the PHP both in human plasma albumin solution and in sodium deoxycholate solution as described for beef brain phosphatidylserine by Silver et al. (19). Solubilization in sodium deoxycholate solution was almost complete at a concentration of 2 mg/ml of PHP in 2 mg/ml of sodium deoxycholate buffered at pH 7.4 with 0.05 M imidazole buffer in 0.154 M sodium chloride solution. The preparation was transparent with a few barely visible particles. Efforts to solubilize the free acid PHP in albumin solution however were unsuccessful. The free acid PHP was therefore converted to the sodium salt to see if it might not be more easily solubilized with albumin. The sodium salt was prepared according to the method of Abramson et al. (20) for the conversion of free acid phosphatidylserine to its salt form. The sodium PHP was partially solubilized by albumin. Fine particles in stable suspension were present in the preparation which was employed for the biological test.

The solubilized PHP preparations were compared with preparations of phosphatidylserine

TABLE I

Activities of Solubilized Synthetic Phosphatidyl(dioleoyl)hydroxy-L-proline and Beef Brain Phosphatidylserine in the Modified Hicks-Pitney Test[a]

Incubation Time (Minutes)			4	6	8	10
Substance tested	Solubilized in	μg in incubation mixture	Substrate clotting times (seconds)			
PS[b]	Desoxycholate	200	> 100	> 100	> 100	> 100
	Desoxycholate	100	> 100	> 100	> 100	> 100
	Desoxycholate	10	> 100	> 100	> 100	32
	Desoxycholate	1	23	8	8	8
PHP[b]	Desoxycholate	200	> 100	> 100	> 100	> 100
	Desoxycholate	100	> 100	> 100	> 100	> 100
	Desoxycholate	10	30	8	8	8
	Desoxycholate	1	8	8	8	8
Desoxycholate controls			8	8	8	8
PS	Albumin	100	> 100	> 100	> 100	> 100
	Albumin	50	> 100	> 100	> 100	> 100
	Albumin	25	> 100	> 100	> 100	> 100
	Albumin	12	> 100	> 100	> 100	> 100
	Albumin	6	> 100	> 100	> 100	> 100
PHP Na[b]	Albumin	100	> 100	> 100	> 100	> 50
	Albumin	50	110	65	20	8
	Albumin	25	37	12	8	8
	Albumin	12	20	8	8	8
	Albumin	6	8	8	8	8
Albumin controls			8	8	8	8
Buffered saline controls			8	8	8	8

[a] The modified Hicks-Pitney test is described in Reference 19. As employed, the controls and all test runs contain all components necessary for rapid plasma thromboplastin formation (substrate clotting times under 10 sec). Long clotting times indicate inhibition of thromboplastin formation.

[b] Abbreviations: PS—phosphatidylserine from beef brain; PHP—phosphatidyl(dioleoyl)hydroxy-L-proline (free acid); PHP Na—phosphatidyl(dioleoyl)hydroxy-L-proline (sodium salt).

from beef brain solubilized in a similar fashion. The blood coagulation tests were the antithromboplastin test (Table II) and the modified Hicks-Pitney test described by Silver et al. (Table I).

The modified Hicks-Pitney test, as used in this study, allows for the measurement of the inhibition of the generation of plasma thromboplastin by the substances under testing. The results (Table I) show that the solubilized test preparations of phosphatidyl(dioleoyl)hydroxy-L-proline (PHP) inhibit plasma thromboplastin formation and have roughly 10% of the activity of the beef brain phosphatidylserine (PS) preparations. For example, in Table I, it can be seen that PS solubilized in desoxycholate was strongly inhibitory with 10 μg in the incubation mixture. Similar inhibitory activity required 100 μg of PHP. The sodium salt of PHP appeared to be less inhibitory when it was solubilized in albumin solution. This may be a reflection of its incomplete solubilization in this medium.

In the antithromboplastin test, results were similar. In Table II it can be seen that 10 μg of PS, solubilized in desoxycholate, gave a 25-second clotting time with a 13-second control. To obtain inhibition of a similar order of magnitude, 100 μg of the PHP were required. The activity of the PHP Na solubilized in albumin was somewhat less than when it was solubilized in desoxycholate. Again, this probably represents incomplete solubilization.

DISCUSSION

The importance of synthesizing phosphatides containing a variety of hydroxy amino acids for biological study arises from the increasing number of functions for phosphatidylserine.

It is an inhibitor of D-gulonolactone oxidase (21) and an activator of an ATPase (22), and it plays a part in the transport of ions across membranes (23,24 i.a.). A phospholipid containing a hydroxy acid other than serine has been found to be a renin activator (5).

The synthesis of the phosphatide started with a *rac*-1,2-diolein which was made by a large-

scale application of the simple method of Krabisch and Borgström (1). Gigg and Gigg (25) have suggested other methods for removing the tetrahydropyranyl group from *rac*-3-(2′-tetrahydropyranyl)-1,2-diolein and have indicated means whereby the dioleoyl-tetrahydropyranylglycerol could be prepared as optically active stereoisomers. The diolein was combined with phosphorus oxychloride and a protected amino-acid. The protecting groups chosen have been discussed earlier (3,4). The protected phosphatide was stripped of its protecting groups by the method employed for the corresponding serine derivative (4), and the phosphatidyl (dioleoyl)-hydroxy-L-proline was purified by chromatography using Rouser's methods (6). TEAE cellulose was more effective for the purification of this phosphatide than DEAE cellulose.

The purified phosphatide was tested for its effect on blood coagulation after solubilization using the modified Hicks-Pitney test (19) and the antithromboplastin test (4,19). In each case the test preparation was compared with phosphatidylserine under similar conditions. As mentioned in the experimental section, the phosphatidyl(dioleoyl)hydroxy-L-proline had about one-tenth of the activity of phosphatidylserine from beef brain.

Since it has been shown that a racemic synthetic phosphatidylserine essentially duplicates the activity of natural phosphatidylserine (4), it seems unlikely that the low activity of the phosphatide studied here is caused by a lack of stereospecificity. The ring nitrogen and the rigidity of the ring system of the phosphatidylhydroxy-L-proline are more likely to influence the colloidal properties of the dispersed phosphatide which are important for clotting activity (19).

TABLE II

Activities of Solubilized Synthetic Phosphatidyl(dioleoyl)-hydroxy-L-proline and Phosphatidylserine from Beef Brain in the Antithromboplastin Test[a]

Micrograms phospholipids in test		Clotting Times in Seconds When Phospholipids Solubilized in: Desoxycholate PS[b]	Desoxycholate PHP[b]	Albumin PS	Albumin PHP Na[b]
200		129	36	—	—
100		86	24	102	18
10		25	14	28	14
1		14	13	—	
Controls					
1. Desoxycholate	200	16			
	100	13			
	10	13			
	1	13			
2. Albumin	1000			13	
	100			13	
	10			13	
3. Buffered saline			13		

[a] The antithromboplastin test gives the clotting time in seconds of a mixture of 0.1 ml of human plasma, 0.1 ml of the test solution of phosphatide or of sodium desoxycholate (100 μg in the buffer), and 0.1 ml of brain thromboplastin. The time is taken from the addition of 0.1 ml of 0.02 M calcium chloride.

[b] Abbreviations: PS—phosphatidylserine from beef brain; PHP—phosphatidyl(dioleoyl)hydroxy-L-proline (free acid); PHP Na—phosphatidyl(dioleoyl)hydroxy-L-proline (sodium salt).

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An Unsaturated Phosphonic Acid Analogue of Phosphatidylethanolamine and Its Activity in Blood-Clotting Systems

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ABSTRACT

An unsaturated phosphonolipid analogous to phosphatidylethanolamine, *rac*-dioleoylglyceryl(2-aminoethyl)phosphonate, was synthesized by a general method introduced by Baer for similar saturated substances. An improvement was made in the preparation of the phthalimidoethyl-phosphonic acid precursor.

The phosphonolipid was purified by DEAE cellulose and silicic acid chromatography. It was tested by comparison with synthetic phosphatidyl (dioleoyl) ethanolamine and phosphatidyl(dilinoleoyl) ethanolamine in the Hicks-Pitney test and in a test for prothrombin conversion by using purified blood coagulation factors. In both tests it had more acceleratory activity than the synthetic phosphatidylethanolamines.

INTRODUCTION

BAER ET AL. (1,2) have recently described the synthesis of a phosphonic acid analogue of phosphatidylethanolamine, dipalmitoyl L-α-glyceryl-(2-aminoethyl)phosphonate. An analogue of a different type has been described by Rosenthal (3). These compounds apparently cannot be dispersed in aqueous media, and all of them have contained totally saturated fatty acid radicals. In the phosphatide series, unsaturation in the fatty acid portion, attached to the glycerol of the phosphatide, makes the compounds dispersible in water with the aid of solubilizing agents like sodium desoxycholate (4-6). It therefore seemed of interest to make dioleoylglyceryl-(2-aminoethyl)phosphonate and, if it were dispersible, to test it for biological activity. The recent report of the occurrence of diacylglyceryl-(2-aminoethyl) phosphonates in Tetrahymena (7) makes the project even more interesting.

Reports (up to 1962) of procoagulant activity of phosphatidylethanolamines, isolated from various natural sources, have been reviewed (8). In 1963 (9) the activity of synthetic *rac* phosphatidyl(dioleoyl)ethanolamine in tests of "thromboplastin generation" was demonstrated. The activity of the same synthetic phospholipid was greater in the modified Hicks-Pitney test (4). Other workers (10,11) have also found synthetic unsaturated phosphatidylethanolamine to be acceleratory in clotting tests, but the activity of natural phosphatides is still controversial (12).

More recently (13) it was reported that phosphatidylethanolamine from egg could provide the phospholipid component required for active conversion of prothrombin to thrombin in a test system consisting of all purified components. Consequently it was considered important to investigate the biological activity of the synthetic phosphonate analogue of phosphatidyl(dioleoyl)ethanolamine and to compare its activity with that of synthetic phosphatidyl (dioleoyl)ethanolamine and phosphatidyl (dilinoleoyl)ethanolamine. The biological tests used determined the activity in blood coagulation in which synthetic *rac*-phosphatidyl (dioleoyl)ethanolamine has been shown to have an accelerating effect (4,9,13).

PROCEDURES AND RESULTS

Materials and Methods

The *rac*-1,2-diolein was synthesized as described (14). The oleic acid for this was the Purum grade of Fluka A.G., Buchs, Switzerland. The linoleic acid used for the synthesis of synthetic phosphatidyl(dilinoleoyl)ethanolamine was made according to the method of Parker et al. (15) except that distillation was omitted. It contained oleic acid as an impurity, amounting to 15.6%. The synthesis of the *rac* phosphatidyl(dioleoyl)ethanolamine is described in Reference 9.

Unisil sicilic acid was obtained from Clarkson Chemical Company, Williamsport, Pa., and silica gel from E. Merck, Darmstadt. The general technique of column chromatography was that of Rouser et al. (16-18). All operations involving unsaturated fatty acids were conducted under nitrogen. Melting points were determined by using the Kofler hot bench.

2 Phthalimidoethylphosphonic Acid Dicyclohexylammonium Salt

The Michaelis-Arbusov reaction was carried out using *N*-(2-bromoethyl)-phthalimide and trimethylphosphite, as described by Baer and

Stanacev (1). However the diethyl 2-phthalimidoethylphosphonate obtained was not hydrolyzed with hydrobromic acid. Instead a milder method was employed, based on the work of Rabinowitz (19). A mixture of 15.5 g of the crude methyl 2-phthalimidoethyl phosphonate and 8.7 g of trimethylchlorosilane was refluxed for 14 hr, during which time the temperature rose from 57C to 105C. Then 3.3 g of trimethylchlorosilane was added, and the heating was continued for 12 hr at 105C. At this time the reaction appeared complete by TLC [using isopropyl alcohol-ammonium hydroxide-water (7:1:2) and the Zinzadze stain (20) on silica gel H].

The mixture was treated with 50 ml of methanol and evaporated to an oil which crystallized. The product was triturated with water containing enough alcohol to free the crystals of oily material. The purified crystals weighed 4.8 g and were found to be 2-bromoethylphthalimide. The mother liquor was evaporated, taken up in acetone, and neutralized with dicyclohexylamine to give crystals weighing 10.0 g. Recrystallization was done from ethanol-acetone, mp 218C (dec.).

Anal. Calcd. for $C_{30}H_{42}N_2O_5P$ (436.47): C, 60.54; H, 7.62; N, 6.41; P, 7.09. Found: C, 60.54; H, 7.95; N, 6.44; P, 7.45.

2-Phthalimidoethylphosphonic Acid

The preceding material was converted to the free acid with Amberlite IR-120 (H^+), as described by Baer and Stanacev (1) for their anilinium salt. The product had mp 204C.

Anal. Calcd. for $C_{10}H_{10}NO_5P$ (255.2): C, 47.07; H, 3.95; N, 5.49; P, 12.13. Found: C, 46.81; H, 4.05; N, 5.46; P, 11.94.

Dioleoylglyceryl-(2-phthalimidoethyl)phosphonate

The procedure of Baer and Stanacev (1) was followed except that *rac*-1,2-diolein (14) was used in place of dipalmitin. The ether-soluble product was treated with Amberlite as described (1), and the crude product was used for the next step after evaporation and drying.

Rac-Dioleoyl 3-Glyceryl(2-aminoethyl)phosphonate

Hydrazinolysis of the preceding material was performed using 95% hydrazine (Eastman) in ethanol at 37C for 2 days, as described in earlier work (6). The product was worked up as described by Baer (1) except that Amberlite IRC-50 was used instead of Amberlite IR 120 H^+. By repeated TLC and comparison of different concentrations of the product with corresponding parallel standards of phosphatidylethanolamine, it was estimated that 3 g of phosphonate was in the mixture. This came from 8 g of diolein. To purify this material it was separated in portions on columns of DEAE cellulose (acetate form), as described by Rouser (16-18) for phosphatidylethanolamine. For the TLC of the fractions from the column, Silica Gel H was used with the solvent system of Grisdale and Okany (21). This showed that the main eluate with a positive ninhydrin reaction contained traces of two impurities, one moving faster and one (lysophosphatide analogue) more slowly than the main product. (For a satisfactory blue ninhydrin test the chloroform-methanol eluates from the column required more heating than corresponding material containing phosphatidyl ethanolamine. The fast-moving spot appeared, by analysis for C,H,N,P, to be an oxidation product of the phosphonolipid rather than an isomer.)

To obtain pure material the substance was rechromatographed on deoxygenated Unisil silicic acid, from which the completely pure phospholipid was eluted with 10% methanol in chloroform as a colorless wax. The recovery (60%) was equal to that found in earlier and similar column operations (5,6,14).

The material was homogeneous by TLC and chromatography on paper impregnated with silicic acid, according to the method of Marinetti (22). With di-isobutyl ketone-acetic acid-water the phosphonolipid migrated on the paper as a single spot in amounts of 250 μg with a mobility distinctly greater than that of phosphatidylethanolamine from beef brain. It also moved slightly beyond synthetic dilinoleoylphosphatidylethanolamine. On TLC with Silica Gel H and the solvent of Grisdale and Okany (21) the phosphonolipid moved with the same mobility as synthetic dilinoleoylphosphatidylethanolamine.

Anal. Calcd. for $C_{41}H_{78}NO_7P$ (728.0): C, 67.64; H, 10.80; N, 1.92; P, 4.25. Found: C, 67.21; H, 10.70; N, 1.93; P, 4.15.

Rac Phosphatidyl(dilinoleoyl)ethanolamine

This was synthesized, as described by Baer and Blackwell (23), except that the product was purified by DEAE cellulose (acetate) chromatography, then by chromatography on Unisil silicic acid. It showed a single spot when chromatographed in amounts of 250 μg on paper impregnated with silicic acid. GLC of the fatty acids from the phosphatide showed 83% linoleic acid, 15.6% oleic acid, 0.37% palmitic acid, and 0.6 stearic acid. This duplicated the composition of the fatty acid which was used as starting material.

TABLE I

Activities of Solubilized[a] *rac*-Dioleoyl 3-Glyceryl (2-Aminoethyl)phosphonate, Dioleoyl PE, and Dilinoleoyl PE in the Modified Hicks-Pitney Test (4)

Incubation time in minutes		2	4	6	8
Substance tested	μg in Incubation mixture	Substrate clotting time in seconds			
Phosphonolipid	100	52.0	8.0	6.0	6.8
	10	22.2	7.0	7.2	8.8
	5	46.2	13.0	9.8	9.8
Dioleoyl PE	100	39.5	7.0	6.8	7.8
	10	59.2	15.2	10.0	7.8
	5	34.5	15.8	11.0	11.0
Dilinoleoyl PE	100	61.2	9.5	7.0	7.5
	10	34.8	15.0	11.0	11.2
	5	40.0	14.8	10.8	11.0
Controls					
Crude phosphatides (24)	6	94.0	9.5	8.0	8.0
Buffered saline (4)	—	90	90	37.3	25.2
Sodium desoxycholate (4)	100	90	88.0	33.0	21.3

[a] Each substance tested was solubilized in a solution of sodium desoxycholate in buffered saline (4).

Anal. Calcd. for $C_{41}H_{74}NO_8P$ (739.99): C, 66.54; H, 10.08; N, 1.89; P, 4.19. Found: C, 66.53; H, 10.19; N, 1.95; P, 4.24.

Action of Phospholipase A (Snake Venom)

A dispersion of 0.5 mg of Russell viper venom ("Stypven," Burroughs Wellcome and Company) in 1 ml of water was used. A drop of this was added to a) a solution of 5 mg of phosphonolipid in 1 ml of ether, b) a solution of 5 mg of phosphonolipid mixed with 5 mg of egg lecithin in 1 ml of ether, and c) a solution of egg lecithin in ether. After standing overnight, the solutions were examined by TLC against standards of phosphonolipid and lecithin. It was found that one-half of the phosphonolipid (R_F 0.52) of a) and b) was converted to material still ninhydrin-positive but of lesser R_F (0.3). All of the lecithin in b) and c) was converted to lysolecithin.

Activity of rac-Dioleoyl-3-glyceryl(2-aminoethyl)-phosphonate

The activity of the synthetic dioleoyl phosphonolipid was compared with those of synthetic phosphatidyl(dioleoyl)ethanolamine and phosphatidyl(dilinoleoyl)ethanolamine in two different tests of blood coagulation. Employed were the modified Hicks-Pitney test (4) and a prothrombin conversion test (13), which will be presented in greater detail in a future publication.

The Hicks-Pitney test evaluates the activity of the synthetic phosphonolipid or phospholipids in the generation of plasma thromboplastin. In Table I the activities of the synthetic phosphonolipid and of the phosphatidylethanolamines are compared with that of a suspension of a crude, mixed phosphatide fraction (24). The phosphonolipid preparation was the most active, behaving like the crude phosphatides at equivalent concentration. Both the phosphatidyl(dioleoyl)ethanolamine and the phosphatidyl(dilinoleoyl)ethanolamine showed less activity. Approximately 10 times as much of these phospholipids was required to obtain activity similar to that of 10 μg of the phosphonolipid.

In the tests of conversion of prothrombin to thrombin with highly purified components (Table II) the synthetic phosphonolipid was more active than the phosphatidylethanolamines. The 10 μg of the phosphonolipid had approximately the same activity as 100 μg of either the phosphatidyl(dioleoyl)ethanolamine or the phosphatidyl(dilinoleoyl)ethanolamine. And 100 μg of the phosphonolipid brought about the conversion of more prothrombin to thrombin than any of the phospholipids studied (thrombin yields of 10.8 units as compared with 8.8 units).

DISCUSSION

Baer and Stanacev (1), Liang and Rosenberg (7) have emphasized the similarity of the phosphonolipid which is analogous to phosphatidylethanolamine to phosphatidylethanolamine itself. Nevertheless there are differences in physical properties since our phosphonolipid migrated slightly farther on paper impregnated with silicic acid than did phosphatidylethanolamine with the same fatty acid component.

TABLE II

Activities of Solubilized[a] *rac*-Dioleoyl Glyceryl (2-Aminoethyl) phosphonate, Dioleoyl PE, and Dilinoleoyl PE in the Conversion of Prothrombin to Thrombin[b]

Incubation time in minutes		0.5	5	10	20
Substance tested	μg in Prothrombin converting mixture	Thrombin units[c] measured			
Phosphonolipid	100	4.3	10.8	10.0	10.8
	10	3.5	8.0	7.0	6.5
	1	0.7	1.1	1.3	1.4
Dioleoyl PE	100	2.6	6.0	6.9	7.9
	10	2.4	5.6	6.0	6.0
	1	0.1	0.2	0.3	0.4
Dilinoleoyl PE	100	3.0	8.4	6.9	6.9
	10	1.5	5.0	5.3	6.0
	1	0.1	0.1	0.1	0.1
Controls					
Crude phosphatides (24)	60	4.7	8.4	8.4	8.8
Buffered saline (4)	—	.01	.01	.01	.01
Sodium desoxycholate (4)	100	.01	.01	.01	.01

[a] Each substance tested was solubilized in a solution of sodium desoxycholate in buffered saline (4).

[b] Test System—Factor V (0.2 ml of a 9.0 μg/ml solution), phosphonate or PE (0.1 ml of a 1, 0.1, or 0.01 mg/ml suspension), and thrombokinase (25) (0.1 ml of a 0.0008 μg/ml solution) were mixed. Then $CaCl_2$ (0.1 ml of a 0.025 M solution) was added, and the entire mixture was incubated for 1 min at 37C. To this mixture (prothrombin converter) was then added 0.5 ml of purified human prothrombin solution (70 U/ml). The conversion of prothrombin to thrombin was finally measured by transferring 0.1 ml of this mixture (Incubation Time in Table II) to 0.3 ml of 0.3% bovine fibrinogen (Behringwerke) and noting the clotting time.

[c] Calculation of Thrombin Units—A standard curve relating known units of thrombin (based on NIH standard) to clotting time was obtained by adding graded amounts of thrombin to fibrinogen solutions under conditions similar to those employed in the prothrombin conversion test. A straight line on log-log paper was obtained. The values given in the column, "Thrombin Units Measured," represent the amount of thrombin obtained from the original amounts of prothrombin which were added to the prothrombin converter in each test. They were obtained by multiplying the thrombin units obtained in each test by eight, which is the number of times the original prothrombin solution was diluted.

When tested with phospholipase A, about one-half of the racemic phosphonate was degraded to lyso compound. This suggests that the enzyme from snake venom was stereospecific and acted only on the stereoisomer which had a stereochemical configuration similar to that of the natural phosphatides. The action of snake venom on racemic synthetic phosphatidyl ethanolamine is similar. The likeness of the unsaturated phosphonolipid to unsaturated phosphatidylethanolamine was emphasized by the study of the effects of both lipids in in-vitro tests of blood coagulation.

The phosphonolipid analogue of phosphatidylethanolamine appeared to have activity qualitatively similar to but quantitatively somewhat greater than the activity of closely related phosphatidylethanolamines. The phosphonolipid and the phosphatidylethanolamines substituted completely for the well-known activity of human platelets in the Hicks-Pitney test. Milstone (25) has shown that microgram quantities of thrombokinase will slowly convert prothrombin to thrombin in the absence of added phospholipids. (Reference 25 shows the relationship between thrombokinase and Factor X.) However he obtained rapid conversion of prothombin to thrombin with nanogram quantities of thrombokinase when crude mixtures of phospholipids were present. Thus the potent acceleratory activity of synthetic phosphatidylethanolamines (containing unsaturated fatty acids) in both the modified Hicks-Pitney test and in the test of the conversion of prothombin to thrombin emphasizes the important role of colloidal dispersions of the phospholipids in the conversion of prothrombin to thrombin.

The greater activity of the phosphonolipid may indicate that this substance is more readily dispersed and yields micelles of the proper charge, size, and shape; hence it exhibits greater activity at the same concentration. This idea has been proposed by several investigators (4, 8,10,26-30) to explain the varying clotting activity of different preparations of various synthetic and natural phosphatidylethanolamines (26-29), phosphatidylserines (4,10), and combinations of these two phospholipids with lecithin (4,10). The same explanation has been

invoked to explain the inhibitory activity of phosphatidylserines (4,10).

It is also possible that the direct carbon-to-phosphorus bond may provide a specific structural configuration which enhances the conversion of prothrombin to thrombin or the formation of a more active prothrombin converter.

ACKNOWLEDGMENT

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Sterol Metabolism. III. Sterols of Marine Waters[1]

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ABSTRACT

The detection and tentative identification of three sterols, cholesterol, stigmasterol, and β-sitosterol, in hexane extracts of Gulf of Mexico waters has been achieved by using thin-layer and gas chromatographic procedures. The identifications are assigned on the basis of chromatographic properties of the free sterols, of their acetates, and of their trimethylsilyl ethers.

INTRODUCTION

THE PRESENCE OF UNSATURATED sterols and their esters in marine waters (1) and in associated aquatic sediments (2) has been suggested by application of the widely accepted Liebermann-Burchard color test to appropriate marine samples. However identification of specific sterols in these samples has not been made, nor have reliable means of estimation of these sterols been devised.

We have attempted to obtain more convincing proof of the presence of specific sterols in marine waters by means of thin-layer and gas chromatographic procedures, coupled with infrared absorption spectra, and to provide a simplified means of analysis of water samples for sterols. The present paper bears on these matters.

EXPERIMENTAL PROCEDURES

Solvents

All solvents used for extractions, for solutions, and for chromatography were of reagent quality, redistilled just prior to use. All concentrations and evaporations of solvent extracts and solutions were conducted in all-glass rotary evaporators under diminished pressure at bath temperatures not exceeding 35C. Evaporated samples were stored under a vacuum in a vacuum desiccator or in a deep freeze until analysis and further processing.

Spectra

Infrared absorption spectra over the range 400–4000 cm^{-1} were recorded from 1.5-mm potassium bromide disks of samples by using a Perkin-Elmer Model 337 infrared spectrophotometer, equipped with a beam condenser. Ultraviolet absorption spectra were recorded on ethanol solutions of samples with the use of a Cary Model 14 spectrophotometer.

Thin-Layer Chromatography

TLC was conducted with 250-μm thick 20 × 20-cm chromatoplates of Silica Gel HF_{254} (E. Merck GmbH, Darmstadt), irrigated with ethyl acetate-heptane (1:1) or acetone-heptane (1:1), as described in detail previously (3). Both one- and two-dimensional techniques were used, with multiple irrigation in each dimension as indicated. Resolved components were routinely examined under ultraviolet light (254 and 366 nm) prior to visualization by spraying with 50% aqueous sulfuric acid and heating. Chromatoplates were heated to full color display and then charred.

Preparative TLC was accomplished by using 1- and 2 mm-thick 20 × 40-cm chromatoplates, prepared with Silica Gel PF_{254}. Samples were applied as a fine line by means of a streaking device (Rodder Streaker, Rodder Instrument Company, Los Altos, Calif.). Irrigation was made with the same solvents as used for analytical TLC. Components were detected by means of reference sterols applied at one edge of the preparative chromatoplate, which were visualized with 50% sulfuric acid.

Gas Chromatography

Gas chromatography of lipid samples dissolved in methylene chloride was conducted on F and M Models 400 and 402 chromatographs by using 1.83-m long, 6-mm O.D. silanized glass columns, packed with either 3% SE-30 (methyl silicone) on 80-100 mesh Gas-Chrom Q or 3% QF-1 (trifluoro propyl methyl silicone) on 100-120 mesh Gas-Chrom Q (all from Applied Science Laboratories, State College, Pa.). Injection port temperature was 250C, and the columns were operated at 245C with nitrogen carrier gas flow-rate of 21 ml/min.

Sterol Derivatives

Mixed sterol and reference sterol acetates were prepared by dissolving approximately 50 μg of sterol in 500 μl of pyridine and 250 μl of acetic anhydride, as previously described

[1] Presented in part at the 7th International Congress of Biochemistry, Tokyo, Japan, August 19-25, 1967; Abstracts, Vol. IV, p. 736.

(4). Trimethylsilyl ethers were prepared by using approximately 100 μg of sterol and 10 μl of a commercially prepared mixture of hexamethyldisilazane and trimethylchlorosilane in dimethylformamide (Tri-Sil DMF of Pierce Chemical Company, Rockford, Ill.).

Water Samples

Water samples (17-45 liters) were taken in lipid-free glass bottles and in Negal plastic bottles at three separated beaches adjacent to Galveston Island over a period of six months. Each water sample was filtered through a layer of Celite 545 diatomaceous earth, and the pH was adjusted to 2.5-3.5 with 6N hydrochloric acid (1). The prepared water samples were extracted by multiple-batch contacting of 10 volumes of sea water with one volume of hexane, repeated until further extractions yielded diminished amounts of extractable matter which showed no sterol components by TLC. The two larger (45 liter) samples (Nos. 10 and 11, Table I) were extracted continuously with hexane for 24-48 hr with a one-tenth volume of hexane, and repeated continuous extraction with fresh hexane yielded no further sterol-like components. Hexane extracts were dried by filtering through a layer of anhydrous sodium sulfate, and the dried hexane extracts were concentrated to near dryness in an all-glass rotary evaporator under diminished pressure. The lipid extract sample was analyzed as such or dried completely under nitrogen to a residue which was redissolved in methylene chloride for analysis.

Extraction with hexane of 40 liters of City of Galveston tap water which had been previously filtered and acidified gave a residue of 8 mg, which did not give color test or chromatographic behavior supporting the presence of unsaturated sterols. Similar extraction of 40 liters of tap water, to which 2.5 mg of cholesterol had been added as an acetone solution, afforded an extractable lipid fraction of 10.9 mg, identified as unsaturated sterol.

The total-extractables fraction from each water sample was initially analyzed qualitatively by one-dimensional TLC with ethyl acetate-heptane (1:1) by using empirically determined amounts of sample to show sterol-like components. Two-dimensional TLC analysis with ethyl acetate-heptane (1:1) and acetone-heptane (1:1) was employed to determine the extent of autoxidation where such was suspected or indicated, and to characterize more satisfactorily the more mobile components of certain samples. Gas chromatography was conducted on suitable dilutions of the initially analyzed preparation, which has evaporated under nitrogen and redissolved in methylene chloride such that 1 μl of methylene chloride sample solution was injected onto the gas chromatographic column.

A 1-μg amount of reference sterol (cholesterol, stigmasterol, and β-sitosterol) was added to a portion of each marine sterol preparation to determine whether or not resolved components of the marine sterol mixtures were resolved in turn from reference sterol. Similar comparison of gas chromatographic behavior of the marine mixed sterol acetates and trimethylsilyl ethers with reference sterol acetates and trimethylsilyl ethers was made.

Sterol Purification

The two larger batches (Nos. 10, 11) and the pooled sterol fractions from combined smaller batches were individually chromatographed further in order to obtain mixed sterol fractions, from which reliable retention data, derivation, and infrared absorption spectra could be obtained. Slightly different chromatographic procedures were used in each of the three cases examined extensively, but the results obtained in each instance support the suggested identification of cholesterol, stigmasterol, and β-sitosterol in detail.

Sample No. 10. The hexane-extractable matter (1.467 g) from this water sample was chromatographed on 60 g of silica gel. Elution with hexane afforded 462 mg of nonsterol material (at high R_f on thin-layer chromatograms), possibly containing hydrocarbons and esters but not further examined. Elution with 50% diethyl ether in hexane gave 798 mg of sterol-like material, characterized by TLC as a mixture of sterols (R_c 1.00)[2] and sterol-like component (R_c 1.3), giving an initial beige color reaction with sulfuric acid, turning rust red (Component No. 5, Table I.) Ten percent methanol in diethyl ether eluted polar matter which did not contain sterols, but it was not examined further. The middle fractions containing sterols were rechromatographed on silica gel, giving largely the R_c 1.3 component No. 5 (of Table I) with 1-2% diethyl ether in hexane, and 427 mg of sterol-containing material (still containing the R_c 1.3 component also) with 4-12% diethyl ether in hexane. TLC of the 1-2%

[2]The term R_c is defined as the TLC mobility of the component described relative to that of cholesterol as unity. The term r_T is similarly the gas chromatographic retention-time relative to that of cholesterol as unity.

TABLE I
Chromatographic Analysis of Sea Water for Sterols

No.	Location	Volume l	Sterol-like Components Detected by Thin-Layer Chromatography[a]								Sterols Detected by Gas Chromatography[e]						Ratio Cholesterol/ β-Sitosterol
			1	2	3	4	5	6	7	Other	1	2	3	4	5	6	
1	South Jetty, Galveston Island 30-70 m offshore	19	+		+			+			–[f]	–[f]	–[f]	–[f]	–[f]	–[f]	–[f]
2		19			+			+	+			+	+	+	+		3:4
3		40	+		+			+		+[b]		+	+		+		30:1
4		40	+	+	+			+				+			+		1:1
5		19		+	+	+		+		+[c]		+		+	+		2:1
6		19		+	+	+		+				+	+		+		1:4
7	East Beach Galveston Island 70 m offshore	17	+		+			+				+		+	+		6:1
8		19			+			+				+	+	+	+		1:1
9		19			+			+		+[d]		+	+		+		2:1
10	East Beach Galveston Island 1 km offshore	45			+		+	+	+			+		+	+	+	1:13
11	San Luis Pass Galveston Island 300 m offshore	45			+		+	+	+		+	+		+	+	+	1:4

[a] As resolved on one- and two-dimensional thin-layer chromatograms, run as described in the text. Components identities (No., identity, R_C vs. cholesterol as unity, color with 50% sulfuric acid): 1, unknown, 0.25, beige; 2, 7-keto-cholesterol (?), 0.8, no response; 3, unsaturated sterols, 1.00, magenta; 4, cholesta-3, 5-dien-7-one (?), 1.1, beige; 5, artifact phthalate esters, 1.3-1.5, beige turning rust red; 6, unidentified, multiple components, 1.2-1.7, beige; 7, unidentified, 1.5-1.7, dark red.

[b] Typical blue-colored spots of 7α- and 7β-hydroxycholesterol in this sample.

[c] A component similar to 25-hydroxycholesterol in this sample.

[d] An unidentified component at R_C 1.3, giving an immediate sky-blue color, in this sample.

[e] Analysis on 3% QF-1 columns as described in the text. Component identities (No., identity, r_T vs. cholesterol as unity): 1, unidentified, 0.78; 2, cholesterol, 1.00; 3, unidentified, 1.23 (range 1.21-1.25); 4, stigmasterol 1.32 (range 1.30-1.37); 5, β-sitosterol, 1.54 (range 1.50-1.57); 6, unidentified, 1.75.

[f] Not analyzed.

diethyl ether in hexane fractions afforded the purified R_c 1.3 component. Similarly the recognized sterols at R_c 1.00 were separated from more of the R_c 1.3 component by preparative TLC of the 4-12% diethyl ether in hexane cuts. A purified mixed sterol preparation (8.8 mg), free from other components, which moved as a single component with color test and mobility behavior identical with those of reference cholesterol, was thus obtained.

Gas chromatographic analysis of the sterol preparation on 3% SE-30 and 3% QF-1 systems established its composition as a mixture of cholesterol, stigmasterol, and β-sitosterol (each identified by their absolute retention-times in comparison with reference sterols, by cochromatography with reference sterols, and by similar data on the mixed steryl acetate and trimethylsilyl ether derivatives), with a trace of a fourth sterol (Table I, Fig. I).

Preparative gas chromatographic separation of 40 μg of the three major sterols on 3% QF-1 (each resolved sterol zone was collected as it eluted from the column in silanized glass capillaries (4)) afforded homogeneous single component preparations of each sterol. These collections did not give crystalline material, however, and infrared spectra of each resolved sterol were not recorded nor were melting points taken. However, rechromatography of the resolved sterols on both 3% SE-30 and 3% QF-1 columns and on thin-layer chromaplates established integrity of the sterol on the preparative QF-1 column, and the suggested identities as cholesterol, stigmasterol, and β-sitosterol were further supported.

From the areas under the peaks of the elution curves (compared with calibration curves constructed with reference sterols) the amounts of sterol in the original water sample was calculated: cholesterol, 10 μg/liter; stigmasterol, 17 μg/liter; and β-sitosterol, 135 μg/liter.

Sample No. 11. The hexane-extractable matter (496 mg) was chromatographed on a silica

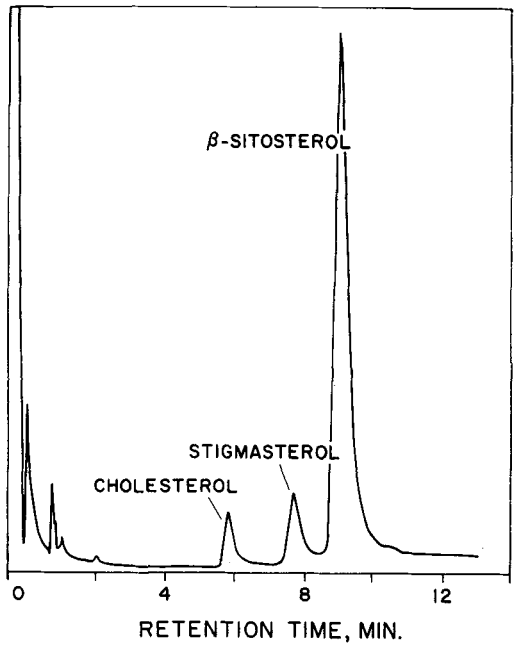

FIG. 1. Gas-chromatographic elution curve of sterol fraction from sea-water extract. (3% QF-1).

gel column. Elution with hexane and with up to 50% chloroform in hexane gave 89.1 mg. of mobile nonsterol material, which was further subjected to preparative TLC, yielding 10.2 mg of material initially suspected of being steryl esters because of the high R_f on the chromatograms and the dark red coloration with 50% sulfuric acid. Gas chromatography of this fraction on 3% QF-1 showed only rapidly eluted components at r_T[2] less than 0.35 and no components at r_T characteristic of steryl esters.

Further elution of the silica gel column with 50-75% chloroform in hexane afforded a fraction rich in the R_c 1.3 component, the chromatographic behavior of which mimicked that of unsaturated sterols. Preparative TLC of this material, followed by column chromatography on alumina (eluted with 5-10% diethyl ether in hexane) and a second preparative TLC operation gave 75 mg of the purified component, homogeneous on thin-layer chromatograms and on gas chromatograms, with r_T 0.4. A small portion of the semisolid material was successfully crystallized from cold (20C) methanol, yielding 1.8 mg, mp (Kofler block) 48.5-51C; $\tilde{\mu}^{\text{KBr}}_{\text{max.}}$ 2960, 2920, 2850, 1720, 1600, 1460, 1380, 1285, 1270, 1120, 1070 cm^{-1}; $\lambda^{\text{EtOH}}_{\text{max.}}$ ($E^{1\%}_{1\,cm}$): 226 (1150), 276 (163), 283 nm (140); λ_{min} 263 nm (117).

The component was recovered unaltered after attempted semicarbazone formation and after attempted sodium borohydride reduction.

Continued elution of the silica gel column with 75% chloroform in hexane afforded the sterol fraction (85 mg), which was chromatographed on 1-mm Silica Gel PF_{254} chromatoplates, yielding 2.18 mg of purified mixed sterols, the composition of which as a mixture of an unidentified mobile sterol, cholestrol, stigmasterol, β-sitosterol, and an unidentified trace of polar sterol (Table I) was demonstrated by gas chromatography on 3% QF-1. Each suggested identification was supported by comparisons with reference sterols, by cochromatography with reference sterols, and by similar evidence obtained on the acetylated and trimethylsilyl ether-derived preparations.

Phthalate Ester Artifacts

The Negal plastic bottles used for collection of the 45-liter water samples were extracted with 5 liters of hexane for 30 hr on a reciprocal shaker. The extractables (294 mg) were semisolid and gave a single component on thin-layer chromatograms, irrigated with ethyl acetate-heptane (1:1) at R_c 1.3, exhibiting an initial beige color with 50% sulfuric acid, turning rust red, unresolved by thin-layer or gas chromatography from the R_c 1.3 component isolated from sea water. Ultraviolet absorption spectra of the two samples were essentially the same and in good agreement with published ultraviolet absorption spectra of dimethyl, dibutyl, and di-isooctyl phthalate (5). Infrared absorption spectra of the R_c 1.3 component from the plastic bottles were indistinguishable from spectra of the sample obtained from sea water and from published infrared spectra of didecyl phthalate (6).

Sterol Autoxidation

Cholesterol was added to sea water so as to give a suspension of ca. 300 μg/ml. These preparations were exposed for 96 hr to light and aeration as follows, and after the experimental period the water was extracted with hexane, the hexane extracts were concentrated in vacuo, and the sterol residue was analyzed by TLC. The experimental conditions and results are: a) water held in daylight with no aeration; no autoxidation, cholesterol only was found; b) water held in daylight and aerated; cholesterol and a trace of 7-ketocholesterol detected; c) water exposed to a sodium vapor lamp with no aeration; cholesterol with traces

TABLE II
Relative Gas Chromatographic Retention-Data of Sterols Identified in Marine Waters

	Relative Retention Times					
	Free sterol		Steryl acetate		Steryl trimethylsilyl ether	
Sterol	3% SE-30	3% QF-1	3% SE-30	3% QF-1	3% SE-30	3% QF-1
Cholesterol	1.00	1.00	1.00	1.00	1.00	1.00
Stigmasterol	1.35	1.31	1.44	1.32	1.48	1.45
β-Sitosterol	1.58	1.52	1.66	1.52	1.59	1.55

of 7-ketocholesterol and the epimeric 7-hydroxycholesterols detected; d) water exposed to sodium vapor lamp and aerated; cholesterol with increased amounts of 7-ketocholesterol and the epimeric 7-hydroxycholesterols detected; e) sea water without any cholesterol added, held in daylight with no aeration; cholesterol only, with no other detectable components. Confirmation of these results by gas chromatography on the hexane extractables was made by using gas chromatographic recognition methods for cholesterol autoxidation products developed in this laboratory (7).

RESULTS

Our evidence for the presence of sterols in sea water, supporting in detail that previously reported (1), is accumulated in Table I. The distribution pattern of sterol components as determined by gas chromatography on 3% QF-1 suggests that Components No. 2 (cholesterol) and No. 5 (β-sitosterol) are present in each of the 10 water samples analyzed. Our extensive chromatographic analysis supporting the specific identification of cholesterol, stigmasterol, and β-sitosterol utilizes gas chromatographic retention data of the mixed sterols, of their acetates, and of their trimethylsilyl ethers, as presented in Table II. Exact correspondence between values obtained in the several marine sterol mixtures with those obtained with reference sterols was obtained for the identified three sterols.

The presence of stigmasterol (No. 4) and of unidentified sterol (No. 3 of Table I), each in about half of the water samples, completes the regular sterol distribution pattern. The additional components detected in Samples 10 and 11 were not seen in the smaller water collections. Of the three unidentified sterols detected in some water samples, the one found with frequency (No. 3) may be campesterol, known to be readily resolved as a more mobile component on gas chromatograms from stigmasterol (8). However campesterol is not resolved on SE-30 or QF-1 columns from its C_{24}-epimer 22, 23-dihydrobrassicasterol (M. J. Thompson, private communication), and no support is offered for identification of Component No. 3 at this point.

A selected gas chromatogram on the purified mixed sterols from Water Sample No. 11 showing cholesterol, stigmasterol, and β-sitosterol, together with a trace polar sterol, is presented in Fig. 1. Although gas chromatography of other less purified water-extract preparations gave well-resolved sterol peaks, base-line separation was not achieved in impure samples so meaningful quantitation was obviated. Although present work was not planned as a quantitative study and no sophistication was involved in these estimates, although no corrections were applied for differential hydrogen flame response (8) and peak areas were not referred to absolute calibration curves, an interpolated base line with associated peak area measurement did afford a relative measure of levels of the two regularly encountered sterols cholesterol and β-sitosterol. These relative values are expressed as a ratio of the amounts of cholesterol to the amount of β-sitosterol and are presented in Table I. Approximately equal amounts of the two sterols were indicated on balance (range 6:1 to 1:4), with the two exceptions of Sample No. 3, which showed a 30:1 ratio of cholesterol to β-sitosterol, and of Sample No. 10, which had a 1:13 ratio. These varying amounts must reflect varying proportions in the sea water since none of the thin-layer and column techniques were adequate for resolution of cholesterol from β-sitosterol, nor were crystallizations or derivations involved which might have afforded partial separation of one sterol from the others.

The absolute levels of sterols determined from Sample No. 11 gave cholesterol, 10 μg/liter; stigmasterol, 17 μg/liter; and β-sitosterol, 135 μg/liter. In model recovery experiments from preparative thin-layer chromatoplates at least a 83% recovery of sterols was estimated. The hexane-extraction methods were designed to lend specificity to the recovery of lipid matter only, and complete recovery of sterols from

water was demonstrated by these means. The recovery of unsaturated sterols from silica gel column chromatography, as practiced herein, was not specifically determined, but the recovery should be high. Examination of eluates obtained from the columns after elution of the sterols by a variety of polar solvents as well as nonpolar solvents failed to give any further sterol material.

Consequently the estimated levels of chromatographically purified sterols represent, within an order of magnitude or so, a fair measure of the sterols in sea water. It should be noted that the cholesterol and stigmasterol levels (10-17 μg/liter) are below the water-solubility levels of pure cholesterol (25-29 μg/liter) (9) but that the β-sitosterol level (135 μg/liter) is somewhat above.

Infrared absorption spectra of the mixed sterol preparations were also in good accord with spectra of the reference sterols cholesterol and β-sitosterol. Notably, infrared spectra are poor means of differentiation among the common sterols; spectra of cholesterol and β-sitosterol are essentially identical (10,11). Thus, although infrared spectra were not recorded satisfactorily on resolved single sterols, spectra of the mixed sterols may be taken as good evidence for the presence of high proportions of the identified sterols in the mixed preparations.

Evidence for the presence of other common sterols in the water preparations was not encountered in these studies. The higher methylated sterols, such as lanosterol, or sterols of higher unsaturation, such as ergosterol or 7-dehydrocholesterol, were not detected on either thin-layer or gas chromatograms. The chromatographic systems would have resolved both of these classes of sterols from monounsaturated C_{27}-, C_{28}-, and C_{29}-sterols were they present in detectable amounts. Although the saturated 5α-stanols: 5α-cholestan-3β-ol, 5α-stigmast-22-en-3β-ol, and 5α-stigmastan-3β-ol would not be resolved from their respective Δ^5-unsaturated analogs, estimates of color intensity with 50% sulfuric acid on thin-layer chromatograms of the marine sterol mixture, together with good infrared absorption at 1600 cm^{-1} in the mixed sterols, precluded major proportions of the stanols in the preparations.

The presence of sterols more mobile than cholesterol on thin-layer chromatograms could not be confirmed. Components which gave red colors with sulfuric acid and which had high R_f values mimicked steryl esters; however gas chromatographic analysis of these components indicated that steryl esters were not present and that more likely lower-molecular-weight esters or hydrocarbons contributed to these components.

The sometime presence of a component giving an initial beige color with sulfuric acid, turning to rust red, at thin-layer chromatographic mobilities between those of authentic steryl esters and cholesterol was shown not to be that of a sterol by a combination of spectral and chromatographic details. The probable identity of this component as an alkyl ester of phthalic acid, derived from plastic water-collection bottles or from plastic tubing and connections used in filtration and extraction procedures, furnishes another example of the inadvertent contamination of samples to be examined for lipids by inexpedient contact or storage in plastic ware (6).

The sometime presence of a sterol-like component more mobile than cholesterol on thin-layer chromatograms, giving a beige coloration with sulfuric acid, indicates that some sterol autoxidation dien-7-ones (cholesta-3,5-dien-7-one, stigmasta-3,5-dien-7-one, stigmasta-3,5,22-trien-7-one) have formed in processing the sterol mixtures. The irregular presence of the suspected sterol 3,5-dien-7-ones implies that these components do not occur as companion trace sterols in sea water.

Other possible sterols thought to have some chance of occurrence in water are the 5β-stanols, ordinarily derived by intestinal microfloral reduction of sterols in mammalian metabolism. These 5β-stanols would be well resolved on thin-layer chromatograms from their respective Δ^5-unsaturated sterols. However no chromatographic evidence for the presence of 5β-stanols was obtained.

Sterols more polar on thin-layer chromatograms than cholesterol, stigmasterol, and β-sitosterol were not encountered in water extracts under circumstances where their presence in the original water sample could be established. Although sterol-like components, the color test behavior and TLC mobilities of which supported their recognition as autoxidation products of the types 7-ketocholesterol and the epimeric 7-hydroxycholesterols, the frequency of appearance of these components was such as to suggest that autoxidation during processing was a more suitable explanation of their presence in the water-extract preparations. Their presence was not examined further. The possibilities that sterols could be autoxidized in sea water are noted in the Experimental Section however.

DISCUSSION

Little prior work is known to us with which to compare these findings of free sterols in marine waters. Although our identifications of specific sterols must be regarded as tentative, based as they are only on chromatographic data, the presence of sterols in marine waters is adequately demonstrated. The question of definitive specific identities must be deferred until suitable mass spectrometric or other means can be applied to the identifications.

The now demonstrated presence of specific sterols in sea water raises the immediate question of their origin, their eventual disposition, and their role in the ecology of the sea. The ultimate origin of the sterols detected in this work remains obscure. In that our water-filtration procedure was simply to remove sand and other visible particulate matter, the inclusion of microflora in the water samples seems assured. Microscopic examination of interfacial emulsion in several extractions gave some evidence of the presence of diatoms and plankton so that lipids extracted must derive in part from this source. Further work is required to determine whether sterols exist in sea water as dissolved or suspended material or whether these sterols derive in whole or in part from plankton and other microflora. Our preliminary attempts to detect sterols in residues from thoroughly filtered water have not met with success; however the well known binding of sterols by microorganisms could readily account for our results to date.

The sterols present, cholesterol, stigmasterol, and β-sitosterol, resemble in their occurrence and distribution the sterols of plankton. A large proportion of cholesterol was found in Monaco plankton, with varying proportions of stigmasterol, β-sitosterol, and campesterol, with occasional detection of 24-methylene-cholesterol and other sterols (12,13). Equally suggestive is the sterol composition of *Escherichia coli* (cholesterol, campesterol, stigmasterol, and β-sitosterol) (14). The presence of cholesterol in red algae (15), in the crustacean *Euphasia pacifica* (16), indeed in many marine protozoa, in invertebrate and vertebrate life (17), and of stigmasterol, campestrol, and β-sitosterol, along with other sterols, in plant life (18) suggests an obvious source of the sterols detected in these studies.

Although the sterols may derive from marine plant and animal sources, they may also derive from fresh water sources carried to the sea. The offshore collections which were made do not permit decision on this point; however, domestic sewage does not appear to be a factor.

From the quantitative data of Jeffrey (1) much more sterol material is found in offshore water than in water distant from land. Much nonsterol material is present in beach and shallow offshore water samples, which mimics sterols in color reaction and TLC behavior, and it is only through extensive purification and attention to specific gas chromatographic analysis that more appropriate measures of the quantity of sterol in a sample may be obtained. The fortuitous collection of water near large chemical plants (Texas City near the Galveston beaches, the Monsanto Chocolate Bayou plant near San Luis Pass, Corpus Christi near Port Aransas) complicates the lipid extractables. The sterol levels of Jeffrey (1) of 90-1990 μg/liter (or 90-470 μg/liter if the two high nearshore samples are deleted) are of the same order as that reported (162 μg/liter total sterols). However, we offer no evidence for the presence of steryl esters in our sea water samples, whereas Jeffrey reported 70-2300 μg/liter.

Regarding the disposition of sterols in sea water, it has been repeatedly suggested that marine organic matter is assimilated by microorganisms which, in turn, feed higher forms of life. The presence of sterols in aquatic sediments (2) may derive in part from sterols in the water; these sediment sterols in turn serve as sources of organic matter for petroleum formation.

Our examination of these sterols has not indicated the presence of oxidized sterol derivatives in sea water. Thus microbial metabolism does not contribute extensively to demonstrable sterol oxidation products, nor does autoxidation by action of air and light contribute. However, we have demonstrated that autoxidation of cholesterol suspended in sea water can be effected by aeration in the presence of light. Further considerations of the disposition of sterols and their role in the ecology of the sea necessarily must await better definition of their nature and origin.

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The Lipids of the Common House Cricket, *Acheta domesticus* L. I. Lipid Classes and Fatty Acid Distribution

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ABSTRACT

The lipids of the common house cricket, *Acheta domesticus* L., have been examined with the following results. The fatty acids associated with the lipid extracts do not change significantly from the third through the eleventh week of the crickets' postembryonic life. The major fatty acids are linoleic (30-40%), oleic (23-27%), palmitic (24-30%), and stearic acids (7-11%). There are smaller amounts of palmitoleic (3-4%), myristic ($\approx$1%), and linolenic acids ($<$1%). The fatty acid composition of the cricket lipids reflects but is not identical to the fatty acids of the dietary lipids: linoleic (53%), oleic (24%), palmitic (15%), stearic (3%), myristic (2%), and linolenic acids (2%).

The amount of triglycerides present in the crickets increases steadily from the second through the seventh or eighth week of postembryonic life, then drops sharply. Other lipid classes, such as hydrocarbons, simple esters, diglycerides, monoglycerides, sterols, and free fatty acids remain about constant. The composition of the fatty acids associated with the tri-, di-, and monoglycerides and the free fatty acid fraction are all about the same. The fatty acids associated with the simple esters are high in stearic acid.

INTRODUCTION

INSECT LIPID metabolism is a topic of considerable current interest, and a number of good reviews covering the subject have appeared in the last five years (1-4). In this and the following two papers, the analysis and characterization of the lipids of the common house cricket, *Acheta domesticus* L., are reported. Because of the relative ease of maintaining laboratory colonies (5,6) and because of the commercial availability in large quantities of *Acheta domesticus*, this species is a particularly appealing one, both for biologically and chemically oriented studies. In this first paper the over-all lipid pattern in crickets at different stages of development is established, and the fatty acids associated with each lipid class are characterized. In the second and third papers the hydrocarbon and sterol fractions are examined in more detail.

MATERIALS AND METHODS

Live crickets were purchased from the Lucky Lure Cricket Hatchery, Whitmore Lake, Mich. The crickets were stored for 24 hr without food and were separated from excretory products prior to being extracted.

Lipid Extraction

In a typical extraction 1,000 g (fresh weight) of crickets, immobilized by quick-freezing, were extracted in a 1-gallon Waring Blendor with two liters of ether-methanol (3:1). The marc was extracted twice with two-liter portions of ether-methanol (3:1). The three extracts were combined and separated into an ether and an aqueous phase. The ether phase was washed once with a saturated sodium chloride solution. To the aqueous phase was added an equal volume of saturated sodium chloride solution. It was then extracted with ether until the extracts were colorless. The combined ether extracts were dried in a preliminary fashion with sodium sulfate and concentrated on a rotary concentrator. The residue was dissolved in methylene chloride and dried over sodium sulfate. Filtration and concentration gave the crude lipid extracts studied in this and the next three papers.

Lipid Separations

Lipid separations were carried out by a modification of the method of Carroll (7). In a typical run, 5.0 g of lipid, dissolved in a minimum of petroleum ether (bp 40-60C, redistilled), was adsorbed on a column of 400 g of Florisil (Fisher), to which 20 g of water had previously been added. Hydrocarbons were eluted with petroleum ether, simple esters with ether-petroleum ether (5:95), triglycerides, and sterols, successively with ether-petroleum ether

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TABLE I
Fatty Acid Composition of Lipid Extracts of *Acheta domesticus* of Different Ages

Postembryonic age of crickets (weeks)	Percentage Composition of Fatty Acids								
	$<C_{14}$	$C_{14:0}$	$C_{14:1}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
3	Trace	1	—	25	4	11	27	30	—
4	Trace	1	—	25	3	9	24	36	—
5	Trace	1	—	24	2	8	26	37	<1
6	Trace	1	—	27	3	8	23	35	1
8	Trace	1	<1	26	2	8	23	40	<1
10½	Trace	1	<1	30	2	7	24	39	—
Cricket food	Trace	2	<1	15	1	3	24	53	2

(15:85), diglycerides with ether-petroleum ether (30:70), monoglycerides with methanol-ether (2:98), and free fatty acids with acetic acid-ether (4:96). Total amounts of each lipid class were determined gravimetrically. Duplicate runs gave results which were within 0.5 absolute per cent of each other.

Free Fatty Acids

These were converted into methyl esters for identification and quantitation determination by gas-liquid chromatography (GLC). Esterification was carried out using 14% boron trifluoride-methanol reagent (Applied Science Laboratories) by the method of Metcalf and Schmitz (8). Fatty acids in esterified lipids were converted into methyl esters for GLC analysis by transesterification using the same procedure as modified by Peterson et al. (9). GLC analyses were conducted on an F and M Model 810 dual-column chromatograph, equipped with a flame ionization detector. The column of choice was a 6-ft × ⅛-in. 6% diethyleneglycol succinate (LAC-728) on acid-washed silanized Chromosorb W. The carrier gas flow rate was 30 cc/min, the detector temperature was 275C, the injection port was 260C, and the column was maintained at a constant temperature between 150C and 175C. Peak areas were determined either by triangulation or with the aid of a disc integrator. Duplicate analyses conducted on crude lipid extracts or lipid fractions gave percentage compositions for major components within two or three absolute per cent of one another and values for minor components within one absolute per cent of one another. Identification of the fatty acids was achieved through a comparison of the retention times of the methyl esters with those of authentic samples (Applied Science Laboratories).

RESULTS AND DISCUSSION

Diet, Age and Fatty Acid Composition

In Table I are tabulated the fatty acid compositions of the total lipid extracts of crickets of different ages. Also included for comparison is the fatty acid composition of the lipids of the cricket food. There appear to be no dramatic differences in the fatty acid composition of the lipid extracts derived from crickets of different ages. The comparatively lower value for linoleic acid in three-week-old crickets is probably real, but even there the change as the insect ages is extremely small. The fatty acid composition of cricket lipids is in no way remarkable and shows a pattern similar to those exhibited by two other Orthopterans, *Melanoplus sanguinipes* (10) and *Gryllus bimaculatus* (11).

The influence of dietary lipids is also apparent. The three major acids of the food are also

TABLE II
Composition by Class of Lipids from *Acheta domesticus* of Different Ages

Postembryonic age of crickets (weeks)	Lipid Class (mg of Fraction/g Fresh Weight)								
	Total	Hydrocarbons	Simple esters	Triglycerides	Sterols	Diglycerides	Monoglycerides	Fatty acids	Unrecovered
2	48.4	2.4	0.4	21.3	2.6	2.5	3.3	2.2	11.9
3	54.8	2.0	0.4	32.1	2.5	1.8	1.0	1.0	12.3
4	60.1	1.9	0.5	40.3	3.1	2.1	0.8	0.8	9.2
5	65.9	2.0	0.4	44.1	2.4	2.6	2.6	2.6	8.2
6	77.1	2.3	1.2	53.2	1.9	3.8	1.5	1.5	10.3
7	78.6	2.6	0.9	52.7	3.7	2.1	3.5	3.5	11.9
8	76.9	3.7	1.5	49.9	2.7	2.9	1.4	1.4	15.0
10½	49.4	2.1	0.6	29.1	2.5	2.2	1.3	1.3	9.1
Average	—	2.4	—	—	2.7	2.5	2.2	1.8	11.0

TABLE III

Composition of Fatty Acids Associated with Lipid Classes from Eight-Week-Old *Acheta domesticus*

Lipid class	Percentage Composition of Fatty Acids								
	$<C_{14}$	$C_{14:0}$	$C_{14:1}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Simple esters	Trace	1.0	Trace	20.5	3.0	19.0	21.5	34.0	Trace
Triglycerides	Trace	1.0	Trace	22.5	3.0	7.0	27.5	37.0	2.0
Diglycerides	Trace	0.5	Trace	25.0	3.0	6.0	26.0	39.0	1.5
Monoglycerides	Trace	1.0	Trace	28.5	3.0	7.0	22.0	37.5	1.5
Free acids	Trace	0.5	—	23.5	2.5	9.0	26.0	37.5	1.0

the three major acids of the cricket. The cricket does seem to store the saturated acids, palmitic and stearic, and utilize the unsaturated acid, linoleic, preferentially since the cricket lipids are higher and lower respectively in these acids than is the cricket food. This is in accord with the data of House et al. (12), who demonstrated that dietary polyunsaturated lipids can increase the degree of unsaturation of depot fat only up to a certain level.

Lipid Composition and Age

In Table II the breakdown of the lipids into classes, that is, hydrocarbons, simple esters, triglycerides, sterols, diglycerides, monoglycerides, and free fatty acids for crickets of varying ages is presented. From the first column of Table II, it is clear that there is a build-up of depot fat during the first seven or eight weeks of the crickets' postembryonic life. This is followed by a loss of lipid between the eighth and eleventh week. From the fourth column it is apparent that it is the triglyceride fraction which is primarily responsible for the changes in total amounts of lipid which are present in the cricket. There also appears to be a small increase in the quantity of simple esters although the magnitude of the effect is on the borderline of experimental significance. The other lipid classes, hydrocarbons, sterols, diglycerides, monoglycerides, simple fatty acids, and highly polar unrecovered lipids appear to remain at fairly constant low levels throughout the crickets' life. The increase in triglyceride between the second and eighth week and the subsequent rapid decline between the eighth and eleventh week would seem most likely to be associated with the processes of egg development and oviposition.

Fatty Acids Associated with Each Lipid Class

In Table III are tabulated the compositions of the fatty acids associated with each lipid class isolated from 8-week-old crickets. It would appear that the free fatty acid fraction and the fatty acids derived from the mono-, di-, and triglyceride fractions are similar. Only the simple ester fraction appears to be distinctive in that it contains an unmistakably higher proportion of stearic acid and also seems to differ from the other fractions in some of the minor components. It contains less linolenic acid and more (1%) of an unknown material which has a retention close to that anticipated for a straight-chain 16-carbon doubly-unsaturated acid, a straight-chain 17-carbon acid, or a branched-chain 18-carbon acid.

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The Lipids of the Common House Cricket, *Acheta domesticus* L. II. Hydrocarbons

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ABSTRACT

The hydrocarbons of the common house cricket *Acheta domesticus* L. consist of *n*-alkanes (4%), 2-methylalkanes (20%), x-methylalkanes (59%), an unidentified series of alkanes (trace), and olefins (16-18%). The major n-alkane is *n*-nonacosane (3.5%). The major 2-methylalkanes are 2-methyloctacosane (8%) and 2-methyltriacontane (11%). The members of the homologous x-methylalkane series consist of mixtures of methylalkanes in which the methyl side-chain is located on the 13th, 15th, and 17th carbon atom in the chain. The major x-methylalkanes are the homologues containing 31 (3%), 33 (6.5%), 35 (12.5%), 37 (27%), and 39 (2.5%) carbon atoms. The olefins are a mixture of straight-chain and 2-methyl-alkenes and alkadienes. The major olefins contain 31 (3%) and 37 (7.5%) carbon atoms.

INTRODUCTION

THE ADVENT OF analytical and preparative gas-liquid chromatography (GLC), coupled with mass spectral techniques, has permitted detailed analyses of naturally occurring hydrocarbon mixtures heretofore considered beyond the realm of possibility. Whereas early studies of the hydrocarbon constituents of various plant and insect waxes had succeeded in recognizing the presence of an homologous series of alkanes, usually characterized as predominantly odd carbon-numbered normal alkanes in the 27 to 33 carbon range, more recent studies have demonstrated a much greater degree of complexity.

Even-numbered as well as odd-carbon-numbered alkanes, alkenes, alkadienes, monomethylalkanes with the methyl side-chain located at a number of different positions, dimethylalkanes, highly branched alkanes, and cycloalkanes have now been recognized as significant components of a number of natural waxes (1-10). By far the most thorough study of a naturally occurring hydrocarbon mixture has been by Mold et al. (11,12), who utilized the hydrocarbon fraction of wool wax. They have characterized 93 components of a hydrocarbon fraction, which constitutes only 32% of the total hydrocarbons present.

The characterization of the hydrocarbons of insect cuticle is of interest for a number of reasons. A knowledge of the constituents of cuticular wax is clearly basic to an understanding of the role of the cuticle in maintaining water balance. Likewise basic to any biochemical study designed to establish the metabolic pathways available for hydrocarbon synthesis is the characterization of the metabolic end-products. Hydrocarbon biosynthesis is an area of lipid metabolism in which little has been done. Robbins et al. (13) and Piek (14) have demonstrated the incorporation of ^{14}C from 1-^{14}C acetate into the hydrocarbons of the wax of *Musca domesticus* and *Apis mellifera* respectively, but any characterization of the anabolic pathways connecting acetate and the many constituents of the hydrocarbon mixtures present is lacking. Finally chemical taxonomy, utilizing hydrocarbon patterns, promises to be a valuable adjunct to classical morphological taxonomy when applied to large groups of similar and closely related species (7, 15).

This paper discusses the characterization of the hydrocarbons of the common house cricket, *Acheta domesticus* L. As a consequence of the relative ease of maintaining laboratory colonies (16,17) and because of its commercial availability, *A. domesticus* is an attractive species for chemical, biochemical, and biological studies.

MATERIALS AND METHODS

Isolation of the Hydrocarbon Fraction

In a typical run, 5.0 g of lipid extract, obtained as described in the previous paper (18), was adsorbed onto a column of 400 g of Flori-

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sil (Fisher) to which 28 g of water had previously been added (19). A hydrocarbon fraction weighing 290 mg was eluted with petroleum ether (40-60C, redistilled). This hydrocarbon fraction showed no absorption in its infrared spectrum characteristic of a carbonyl or hydroxyl function.

Separation of Saturated from Unsaturated Hydrocarbons (20)

In this and all subsequently described operations anhyrous reagent grade ether and redistilled reagent grade petroleum ether were utilized. The glassware was carefully cleaned. In a typical run 1.15 g of cricket hydrocarbons was placed on a column of 50 g of silica gel, impregnated with 25% silver nitrate (Adsorbosil-CABN, Applied Science Laboratories). Saturated hydrocarbons (0.97 g) were eluted with petroleum ether, unsaturated hydrocarbons (0.19 g) with ether: petroleum ether (10:90). Infrared spectra of both fractions indicated an efficient separation of saturated from unsaturated hydrocarbons.

Hydrogenation of Unsaturated Hydrocarbons

The unsaturated hydrocarbon fraction (100 mg), dissolved in 25 ml of acetic acid to which just enough petroleum ether was added to effect solution, was hydrogenated at room temperature and atmospheric pressure for 2 hr using 100 mg of 5% platinum on carbon (Engelhard Industries Newark, N. J.) as catalyst. The mixture was filtered, water was added, and the hydrogenated product, was extracted with petroleum ether. Removal of the petroleum ether solvent gave a hydrocarbon product devoid of any infrared absorption attributable to a double bond.

GLC Conditions

The GLC analyses were conducted on a dual column F & M Model 810 gas chromatograph, equipped with dual flame ionization detectors. Analyses were run isothermally at temperatures between 220C and 330C on 6 ft. × ⅛ in. o.d. 10% Apiezon L on acid-washed silanized Chromosorb W columns. High operating temperatures were advantageous if the analyses were concerned with higher homologues, but lower temperatures were used for the lower homologues.

Determination of Amounts of Each Component

The relative amounts of the various components in the saturated and unsaturated hydrocarbon mixture were determined by quantitative GLC. The higher homologues in these mixtures gave broad, rounded peaks, and by analyses of prepared mixtures of known composition it was found that the most accurate quantitative data were obtained by cutting out the individual peaks and weighing them. Such a procedure was far superior to triangulation or disc integration. The quantitative data presented in Table I are averages of three or four different determinations run at different temperatures.

Removal of n-Alkanes from an Alkane Mixture

In order to confirm the identification of the *n*-alkane series in the saturated alkane mixture, *n*-alkanes were removed by treatment with a molecular sieve (Linde, 5 A). Those peaks absent from the GLC of a sample so treated corresponded exactly to those which had been identified as *n*-alkanes by comparison with standards and by graphing of the logarithms of retention times against carbon number. In a typical experiment 101 mg of an alkane mixture, from which unsaturates had been removed, was dissolved in 7 ml of isooctane, and the solution was shaken with 6 g of Linde 5 A molecular sieves (1/16-in. pellets) overnight. The mixture was then filtered, and the sieves were washed twice with an equal volume of isooctane. The filtrate was concentrated to 7 ml, 6 g of Linde 5 A molecular sieve were added, and the mixture was shaken for 24 hr, after which it was filtered and washed as described previously. Upon concentrating the isooctane filtrates, a hydrocarbon mixture was obtained which contained only the Series II and the Series III alkanes. The largest peaks in the gas chromatogram which were removed were the two with retention times identical to n-octacosane and *n*-nonacosane.

Oxidative Cleavage of the Unsaturated Hydrocarbons (21)

To a solution of 135 mg of unsaturated hydrocarbons in 50 ml of *t*-butyl alcohol was added a solution of 600 mg of sodium periodate and 40 mg of potassium permanganate dissolved in 50 ml of water. The pH of the mixture was adjusted to 8-9 by the addition of aqueous potassium carbonate, then the solution was stirred for four days. The solution was extracted with methylene chloride to remove any neutral materials, of which there appeared to be none. The aqueous solution was then decolorized and neutralized with an aqueous solution of sodium bisulfite and extracted with chloroform. The chloroform solution was dried over sodium sulfate, filtered, and evaporated to dryness, leaving 117 mg of acids. This residue was esterified using boron trifluoride/

TABLE I

The Hydrocarbons of the Common House Cricket, *Acheta domesticus* L., Percentage Composition[a]

Carbon Number	Series I (n-alkanes)	Series II (2-methyl-alkanes)	Series III (x-methyl-alkanes)	Series IV (unidentified)	(Olefins)
15	Trace	Trace	—	Trace	—
16	Trace	Trace	—	Trace	—
17	Trace	Trace	—	Trace	—
18	Trace	Trace	—	Trace	Trace
19	Trace	Trace	—	Trace	Trace
20	Trace	Trace	—	Trace	Trace
21	Trace	Trace	—	—	Trace
22	Trace	Trace	—	—	Trace
23	Trace	Trace	—	—	Trace
24	Trace	Trace	—	—	Trace
25	Trace	Trace	—	—	Trace
26	Trace	Trace	—	—	Trace
27	Trace	Trace	Trace	—	Trace
28	<0.5	Trace	—	—	Trace
29	3.5	8.0	Trace	—	1.5
30	Trace	0.5	Trace	—	<0.5
31	—	11.5	3.0	—	3.0
32	—	—	0.5	—	Trace
33	—	0.5	6.5	—	1.0[b]
34	—	—	1.5	—	Trace
35	—	—	12.5	—	0.5[c]
36	—	—	4.0	—	<0.5
37	—	—	27.0	—	7.5[d]
38	—	—	1.5	—	1.5
39	—	—	2.5	—	1.5
Total %	4.0	20.5	59.0	Trace	17.5

[a] Accurate to 2-3 absolute percentage for components present to the extent of 3% or more. Percentages for minor components have a larger error associated with them.

[b] Three overlapping peaks on GLC, one minor and two of equal magnitude.

[c] Two overlapping peaks of equal magnitude on GLC.

[d] Two peaks on GLC, one very minor, the other major (> 97%).

methanol (22) and analyzed by GLC with the use of 6-ft. × ⅛-in O.D. 6% LAC 728 columns.

RESULTS AND DISCUSSION

In Table I is presented the distribution of the cricket hydrocarbons. The weight percentage composition values have been rounded off to the nearest half per cent. Those components designated as "trace" constituents were clearly identifiable peaks on the gas-liquid chromatograms and fit nicely onto the logarithmic plots but were so minor as to make any attempt at quantitation pointless.

The hydrocarbon mixture initially obtained from the crickets was subdivided into a saturated and an unsaturated fraction by column chromatography on silica gel impregnated with silver nitrate. GLC of the saturated hydrocarbon fraction indicated a complex mixture. However, by plotting the logarithm of the retention times of the various components against a linear ordinate representing the number of carbon atoms present in the hydrocarbon, three parallel lines resulted, indicating that the saturated hydrocarbon mixture consists of three homologous series, designated Series I, II, and III.[3]

Series I was identified as homologous normal alkanes; every carbon number from 15 to 30 was represented although most of them were only in trace amounts. The line resulting from the logarithmic plot coincided precisely with one constructed from authentic reference samples. Furthermore the two major components of the Series I hydrocarbons had retention times on GLC identical to authentic *n*-octacosane and *n*-nonacosane. Treatment of the saturated hydrocarbon mixture with the Linde 5 A molecular sieve completely removed all of the components present in Series I. These hydrocarbons are therefore normal alkanes. The

[3] Authentic samples utilized for direct comparison and for the construction of logarithmic plots included *n*-hexane, *n*-octane, *n*-decane, *n*-tetradecane, and *n*-hexadecane (F & M Scientific Corporation); *n*-octadecane and *n*-tetracosane (Applied Science Laboratories); *n*-octacosane, *n*-dotriacontane, and *n*-hexatriacontane (Aldrich Chemical Company); *n*-nonacosane and *n*-hentriacontane (donated by E. Ritchie, Department of Organic Chemistry, University of Sydney, Sydney, Australia); 2-methyltricosane, 2-methyloctacosane, 3-methyltetracosane, and 12-methylnonacosane (donated by James Mold, Research Department, Liggett and Myers Tobacco Company, Durham, N. C.).

n-alkanes, which so often constitute the bulk of naturally occurring hydrocarbon mixtures, make up only 4% of the hydrocarbons of *Acheta domesticus* L.

Series II was identified as homologous 2-methylalkanes (isoalkanes). An authentic sample of 2-methyloctacosane had a retention time identical to the 29-carbon Series II hydrocarbon. An authentic sample of 3-methyltetracosane had a different retention time from the trace Series II component with 25 carbons. Also, a mass spectrum of the saturated hydrocarbon mixture had parent peaks at 408 and 436 mass units, corresponding to $C_{29}H_{60}$ and $C_{31}H_{64}$ hydrocarbons (the two major Series II alkanes); large fragment peaks at 393 and 421, corresponding to the loss of 15 mass units; a methyl group from each of parent ions but not fragment peaks at 379 and 407, which would have resulted from the loss of 29 mass units; an ethyl group from the parent ions of 3-methyloctacosane and 3-methyltriacontane. Thus it can be concluded that the Series II hydrocarbons are 2-methylalkanes (isoalkanes) and that 3-methylalkanes (anteisoalkanes) are absent from the hydrocarbon mixture. The isoalkanes, a class of hydrocarbons which is being encountered more and more frequently in naturally occurring hydrocarbon mixtures, constitutes 20.5% of the cricket hydrocarbons.

The Series III hydrocarbons constitute 59% of cricket hydrocarbons. The 37-carbon member of this series alone constitutes 27% of the total. This series was established to consist of methylalkanes having the methyl side-chain toward the middle of the carbon chain. In fact, each homologous member of this series consists of a mixture of alkanes having the methyl side-chain at different positions in the carbon chain. This was established by collecting and submitting to mass spectral analysis the 35, 36, and 37 carbon members of the series. The mass spectra were interpreted in the same way that Mold et al. (12) interpreted mass spectra of similar hydrocarbons.

The 37-carbon homologue, with a parent peak at 520 ($C_{37}H_{76}$), had prominent fragmentation peaks at 323 ($C_{23}H_{47}$), 295 ($C_{21}H_{43}$), 253 ($C_{18}H_{37}$), and 225 ($C_{16}H_{33}$). This pattern suggests that the sample is a mixture of 15-methylhexatriacontane and 17-methylhexatriacontane. The major fragment ions result from cleavage of the parent ion on either side of the methyl group. There may be traces of 13-methylhexatriacontane since there are slightly enhanced fragment peaks at 351 ($C_{25}H_{51}$) and 197 ($C_{14}H_{29}$).

The 36-carbon homologue submitted for mass spectral analysis was unfortunately contaminated with significant quantities of 35-carbon Series III alkanes. Thus the mass spectrum is less definitive than the one discussed above. Nonetheless it is informative. Prominent fragmentation peaks at 337 ($C_{24}H_{49}$) and 197 ($C_{14}H_{29}$) indicate the presence of 13-methylpentatriacontane; peaks at 309 ($C_{22}H_{45}$) and 225 ($C_{16}H_{33}$) indicate 15-methylpentatriacontane; and peaks at 281 ($C_{20}H_{41}$) and 253 ($C_{18}H_{37}$) indicate 17-methylpentatriacontane. The 35 carbon contaminants appear to be 13-methyltetratriacontane, 15-methyltetratriacontane, and 17-methyltetratriacontane.

Clearly the mass spectrum of a mixture as complex as this one is not subject to rigorous interpretation. However the absence of prominent fragmentation peaks at 155 ($C_{11}H_{23}$), 183 ($C_{13}H_{27}$), 211 ($C_{15}H_{31}$), and 239 ($C_{17}H_{35}$) requires that no methyl branches occur on carbon atoms number 10, 12, 14, or 16 in the alkane chain. Likewise the absence of any major fragment peaks above 337 ($C_{24}H_{49}$) requires that no methyl group occur closer to the end of a 35-carbon chain than carbon atom number 13.

The mass spectrum of the 35-carbon Series III alkane, which is contaminated with 34- and 36-carbon homologues, is even more complex and less rigorously interpreted. However the pattern is the same. The mass spectrum is consistent with a mixture of 13-methyltetratriacontane (peaks at 323 and 197 owing to $C_{23}H_{47}$ and $C_{14}H_{29}$), 15-methyltetratriacontane (peaks at 295 and 225 owing to $C_{21}H_{43}$ and $C_{16}H_{33}$), 17-methyltetratriacontane (peaks at 267 and 253 owing to $C_{19}H_{39}$ and $C_{18}H_{37}$), 13-methyltritriacontane (peaks at 309 and 197 owing to $C_{22}H_{45}$ and $C_{14}H_{29}$), 15-methyltritriacontane (peaks at 281 and 225 owing to $C_{20}H_{41}$ and $C_{16}H_{33}$), 17-methyltritriacontane (one peak at 253 owing to $C_{18}H_{37}$), and 13-methylpentatriacontane (peaks at 337 and 197 owing to $C_{24}H_{49}$ and $C_{14}H_{29}$).

Although the suggested composition of this mixture may be subject to debate, what the mass spectrum indicates incontestably is that there are no major fragment ions with the formulas $C_{11}H_{23}$ (155), $C_{13}H_{27}$ (183), $C_{15}H_{31}$ (211), and $C_{17}H_{35}$ (239), thus reinforcing the conclusion arrived at earlier that, in the Series III alkanes, methyl groups occur at the odd-numbered carbon atoms 13, 15, and 17 and not at the even-numbered carbon atoms 10, 12, 14, and 16.

Hydrocarbons of this type, in which the

methyl group is located at a carbon atom toward the center of the chain, have been identified in wool wax (12), where they comprise 4.5% of the total saturated hydrocarbons, and may have been detected in sugar cane wax (9) and in the waxes derived from three plants, *Humulus lupulus, Populus nigra, and Rosa spec.* (5, 6), but in none of these cases do they constitute the major hydrocarbon type.

The Series IV hydrocarbons, which are trace components in the 15 to 20 carbon range were not identified. They fall on the same logarithmic plot as the Series III hydrocarbons, but since no Series III or IV hydrocarbons in the 21 to 26 carbon range could be detected even as trace components, it does not seem likely that the two series are related anabolically. Besides, since the Series III alkanes have their methyl group at least 13 carbon atoms from the nearest end, no 15 or 20 carbon compound could class as a member of such a series. No attempt was made to characterize these trace constituents.

The olefinic hydrocarbon fraction constitutes 16-18% of the total hydrocarbons. The GLC of the olefin fraction clearly indicated one major and possibly two minor homologous series. Hydrogenation of the olefin mixture gave an alkane mixture composed of *n*-alkanes from 18 through 39 carbon atoms and 2-methylalkanes from 22 through 39 carbon atoms. The n-alkanes made up about 67% of the hydrogenation mixture, the isoalkanes about 33%. No 3-methylalkanes or Series III alkanes were detectable. The major n-alkanes were *n*-nonacosane, *n*-hentriacontane, *n*-heptatriacontane, *n*-octatriacontane, and *n*-nonatriacontane. All of the isoalkanes were present in small quantities except for 2-methyldotriacontane, 2-methyltetratriacontane, and 2-methylhexatriacontane, which were the three major isoalkanes.

A mass spectrum of the hydrocarbon mixture obtained initially from the crickets had parent peaks at 544 ($C_{39}H_{76}$), 530 ($C_{38}H_{74}$), 516 ($C_{37}H_{72}$), 460 ($C_{33}H_{64}$), 432 ($C_{31}H_{60}$), and 404 ($C_{29}H_{56}$), indicating a prevalence of dienes. An ultraviolet spectrum of the olefin mixture had a weak maximum at λ_{max} of 230 μ ($\varepsilon \sim 500$, assuming an average molecular weight of 486). Thus there is less than 5% conjugated diene present. The oxidative cleavage of the olefin mixture is informative although the complexity of the mixture precluded complete structural assignment. Straight-chain acids from 6 to 26 carbon atoms were obtained. The absence of lower acids is not significant since they would have been lost in the work-up The predominant straight-chain acids were the 7, 12, 14, 24, and 26 carbon acids.

Methyl-substituted acids with from 20 to 26 carbon atoms were also produced; the 22 and 24 carbon homologues predominated. Thus in the 2-methylalkadiene series it appears that unsaturation gets no closer to the branched end of the chain than 19 carbon atoms. The straight-chain acids suggest a similar location of the unsaturation in the normal alkenes and alkadienes. A more complete structural analysis of this olefin mixture would require isolation of individual homologues. Alkanes and alkadienes have been isolated from a number of natural sources, but 2-methylalkenes and 2-methylalkadienes do not appear to have been reported previously.

The hydrocarbons of the crickets' food were also surveyed. In addition to large amounts of highly branched material, probably largely isoprenoid in nature, which would not form a soluble urea clathrate compound, there was also present some material which did form a soluble clathrate. This material was almost entirely composed of n-alkanes, from n-nonadecane through n-tritriacontane. At most only traces of Series II isoalkanes were present, and none of the Series III hydrocarbons were detectable. Thus the n-alkanes in the cricket may be present as a consequence of the selective up take of dietary hydrocarbons. However the Series II hydrocarbons appear to be, and the Series III hydrocarbons most certainly are, products of anabolic hydrocarbon metabolism.

So little is known about hydrocarbon biosynthesis that it is hardly even fruitful to speculate about the origin of the cricket hydrocarbons. Long-chain fatty acids are plausible precursors. Because of the ease of raising *A. domesticus,* the ease with which radioactive fatty acids could be fed to them, and the ease with which the various hydrocarbon classes can be separated, crickets would appear to be an ideal organism for the study of hydrocarbon metabolism.

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The Lipids of the Common House Cricket, *Acheta domesticus* L. III. Sterols

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ABSTRACT

Sterols constitute 1.95% of the total extractable lipids of *Acheta domesticus* L., of which 18% are esterified. The free sterols consist of cholestane-3β-ol (0.5%), Δ^5-cholestene-3β-ol (83.5%), Δ^7-cholestene-3β-ol (2.3%) $\Delta^{5,7}$-cholestadiene-3β-ol (3%), $\Delta^{5,22}$-cholestadiene-3β-ol (4%), $\Delta^{5,7,22}$-cholestatriene-3β-ol (0.2%), campestane-3β-ol (0.03%), Δ^5-campestene-3β-ol (1.0%), Δ^7-campestene-3β-ol (trace), $\Delta^{5,7}$-campestadiene-3β-ol (0.2%), stigmastane-3β-ol (0.09%), Δ^5-stigmastene-3β-ol (2.1%), Δ^7-stigmastene-3β-ol (0.04%), $\Delta^{5,7}$-stigmastadiene-3β-ol (0.4%), $\Delta^{5,22}$-stigmastadiene-3β-ol (0.1%). The same sterols are present in the esterified sterol fraction. Δ^7-Sterols and $\Delta^{5,7}$-sterols are present in significantly larger amounts in the esterified fraction than in the free sterol fraction. By a comparison with the sterols of the cricket food, it is clear that *A. domesticus* is capable of removing methyl and ethyl groups from C-24 of sterols of the campestane and stigmastane type. The ability to introduce a Δ^7 double bond into saturated and Δ^5-sterols is indicated, and it is suggested that Δ^7-sterols of the C_{27}, C_{28}, and C_{29} sterol series may be intermediates in the conversion of Δ^5-sterols to $\Delta^{5,7}$-sterols.

INTRODUCTION

UNLIKE VERTEBRATES, insects are unable to synthesize sterols from such precursors as acetate, mevalonate, squalene or lanosterol (1-7). Consequently, insects provide an excellent system for studying such fundamental problems as sterol function in biological membranes (8,9) and metabolic transformations which involve skeletal modifications of the preformed steroid system, uncomplicated by the multitude of possible reactions which do or can occur in plants and animals during sterol biogenesis.

Cholesterol is the major sterol of insects. Other sterols which have been encountered in smaller amounts or in only certain species include 7-dehydrocholesterol, cholestanol, 22-dehydrocholesterol, Δ^7-cholestenol and β-sitosterol (10,11). In addition, in a number of the more careful studies, a small fraction of polar sterols has been reported but never characterized (3, 12-14). Since insects are unable to synthesize sterols, they require a dietary source. Phytophagous insects are generally able to transform the common plant sterols, such as β-sitosterol and stigmasterol, into cholesterol by removal of the ethyl group from C-24, whereas carnivorous insects have lost this metabolic capability and require cholesterol or 7-dehydrocholesterol in their diets (10,15). The ability to utilize ergosterol is less widespread (5, 16-18).

In this study the simple sterols of the common house cricket, *Acheta domesticus* L., are characterized and analyzed quantitatively. A much less complete study of the sterols of *A. domesticus* has appeared (19). This study is preliminary to a more ambitious effort to characterize the minor sterols of the polar sterol fraction. It is in this fraction that important sterol metabolites, either derived from dietary sterols or from cholesterol, are likely to be found. *Acheta domesticus* has been chosen for this study as a consequence of its commercial availability in quantities necessary to permit a successful isolation of minor components.

MATERIALS AND METHODS

Lipid Extraction

Lipids were extracted from 8 to 8½ week old crickets by the procedure described in the first paper of this series (20).

Separation of Sterols

In a typical run, 25.25 g of lipid extract was chromatographed over 1275 g of Florisil (Fisher) containing 7% water. Hydrocarbons were eluted with 4 liters of petroleum ether (bp 40-60, redistilled). A simple ester fraction weighing 2.356 g, which contained the sterol esters, was eluted with 7 liters of ether-petroleum

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ether (5:95). There was some contamination by triglycerides. Triglycerides were eluted in 7 liters of ether-petroleum ether (15:85), and 1.086 g of sterols were eluted with 8 liters of ether-petroleum ether (25:75).

Purification of the Sterols

The simple ester fraction, 2.356 g, was saponified by refluxing for 3 hr under nitrogen in 25 ml of methanol containing 1.5 g of 85% potassium hydroxide and 1.5 ml of water. A neutral fraction weighing 225 mg was isolated by conventional techniques. This material consisted of a mixture of sterols, simple alcohols and fatty acid contaminants which persisted through the purification procedure applied to the saponification mixture. Fatty acids were removed by passing the mixture through an ion exchange column. The column was prepared by stirring 50 g of Amberlite IRA-400 ion exchange resin with 100 ml of 0.5*N* sodium hydroxide. This suspension was packed into the column, and washed with distilled water until neutral. The residue from the saponification, 225 mg, dissolved in ether saturated with water, was applied to the column. Slow elution with 1 liter of wet ether removed neutral material. The eluant was dried over sodium sulfate and evaporated, leaving 167 mg of residue. Final purification of the 3β-hydroxysterols was achieved by a digitonide precipitation using the method of Schoenheimer and Dam (21), which was found to work better than the more recent modification of Bergman (22). This procedure gave 88 g of purified sterols (100% of the theoretical amount which should be liberated from the 367 mg of digitonide obtained). Sterol esters, therefore, constitute 0.35% of the total lipids of *A. domesticus*.

Purification of the Free Sterol Fraction

The same three steps, saponification, ion exchange chromatography and digitonide precipitation were utilized to purify the free sterols. From 1.086 g of free sterol fraction, saponification gave 997 mg of a mixture which was reduced to 452 mg of neutral material by ion exchange chromatography. From these 452 mg of material, 1.687 g of digitonide was obtained, from which 402 mg of pure sterols was liberated. Free sterols, therefore, constitute 1.6% of the total lipids of *A. domesticus*.

GLC of Sterol Mixtures

The sterols were converted into their trimethylsilyl ethers for analysis by gas-liquid chromatography (GLC). For this transformation 0.1 ml of TRI-SIL (Pierce Chemical Company) Solvent-Reagent-Catalyst formulation was used per milligram of sterol mixture, according to the procedure suggested on the bottle. Reaction was complete within 5 min.

GLC Conditions

GLC analyses were conducted on a dual column F&M Model 810 chromatograph equipped with dual flame ionization detectors. Two columns were found to be satisfactory for these analyses. An 8 ft × ⅛ in O.D. SE-30 Hi-Pak column (F and M Scientific Corporation), conditioned for 3 hr at 300C and deactivated by repeated injections of tetramethyldisilazane separated the sterols very well, and gave symmetrical peaks. The operating conditions were as follows: column temperature, 250C; injection port temperature, 330C; detector temperature, 310C; helium flow rate, 35 ml/min; sample size, 0.8-1.0 μl. A 6 ft × ⅛ in O.D., 3% QF-1 on 100-120 mesh Gas-Chrom Q (Applied Science Laboratories) column, conditioned for 3 hr at 260C also served well. Operating conditions were as follows: column temperature, 200C; injection port temperature, 265C; detector temperature, 310C; helium flow rate, 25-30 ml/min; sample size, 0.3-1.0 μl.

Sterols were identified by comparing retention times of the components with authentic samples of cholesterol, cholestanol, Δ^7-cholestenol, β-sitosterol, β-sitostanol, stigmasterol, campestanol and campesterol (generously provided by Dr. Richard Rapala of Eli Lilly Company) on both columns. Mixtures were analyzed quantitatively by determining peak area, using triangulation, of duplicate chromatograms on the SE-30 Hi-Pak column.

Determination of Sterol Distribution

A complete analysis of the sterol mixtures by GLC is complicated by the fact that the separation of saturated, Δ^5- and $\Delta^{5,7}$-sterols for each series is inadequate to permit acceptable quantitation. On the SE-30 column the saturated and Δ^5-enol of a given series have virtually identical retention times, and on the QF-1 column the stanol is a shoulder on the trailing side of the stenol peak. The $\Delta^{5,7}$-dienols have only slightly greater retention times on the SE-30 column.

In order to obtain a complete quantitative analysis of a sterol mixture, the following series of operations was conducted. First the entire sterol mixture was analyzed by GLC. This gave an accurate value for the Δ^7-enol and a composite value for the saturated, Δ^5- and $\Delta^{5,7}$-sterols. Then a portion of the sterol mixture was oxidized with performic acid, a proc-

TABLE I
Free and Esterified Sterols of *A. domesticus* and Its Food

Sterol	Percentage Composition			
	Cricket Sterols		Cricket Food Sterols	
	Free	Esterified	Free	Esterified
1. $\Delta^{5,22}$-Cholestadiene-3β-ol	4.0	5.5	0.0	0.0
2. Cholestane-3β-ol	0.5	1.5	0.4	0.5
3. Δ^{5}-Cholestene-3β-ol	83.5	69	6.0	4.0
4. $\Delta^{5,7}$-Cholestadiene-3β-ol	3.0	6.5	<0.7[a]	<0.6[a]
5. Δ^{7}-Cholestene-3β-ol	2.3	11	0.0	0.0
6. $\Delta^{5,7,22}$-Cholestatriene-3β-ol	0.2	0.4	<0.7[a]	<0.6[a]
7. Campestane-3β-ol	0.03	0.08	1.0	1.0
8. Δ^{5}-Campestene-3β-ol	1.0	0.9	19	17
9. $\Delta^{5,7}$-Campestadiene-3β-ol	0.2	0.4	<0.7[a]	<0.6[a]
10. Δ^{7}-Campestene-3β-ol	trace	~0.1	0.0	0.0
11. $\Delta^{5,22}$-Stigmastadiene-3β-ol	0.1	~0.05	6.0	4.0
12. Stigmastane-3β-ol	0.09	0.4	2.5	3.5
13. Δ^{5}-Stigmastene-3β-ol	2.1	1.5	64	64
14. $\Delta^{5,7}$-Stigmastadiene-3β-ol	0.4	0.8	<0.7[a]	<0.6[a]
15. Δ^{7}-Stigmastene-3β-ol	0.04	1.0	0.0	0.0
16. Unknown A (sat'd) R_S 3.9 on SE-30	0.03	0.02	0.0	0.2
17. Unknown B (unsat'd) R_S 3.9	0.04	1.0	0.7	3.8
18. Unknown C	0.0	~1.5	0.0	<0.4[a]
19. Other Unknowns	0.0	0.0	0.0	1.5[b]

[a] By ultraviolet analysis.

[b] Three components: R_S 4.1, 4.4, 4.9 on SE-30.

ess which completely removed all of the unsaturated sterols (23). The saturated sterol mixture which remained was then analyzed by GLC. The total $\Delta^{5,7}$-sterol content was then established by spectral examination of the entire sterol mixture at 272 mμ, 282 mμ, and 294 mμ (4% of the free sterols, 8% of the sterol esters). The $\Delta^{5,7}$-dienols were then separated from the rest of the sterols by conversion to their peroxides (24). The peroxides were purified by chromatography on Florisil (7% water), eluting with ether-petroleum ether (40:60). The peroxide mixture was then reduced with sodium and ethanol (25) to a 9:1 mixture of the Δ^{7}-enol and Δ^{5}-enol. The product mixture of the reduction was analyzed by GLC. Thus, the amount of Δ^{5}-enol present could be obtained by subtracting the values for $\Delta^{5,7}$-dienol and saturated sterol from the composite value obtained from the GLC analysis of the entire mixture.

Identification of 22-Dehydrocholesterol

One component of the sterol mixture had a smaller retention time (1.98 relative to cholestane) than cholesterol (2.23 relative to cholestane). 22-Dehydrocholesterol is the only sterol which has been reported to have a shorter retention time than cholesterol under GLC conditions comparable to those used in this work (26,27). The presence of 22-dehydrocholesterol in the sterol mixture was confirmed by subjecting the mixture to oxidative cleavage (28), and detecting isovaleric acid in the product.

Analysis of Dietary Sterols

Isolation, separation, purification and analysis of the dietary sterols were conducted by the same series of operations that were applied to the cricket sterols.

RESULTS AND DISCUSSION

The results of the characterization of the sterols of *A. domesticus* and its food are presented in Table I. Cholesterol (entry 3) is the major sterol of *A. domesticus*. The cricket, a phytophagous insect, has a dietary sterol supply which consists of over 90% sterols with methyl or ethyl groups attached to C-24 of the sterol side chain (entries 7-15), whereas over 90% of the sterols of the adult cricket are unsubstituted at C-24 (entries 1-6). Only small amounts of the major dietary plant sterols (entries 8, 11, 12, 13 and 17) persist in the cricket sterols. The ability on the part of the cricket to dealkylate C-24 is clearly indicated.

Several other metabolic capabilities are indicated by the results summarized in Table I. The Δ^{7}-enols (entries 5, 10 and 15) are completely absent in the food, but are present in the significant amounts, especially Δ^{7}-cholestene-3β-ol, in the cricket. Irreversible conversion of cholestane-3β-ol to Δ^{7}-cholostene-3β-ol has been demonstrated in *Eurycotis floridana*

(29, 30) and *Blatella germanica* (29-31), and the dealkylation of saturated sterols of the stigmastane-3β-ol type has been demonstrated in *Blatella* (32). Thus the saturated sterols (entries 2, 7 and 12) are likely precursors to the Δ^7-cholestene-3β-ol, by way of dealkylation and oxidation.

$\Delta^{5,7}$-Dienes, particularly $\Delta^{5,7}$-cholestadiene-3β-ol (entry 4), are also present in larger amounts in the cricket than in its food. Desaturation of Δ^5-cholestene-3β-ol to $\Delta^{5,7}$-cholestadiene-3β-ol by *Musca domesticus* has been demonstrated (12), and such a process may represent the origin of the $\Delta^{5,7}$-dienes in the cricket. Desaturation of a Δ^7-enol to a $\Delta^{5,7}$-dienol has never been suggested as a possible route to the $\Delta^{5,7}$-dienols, but is a definite possibility.

Δ^7-Cholestene-3β-ol and $\Delta^{5,7}$-cholestadiene-3β-ol are seen to be concentrated in the esterified sterol fraction. $\Delta^{5,7}$-Cholestadiene-3β-ol is believed to be a precursor of physiologically active growth and fertility hormones (26, 33). This may suggest that subsequent transformations of the $\Delta^{5,7}$-dienol occur in the esterified compound. Similarly, the concentration of the Δ^7-enol in the esterified fraction and Δ^5-enols in the free sterol fraction may suggest that Δ^7-enols are closer to $\Delta^{5,7}$-dienols than the Δ^5-enols in a biosynthetic pathway, as would be the case if the sequence Δ^5-enol $\rightarrow$ saturated sterol $\rightarrow$ Δ^7-enol $\rightarrow$ $\Delta^{5,7}$-dienol were operative. These are suggestions which will require testing by suitable labeling experiments.

Finally, $\Delta^{5,22}$-cholestadiene-3β-ol (entry 1) is present in the crickets and not in the food. It probably arises through the dealkylation of $\Delta^{5,22}$-stigmastadiene-3β-ol (entry 11). A similar conversion of ergosterol to $\Delta^{5,22}$-cholestadiene-3β-ol has been demonstrated in *B. germanica* (2, 34). Subsequent reduction of the Δ^{22}-unsaturation by *germanica* was not observed.

$\Delta^{5,24}$-Cholestadiene-3β-ol, or desmosterol, recently shown to be an intermediate in the dealkylation process by which sitosterol is converted to cholesterol (27) was not detected in the cricket sterols. Its presence in small amounts would have gone undetected, however.

ACKNOWLEDGMENT

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Fatty Acid Composition of *Claviceps* Species. Occurrence of (+)-*threo*-9, 10-Dihydroxystearic Acid

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ABSTRACT

The fatty acids from sclerotia of *C. purpurea* and of four other *Claviceps* species have been examined chromatographically, and all contain greater or lesser amounts of various oxygenated fatty acids. *C. sulcata* was the most unusual in that it contained some 60% of (+)-*threo*-9,10-dihydroxystearic acid.

INTRODUCTION

ERGOT OIL, THE FATTY OIL produced in sclerotia of the fungus *Claviceps purpurea*, has long been known to contain substantial proportions of ricinoleic (D-12-hydroxy-*cis*-9-octadecenoic) acid (1,2). This is derived biogenetically from linoleic acid by a mechanism which does not require oxygen (3) and is combined in unique estolide-triglyceride structures with structural specificities which suggest that the mechanism of biosynthesis may be quite novel (4). The only other *Claviceps* species, the fatty acid composition of which appears to have been examined is *C. paspali;* this is reported to contain no ricinoleic acid in its sclerotial lipids (5).

This communication describes the fatty acid composition of the sclerotial lipids of five *Claviceps* species and the characterization of a dihydroxy acid from *C. sulcata.* A more detailed report will be published when the structures of more of the oxygenated acids which are present have been fully elucidated.

PROCEDURES AND RESULTS

P. G. Mantle, Department of Biochemistry, Imperial College, London, supplied small samples of the lipids extracted from sclerotia of *C. paspali, C. gigantea, C. sulcata,* and an unclassified *Claviceps* species, the sclerotia of which were collected from infected plants of *Pennisetum typhoideum* (hereafter referred to as *C.* (ex-*Pennisetum*)). The lipids had been extracted from the crushed sclerotia with chloroform-methanol (2:1) or with diethyl ether. Fatty acids were released from the lipids by hydrolysis with methanolic potassium hydroxide and, after the removal of unsaponifiable components, methyl esters were prepared from the acids by reaction with diazomethane.

The mixed ester samples were examined by thin-layer chromatography (TLC) on silica gel by using a variety of diethyl ether-light petroleum mixtures as solvents. Besides a major component corresponding to normal methyl esters, Fig. 1a shows that all the *Claviceps* species had a minor component similar in migration to ricinoleate which was, of course, a major constituent of *C. purpurea.* Moreover all the species had a component corresponding to a dihydroxy ester, and this was the main constituent of *C. sulcata.* All of these "dihydroxy ester" components migrated with 9,10-dihydroxystearate under conditions where this was separated from the 6,7- and 12,13-dihydroxy positional isomers (6,7). On silica impregnated with boric acid (Fig. 1b) they all appeared to complex and migrate like authentic *threo*-9,10-dihydroxystearate but unlike the *erythro*-isomer (7). The TLC studies therefore seemed to indicate that *threo*-9,10-dihydroxystearic acid was a component of all of these *Claviceps* species and the major component of *C. sulcata.* Not evident from Fig. 1 is the fact that *C. sulcata* and *C.* (ex-*Pennisetum*) contained trace amounts of a component which, on TLC and on GLC (carbon number = 19.50 on an SE-30 stationary phase), was identical to methyl *cis*-9,10-epoxystearate.

The nonoxygenated methyl esters were isolated from each sample by preparative-TLC and were analyzed by GLC on an ethyleneglycol-adipate polyester (EGA) stationary phase. The fatty acid compositions of these *Claviceps* species, including the various oxygenated components which were semiquantitatively estimated by TLC, are summarized in Table I.

The dihydroxy component of *C. sulcata* esters was isolated by preparative-TLC and was found gravimetrically to comprise 63.6% of the total methyl esters. GLC on an SE-30 stationary phase showed it to have a carbon number of 20.85, identical to that of authentic methyl *threo*-9,10-dihydroxystearate. The purified ester had mp 58-60C and was optically active with $[\alpha]^{26C}_{546.1\ m\mu} = +22.5°$ (c = 1.2%

TABLE I

Fatty Acid Compositions of the Sclerotial Lipids of Five *Claviceps* Species (wt % as Methyl Esters)

	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	epoxy-18[a]	OH-18[a]	diOH-18[a]
C. purpurea	0.1	19.9	6.5	4.3	22.5	14.3	tr.	tr.	—	—	32.3	tr.
C. paspali	0.2	19.0	7.4	2.1	56.7	14.5	—	tr.	—	—	tr.	tr.
C. gigantea	0.2	17.2	2.6	2.7	55.6	19.6	tr.	tr.	—	—	~1[b]	~1[b]
C. (ex-*Pennisetum*)	0.3	34.1	2.4	7.3	38.6	14.4	—	tr.	—	tr.	~1[b]	~2[b]
C. sulcata	0.1	11.1	0.8	4.9	12.8	5.1	0.1	1.4	0.2	tr.	tr.	63.6

[a] The structure of these components has not yet been proved (except for ricinoleate from *C. purpurea* and dihydroxystearate from *C. sulcata*) but is implied by their behavior on TLC and, in some cases, on GLC.

[b] The content of these components was estimated by comparisons with known amounts of standards on TLC.

in methanol); literature values for methyl (+)-*threo*-9,10-dihydroxystearate are mp 62C and $[\alpha]_D$ + 22.5° in methanol (8) or mp 58-60C and $[\alpha]_D$ + 23.6° in ethanol (9). Oxidative cleavage and analysis of the esterified products by GLC showed that only nonanoate and nonandioate were formed. The dihydroxy acid from *Claviceps sulcata* is thus proved to be (+)-*threo*-9,10-dihydroxystearic acid.

DISCUSSION

This acid has been shown by Tulloch (10-12) to be present in the spores of a wide variety of plant rusts. In these spore lipids it accompanies (-)-*cis*-9,10-epoxystearic acid, and Tulloch demonstrated (9,12) that an epoxide-hydrolyzing enzyme system was present in the spores of a number of these fungi which catalyzed the conversion of the epoxy acid to the optically active dihydroxy acid. A similar type of enzyme appears to be present in the seeds of *Vernonia anthelmintica* which, on incubation of the moist ground seeds, results in the conversion of the constituent 12,13-epoxyoleic acid to (+)-*threo*-12,13-dihydroxyoleic acid (13). Recently Niehaus and Schroepfer (14) have shown that the enzyme preparation produced from a *Pseudomonas* species (NRRL-2944), which catalyzes the hydration of oleic acid to D-10-hydroxystearic acid, also catalyzes the hydration of *cis*-9,10-epoxystearic acid to (+)-*threo*-9,10-dihydroxystearic acid and of *trans*-9,10-epoxystearic acid to (+)-*erythro*-9,10-dihydroxystearic acid.

We consider it likely that the (+)-*threo*-9,10-dihydroxystearic acid now shown to occur in *Claviceps sulcata* sclerotia has been similarly biosynthesized by hydration of a *cis*-9,10-epoxystearic acid, particularly as we find that a trace amount of the presumed epoxy intermediate appears to co-occur with it in *C. sulcata* and also in *C.* (ex-*Pennisetum*).

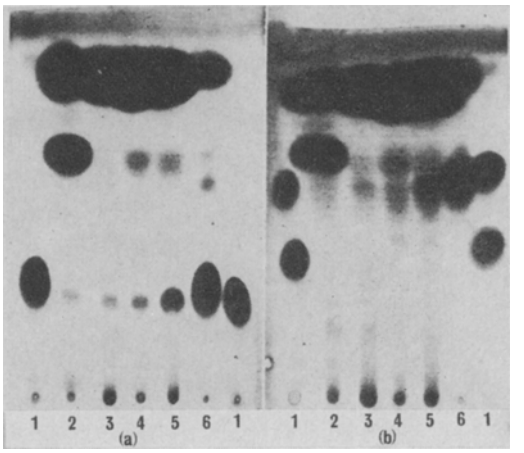

FIG. 1. Thin-layer chromatograms of mixed methyl esters from sclerotial lipids of various *Claviceps* species, (a) on Silica Gel G and (b) on Silica Gel G impregnated (10%, w/w) with boric acid. The developing solvent was diethyl ether-light petroleum (4:1), and spots were located by spraying with 5 N sulphuric acid and charring and were reproduced by photocopying on blue-line diazo paper.

Samples are: 1 = mixture of authentic *threo*-9,10-dihydroxystearate (upper component in (b)) and *erythro*-9,10-dihydroxystearate; 2 = *C. purpurea* mixed esters; 3 = *C. paspali* mixed esters; 4 = *C. gigantea* mixed esters; 5 = *C.* (ex-*Pennisetum*) mixed esters; 6 = *C. sulcata* mixed esters.

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Isolation of Cerebroside Containing Glucose (Glucosyl Ceramide) and Its Possible Significance in Ganglioside Synthesis

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ABSTRACT

A small amount of cerebroside containing glucose (glucosyl ceramide) was isolated from bovine brain by Florisil column chromatography and thin-layer chromatography. The fatty acids of the glucosyl ceramide were palmitic and stearic acids; small amounts of oleic and linoleic acids were present.

Rat brain tissue slices, incubated with U-^{14}C-glucose, incorporated more radioactivity into glucosyl ceramide than into galactosyl ceramide. From these results the possible metabolic significance of the brain glucosyl ceramide in ganglioside metabolism is discussed.

INTRODUCTION

WE HAVE PREVIOUSLY reported that tissue slices from the whole brain of young rats and from the cerebral cortex of adult guinea-pig incorporated ^{14}C-glucose and ^{14}C-galactose into the hexose component of cerebroside fractions separated by silicic acid column chromatography (1). Gas-chromatographic analysis of the trimethylsilane derivatives of the sugar component of cerebroside fractions revealed a small, but definite amount of glucose having a considerable portion of the radioactivity incorporated from ^{14}C-glucose and ^{14}C-galactose into the nonhydroxylated cerebrosides (so-called kerasine).

Although several authors have indicated the presence of glucosyl ceramide in brain tissue (2-4), it has never been isolated because of its low concentration and because of the difficulty in separating it from galactosyl ceramide.

In the present study, glucosyl ceramide was isolated from a large amount of bovine brain, and its possible metabolic significance in the brain is discussed with a consideration of both analytical data and tissue-slice incubation studies.

EXPERIMENTAL

Extraction of Glycolipids

Adult bovine brain was obtained at a slaughterhouse. Lipids were extracted from the tissue with chloroform-methanol (2:1, v/v), and the extract was subjected to partition dialysis by the method of Folch et al. (7). After the lower chloroform phase was concentrated to a small volume under reduced pressure, most of the neutral lipid and phosphoglycerides were removed by treatment with acetone and diethyl ether. The crude sphingolipid fraction, insoluble in these solvents, was subjected to Florisil column chromatography.

Florisil (Floridin Company), activated by heating at 120C for 3 hr, was suspended in chloroform-methanol (93:7, v/v) and packed into a column, 2 × 60 cm. The lipids, dissolved in the same solvent mixture, were mixed with a small amount of Florisil and loaded onto the column. Elution of lipids from the column was carried out by stepwise elution using chloroform-methanol (93:7, 92:8, 9:1, and 8:2). Glycolipid content in an aliquot of each fraction was determined by the anthrone method (8).

The gradient elution column chromatography with silicic acid was carried out as described previously (9).

Preparation of Gaucher's Sample

Spleen, from a patient with Gaucher's disease, was extracted with chloroform-methanol (2:1) and subjected to solvent fractionation. The acetone and ether-insoluble fraction was loaded onto a Florisil column and eluted by chloroform, then by chloroform-methanol (2:1). The effluent of chloroform-methanol (2:1) was rechromatographed on a silicic acid column.

Thin-Layer Chromatography

Activated Silica Gel G (Merck AG, Darmstadt) plates of 0.5-mm thickness were used for the separation of glycolipids with the solvent system of chloroform-methanol-water (90:12:1, v/v/v) (10).

The separation of glucosyl ceramide from galactosyl ceramide was accomplished by using borax-impregnated silica gel plates (BISP). BISP were prepared from Silica Gel G, as described by Young and Kanfer (5). The solvent system for the development was chloroform-methanol-14% aqueous ammonia (40:10:1, v/v/v), according to a modification of Kean (6).

The measurement of the radioactivity, after thin-layer chromatography (TLC), was performed on aliquots of the chloroform-methanol extract from the silica gel. The lipid extracts were dried in aluminum planchets, and radioactivity was determined with a windowless gas-flow type of GM counter RSC-2E4 (Riken K. K.).

Gas-Chromatographic Analysis

A portion of the extract from TLC plates was repeatedly evaporated to dryness from dilute hydrogen chloride in methanol to remove contaminating borax and then methanolyzed with 3% hydrogen chloride in methanol for 3 hr in a sealed tube in a boiling-water bath.

The methanolysate was shaken three times with petroleum ether. The petroleum ether phase was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. The fatty acid methyl ester thus obtained was subjected to gas-liquid chromatography (GLC).

The methanol phase was deionized with Amberite IR4B (hydroxyl form), and the filtrate was evaporated to dryness. Methyl hexoside, thus obtained, was trimethylsilylated by the usual procedure (11) and gas-chromatographed.

A Hitachi Perkin-Elmer, Model F-6 gas chromatograph, equipped with a flame ionization detector, was used for analyses. Separation of fatty acid methyl esters was made with a stainless steel column (2 m × 3 mm), packed with 15% EGSS-X coated on Gas-Chrom RP at 150C. Hexose analysis was performed with a stainless steel column, packed with 5% Ucon LB550-X coated on Gas-Chrom CLH at 190C.

RESULTS AND DISCUSSION

In a previous gas-chromatographic analysis of cerebroside sugars, a small but definite amount of glucose, 2-3% of the total hexose, was detected in addition to galactose from the brain cerebroside fraction containing only nonhydroxylated fatty acids (1). As the first step, separation of this still unidentified glucosyl ceramide from the bulk of the galactosyl ceramide was attempted by using Florisil column chromatography.

In a preliminary experiment, a mixture of approximately equal amounts of glucosyl ceramide, obtained from the spleen of a patient with Gaucher's disease, and galactosyl ceramide, obtained from bovine brain, was applied to a Florisil column (1 × 60 cm) and

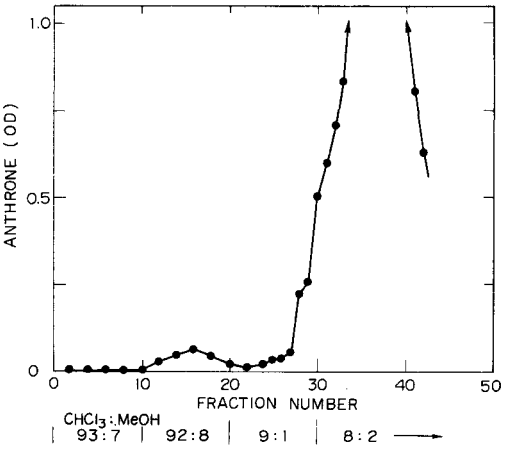

FIG. 1. Florisil column chromatography of bovine brain glycolipids. About 3 g of bovine brain lipid mixture, prepared as described in the text, were loaded onto 150 g of Florisil and eluted with 500 ml each of C-M 95:5, C-M 92:8, C-M 9:1, and C-M 8:2. Hexose content of an aliquot from each 50-ml fraction was measured by the anthrone method.

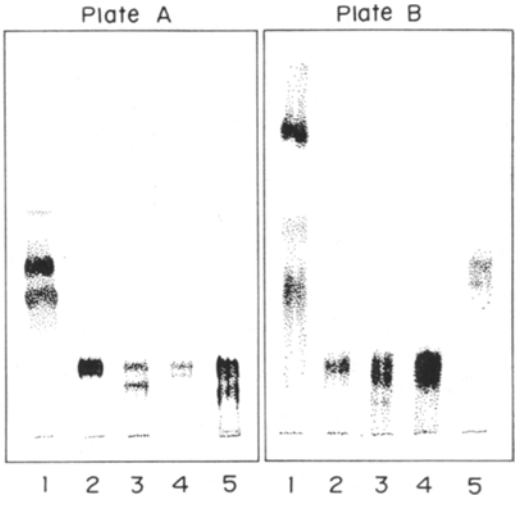

FIG. 2. Thin-layer chromatography of fractions from Florisil column chromatography. A) Silica Gel G plate; solvent system, chloroform-methanol-water (90:12:1, by volume). B) borax-impregnated Silica Gel G plate; solvent system, chloroform-methanol-14% aqueous ammonia (40:10:1, by volume).

Spots were detected by spraying with anthrone reagent: a)1, b)1, fraction of No. 10-21; a)2, b)2, fraction of No. 22-34; a)3, b)3, fraction of No. 35-40; a)4, b)5, Gaucher's spleen glucosyl ceramide; b)4, bovine brain cerebroside with nonhydroxylated fatty acids; a)5, bovine brain cerebrosides with hydroxylated and nonhydroxylated fatty acids.

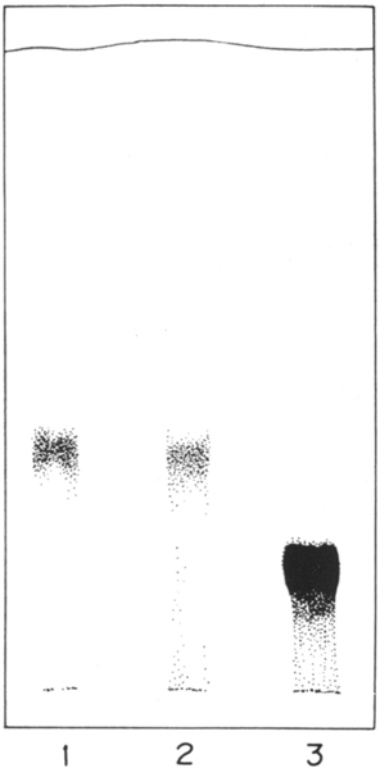

FIG. 3. Thin-layer chromatography of glycolipids on borax-impregnated Silica Gel plate developed in a solvent system of chloroform-methanol-14% aqueous ammonia (40:10:1, by volume). Spots were detected by spraying with anthrone reagent. Sample 1, glucosyl ceramide from Gaucher's spleen; Sample 2, glucosyl ceramide from bovine brain; Sample 3, bovine brain cerebrosides with nonhydroxylated fatty acids.

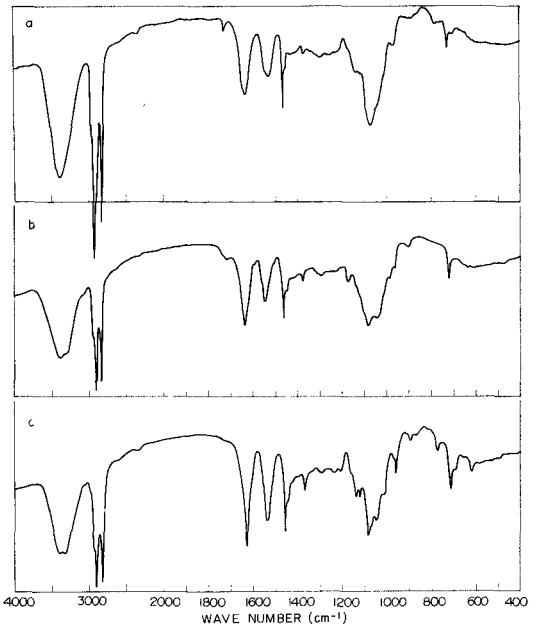

FIG. 4. Infrared spectra of glycolipids: (a) glucosyl ceramide from bovine brain; (b) glucosyl ceramide from spleen of a patient with Gaucher's disease; (c) bovine brain cerebroside with nonhydroxylated fatty acids.

eluted with mixtures of chloroform-methanol. Glucosyl ceramide was eluted in a position preceding galactosyl ceramide by using chloroform-methanol (9:1).

About 3 g of a sphingolipid fraction from bovine brain were loaded onto a large Florisil column (150 g) and eluted by stepwise elution of chloroform-methanol. As illustrated in Figures 1, 2a, and 2b, a group of faster-running glycolipids was eluted with chloroform-methanol (92:8), as previously reported by several authors (10,12,13). Fractions No. 22-34 gave a single spot on Silica Gel G; however two spots were obtained on BISP. The major spot was identified as cerebroside that contained nonhydroxylated fatty acids, and the other, faster-running faint spot had the same R_f value corresponding to Gaucher's glucosyl ceramide. A large amount of cerebrosides with hydroxylated and nonhydroxylated fatty acids was eluted after Fraction No. 35. Fractions containing cerebrosides with nonhydroxylated fatty acids and the undetermined glycolipid were collected from several runs by using Florisil column chromatography and were chromatographed again on a silicic acid column by using a gradient elution with chloroform-methanol. Although the cerebroside peak obtained was apparently symmetrical, it still gave a second spot on BISP, which corresponded to the Gaucher's glycolipid. From 26.6 mg of cerebroside thus obtained, the glycolipid with an R_f value on BISP similar to that of Gaucher's glucosyl ceramide was isolated by preparative TLC. Silica gel of the region corresponding to the reference glucosyl ceramide was scraped off and extracted overnight with a small amount of chloroform-methanol (2:1) and washed with water.

The lipid thus obtained (1.8 mg) gave a single spot with a slightly lower R_f on BISP value than Gaucher's glucosyl ceramide (Fig. 3).

The infrared spectrum of the glycolipid thus isolated is shown in Fig. 4 together with spectra of bovine brain cerebrosides containing nonhydroxylated fatty acids and Gaucher's glu-

Table I

Percentage Distribution of the Radioactivity of Cerebroside Fraction Containing Nonhydroxylated Fatty Acids from Rat-Brain Slices Incubated with Glucose-U-^{14}C

R_f	Age of Rats and Percentage		
	9 days[a]	17 days[b]	Adult[c]
0—0.1	5.6	0.4	8.8
—0.28 (galactosyl ceramide)	24.9	41.8	20.0
—0.42	5.1	0	5.1
—0.59 (glucosyl ceramide)	56.9	42.8	52.4
—0.76	2.6	0	4.7
—front	1.9	4.3	8.8

[a]The 1.8-mg glycolipids (19,436 count/min) were loaded.
[b]The 1.2-mg glycolipids (1,989 count/min) were loaded.
[c]The 5.4-mg glycolipids (1,772 count/min) were loaded.

cosyl ceramide. No major difference was found between the spectra except the absorption pattern between 1200 cm^{-1} and 900 cm^{-1} owing to OH moieties.

Only glucose was identified as the hexose component of the glycolipid by gas chromatography (Fig. 5a). The fatty acids 16:0 (22%), 18:0 (47%), 18:1 (20%), and 18:2 (12%) were found in this glucosyl ceramide from bovine brain (Fig. 5b). Fatty acids longer than 20 carbons, the constituents of the usual galactosyl ceramides from animal brain or of glycosyl ceramide from Gaucher's spleen, were not found in this glucosyl ceramide from bovine brain.

Glucose Incorporated into Glucosyl Ceramide

It has previously been found (1) that brain-tissue slices incorporate ^{14}C-glucose into the glucose fraction of cerebrosides much more actively than into the galactose fraction. In order to confirm the incorporation of ^{14}C-glucose into the glucosyl ceramide and to re-examine whether or not galactosyl ceramide was radioactive, the radioactive cerebroside fraction obtained by the same procedure used in the foregoing experiment was applied on BISP, and the distribution of the radioactivity was determined. The TLC plate was divided into six regions, from which lipids were eluted with chloroform-methanol (2:1), and their radioactivity was measured.

In agreement with the previous results obtained by the GLC analysis of cerebroside hexose, the glucosyl ceramide fraction was found to have more radioactivity than the galactosyl ceramide fraction (Table I).

The radioactivities of both fractions were rather similar in rats 17 days old, in the maximal stage of myelination, when galactosyl ceramide is considered to be most rapidly synthesized. In the nine-day-old rats and in the adult rats, the glucosyl ceramide fraction had more than two times the radioactivity of galactosyl ceramide. Apparently glucose is actively incorporated into brain glucosyl ceramide throughout the life of the animal.

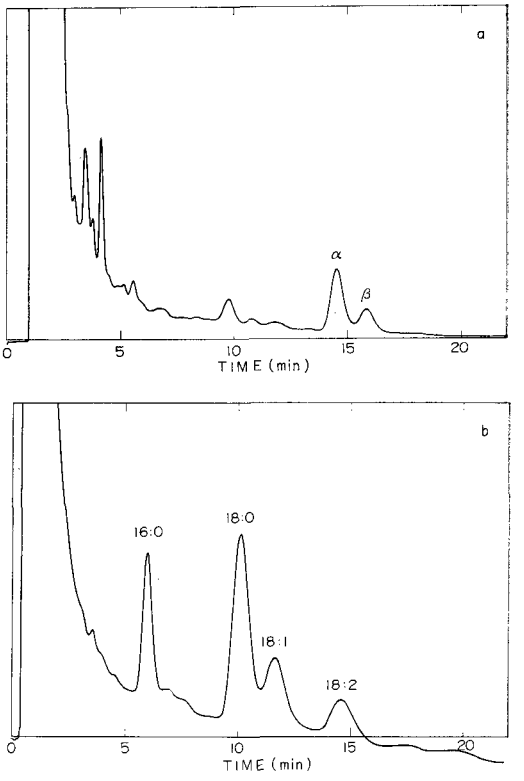

FIG. 5. Gas-chromatography of the glucosyl ceramide from bovine brain: a) carbohydrate composition as trimethylsilylated methyl-hexoside—*alpha* is *alpha*-methyl glucopyranoside, *beta* is *beta*-methyl glucopyranoside .5% Ucon on Gas-Chrom CLH, 190C; b) fatty acid composition—15% EGSS-X on Gas-Chrom RB, 195C. Apparatus: Hitachi Perkin-Elmer Type F-6, equipped with flame ionization detector.

Previously Schwarz et al. (2) found a trace of glucose as a hexose component of cerebroside from the brains of an old human subject and a patient with multiple sclerosis. Later Svennerholm (3) and Pilz et al. (4) reported its presence by TLC isolation from human fetal brain and from the pathological cortex of an infant brain. In this study, glucosyl ceramide was isolated from normal adult bovine brain.

Cerebrosides of white matter, a lipid typical of the myelin sheath, are actively synthesized

during myelination, and the biosynthesis of ganglioside precedes that of myelin cerebroside (14-16). In this experiment the incorporation of glucose into glucosyl ceramide appeared to be active before myelination. As discussed in the previous report, active incorporation of ^{14}C-glucose into glucosyl ceramide, as indicated by a high specific activity, is indicative of its high metabolic activity in brain tissue. The absence of long-chain fatty acids (>20), both in glucosyl ceramide and brain gangliosides, suggests that a metabolic relationship might exist between them.

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Studies on the Lipids of Sheep Red Blood Cells. II. The Incorporation of Phosphorus into Phospholipids of HK and LK Cells

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ABSTRACT

The incorporation of inorganic phosphate (as NaH_2PO_4) into the phospholipids of sheep red blood cells was studied in vitro in blood samples from five high-potassium (HK) and five low-potassium (LK) sheep. The erythrocytes from HK sheep incorporated more activity in 4 hr than those from the LK sheep. However no activity was incorporated into the major phospholipids of the cells (phosphatidyl ethanolamine, phosphatidyl serine, and sphingomyelin) of either group. The phosphatidic acid fraction was labeled in both groups and to a significantly greater extent in the HK samples. However the highest activity in the phospholipid of sheep red-cells was located in three unknown compounds not previously detected. Their specific activities were the same in the HK and the LK samples although they were present in slightly larger amounts in the HK samples. In general, incorporation was at a rather low level, and from stoichiometric considerations it was concluded that the metabolism in the red-cell phospholipids could not be directly involved in the active transport of ions across the cell membrane. This work also confirmed a previous report that no quantitative differences exist among the major phospholipid classes in the two types of cells.

INTRODUCTION

SEVERAL REPORTS (1-5) by previous investigators indicated that the mature, circulating red blood cell of mammals cannot incorporate inorganic phosphate into its phospholipids in vitro. Although the situation in vivo has remained somewhat confused because of various exchange phenomena (6-9) that complicate the design and interpretation of such experiments, the general conclusion has been that there is only limited turnover of the major lipid components of the erythrocyte during its life span and that any turnover is the result of exchange rather than net synthesis. However some more recent observations have indicated that there is indeed net synthesis of one or more phospholipids in mature erythrocytes (10-13).

Jacobs and Karnovsky (13), in particular, have reported that inorganic phosphate is specifically incorporated into the phosphatidic acid and phosphatidyl serine of normal and spherocytotic human erythrocytes and have found that cells from patients with hereditary spherocytosis incorporated more activity than those from presumably normal individuals. This observation holds great interest because the transport of sodium ions is markedly elevated in spherocytotic erythrocytes (14-15); hence it is possible that the turnover of the phosphatidyl serine is related to an active transport mechanism in the erythrocyte membrane.

An analogous situation to that in normal and spherocytotic erythrocytes in human beings is found in high-potassium (HK) and low-potassium (LK) erythrocytes in sheep (16, 17): the sodium flux in HK cells is markedly higher than that in the LK cells (18). In a previous communication it was reported that the total lipid composition of HK and LK erythrocytes did not differ significantly (19). It was concluded that a simple stoichiometric difference in the proportion of lipids in the two types of membranes could not account for the observed differences in the cation concentration in the cells. The possibility still remained however that metabolic differences in the turnover of membrane phospholipids might be related to sodium and potassium transport since the static analyses (19) would not reflect differences in metabolic activity.

Thus, in the light of recent reports of net phospholipid synthesis (10-12) in red cells, and of Jacobs and Karnovsky's work (13) on human red cells *in vitro*, it was decided to measure the incorporation of inorganic phosphate into HK and LK sheep, in an attempt to determine whether indeed incorporation did take place and whether a different incorporation pattern could be observed in the two types of cells. This paper reports the methods and results of the investigation.

MATERIAL AND EQUIPMENT

A flock of purebred Hampshire sheep is maintained at this laboratory, and each animal is specified as HK or LK on the basis of analyses by the flame spectrophotometric techniques described previously (19).

Standard phospholipid preparations were obtained from General Biochemicals, Chagrin Falls, O., and Applied Science Laboratories, State College, Pa. Myophosphatidyl inositol was a gift of C. E. Ballou. These phospholipids were isolated from plant or animal tissues and had mixed fatty acid composition. The purity of the standards was checked by two-dimensional (2D) thin-layer chromatographic (TLC) techniques in all cases. All solvents were redistilled from glass stills and deoxygenated by bubbling nitrogen through them before use.

Silica Gel HR, 20 × 20-cm TLC plates, plate spreader, and spreading template were obtained from Brinkman Instruments Inc., Westbury, N. J. $MgSiO_2$ was obtained from Allegheny Chemical Company, Butler, N. J. Sephadex was purchased from Pharmacia Fine Chemicals, New York, N. Y. Carrier-free P^{32} (as NaH_2PO_4) was obtained from New England Nuclear Corporation, Boston, Mass. Sheep serum albumin (fatty acid-free) was obtained from Nutritional Biochemicals, Cleveland, O. The 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(4-methyl-5-phenyloxazole-benzene (di-methyl POPOP), both "scintillation grade," were purchased from Packard Corporation, Downers Grove, Ill.

Optical densities for the phosphorus determinations were measured with a Zeiss PMQ-II spectrophotometer. Radioactivity was counted with a Nuclear-Chicago Model-702 low-temperature, liquid scintillation counter. Radioautographs were made by using Eastman Kodak No-Screen Medical x-ray film, 8 × 10 in.

All radioactive samples were counted in the same scintillating solution. This was prepared according to Snyder (20) and had the following ingredients: PPO, 10.5 g; di-methyl-POPOP, 0.45 g; naphthalene, 150 g; dioxane, to make 1,500 ml; and H_2O to make 1,800 ml. The presence of water in the scintillator tended to reduce the efficiency of the counter by 20 to 30% over that obtained by a scintillator such as PPO and POPOP in toluene, but, with an energetic isotope like ^{32}P, this was not a serious problem. Table I lists some data on the efficiency of the counting procedure.

TABLE I

Counting Efficiency of Liquid Scintillation Method for P^{32} (Calculated Activity Approximately 10,000 cpm) in the Presence of TLC Absorbent

Sample	Absorbent added (mg)	Activity (cpm)[a]	Efficiency % of Sample 1
1	None	7860 ± 75	100
2	100	5420 ± 93	69
3	250	5403 ± 88	69
4	1000	5698 ± 72	72
5	Recovered from TLC plate in the presence of approximatelv 500 mg absorbent	5538 ± 89	71

[a] Average of triplicate samples ± standard deviation, uncorrected, background ~ 20 cpm.

EXPERIMENTAL PROCEDURE

Blood was drawn into sterile plastic blood bags by venipuncture and immediately cooled to 0C in an ice bath with heparin as an anticoagulant as previously described (19). Usually 100 ml of whole blood were drawn from each animal. The cells were separated from the plasma and thoroughly washed to free them of plasma and leucocytes by the procedures reported previously (19,21). The wash solution was 310 milliosmolar NaCl-KCl buffered with 0.001 M Tris-HCl at pH 7.45. This solution contained 150 milliosmoles of Na^+ and 5 milliosmoles of K^+, which are approximately the concentrations reported (22) for sheep plasma. It was hoped that the ionic composition of the washed cells would not be greatly disturbed. The final washed-cell preparation had less than one leucocyte per 10^4 erythrocytes on the basis of smears prepared with Giemsa stain. The washed cells were stored over-night at 4C under nitrogen except for a 15-ml portion that was extracted immediately to determine the quantitative composition of the phospholipids before incubation.

Incubation

The incubation solution consisted of a 5% solution (w/v) of sheep serum albumin, which was 2.5×10^{-3} M in glucose, 1.98×10^{-2} M in $NaHCO_3$, 1.23×10^{-3} M in $MgCl_2$, 5.1×10^{-3} M in KH_2PO_4, 1.23×10^{-1} M in NaCl, and 7×10^{-3} M in Tris-HCl. The pH was adjusted to 7.44 at 22C with HCl. Chloromycetin (2 mg/liter) was added to retard bacterial growth. The incubation mixture was approximately isotonic to sheep red-cells and caused no noticeable hemolysis in 30 min at 4C.

Ten milliliters of washed cells were mixed gently with an equal volume of the incubation

solution at 0C in a 50-ml, glass-stoppered Erlenmeyer flask. To this solution was added 0.5 mc (0.1 ml) of carrier-free P^{32} as NaH_2PO_4. The incubation flasks were then transferred immediately to a 37C shaker bath and incubated for 4 hr. At various intervals up to 4 hr, 100-μl samples were removed from the incubation mixtures to determine the time sequence of incorporation of ^{32}P into the phospholipids of the erythrocytes.

After 4 hr the incubation mixtures were immediately cooled to 0C and centrifuged at 1570 × g to remove the excess activity in the incubation solution. Moderate hemolysis was observed at this time and may have been caused by radiation damage to the cells. The incubated cells were washed twice with isotonic NaCl-KCl solution, the volume of cells was measured, and their lipids were extracted at 0C under nitrogen by the methods reported in detail elsewhere (21,23,24).

Chromatographic Methods

The chloroform-methanol, 2:1 (v/v), extracts were reduced in volume by rotary evaporation under a reduced pressure of nitrogen below 15C. The extracts were not allowed to reach dryness at any time during extraction or chromatography. This helped reduce the chance of spontaneous decomposition, which can be greatly accelerated in the dry state (25, 26). The total lipid extracts were purified by column chromatography on Sephadex by using the procedures of Siakotos and Rouser (27) as modified slightly (24,28). Details of the method and solvents used for elution of the various fractions are reported in the literature (24, 27, 28) and will not be repeated.

Fraction I contained all of the phospholipids in the initial chloroform-methanol extract and was practically free of nonlipid contaminants. Fraction II contained any gangliosides present in the extract and some traces of other organic compounds. Fraction III consisted of both organic and inorganic matter extracted from the whole cells but had no lipoidal material of any type.

The elution solvent was removed from Fraction I as described for the initial extract; again the residue was not allowed to become entirely dry at any time. The lipids were transferred with chloroform to a 5-ml, glass-stoppered, graduated cylinder, and aliquots were taken for counting, total weight, and phosphorus determination. The remainder of the sample was stored at –10C under N_2 until subjected to the 2D-TLC listed below. All procedures were, of course, carried out as rapidly as possible.

Fractions II and III were taken to dryness on the rotary evaporator and transferred with an appropriate solvent to tared vials for a total weight determination. After the weighing a portion of each sample was counted, and another portion was used to determine the phosphorus content of the fraction. The remainder was usually discarded without further analysis.

The 2D-TLC separations were performed by the methods developed by Rouser et al. (29, 30) with the use of Silica Gel mixed with 10% $MgSiO_3$. Details of the procedures listing plate-preparation techniques, development solvents, and visualization methods have been reported in detail elsewhere (23, 24, 29, 30). The quantitative determinations of percentages of the various phospholipids in the samples were carried out by spectrophotometric phosphorus determinations of the individual phospholipid spots which were scraped from the charred TLC plates without prior removal of the absorbent. The method is essentially that described by Rouser et al. (31). The color was developed by the procedure of Bartlett (32) with only slight modifications.

Radioactivity in the sample spot on the 2D-TLC plates was counted as described by Snyder (20). The plates were first stained with I_2 vapors, and the spots were outlined. The I_2 was removed in vacuum, and the spots were scraped directly into scintillation counting vials. Approximately 40 μg of P was applied to each 2D-TLC plate to be used for counting or radio-autography; 15 to 20 μg P were applied to plates used for quantitative phospholipid analysis. Separate plates were prepared for counting and radio-autography at the same time. The radio-autographs were exposed for periods up to four weeks. Standards were prepared and counted along with the samples to simplify corrections for decay of P^{32}.

Standards to check the efficiency of counting procedures were prepared as follows: Sample 1—triplicate samples diluted to 10,000 cpm based on the initial activity reported by the supplier and uncorrected for decay; (2) Samples 2, 3, and 4—three sets of triplicate samples containing 10,000 cpm, to which were added 100, 250, or 1,000 mg of Silica Gel-$MgSiO_3$ absorbent; and Sample 5—10,000-cpm samples spotted in triplicate on a TLC plate and then scraped into a scintillation vial. Table I lists the counts obtained from the various standard samples. Only a small decrease in counting rate was observed in the presence of the TLC absorbent, and recovery from the TLC plate was close to 100%. Hence it was

TABLE II
Activities of Sephadex Fractions of the RBC Lipids After Incubation with Inorganic ^{32}P

Sample	Volume RBC extracted (ml)	Wt of Sephadex fractions[a] (mg) I	II	III	Activity of Sephadex fractions (cpm)[b] I	II	III
HK-1	8.4	35.9	1.32	11.18	12.0×10^4	14.0×10^2	12.4×10^5
HK-2	8.9	37.4	1.50	8.23	15.8×10^4	10.8×10^2	11.1×10^5
HK-3	9.3	39.0	1.76	13.82	14.6×10^4	23.8×10^2	12.5×10^5
HK-4	9.2	41.0	2.12	9.03	14.1×10^4	31.6×10^2	7.8×10^5
HK-5	9.4	39.5	1.20	6.89	10.8×10^4	6.5×10^2	5.4×10^5
LK-1	8.9	36.9	1.96	7.29	7.0×10^4	4.4×10^2	2.2×10^5
LK-2	9.2	36.3	2.03	8.23	6.0×10^4	5.9×10^2	5.0×10^5
LK-3	9.2	33.6	1.84	13.90	6.0×10^4	5.7×10^2	7.2×10^5
LK-4	9.2	36.0	1.53	11.32	10.0×10^4	20.7×10^2	12.5×10^5
LK-5	9.3	38.0	1.67	10.30	10.6×10^4	12.6×10^2	5.2×10^5

[a] See definition of Sephadex fractions under Chromatographic Methods in text. Fraction I contained essentially all of the phospholipids in the total lipid extract.

[b] Total counts uncorrected for decay or background. All three Sephadex fractions for each sample were counted at the same time.

felt that the lipid need not be eluted from the absorbent before counting, at least for energetic β particles from P^{32}.

RESULTS

Incorporation of inorganic ^{32}P was higher in the HK than in the LK cells. Figure 1 presents a plot of the data for the time sequence of incorporation into the material extracted from the cells with chloroform-methanol (2:1). In this part of the experiment the cells were thoroughly washed to remove all residual activity contributed by the incubation mixture before extraction, but the lipid extract was not further purified. It is possible that some of the activity may be nonlipid and represent the equilibration of interior and exterior phosphate of the cell. In any event the HK cells clearly incorporated more P^{32} than the LK cells. The curve continues to rise even at the 4-hr mark which tends to argue against simple equilibration phenomena.

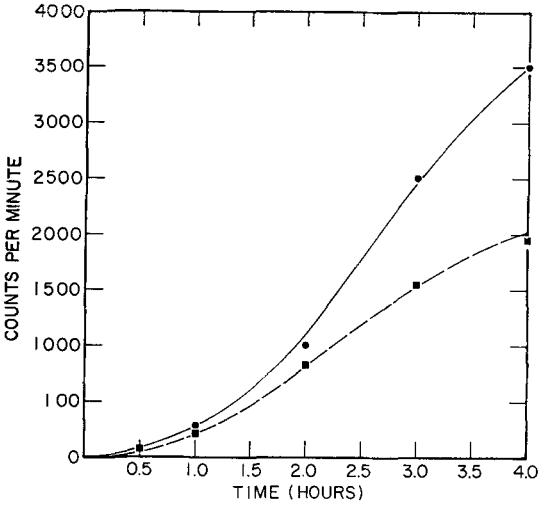

FIG. 1. Rate of incorporation of inorganic phosphate into sheep erythrocytes in vitro. Solid line represents HK cells and dotted line LK cells incubated under identical conditions at 37C. Each point is the average of three separate determinations. Samples in all cases were 100 μl of washed, packed cells.

Table II lists the data for five HK and five LK samples incubated for 4 hr: the total weight of each Sephadex fraction along with the total counts and specific activity in each fraction. It is obvious that considerable incorporation into compounds other than phospholipids has occurred. It is also possible that some inorganic phosphate is extracted with chloroform-methanol (2:1). In all samples the third fraction contained activity in an order of magnitude greater than that found in Fraction I with the phospholipids. Fraction II contained little of the total activity and had a negligible phosphorus content, 0.05%.

The data in Table II and Fig. 1 both indicate that HK cells incorporate more inorganic phosphate in a given time interval than LK cells. However less than 10% of the total activity extracted was recovered in the lipid fraction (Fraction I). Indeed, when the phospholipids were subjected to fractionation on 2D-TLC and the activities of the individual phospholipids were determined, little or no activity was associated with the major phospholipids of the erythrocyte membrane. Fig. 2 is a photograph of a typical TLC separation of the erythrocyte phospholipids along with a radio-autograph made from the plate (exposed for four weeks). It is immediately obvious that the activity is

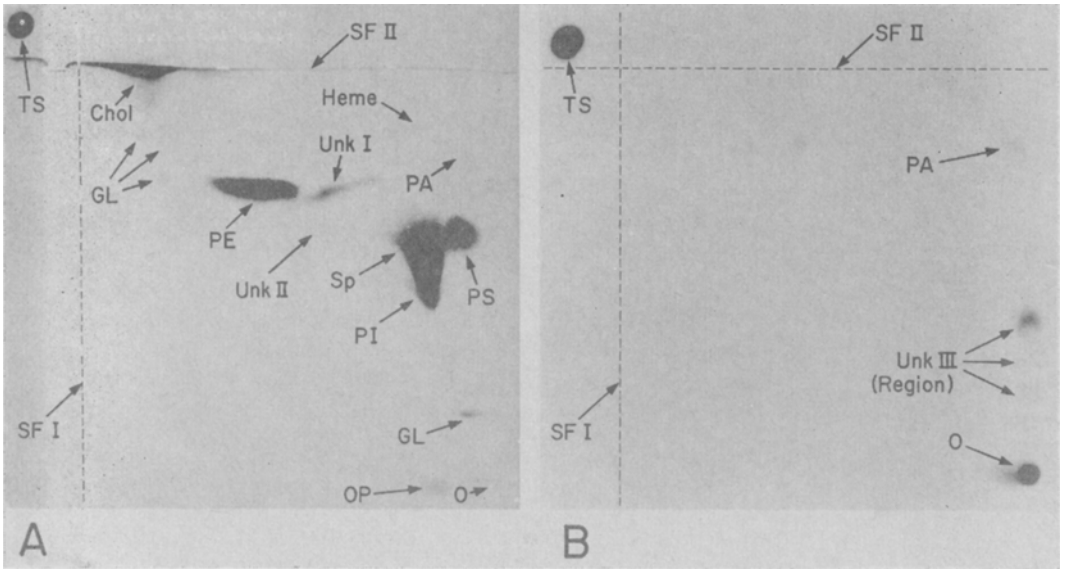

FIG. 2. (A) Thin-layer chromatographic (TLC) separation of the total lipid extract from sheep erythrocytes after a 4-hr incubation with inorganic phosphate ^{32}P, (B) the radioautograph of the same sample prepared concurrently and exposed for four weeks. Spots were visualized on the thin-layer plate by charring with sulfuric-acid/dichromate reagent. It is obvious that the major phospholipids do not incorporate inorganic phosphates to any appreciable extent in vitro.

Abbreviations SF I, first solvent front; SF II, second solvent front; TS, total sample spot; O, origin; Chol, cholesterol; Heme, heme pigments; Unk, unidentified phospholipids; GL, glycolipids; OP, oxidized phospholipids and pigments; PE, phosphatidyl ethanolamine and ethanolamine glyceryl ethers; PA, phosphatidic acid; SP, sphingomyelin; PS, phosphatidyl serine; PI, phosphatidyl inositol. The first solvent was chloroform-methanol—NH_4OH (65:35:5), the second was chloroform–acetone–methanol–acetic-acid–water (5:2:1:1:0.5). Sample: LK sheep No. 3.

not associated with any of the major spots. In fact, the highest activity on the plate remained at the origin. Of the known phospholipids only phosphatidic acid displayed enough activity to darken the film appreciably. The other regions of darkening were not associated with any known phospholipids. Three distinct spots can be observed and were found in all samples. However the total amount of material present in these spots was below the limits of detection

TABLE III

Average Phospholipid Distribution and Specific Activities of Individual Phospholipids in RBC Samples After Incubation with Inorganic ^{32}P

Phospholipid	% of Total phospholipid phosphorus[a] HK n = 5	LK n = 5	Specific activity cpm/μg P HK	LK
Phosphatidic acid	0.30 ± 0.12	0.33 ± 0.11	2.0×10^3	1.2×10^3
Phosphatidyl ethanolamine[b]	28.79 ± 0.28	30.73 ± 0.83	N.A.[c]	N.A.
Phosphatidyl serine	14.29 ± 1.11	13.51 ± 1.18	N.A.	N.A.
Phosphatidyl inositol	3.88 ± 0.48	3.99 ± 0.26	83	75
Sphingomyelin	49.09 ± 1.27	47.00 ± 1.79	N.A.	N.A.
Unknown I	2.22 ± 0.22	3.95 ± 0.17	120	70
Unknown II	0.31 ± 0.09	0.20 ± 0.10	N.A.	N.A.
Unknown Region III	0.66 ± 0.20	0.55 ± 0.22	6.4×10^3	5.7×10^3
Origin	0.43 ± 0.11	0.24± 0.07	23.2×10^3	23.8×10^3

[a] Phospholipid distribution was determined by 2D-TLC by using two solvent systems for each sample. Recovery of phosphorus from the plates was over 95% in all cases. Percentages are mean of the two TLC determinations on each sample; means were then averaged for the five animals in each group. Values are listed with their standard deviation.

[b] Also contains the glyceryl ether analogue.

[c] N.A., no activity.

of charring techniques or charring combined with ultraviolet viewing (33). These limits were at least 0.5 μg. Hence the specific activity of these unknown compounds was considerably above the specific activity of the phosphatidic acid fraction.

The major phospholipids of red-cell membrane do not show any significant stoichiometric differences, a conclusion similar to that reached in the previous study (19), which used column rather than thin-layer chromatographic methods. Table III lists the average phospholipid composition of the five HK and five LK samples and the specific activities of the individual phospholipids as determined by 2D-TLC. The specific activity is corrected for decay and background. The liquid scintillation counting, which is more sensitive than radioautography, showed some evidence of activity in the phosphatidyl inositol spot and another unknown (Unk I) phospholipid previously reported to exist in sheep erythrocytes (24), but no activity was detected in the phosphatidyl ethanolamine spot (which also contains glyceryl ether ethanolamine phospholipid), the phosphatidyl serine spot, or the sphingomyelin spot. No lecithin or lysolecithin could be detected in erythrocyte samples used in this experiment, in agreement with previous findings (24). No activity was detected in the phospholipids that contain more than 90% of the total phospholipid phosphorus of sheep red cells.

The specific activities of the phosphatidyl inositol and unknown phospholipids were very low, and the possibility that this activity was contributed by the few leukocytes remaining in the red-cell incubation sample cannot be entirely eliminated. The small differences between the incorporations of the two types of cells are not significant at this low level of activity.

The specific activity of the phosphatidic acid spot was almost twice as great in the HK as in the LK incubations and was significant at the $P = 0.05$ level. The specific activities of the Unknown Region III and the origin were essentially the same in both types of cells although the total activity was higher in HK than LK cells. This indicates that there was a slightly larger amount of these components in the HK species although the total amount present was less than 1% of the total phosphorus. Also, because the individual components in Unknown Region III could not be located exactly, specific activities for each band were not determined and only the total activity was measured. There may have been variations in specific activities between HK and LK cells in this region although on the basis of the radio-autographs such variations were not readily apparent. No attempt was made to determine the identity of the unknown compounds in Unknown Region III of the 2D-TLC separations because of the small quantities present. The TLC migration patterns however indicate that these compounds are acidic; they remain at the origin in basic solvents.

In a few cases 2D-TLC of Sephadex Fractions II and III were prepared and the radioautographs were made. All of the activity remained at the origin in these runs, and it was considered unlikely to be lipoidal in nature.

DISCUSSION

The Sephadex purification of the initial extract of the cells was an essential step in this experiment. The total amount of nonlipid activity extracted was at least 10 times as great as the activity in the lipid fraction. It is necessary to be extremely careful in evaluating specific activity data on lipid samples which have not been thoroughly purified to remove nonlipid activity. A simple aqueous wash of the organic extract (34) is totally inadequate for removing such contamination from the lipids in solutions. Even the Sephadex procedure does not seem to insure complete elimination of all contaminants in our hands although the amount of remaining contamination is low and presents a problem only with compounds of high specific activity. The material remaining at the origin on the TLC plates has yet to be identified, but it may be inorganic phosphate carried through the wash, extraction, and Sephadex chromatography.

Contrary to the observation of Jacobs and Karnovsky (13) on human erythrocytes, sheep erythrocytes seem incapable of incorporating inorganic phosphate into phosphatidyl serine in vitro. Yet, like human red-cells, sheep cells do incorporate some inorganic phosphate into phosphatidic acid. However the main labeled lipids were unidentified compounds not previously detected in sheep cells.

Hokin and Hokin (11) have reported that human erythrocyte ghosts incorporate phosphorus from ATP into at least four compounds, phosphatidic acid, di- and tri- phosphoinositide, and an unknown compound. The three unknown spots located in this work may well correspond to compounds similar to those reported by Hokin and Hokin. Additional experiments would be necessary to substantiate this speculation however. Hokin and Hokin

(11) found no evidence for the incorporation of activity into the phosphatidyl serine fraction of human erythrocytes. Also, previous investigators were unanimous in concluding that phosphatidyl serine would not incorporate inorganic phosphate in mature erythrocytes (1-5, 10-12).

The data which were obtained indicate that the major phospholipids of sheep red-cells do not turn over appreciably during the life span of the mature cell. There were no differences in this respect between KH and LK cells. Oliveira and Vaughan (35), Mulder et al. (36), Mulder and van Deenen (37) showed that fatty acids of lecithin, and perhaps lecithin itself, can be exchanged in certain species including human beings. Oliveira and Vaughan (35) found little fatty acid exchange in sheep erythrocytes however. Thus sheep red-cells do not exhibit any turnover of their phospholipids, which is consistent with the idea that the role of main phospholipids in the membrane is primarily structural (38). Together these phospholipids, [phosphatidyl ethanolamine (including the glyceryl ether analogue), phosphatidyl serine, and sphingomyelin] constitute approximately 90% of the total phospholipid of the cell membrane.

The same kind of reasoning, applied to the remaining phospholipids of the membrane, such as phosphatidic acid, phosphatidyl inositol and polyphosphoinositides, and other unknown compounds present in relatively small amounts, suggests that they may have a different function in the membrane, which might be related to intermediary metabolism. In the red cell this could well be indirectly related to the mechanism of active transport. However the stoichiometry of the transport system, as pointed out by several authors (11, 39-41), makes it highly unlikely that the low level of lipid metabolism reported for sheep cells and by other authors for human red cells (10-13) is directly related to the transport mechanism.

It is quite interesting that the HK cells exhibited a significantly higher incorporation than the LK cells. This result is consistent with, and might be related to, the observations of Tosteson et al. (18, 42), who studied ATPase in HK and LK erythrocytes and found that HK cells exhibited higher ATPase activity than LK cells. Any simple interpretation is complicated by the recent report of Tosteson et al. (43), in which they concluded that ATPase activity is probably controlled by another mechanism in the intact cell and that the actual levels of the enzyme may be equal in both types of cells.

Thus the unknown compounds labeled in sheep red-cells may be phosphorylated metabolic intermediates involved in the over-all metabolism of the cell (44, 45). The increased incorporation exhibited by the HK erythrocyte may merely reflect the generally higher level of metabolic activity necessary for the HK cells to maintain their potassium gradient in contrast to the lack of such a gradient in LK cells (18) and may not be connected to cation transport *per se*.

This investigation also confirmed the previous observation that no differences exist in phospholipid class distribution between HK and LK red cells. The data presented are more accurate than those previously reported (19) because of the recent improvements in methodology involving primarily the Sephadex and 2D-TLC procedures. The data given in Table III differ in several respects from those reported earlier, particularly for the glycerol phosphatides. The earlier work was done by using silica gel columns which do not yield an adequate separation of phosphatidyl ethanolamine and phosphatidyl serine. This accounts for the earlier low value for phosphatidyl serine and the high value for phosphatidyl ethanolamine. The earlier high value for phosphatidic acid (3.1%) is believed to have resulted from nonlipid phosphorus in the initial extract, which was eluted with phosphatidic acid from the column.

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The *alpha*-Oxidation System of Brain Microsomes. Cofactors for *alpha*-Hydroxy Acid Decarboxylation[1]

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ABSTRACT

A one-carbon degradation of long-chain fatty acids, which was found to occur in the brains of rats in vivo, has been investigated in a brain microsomal fraction in vitro. Decarboxylation of the α-hydroxy acid, a possible intermediate product between the substrate and the next shorter acid, in the presence of brain microsomal fraction was enhanced by ATP, NAD, and a dialyzable fraction from the supernatant fraction. The cofactor requirement for the decarboxylation of the α-hydroxy acids provided by the dialyzable fraction can be met by several reducing agents or ferrous ion. The effectiveness of several possible cofactors for the decarboxylation of α-hydroxy acids has been evaluated.

It is concluded that the decarboxylation of the α-hydroxystearic acid may be a reaction with molecular oxygen catalyzed by an oxidase or oxygenase that requires iron in the reduced state for activity. The possibility that the reaction proceeds through an α-keto acid intermediate has been examined in the light of new knowledge of the conditions for decarboxylation. It is concluded that a short-lived keto acid is a possible intermediate. Definitive proof however is lacking because the characteristics of the reaction require that such an intermediate decarboxylate without dissociating from the enzyme.

INTRODUCTION

In an investigation of the enzymes concerned with α-oxidation it was recently found (1) that a particulate fraction from rat brain in the presence of ATP, NAD, and a dialyzable supernatant fraction from the sedimentation of the particles could catalyze the decarboxylation of long-chain α-hydroxy acids and give rise to unsubstituted acids shorter by one carbon. This reaction was taken to be part of the pathway that, in vivo, leads to odd-chain fatty acids of the brain sphingolipids (2). However neither ATP nor NAD proved to be absolute requirements, the nature of the supernatant factor was not ascertained, and, in general, only one product, the odd-chain acid, could be isolated. Thus the exact nature of the several possible reactions leading to decarboxylation remained obscure.

The present communication describes experiments leading to further elucidation of the nature of the enzymes involved in the decarboxylation of the alpha-hydroxy acids and to clarification of the reactions they catalyze.

EXPERIMENTAL PROCEDURES

Materials and Substrates

All inorganic chemicals and all solvents were reagent grade unless specified otherwise. Solvents, except for pentane, were redistilled before use. Catalase, ascorbic acid, $DMPH_4$[4], glucose, ATP, NAD, NADH, NADP, NADPH were all obtained from the California Corporation for Biochemical Research. Glucose oxidase was purchased from the Worthington Biochemical Corporation. The 1-^{14}C-stearic acid was purchased from New England Nuclear Corporation and was found to be 99% pure by radio-gas chromatography.

The 1-^{14}C-α-hydroxystearic acid was prepared by bromination of the 1-^{14}C stearic acid, followed by alkaline hydrolysis (3). It was purified by chromatography of the methyl ester on silicic acid and was pure as determined by radio-gas chromatography of the acetoxymethyl ester. Its specific activity was 5×10^6 cpm/μmole. The 9,10-^{3}H-α-hydroxy stearic acid was prepared and purified in essentially the same manner. Radio-gas chromatography showed it to be better than 99% pure. Its specific activity was 330 mc/mM.

The 1-^{14}C-α-ketostearic acid was a gift from N. S. Radin and A. Hajra. The 1-^{14}C-tetraco-

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[4]Abbreviations used: $DMPH_4$ is 6,7-dimethyl-5,6,7,8-tetrahydropterine; NAD (or DPN in Fig. 7) is nicotinamide, adenine dinucleotide; NADP is nicotinamide adenine dinucleotide phosphate.

sanoic acid was prepared by the method of Anker (4), which involves decarboxylation of inactive tetracosanoic acid, followed by reaction of the resulting bromotricosane with $K^{14}CN$ and hydrolysis of the resulting nitrile. The resulting 1-^{14}C tetracosanoic acid was purified by gas chromatography to 99% purity.

The 1-^{14}C-α-hydroxytetracosanoic acid was prepared from 1-^{14}C-tetracosanoic acid in essentially the same manner as described for α-hydroxystearic acid.

Tritiated tetracosanoic acid was prepared by the Wilzbach process by the New England Nuclear Corporation and was purified by silicic acid and gas-chromatographic methods. Tritiated α-hydroxytetracosanoic acid was prepared from it in the manner outlined above and was purified by silicic acid and gas chromatography.

Tritiated tricosanal was prepared from tritiated α-hydroxytetracosanoic acid by lead tetraacetate oxidation by the method of Criegee (5) and was purified by silicic acid column chromatography. Heptadecanal was prepared from α-hydroxystearic acid by the same method.

Methods

Preparation of the Enzyme. The method used was similar to that described previously (1). Fresh brains from 250-g rats, or beef brain obtained from the slaughterhouse and used immediately, were homogenized in a large Potter-Elvejhem type homogenizer. Homogenization was carried out in the cold with 9 volumes of 0.2 M sucrose solution. The homogenate was spun for 30 min at 13,750 rpm on the Spinco Model L. centrifuge using a No. 21 rotor. The pellet was discarded, and the supernatant was spun for an additional 2 hr at 21,000 rpm (59,110 × *g*, max radius). The resulting pellet was suspended in a volume of sucrose solution such that 0.1 ml of this suspension contained the same quantity of particles as were contained in 1.5 ml of the original homogenate. The dry weight (exclusive of sucrose) of 0.1 ml of a microsomal suspension, prepared in this way, is between 0.9 and 1.1 mg. It should be recognized that the term "microsomes" is used only in an operational sense and that the fraction obtained from brain in this manner is grossly inhomogeneous.

The almost clear supernatant from this precipitation was used without alteration or was subjected to various treatments as described below.

Enzyme Assay Method. For the assay of carboxy-labeled substrates, incubations were carried out in 10-dram vials fitted with serum stoppers and 1-ml glass cups containing Hyamine solution. The cups were supported above the level of the incubation medium in the bottom of a short section of a polyethylene centrifuge tube, which had a section of the side cut out for easy removal of the cup. The tube itself was suspended by inserting the narrow part of the serum stopper into its top. At the end of the incubation period (carried out in a Dubnoff shaker for 30 min at 37C) Hyamine (1 M in methanol) was injected into the cup. Dilute sulfuric acid was injected into the reaction medium to stop the reaction and liberate CO_2. After they were shaken for an additional 90 min at room temperature to permit the absorption of the CO_2 by the Hyamine, the cups were removed to vials of scintillation solution for counting. The incubation times used in the standard assay are sufficiently longer than the linear part of the reaction (approx. 10 min) that the data have no further meaning than the amount of substrate converted per 30 min. Since the conclusions are based on all-or-none effects, it is not apparent that measurements of the initial rates would have any particular advantage.

For assay of tritium-labeled substrates, the incubation was carried out in a similar manner except that the Hyamine was omitted. After the reaction was stopped with H_2SO_4, the flask contents were extracted with 20 volumes of methylene chloride-methanol (2:1, v/v), and the organic phase was washed with water and evaporated to dryness under reduced pressure. The residue was diluted with appropriate inactive carriers, then dissolved in 4% methanolic HCl and heated in a screw-cap tube for one hour at 80C. The resulting esters were diluted with pentane, and the less polar products were separated from the hydroxy esters by Florisil or silicic acid column chromatography. The separated esters were analyzed on the Cary-Loenco gas-liquid chromatograph, which permits the simultaneous recording of mass and radioactivity of each fraction.

RESULTS

Nature of the Enzyme

Location. It was found in the earlier study (1) that there was a clear separation of enzyme activity in the 100,000 × g precipitate and cofactor in the supernatant. In the present experiments such a clear separation was not found. The need for a more convenient method for producing larger amounts of enzyme required that the centrifugation and homogenization procedures of Reference 1 be modified.

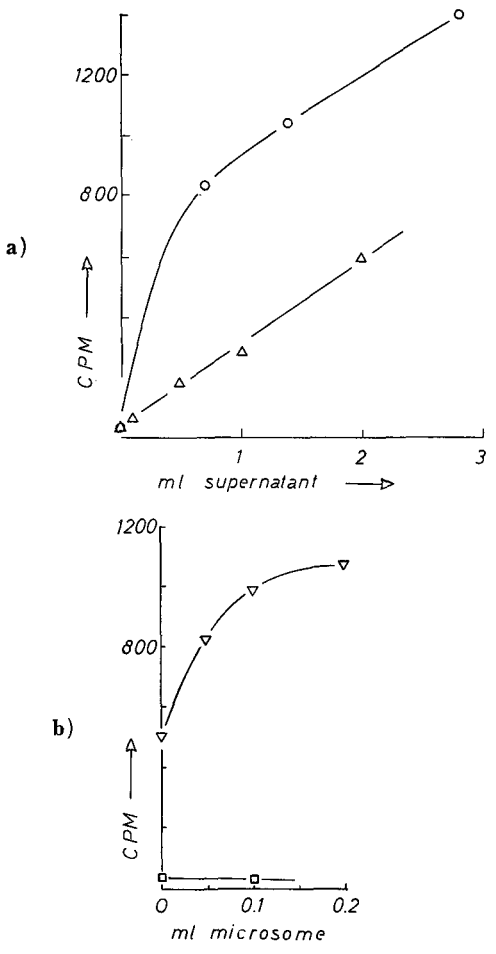


FIG. 1. Dependence of decarboxylation of α-hydroxystearic acid on enzyme/cofactor ratio. Beef brain supernatant and microsomes were prepared as described in text. In addition to the volumes of supernatant and microsomal suspension shown in the figure, each sample contained, in a volume of 1.3 ml, 4 μM NAD, 5 μM ATP, 100 μM Tris-HCl (pH 8.4), 50 mμM ^{14}C-2-OH-stearic acid (emulsified in 1% Tween 20) containing 1.3×10^4 cpm. The upper curve in a) represents activity found with 0.1 ml microsomal suspension augmented with the volume of supernatant shown on the abscissa. The lower curve represents activity found with the volume of supernatant shown on the abscissa. The upper curve of b) represents activity found with 1.4 ml of supernatant augmented with the volume of microsomal suspension shown on the abscissa. The lower curve represents activity found with the volume of microsomal suspension shown on the abscissa.

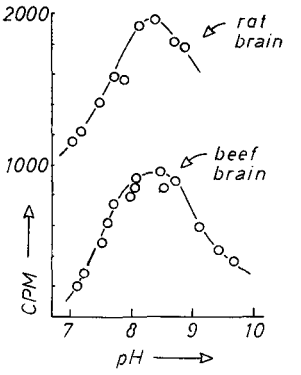


FIG. 2. The pH dependence of beef- and rat-brain α-hydroxy fatty acid decarboxylase. Supernatant and microsomes were present in volumes of 1.4 and 0.1 ml respectively. Each sample contained, in addition, ATP, NAD, buffer, and substrate in the quantities given in the legend for Fig. 1. Final volume was 3.3 ± 0.2 ml. The pH of the incubation medium was adjusted to the values shown in the figure by addition of dilute HCl or NaOH.

The activity distribution found with the present method is given in Fig. 1, which shows the activities of increasing amounts of supernatant and microsomes compared with the activities found when each of these is supplemented with the normal amount of the other component.

As shown in Fig. 1a, the microsomes alone are devoid of activity unless supplemented with supernatant. In contrast to this, the supernatant alone contains both cofactor and enzyme activity; moreover activity is proportional to the amount of supernatant. Addition of microsomes to the supernatant causes an increase in product activity of about 500 cpm, and further increases in supernatant elicit increases in activity parallel to those found with supernatant alone. This behavior implies that, under these conditions of assay, it is the enzyme and not the cofactor that is limiting. Although such behavior could also be expected from a two-enzyme system, the results of earlier work (1) and results presented below indicate that, if more than one enzyme is involved, the activities are not separable by centrifugation.

Evidently the enzyme activity can be associated with particles of varying sizes, and consequently of varying sedimentation rates. Such behavior is to be expected from a membrane-bound enzyme system, and it is quite reasonable to expect this type of enzyme closely associated with the membranes that contain its substrate as a constituent.

pH Optimum. Fig. 2 shows the pH curves of both the rat-brain and beef-brain enzyme systems, indicating that, although the rat-brain enzyme is considerably more active, both have optima close to pH 8.

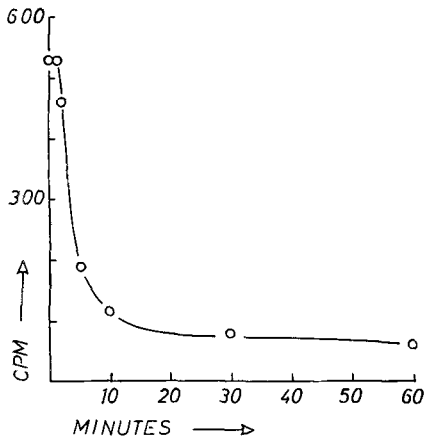


FIG. 3. Heat inactivation of supernatant. Each sample contained 1.4 ml of rat brain supernatant which had been exposed to 60C for the time periods shown in the figure, 50 μM Tris-HCl (pH 7.6), 4 μM NAD, 5 μM ATP, and 50 mμM 1-^{14}C-2-OH-stearic acid containing 1.3×10^4 cpm (in 1% Tween emulsion). Total incubation volume was 2.7 ml.

Stability. Previous work (1) has recognized the extraordinary heat-stability of these microsomal enzymes. The present experiments have confirmed these results. However it has been found that the supernatant preparation, which contains both enzyme and cofactor activity, is quite labile to moderate heat. Fig. 3 shows the loss of activity of the supernatant during exposure to 60C temperature. It can be seen that after 5 min about 60% of the activity has been lost. Although the stability of the complete system (heated microsomes plus heated supernatant) is greater, it too may be inactivated by incubation at relatively low temperatures for extended periods of time. A possible explanation for this inactivation is that the supernatant may contain a heat-stable protease capable of attacking the enzyme.

The enzyme system was inactivated by a bacterial protease (Calbiochem No. 53913), as shown in Table I. In the same table it can be seen that some loss of activity occurred during incubation with boiled protease but that this was about the same as that which occurred with distilled water.

Other inhibitory factors are EDTA (essentially complete inhibition at 2×10^{-5} M), a nitrogen atmosphere, parachloromercuribenzoate (55% inhibition at 2×10^{-3} M), and excess protein (variable, probably because of substrate binding).

Cofactor Requirements

Complete elucidation of the cofactor requirements was made difficult by the impurity of the enzyme activity, by the nature of the over-all reaction, which might encompass several steps, and by the fact that the activity of one cofactor might be altered by the presence of others. The original observations (1) indicated that ATP, NAD, and the dialysate of the supernatant from centrifugation of the microsomes were necessary for maximum activity but that either ATP or NAD alone could promote moderate decarboxylation. The supernatant factor however was indispensable. Although the exact nature of the supernatant factor, other than contribution of a fraction of the enzyme activity, is not known, Levis (6) found that ascorbate could replace it for an α-hydroxy acid decarboxylating system from pig brain, and Davies et al. (7) showed it to be active in α-keto acid decarboxylation.

In the present studies a reducing agent was implicated in at least a part of the action of the supernatant since its inactivation on standing at room temperature was prevented by thiols. In fact, in some cases activity was increased after incubation with thioglycolate, perhaps implying reduction of a partially oxidized cofactor. A trial of ascorbic acid in the microsomal hydroxy acid decarboxylation system showed it to be an effective cofactor in the absence of supernatant. The association of ascorbate and ferrous ion in a number of oxygenase reactions as well as the marked inhibition of the reaction by EDTA led to an investigation of the effect of ferrous ion. This metal proved to be capable of substituting for the supernatant and was even more effective than ascorbate, only however in the presence of ATP. Of the other metals tested, none was

TABLE I

Effect of Protease on α-Hydroxy Acid Decarboxylase[a]

Treatment	CPM in CO_2
None	1674
Preincubate with protease	292
	272
Preincubate with boiled protease	1159
	1164
Preincubate with dist. H_2O	1198
Omit microsomes	124

[a] Preincubations of 0.1 ml of microsomal suspension were carried out in 1 ml of water for 1 hr at 37C. Where indicated, 1 mg of bacterial protease was included. Boiled protease was subjected to 100C for 1 hr. After the preincubation, the rest of the components of the assay were added; 100 μM Tris-HCl (pH 8.4), 5 μM ATP, 0.9 μM $FeSO_4$, and 100 mμM 1-^{14}C-2-hydroxystearic acid containing 5×10^4 cpm (in 0.1 ml % Tween 20). Final volume was 2.6 ml. Where indicated the assay was done in duplicate.

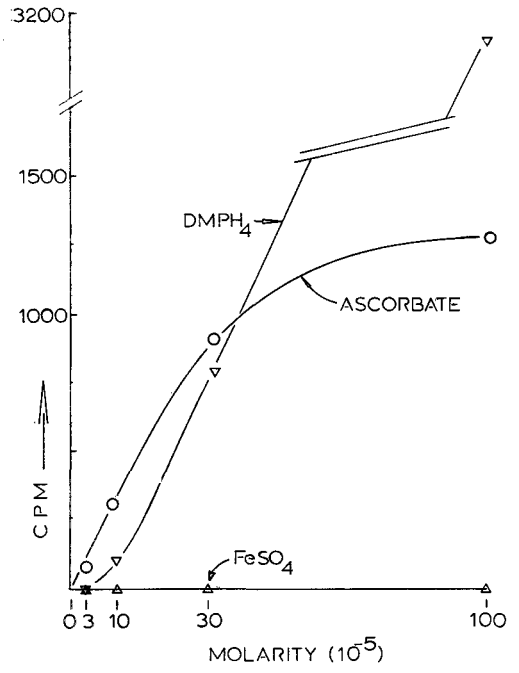


FIG. 4. Effect of ascorbate, $DMPH_4$, and $FeSO_4$, decarboxylation of α-hydroxystearate. Each incubation contained, in a total volume of 3.0 ml, 0.1 ml of microsomal suspension (beef brain), 100 μM Tris-HCl buffer (pH 8.4), 50 mμM 1-^{14}C-2-OH-stearic acid containing 5×10^4 cpm (emulsified in 1% Tween 20), and one of the three substances which were tested for cofactor activity. Data were corrected for controls as explained in text. Concentrations of ascorbate, $DMPH_4$, and $FeSO_4$ are expressed in molarity.

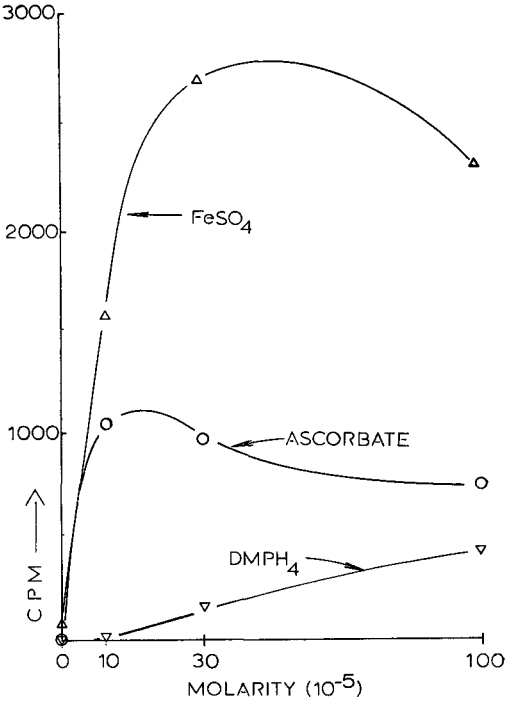


FIG. 5. Effect of ascorbate, $DMPH_4$, and $FeSO_4$ in conjunction with ATP and DPN on the decarboxylation of α-hydroxy stearic acid. Conditions were as listed under Fig. 4 except that ATP (5 μM) and NAD (4 μM) were included.

stimulatory at reasonable concentrations and some were even inhibitory.

The discovery that both of these markedly different substances could replace the supernatant cofactor suggested that this requirement is not rigid. Consequently several other reducing substances were tested, in particular, 6,7-dimethyl-5,6,7,8-tetrahydropterine ($DMPH_4$), an analog of the cofactor involved in phenylalanine hydroxylation (8), which proved to be remarkably active as a cofactor. The results of some of these experiments are shown in Fig. 4 and 5. It is apparent that all these agents are stimulatory under the appropriate conditions but that their effectiveness is dependent upon the presence or absence of ATP and NAD. Fig. 4, showing the effect of increasing concentrations of the three agents tested, is to be compared with Fig. 5, which shows the results of the same experiments carried out in the presence of ATP and NAD.

The data of Figs. 4 and 5 are corrected for controls, which were the same as the experimental incubations except that they contained no microsomes. Only under one set of conditions did decarboxylation in the control exceed 300 cpm. In a few instances, where there was little or no stimulation by the cofactor(s), the difference between experimental and control was a negative number. This discrepancy never exceeded 100 cpm, and since the precision of the assay is less than this (~ 200 cpm), these are shown on the base line (0 cpm) in the figures. The one situation in which there was a significant reaction in the control was the test of $FeSO_4$ in the absence of nucleotides. The values for decarboxylation at concentrations of $FeSO_4$ of 1, 10, and 100×10^{-5} M were 125, 200, and 500 cpm respectively in the presence of microsomes and 300, 350, and 500 cpm respectively in the absence of microsomes.

In Table II the effect of one of these nucleotides in the absence of the other is shown. Ferrous ion is affected to the greatest extent by the addition of the nucleotides. That it is the ATP and not the NAD which is effec-

TABLE II

Effect of ATP and NAD on Decarboxylation of α-Hydroxystearic Acid Stimulated by Ascorbate, $DMPH_4$, or $FeSO_4$[a]

Cofactor	CPM in CO_2		
	Control	ATP	NAD
$FeSO_4$	276	3180	280
Ascorbate	1121	1060	650
$DMPH_4$	993	350	720

[a] Incubations were carried out for 30 min at 37C. Each incubation medium contained, in a total volume of 3.0 ml, 0.1 ml of beef brain microsomal suspension, 100 μM Tris-HCl (pH 8.4), and 50 mM 1-^{14}C-2-hydroxystearic acid containing 5.0 x 10^4 cpm (emulsified in 0.1 ml 1% Tween 20). When indicated, samples contained either 5 μM ATP or 4 μM NAD. Control samples contained neither nucleotide. Ascorbate, $FeSO_4$, and $DMPH_4$ were present in quantities of 900 mμM.

tive in this regard is seen by comparing the results of Fe^{++} + NAD (280 cpm) with those of Fe^{++} + ATP (3180 cpm) in Table II. ATP also affects greatly the activity of $DMPH_4$ but, in this case, is strongly inhibitory. A curious effect of ATP and NAD is that, in their presence, ascorbate becomes inhibitory at high concentrations. In the absence of NAD and ATP, $DMPH_4$ was the most effective cofactor tested. However, in the presence of these nucleotides, the effect of the pteridine was almost completely eliminated.

In view of the fact that the reducing agents tested here as cofactors are all capable of reaction with oxygen to produce peroxide, the possibility was considered that autoxidation of these materials could provide substrate for a peroxidase. Such a reaction is, in fact, known to occur in a plant α-oxidation system (9). Consequently the effect of the H_2O_2-generating system, glucose plus glucose oxidase, was investigated. It was found that, although a small degree of nonenzymatic decarboxylation occurred under these conditions, there was no evidence to suggest that the hydroxy acid decarboxylase is a H_2O_2-linked peroxidase.

Nature of the Products

As discussed in the previous report (1), the only product consistently obtained in these reactions, regardless of cofactors, has been the acid one carbon shorter than the starting material. Under unusual conditions the keto acid was detected, leading to the proposal that it was at least in equilibrium with the true (bound) intermediate. In the present experiments as well, heptadecanoic acid was the chief product of the decarboxylation of α-hydroxystearate. It was considered however that, in the presence of Fe^{++} as a cofactor, a different product might result. Consequently the products of the standard assay procedure with tritium-labeled hydroxystearate as substrate were analyzed by radio-gas chromatography. Again it was found that the dominant product was heptadecanoic acid, but a small peak (less than 0.4% of the substrate) appeared in the position of heptadecanal. The possibility therefore remains that at least a part of the reaction could proceed through a pathway with this compound as intermediate.

Decarboxylation of α-Keto Acids

In the previous report (1) it was demonstrated that α-ketostearate would undergo decarboxylation under the same conditions which lead to decarboxylation of α-hydroxystearate. The suggestion was made then that the keto acid would be a likely intermediate in the hydroxy acid reaction. To explore this possibility further, a number of the investigations which were made on the hydroxy acid system were done in parellel by using the corresponding α-keto acid as substrate. In general, there were considerable similarities between the two decarboxylation reactions with respect to the distribution of enzyme activity between pellet and supernatant, and stability to inactivation. Although perhaps suggestive, these observations do not, in themselves, shed any light on the relationship between the two activities.

Since more extensive investigations of α-keto acid decarboxylation have appeared elsewhere (7), this discussion will be limited to a comparison of the effectiveness in the keto acid reaction of those substances which were found to be active as cofactors in the decarboxylation of the hydroxy acid. Studies by Davies et al. (7) and by Levis (6) have shown that ascorbate is an effective cofactor for the decarboxylation of α-ketostearate. In agreement with this work, ascorbate, in the presence of ATP and NAD, proved to possess considerable cofactor activity. In contrast to the situation with the hydroxy acid, $FeSO_4$ is a poor cofactor substitute. Also in contrast to hydroxy acid decarboxylation, the H_2O_2-generating system promoted keto acid decarboxylation under essentially all conditions which were tested. The influence of the nucleotides is marked only in the case of ascorbate as a cofactor. The requirement for both nucleotides is not absolute. At a concentration of ascorbate three times higher than that shown (Fig. 6) decarboxylation takes place to the extent of 11,000 cpm. In the presence of only ATP or NAD this drops to 8,400 and 6,300 cpm respectively.

The degree of spontaneous decarboxylation of α-ketostearate (incubation of substrate and buffer alone) may be considerable. This is

probably because of slow decomposition of the substrate in handling and storage. The CO_2 from such decomposition would be trapped in solution as HCO_3^- and not released until acidification of an incubation. This occurred to the extent of 1800 ± 200 cpm in the experiments shown in Fig. 6 and is indicated by the dotted line. This value, to the precision of the assay (± 400 cpm), encompasses the values of all of the controls (no enyzme, with and without ATP and NAD) for this experiment so that this value may be taken as the control for the entire set of data.

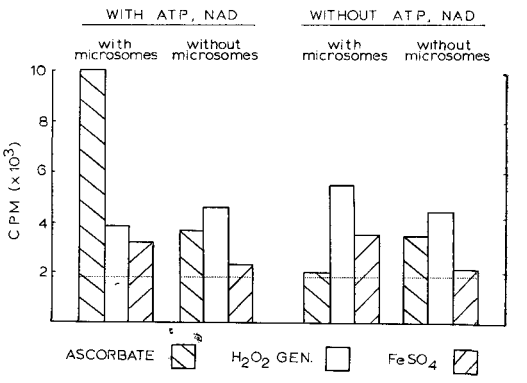


FIG. 6. Decarboxylation of α-ketostearic acid under various conditions. Each incubation contained, in a total volume of 3.0 ml, 6.1 ml of beef brain or 0.1 ml, 0.25 sucrose (as indicated), 100 μM Tris-HCl (pH 8.4) and 50 mμM 1-^{14}C-2-keto searic acid containing 5.0 × 10^4 cpm (emulsified in 0.1 ml 1% Tween 20). Where indicated, samples contained 5 μM ATP and 4 μM NAD. Ascorbate and $FeSO_4$ were present in quantities of 900 mμM. The H_2O_2-generator consisted of 0.1 mg of glucose oxidase (17,000 mM units/mg) and 100 μM glucose.

DISCUSSION

The present experiments have served to increase the knowledge of the nature and requirements of the enzymes involved in α-oxidation. They have set some limits on the nature of the reaction although they have not specifically defined the mechanism which contributes to the over-all result. To place the discussion in perspective and to indicate the possible complexity of the system, the possible pathways of α-oxidation are presented in Fig. 7.

Whatever the uncertainties in the number of possible reactions leading eventually to the production of odd-chain acids, it is evident that the reaction which predominates under the conditions in the present experiments is one that involves reaction with oxygen. In addition to the requirements for an aerobic atmosphere, the inhibition of the reactions by EDTA and the strong stimulation by ferrous ion are compelling indications of an oxidase or oxygenase reaction. Iron is almost universally found to be required for dioxygenases and is frequently associated with monoxygenases (10-12) and sometimes oxidases (13).

In addition to the reaction with oxygen, any reasonable hypothesis for the mechanism of the decarboxylation reaction must take into consideration the question of the intermediacy of an α-keto acid. The original observation of Levis and Mead (1) was that, although the α-hydroxy acid underwent decarboxylation in the presence of a considerable excess of keto acid, a small amount of radioactivity from the hydroxy acid nevertheless appeared in the keto acid. The explanation may be that the keto acid is simply too rapidly oxidized to interfere with the reaction of the hydroxy acid.

While there are similarities between α-hydroxy- and α-keto acid decarboxylation, there are also distinct differences in cofactor requirements. Such differences should be reconciled before a keto acid can be accepted as an obligatory intermediate. The hydroxy acid requires for decarboxylation either one of several reducing agents or ferrous ion. Although all systems (except that containing $DMPH_4$) are stimulated by ATP, its presence is a requirement for $FeSO_4$ activation of the enzyme. The presence of iron in oxygenases or oxidases is a common finding, but it is also known that reducing agents may activate these enzymes even though they are not required as cofactors per se. This is, for example, the case with meta pyrocate-

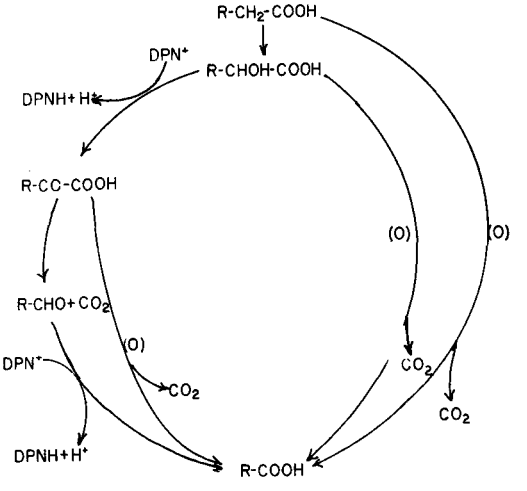


FIG. 7. Alternate pathways of α-oxidation.

chase, which requires ferrous ion for activity. It may be inactivated by oxidation and then reactivated (12) by ascorbate or cysteine (complete reactivation requires the presence of iron as well). A similar situation may obtain in the case of α-hydroxy acid decarboxylation.

The requirement of ATP for full activity in the supernatant-substitution experiments is in accord with the stimulation afforded by ATP in the whole homogenate (1). Although at present its function cannot be confidently assigned, it appears to be unrelated to an activation reaction since neither CoA nor Mg^{++} are required for its stimulation. At present, the best explanation appears to be that it functions as a labile chelating agent to protect and perhaps slowly liberate ferrous ion. This function is suggested by the observation that, in the absence of ATP, a precipitate or ferric hydroxide rapidly forms in a solution of $FeSO_4$ at the pH of the incubation.

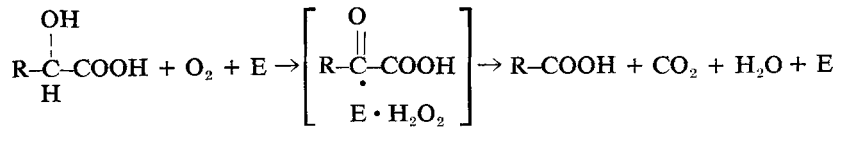
In contrast to the hydroxy acid, the keto acid requires the presence of ascorbate for maximal decarboxylation. Iron, although implicated in the reaction (7), is not nearly so effective. A reducing agent and oxygen are common requirements for a monoxygenase reaction. A reaction in which two reducing equivalents from the substrate and two from ascorbate react with an O_2 molecule was in fact suggested by Davies et al. (7). But the requirement for O_2 and an oxidizable reducing agent could equally well implicate a peroxidase reaction. This is the case in a plant α-oxidation system which results in the decarboxylation of fatty acids to aldehydes (9). The primary enzyme is a peroxidase, and a number of peroxide generators may be coupled to it.

The well-known lability of α-keto acids to oxidation by H_2O_2 as well as the present demonstration that glucose and glucose oxidase may partially substitute for ascorbate indicate that such a reaction could account for the decarboxylation of the α-keto acid in the brain system. Since Levis (7) has shown that, on a molar basis, the amount of ascorbate exceeds by three- to five-fold the amount of keto acid oxidized, it is evident that oxidation of ascorbate (which may be either enzymatic or spontaneous), could provide sufficient H_2O_2 for a peroxidase-catalyzed decarboxylation of α-keto stearate. Davies et al. (7) considered this possibility but discarded it on the basis that catalase was not inhibitory to the reaction. Since the publication of this report it has been found that ascorbate is a potent inhibitor of catalase, particularly under oxidizing conditions (14). Thus it would appear that there is no evidence against a peroxidase, and some circumstantial evidence for one.

One of the distressing problems in the consideration of a keto acid intermediate is that there has been no evidence for its production under normal conditions. There is one formulation of the reaction scheme which would account for the difficulty in detecting such an intermediate as well as explain the different cofactor requirements for the two decarboxylation reactions. If the initial reaction of the α-hydroxy acid with O_2 produced the keto acid and H_2O_2, the latter because of proximity and simultaneity of production would react rapidly.

Such a formulation requires an enzyme capable of the dual function of oxidation and oxidative decarboxylation. In at least one case a purified enzyme has been shown to have such a dual role. This enzyme is lactic oxidative decarboxylase, a reaction analogous to the decarboxylation of hydroxy acids, namely, the oxidation of lactic acid to acetic acid and CO_2. It has been shown that pyruvate and H_2O_2 are intermediates in this reaction although they are not detectable under normal conditions (15). There also exists L-α-hydroxy acid oxidases in kidney, which catalyze an α-oxidative sequence of this type. They differ from lactic oxidative decarboxylase in that the second step of the reaction can be inhibited with catalase (16). Another enzyme which catalyzes a reaction similar to that under study is the inositol cleavage enzyme. This iron-containing enzyme is thought to be a monoxygenase, which is activated by certain reducing agents (17).

Based on analogy with the enzymes mentioned above, previous knowledge of the reaction of α-oxidation, and the data presented herein, the following formulation of the oxidative decarboxylation of α-hydroxy acid in brain may be suggested:



This scheme, or a similar one involving another reactive species of oxygen besides peroxide, is in accord with the requirements of the enzyme activities responsible for decarboxylating both hydroxy and keto acids and also suggests the reason for the association of the two activities.

ACKNOWLEDGMENTS

Amiya Hajra and Norman Radin gave labeled α-hydroxy and α-ketostearic acid; Armand Fulco, unlabeled samples of the same compounds. G. M. Levis permitted the reading of his manuscript prior to its publication. Dr. Radin also provided many helpful discussions, particularly regarding the effects of ascorbic acid.

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SHORT COMMUNICATIONS

Curvilinear Regression Course of Human Brain Lipid Composition Changes With Age

WE HAVE RECENTLY COMPLETED a detailed study of human brain lipid composition changes with age. The unexpected nature of the changes and their obvious general importance prompted us to report at once findings derived from the data. In our study, lipid class composition, including most of the minor components, and the fatty acid composition of each lipid class were determined in 13 normal, male, human, whole brains (age range, 6 months fetus to 98 years). Accuracy and precision were insured by determination of lipid class composition of each sample by at least two different methods involving spectrophotometric assay following separation by two-dimensional thin-layer chromatography alone or in combination with column chromatography (1-3) and by repeat analysis of each sample by each method. The data were then submitted to various forms of graphic analysis.

Semilogarithmic plots of lipid values at different ages were found to give a series of straight lines, indicating periods following curvilinear regression of a very precise nature. A typical semilogarithmic plot is shown in Fig. 1 where the steady decline of total phospholipid as percentage of the total lipid (excluding gangliosides) is apparent, up to about 33 years of age. Similar results were obtained with the individual lipid classes when values were plotted as weight percentage of the total lipid, micromoles/100 mg of lipid, or millimoles/100 g fresh weight or dry weight. Use of the log scale for age is convenient because the scale is expanded at the early ages where changes are greatest. Plots of lipid data show that development and aging of brain are continuous processes without a fully stable intermediate period.

Once the straight line periods are recognized from plots on semilogarithmic paper, regression equations can be calculated for each period. The equations (Table I) calculated for water content and total lipid as percentage of the fresh weight, and for lipid classes as millimoles/100 g fresh weight, were found to give values with a maximum deviation of ± 2% of the measured values. Values as millimoles/100 g fresh weight calculated from the equations of Table I are convenient for general use, since from them values in terms of dry weight, percentage of the total lipid, etc. can be calculated.

Plots of lipid data show inflection points, i.e. ages at which the slope of the regression line changes. These ages are important because a change of rate may reflect differences in regional development of the brain or changes in physiological control factors such as cerebral blood flow and hormonal influences. Inflection points can be observed by examination of the equations in Table I, but are best seen when the amount of increase or decrease of a lipid class is plotted. The equations of Table I were used to calculate (by computer) the annual change in the amounts of various lipids. The values obtained up to 40 years of age, the last inflection point seen, are shown in Table II. When these values are plotted on

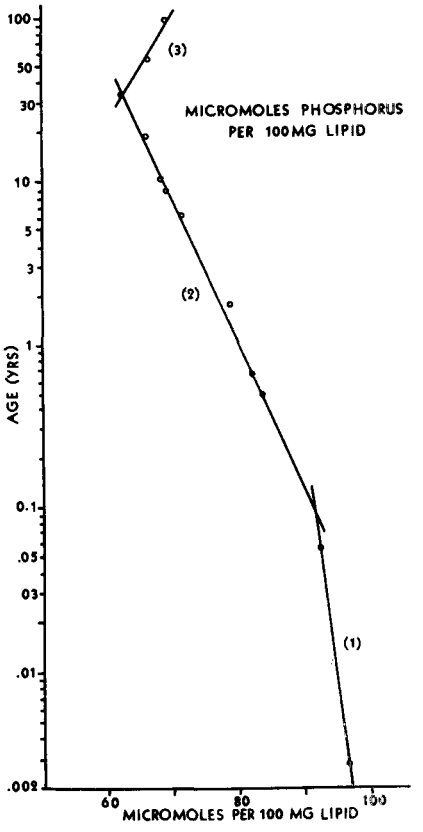

FIG. 1. Micromoles phosphorus per 100 mg lipid.

TABLE I

Equations for Calculation of Normal, Male, Human, Whole Brain Water and Lipid Composition[a]

Component	Period	Equation
Water (% fresh wt)	(1) 1D-3W	(1) 85.92–1.374 (log age)
	(2) 3W-18Y	(2) 82.69–3.931 (log age)
	(3) 18-33Y	(3) 76.36+0.9514 (log age)
	(4) 33-98Y	(4) 72.36+3.551 (log age)
Total lipid (% fresh wt)	(1) 1D-6M	(1) 5.94+1.30 (log age)
	(2) 6M-26Y	(2) 6.69+3.50 (log age)
	(3) 26-98Y	(3) 18.6–4.85 (log age)
Lipid phosphorus	(1) 1D-3W	(1) 3.93+0.393 (log age)
	(2) 3W-23Y	(2) 5.30+1.55 (log age)
	(3) 23-98Y	(3) 10.1–1.94 (log age)
Cholesterol	(1) 1D-3W	(1) 2.379+0.2567 (log age)
	(2) 3W-6Y	(2) 3.988+1.633 (log age)
	(3) 6-8.5Y	(3) 8.330 (log age) –1.202
	(4) 8.5-33Y	(4) 5.894+0.6288 (log age)
	(5) 33-98Y	(5) 11.27–2.900 (log age)
Cholesterol ester	(1) 1D-3W	(1) 0.109+0.031 (log age)
	(2) 3W-6M	(2) 0.0051–0.0530 (log age)
	(3) 6M-33Y	(3) 0.0176–0.0077 (log age)
	(4) 33-98Y	(4) 0.0520 (log age) –0.0713
Cerebroside	(1) 1D-3W	(1) 0.1056+0.0365 (log age)
	(2) 3W-6M	(2) 0.5921+0.4273 (log age)
	(3) 6M-2Y	(3) 0.6848+0.7173 (log age)
	(4) 2-6Y	(4) 0.5819+1.1034 (log age)
	(5) 6-21Y	(5) 2.171 (log age) –0.2312
	(6) 21-98Y	(6) 4.511–1.384 (log age)
Sulfatide	(1) 1D-3W	(1) 0.0235+0.0076 (log age)
	(2) 3W-6M	(2) 0.1749+0.1293 (log age)
	(3) 6M-6Y	(3) 0.2008+0.2100 (log age)
	(4) 6-10Y	(4) 0.8687 (log age) –0.3230
	(5) 10-40Y	(5) 0.2427+0.3212 (log age)
	(6) 40-98Y	(6) 2.332–0.9606 (log age)
Ganglioside	(1) 1D-8M	(1) 0.155+0.0181 (log age)
	(2) 8M-98Y	(2) 0.146–0.0298 (log age)
Ceramide	(1) 1D-3W	(1) constant at 0.046±0.05
	(2) 3W-6M	(2) 0.142+0.0731 (log age)
	(3) 6M-1Y	(3) (–) 0.0008–0.401 (log age)
	(4) 1-33Y	(4) 0.0354+0.0284 (log age)
	(5) 33-98Y	(5) 0.281–0.123 (log age)
Triglyceride	Birth-98Y	(1) 0.016+0.0038 (log age)
Free fatty acid	(1) 1D-10Y	(1) 0.429+0.120 (log age)
	(2) 10-18Y	(2) 0.684–0.157 (log age)
	(3) 18-33Y	(3) 1.26 (log age) –1.10
	(4) 33-98Y	(4) 2.59–1.17 (log age)
Sphingomyelin	(1) 1D-3W	(1) 0.1608+0.0264 (log age)
	(2) 3W-38Y	(2) 0.5040+0.3244 (log age)
	(3) 38-98Y	(3) 1.782–0.4903 (log age)
Phosphatidyl choline	(1) 1D-33Y	(1) 1.892+0.2121 (log age)
	(2) 33-98Y	(2) 2.760–0.4231 (log age)
Phosphatidyl ethanolamine	(1) 1D-3W	(1) 1.221+0.1291 (log age)
	(2) 3W-30Y	(2) 1.804+0.6071 (log age)
	(3) 30-98Y	(3) 4.358–1.186 (log age)
Phosphatidyl ethanolamine plasmalogen	(1) 1D-3W	(1) 0.5969+0.1109 (log age)
	(2) 3W-30Y	(2) 1.031+0.4614 (log age)
	(3) 30-98Y	(3) 3.303–1.101 (log age)
Phosphatidyl serine	(1) 1D-3W	(1) 0.5145+0.0687 (log age)
	(2) 3W-6Y	(2) 0.7549+0.2603 (log age)
	(3) 6-10Y	(3) 0.0408+1.160 (log age)
	(4) 10-33Y	(4) constant at 1.23±0.02
	(5) 33-98Y	(5) 1.960–0.4798 (log age)
Phosphatidyl inositol	(1) 1D-6Y	(1) 0.1327+0.0194 (log age)
	(2) 6-13Y	(2) 0.0223+0.1603 (log age)
	(3) 13-37Y	(3) 0.3459–0.1290 (log age)
	(4) 37-98Y	(4) 0.1673 (log age) –0.1161
Phosphatidic acid	(1) 1D-98Y	(1) 0.0268+0.0098 (log age)
Diphosphatidyl glycerol	(1) 1D-2Y	(1) 0.0490+0.0167 (log age)
	(2) 2Y-98Y	(2) 0.0619–0.0159 (log age)

[a]Millimoles/100 g fresh weight. Abbreviations: D, W, M, Y: days, weeks, months, years. Log age refers in each case to $\log_{10}$ age in years.

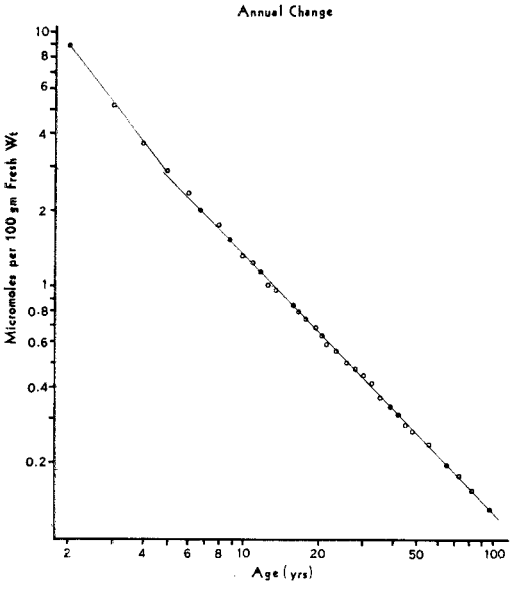

FIG. 2. Human brain total gangliosides.

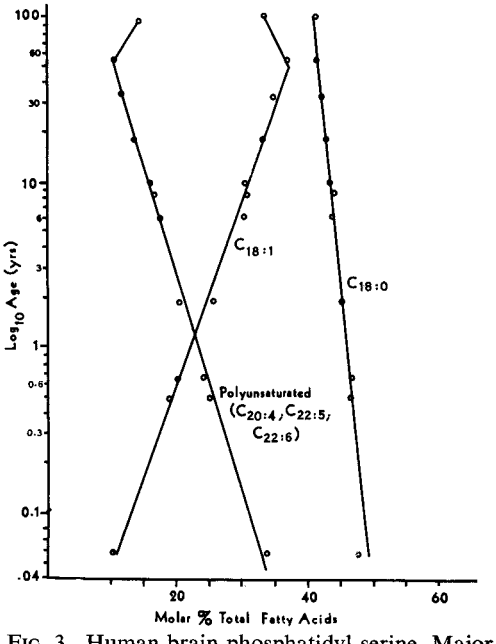

FIG. 3. Human brain phosphatidyl serine. Major fatty acids.

TABLE II

Annual Changes in the Amounts of Human Brain Lipids[a,b]
(Micromoles/100 g Fresh Weight)

Year	Phos	Chol	Gang	Cer	Sulf	Sph	PC	PE	PS	PI	PA	PE Plas
1	2400	2140	(+)42.0	673	197	1350	510	919	419	42.7	16.8	719
2	465	489	(–) 8.93	215	62.9	97.2	63.6	182	78.0	5.8	2.93	138
3	273	288	5.25	194	37.0	57.1	37.4	107	45.8	3.4	1.73	81.3
4	194	205	3.74	138	26.3	40.7	26.6	76.1	32.6	2.4	1.22	57.9
5	150	158	2.89	107	20.4	31.5	20.6	58.9	25.2	1.9	0.95	44.7
6	123	129	2.36	87	16.6	25.7	16.8	48.1	20.6	1.5	0.78	36.5
7	103	556	1.99	145	57.9	21.6	14.1	40.5	77.4	7.1	0.65	30.8
8	90	482	1.72	126	50.2	18.8	12.3	35.1	67.1	6.1	0.57	26.7
9	80	430	1.54	112	44.8	16.7	10.9	31.3	59.8	5.5	0.51	23.8
10	70	29	1.35	98	39.4	14.7	9.6	27.5	52.6	4.8	0.44	20.9
11	65	26	1.25	91	13.4	13.6	8.9	25.4	c	4.4	0.41	19.3
12	59	24	1.14	83	12.3	12.4	8.1	23.2		4.1	0.38	17.6
13	53	21	1.01	73	10.9	11.0	7.2	20.5		3.6	0.33	15.6
14	51	21	0.98	71	10.6	10.7	7.0	20.0		(–)4.2	0.32	15.2
15	45	18	0.88	64	9.4	9.5	6.2	17.8		3.8	0.29	13.5
16	44	18	0.85	62	9.1	9.2	6.0	17.3		3.7	0.28	13.1
17	42	17	0.80	58	8.6	8.7	5.7	16.2		3.4	0.26	12.3
18	38	16	0.74	54	8.0	8.1	5.3	15.1		3.2	0.24	11.5
19	35	14	0.66	48	7.1	7.2	4.7	13.5		2.9	0.22	10.3
20	36	14	0.69	50	7.4	7.5	4.9	14.0		3.0	0.23	10.7
21	33	13	0.64	46	6.9	6.9	4.5	13.0		2.8	0.21	9.8
22	30	12	0.58	(–) 27	6.3	6.3	4.2	11.9		2.5	0.19	9.0
23	30	12	0.58	27	6.3	6.3	4.2	11.9		2.5	0.19	9.0
24	(–)36	12	0.56	26	6.0	6.1	4.0	11.3		2.4	0.18	8.6
25	35	11	0.53	25	5.7	5.8	3.8	10.8		2.3	0.17	8.2
26	33	11	0.50	23	5.4	5.5	3.6	10.3		2.2	0.17	7.8
27	31	10	0.48	22	5.1	5.2	3.4	9.7		2.1	0.16	7.4
28	31	10	0.48	22	5.1	5.2	3.4	9.7		2.1	0.16	7.4
29	29	10	0.45	21	4.9	4.9	3.2	9.2		2.0	0.15	7.0
30	29	10	0.45	21	4.9	4.9	3.2	9.2		2.0	0.15	7.0
31	28	9	0.42	20	4.6	4.6	3.0	(–)16.9		1.8	0.14	(–)15.7
32	26	8	0.40	18	4.3	4.3	2.8	15.8		1.7	0.13	14.7
33	27	9	0.42	20	4.6	4.6	3.0	16.9		1.8	0.14	15.7
34	25	(–)36	0.37	17	4.0	4.0	(–)5.3	14.8	(–)6.0	1.6	0.12	13.7
35	24	36	0.37	17	4.0	4.0	5.3	14.8	6.0	1.6	0.12	13.7
36	24	36	0.37	17	4.0	4.0	5.3	14.8	6.0	1.6	0.12	13.7
37	22	34	0.35	16	3.7	3.8	4.9	13.7	5.5	1.5	0.11	12.7
38	23	34	0.34	16	3.7	3.8	4.9	13.7	5.5	1.9	0.11	12.7
39	22	34	0.35	16	3.7	(–)5.7	4.9	13.7	5.5	1.9	0.11	12.7
40	21	31	0.32	15	3.4	5.2	4.5	12.7	5.1	1.8	0.10	11.8
41	21	31	0.32	15	(–)10.3	5.2	4.5	12.7	5.1	1.8	0.10	11.8

[a] Abbreviations: Phos (total lipid phosphorus); Chol (cholesterol); Gang (total gangliosides); Cer (cerebroside); Sulf (sulfatide); Sph (sphingomyelin); PC (phosphatidyl choline); PE (phosphatidyl ethanolamine; PS (phosphatidyl serine); PI (phosphatidyl inositol); PA (phosphatidic acid); PE Plas (phosphatidyl ethanolamine plasmalogen). The term phosphatidyl refers to all forms of the lipid class without regard to differences in chain length, unsaturation, or type of linkage (ester, vinyl ether, ether) of the hydrocarbon chain.

[b] Values preceded by (–) are negative and indicate the first year at which a decrease of lipid was noted. All values below the (–) in the same column also represent decreases in total amount.

[c] No change from 10-33 yr.

logarithmic paper, straight lines are observed as illustrated in Fig. 2 by the values for total gangliosides. The inflection points observed from all the plots are shown in Table III. Additional work is required to establish the basis for these rate changes taking place up to 40 years of age.

Somewhat surprisingly, semilogarithmic plots of the fatty acid composition of each lipid class were found to give long straight line periods. One plot (Fig. 3) illustrates the nature of the changes with values as molar percentages of the total fatty acids. Calculation of regression equations from our fatty acid data have not been completed.

The changes with age of human brain lipid composition appear to be very orderly, predictable, and continuous. The changes were not found in previous investigations in which separated grey and white matter rather than the whole brain were analyzed. Since changes in whole brain are orderly, it is apparent that different parts of the brain (cerebral hemispheres, cerebellum, etc.) should show age changes of a similar nature. Our study was confined to males. A sex difference is possible since Bürger (4) has shown that changes with age of brain weight and total solids are different for males and females. From the practical standpoint, the equations presented in Table I will be useful for calculation of brain lipid values not available from direct analysis. The recognition

TABLE III
Inflection Points for Rate of Change of Human Brain Lipids

Year	Phos[a]	Chol	Gang	Cer	Sulf	Sph	PC	PE	PS	PI	PA	PE Plas
1	↑	↑	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑
2	↑	↑	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑
3	↑	↑		↑	↑			↑				
5			↓			↑						↑
6		↑		↑	↑				↑	↑		
7							↑					
9		↑										
10					↑				↔[b]			
13										↓		
21				↓								
23	↓											
29								↓				
30												↓
33		↓					↓		↓			
37										↑		
38						↓						
40					↓							

[a] Abbreviations: see legend for Table II.
[b] Constant from 10-33 years.

of inflection points should stimulate a search for the morphological and physiological basis for the changes. Additional work is required to establish the course of change for females and different parts of the brain of both sexes.

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4. Bürger, M., Medizinische *1*, 561-567 (1956).

Accumulation of a Glycerolphospholipid in Classical Niemann-Pick Disease

ACCUMULATION OF SPHINGOMYELIN in organs (1) and a deficiency of sphingomyelinase (2) have been established in classical (infantile) Niemann-Pick disease. We report here the accumulation of a glycerolphospholipid, tentatively identified as lyso bisphosphatidic acid, in classical Niemann-Pick disease and some apparently related disorders.

Autopsy specimens of brain, heart, kidney, liver, lung, and spleen were obtained from two cases of classical (infantile) Niemann-Pick disease which were described by Knudson and Kaplan (3). The same organs were obtained from a third, entirely similar case. Liver biopsy specimens were obtained from two additional cases corresponding closely to the other three. Organs were also obtained from eight other disorders: acute (infantile) Gaucher's disease (three cases); chronic Gaucher's disease (three cases); infantile metachromatic leukodystrophy (two cases); Tay-Sachs disease (five cases); late infantile amaurotic familial idiocy (one case); juvenile amaurotic familial idiocy (one case); three cases of uncertain diagnosis but with early death (3, 3½, 9 years), neurological involvement, and storage cells suggesting a lipidosis; and one adult case (male, age 65 yr) resembling literature descriptions of the adult

TABLE III
Inflection Points for Rate of Change of Human Brain Lipids

Year	Phos[a]	Chol	Gang	Cer	Sulf	Sph	PC	PE	PS	PI	PA	PE Plas
1	↑	↑	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑
2	↑	↑	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑
3	↑	↑		↑	↑			↑				
5			↓			↑						↑
6		↑		↑	↑				↑	↑		
7							↑					
9		↑										
10					↑				↔[b]			
13										↓		
21				↓								
23	↓											
29								↓				
30												↓
33		↓					↓		↓			
37										↑		
38						↓						
40					↓							

[a] Abbreviations: see legend for Table II.
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Autopsy specimens of brain, heart, kidney, liver, lung, and spleen were obtained from two cases of classical (infantile) Niemann-Pick disease which were described by Knudson and Kaplan (3). The same organs were obtained from a third, entirely similar case. Liver biopsy specimens were obtained from two additional cases corresponding closely to the other three. Organs were also obtained from eight other disorders: acute (infantile) Gaucher's disease (three cases); chronic Gaucher's disease (three cases); infantile metachromatic leukodystrophy (two cases); Tay-Sachs disease (five cases); late infantile amaurotic familial idiocy (one case); juvenile amaurotic familial idiocy (one case); three cases of uncertain diagnosis but with early death (3, 3½, 9 years), neurological involvement, and storage cells suggesting a lipidosis; and one adult case (male, age 65 yr) resembling literature descriptions of the adult

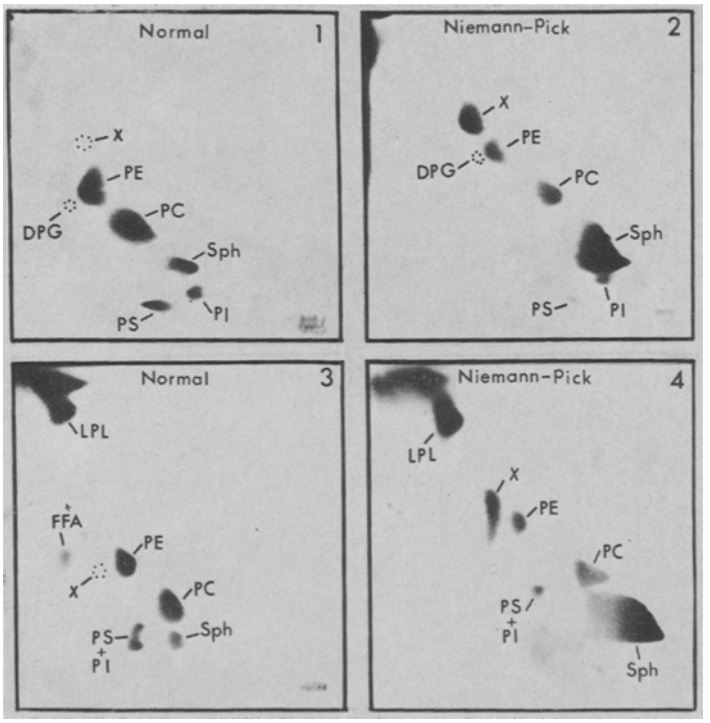


FIG. 1 and 3. Normal liver lipid (800 μg spotted on the lower right). Fig. 1 developed in the vertical direction with chloroform-methanol-28% aqueous ammonia (65:35:5), followed by air-drying (10 min) and development in the horizontal direction with chloroform-acetone-methanol-acetic acid-water (5:2:1:1:0.5). Fig. 3 developed in the vertical direction with chloroform-methanol-water (65:25:4), followed by air-drying (10 min) and development with 1-butanol-acetic acid-water (3:1:1). Spots located by charring at 180C for 30 min after spraying lightly with a mixture of 3 volumes of 37% formaldehyde solution and 97 volumes of 98% sulfuric acid.

FIGS. 2 and 4. Niemann-Pick liver lipid (600 μg spotted at the lower right). Fig. 2 developed as described for Fig. 1, and Fig. 4 developed as for Fig. 3. The large spot marked X (see text for details) that accumulates in Niemann-Pick liver as well as the large sphingomyelin spot will be noted.

Abbreviations: LPL, less polar lipids (cholesterol, triglycerides); X, new lipid; PE, phosphatidyl ethanolamine; DPG, diphosphatidyl glycerol (cardiolipin); PC, phosphatidyl choline (lecithin); Sph, sphingomyelin; PI, phosphatidyl inositol; PS, phosphatidyl serine; FFA, free fatty acid.

form of Niemann-Pick disease. Case descriptions in the first four categories were presented by Knudson and Kaplan (3).

Organs were obtained as soon as possible, frozen in dry ice, and stored at –20C. Lipids were extracted, determined by quantitative thin-layer and column chromatographic procedures, and characterized as previously described (4-6).

In classical (infantile) Niemann-Pick disease, thin-layer chromatographic (TLC) examination disclosed a large accumulation of a new phospholipid in liver, heart, kidney, spleen, and lung. Fig. 1-4 show TLC findings for normal and Niemann-Pick liver. In normal liver the new lipid is a minor component not visible on photographic reproductions. When ion-exchange cellulose column chromatography was first used to concentrate acidic lipids into one fraction, TLC disclosed accumulation of the new lipid in brain. Quantitative values for liver phospholipids (Table I) show clearly that the percentage increase of the new lipid in liver is similar to that of sphingomyelin. On a fresh-weight basis, sphingomyelin was elevated 59-fold (from 0.16 to 9.38%), and the new lipid was elevated 85-fold (0.025 to 2.13%) while other phospholipids were present at normal levels.

No increase of the new phospholipid was

TABLE I
Phospholipid Composition of Normal and Niemann-Pick Disease Livers[a]

Phospholipid	Normal		Niemann-Pick Disease		
	Biopsy	Autopsy[b]	Case 1 Biopsy	Case 2 Autopsy[c]	Case 3 Autopsy[d]
Sphingomyelin	6.4 ±0.15	4.8 ±0.25	62.8 ±1.10	74.6 ±1.21	76.4 ±.92
X	0.9 ±0.03	0.8 ±0.03	14.4 ±0.45	8.1 ±0.26	10.8 ±0.32
Cardiolipin	3.0 ±0.12	3.9 ±0.03	0.5 ±0.02	0.4 ±0.25	0.3 ±0.04
Phosphatidyl choline	47.4 ±0.65	45.8 ±0.96	11.4 ±0.75	6.4 ±0.01	5.8 ±0.05
Phosphatidyl ethanolamine	26.4 ±0.75	29.3 ±0.36	7.2 ±0.20	3.5 ±0.06	3.6 ±0.03
Phosphatidyl serine	4.3 ±0.05	3.4 ±0.02	1.4 ±0.05	0.7 ±0.03	0.5 ±0.04
Phosphatidyl inositol	8.8 ±0.50	9.3 ±0.04	2.2 ±0.04	1.5 ±0.05	1.1 ±0.04
Phosphatidic acid	1.4 ±0.06	0.7 ±0.05	N.D.	0.1 ±0.01	N.D.
Lysophosphatidyl choline	1.4 ±0.05	1.1 ±0.06	0.4 ±0.03	1.4 ±0.07	1.0 ±0.07
Lysophosphatidyl ethanolamine	N.D.	0.4 ±0.01	N.D.	1.3 ±0.03	0.7 ±0.03

[a] Values obtained by phosphorus determination in quadruplicate of spots after two-dimensional TLC (5) and expressed as percentage of the phospholipids shown ± standard deviation. N.D. = not detected.

[b] One hr, 40 min post-mortem.

[c] About 4 hr post-mortem.

[d] About 3 hr postmortem.

seen in acute or chronic Gaucher's diseases, Tay-Sachs disease, metachromatic leukodystrophy, or juvenile amaurotic familial idiocy. Elevation of the phospholipid was seen however in the three cases of uncertain diagnosis, in a case resembling descriptions of adult Niemann-Pick disease (but without elevation of sphingomyelin), and in late infantile amaurotic familial idiocy. One of the cases of uncertain diagnosis (death at 9 yr) also showed an elevation of sphingomyelin in spleen equivalent to that of infantile Niemann-Pick disease; accumulation in other organs was only about 2-fold. The other two cases showed normal or below normal levels of sphingomeylin.

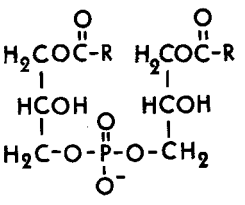
Phospholipids of normal liver undergo rapid post-mortem breakdown with little change in the relative amounts (values may be compared in Tables I and II) and release of free fatty acids. Niemann-Pick liver presents a striking contrast. With autopsy specimens, no increase of free fatty acid or phospholipid degradation

TABLE II
Post-mortem Decline of Phospholipids in Normal Human and Infantile Niemann-Pick Disease Livers[a]

Phospholipid	Normal				Niemann-Pick	
	Zero time (biopsy)	1.7 hr 24C	7 hr 24C	7 days[b] at 4C	Zero time (biopsy)	18 hr 23C
Sphingomyelin	0.880	0.270	0.202	0.184	11.86	11.96
X	0.094	0.080	0.044	N.D.	2.66	1.93
Cardiolipin	0.314	0.223	0.187	0.143	0.10	0.03
Phosphatidyl choline	6.482	2.714	1.642	1.523	2.12	1.86
Phosphatidyl ethanolamine	3.615	1.732	1.184	1.093	1.34	1.15
Phosphatidyl serine	0.587	0.190	0.132	0.143	0.25	0.14
Phosphatidyl inositol	1.196	0.534	0.341	0.308	0.42	0.31
Lyso PC	0.066	0.080	0.146	0.066	N.D.	0.23
Lyso PE	N.D.	N.D.	0.101	0.212	N.D.	0.32

[a] Values (μg phosphorus/800 μg of lipid), obtained by phosphorus determination in duplicate of spots after TLC.

[b] The sample first stood for 7 hr at room temperature. N.D. = not detected.



Lyso Bisphosphatidic Acid

was observed. The phospholipids in a liver biopsy specimen obtained at 13 months of age were degraded at a slower-than-normal rate (Table II). These observations suggest a generalized deficiency of phospholipase activity.

The new lipid was isolated by using the sequence: a) Sephadex column chromatography for removal of nonlipid contaminants; b) DEAE column chromatography for recovery of the strongly acidic lipid fraction eluted with chloroform-methanol-ammonia-ammonium acetate, in which the new lipid appeared; and c) TLC for final separation free of other lipids. The infrared spectrum of the new lipid was almost identical with that obtained from diphosphatidyl glycerol (cardiolipin), which was isolated from mitochondrial lipid extracts (7). Mild alkaline deacylation gave a water-soluble phosphate ester with paper chromatographic migration of the deacylation product of phosphatidyl glycerol. After acid hydrolysis, fatty acids and glycerophosphate were found, but paper chromatography and TLC failed to disclose the presence of ethanolamine, serine, choline, or sphingosine. The ester:phosphorus ratio was 1.8:1.0. Acetolysis with acetic acid-acetic anhydride (3/2, v/v, 145C, 14 hr) gave a single product migrating on TLC (n-hexane-diethyl ether, 70/30 as solvent) with authentic monoacylglyceroldiacetate prepared from lysophosphatidyl choline, without formation of diacylglycerolmonoacetate as obtained from phosphatidyl choline, phosphatidyl glycerol, and diphosphatidyl glycerol. Acetolysis of authentic samples of other phospholipids gave results corresponding to those reported by Renkonen (8). The new lipid is tentatively identified as a lyso bisphosphatidic acid of the type shown in which the fatty acids have been assigned arbitrarily to the primary hydroxyl groups.

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Structure of Bovine Milk Fat Triglycerides. I. Short and Medium Chain Lengths[1]

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ABSTRACT

The triglycerides of bovine milk fat globules were isolated and separated into short, medium and long chain lengths by thin-layer chromatography. The molecular weight distribution and the fatty acid composition of the component triglycerides was then separately determined by gas chromatography following argentation-thin-layer and preparative gas chromatography. Some 38 triglyceride types (28% of total), of which there could be up to 6 isomers, were specifically identified and quantitatively estimated. The quantitative estimates for the rest of the milk fat triglycerides were limited to much more complex glyceride groups. The results confirm the earlier claim that butyric and caproic acids occur in milk fat almost exclusively in combination with medium and long chain fatty acids.

INTRODUCTION

RUMINANT MILK FATS are characterized by a high proportion of short chain fatty acids which account for the finding of large quantities of triglycerides with 26-44 acyl carbons (1). Gross analysis of butterfat triglycerides have shown (2,3) that the short chain acids in these glycerides are distributed in a nonrandom manner, with only one short chain fatty acid per triglyceride molecule. Furthermore, studies of the intramolecular structure of milk fat triglycerides by gas chromatography (2,4), thin-layer chromatography (TLC) (5), countercurrent distribution (6) and selective lipase hydrolysis (7,8) have shown that the other fatty acids also are specifically esterified in the primary and secondary positions of the glycerol molecule. This suggests that the processes involved in the final assembly of the milk fat glycerides are highly specific. It was therefore felt that a determination of the association of the fatty acids in the individual molecules of the milk fat triglycerides might yield valuable clues to the mechanism of their biosynthesis, as this would allow comparisons with the glyceride structure of potential phosphorylated precursors in the mammary gland.

In the present investigation the intraglyceride association of fatty acids was determined in some 38 triglyceride types accounting for about 28% of total milk fat and about 77% of total milk fat butyrate.

MATERIALS AND METHODS

Reagents and Standards

All chemicals were of reagent grade quality and were used without purification except as indicated below. Petroleum ether (bp 30-60C) was redistilled and the fraction which boiled between 40-50C was collected and used as solvent for lipid samples. Silica Gel G (Merck & Co.) was washed with methanol before use. Adsorbosil-3 (Applied Science Laboratories, Inc., State College, Pa.) was washed with chloroform-methanol-acetic acid (2:1:1, v/v-/v) and methanol. The fluorescent dye, 2',7'-dichlorofluorescein, was purified by dissolving it (0.4 g) in 50% methanol (400 ml) and extracting the solution with hexane (3 × 100 ml).

Methyl esters of standard fatty acids (Mixtures A,B,C,D,E and F) were supplied by the National Heart Institute (Bethesda, Md.). High purity (99%) monoacid triglycerides (tributyrin through tristearin) were purchased from Applied Science Laboratories. A randomized mixture of equal parts of trimyristin and triolein was a gift from F. H. Mattson (Procter & Gamble Co., Cincinnatti, Ohio). It contained triglycerides with 0,1,2 and 3 double bonds per molecule.

Samples

Raw milk was obtained from Holstein cows from a local farm. The milk sample which was analyzed in detail was collected from one cow 1 month after parturition. The milk fat triglycerides were isolated from the milk fat globules (9) by extraction with chloroform-methanol (2:1, v/v). The extract was taken to dryness in a rotary evaporator (40C) and the residue redissolved in chloroform and stored at 2C.

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

TLC

Silicic acid TLC was used to purify all lipid samples and to separate milk triglycerides into groups of short, medium and long chain lengths. For this purpose plates of Adsorbosil-3 (20 × 20 cm, 250 or 500 μ thick) were prepared by standard methods (10) using Desaga equipment (C. Desaga, G. M. B. H., Heidelberg, Germany). Approximately 12-15 mg of milk fat, in 4 ml of petroleum ether, was applied as a narrow band about 2.5 cm from the edge to two plates at a time by means of the Desaga automatic sample applicator. The plates were developed in heptane-isopropyl ether-glacial acetic acid (60:40:4, v/v/v). Lipid bands were located by spraying the plate with a 0.05% solution of dichlorofluorescein in 50% aqueous methanol and viewing it under UV light.

In the preliminary resolution of the milk fat triglycerides, the location of the bands corresponding to long, medium and short chain lengths respectively were marked, and the silica gel of each area was immediately scraped off the plate, collected into glass-stoppered Erlenmeyer flasks, and covered with a solution of 5% methanol in diethyl ether. Usually 6 plates were prepared at a time in order to obtain sufficient material for further analysis. After filtration of the suspension, the filtrate was evaporated to dryness and the residue taken up in chloroform and stored at 2C until required. Aliquots were then taken for GLC of triglycerides, fatty acids, and for further resolution by argentation TLC. The proportions of the short, medium and long chain triglycerides in the milk fat were determined by GLC of these fractions in the presence of trioctanoin (about 10% of the total lipid) as an internal standard.

For argentation TLC the plates were prepared with Silica Gel G containing 10% silver nitrate. The plates were allowed to dry in the dark for 5 hr prior to activation at 120C for 40 min. Immediately after cooling, 5-10 mg of purified triglyceride was applied to each plate with the automatic applicator. The randomized mixture of trimyristin and triolein was applied near the edge of each plate as a marker. The plates were developed with 0.65% (v/v) methanol in chloroform. The bands were located and the lipids recovered as above. The triglycerides of corresponding degree of unsaturation were pooled, internal standards added, and aliquots taken for triglyceride and fatty acid analysis. The accuracy of the method was determined by analyzing the randomized mixture of trimyristin and triolein in conjunction with the methods described in the next section.

GLC

The analytical GLC of triglycerides was performed under the general conditions previously described (11). Only those columns which gave complete recoveries of all triglycerides through tristearin were used. The temperature programs are given in the figures.

Preparative GLC separations of triglycerides were done using a specially modified Aerograph Autoprep 700 (Wilkens Instrument and Research, Walnut Creek, Calif.) as reported (12). For the present study the injector was modified to allow on-packing application of the sample, and the Powerstat was replaced with a linear temperature programmer (Model 240, F & M Scientific Corp., Avondale, Pa.). The operation conditions are given in the figure. Approximately 10-15 repeat injections (0.5 mg/5 μl each injection) were required to obtain sufficient sample (1-10 mg) for further analysis. The samples were removed from the glass wool in the collection vial by washing with petroleum ether. They were rechromatographed on an analytical GLC column to determine purity. Fractions of less than 95% purity were recollected to decrease crosscontamination, except when insufficient material was available.

The fatty acids of all triglyceride samples were determined by GLC of their butyl esters (1), using an F & M high efficiency gas chromatograph equipped with dual glass columns (4 ft x ¼ in O.D.) containing 15% diethylene glycol succinate (DEGS) on 60-80 mesh Gas-Chrom P (Applied Science Laboratories). Samples containing C_4-C_{18} fatty acids were determined by temperature programming from 70-220C at 4C/min. Long chain fatty acids were determined isothermally at 200C. The instrument was calibrated with mixtures of standard butyl esters prepared by transbutylation of known amounts of high purity triglycerides (tributyrin through tristearin). Values obtained from duplicate analyses showed a relative error of less than 3% for any peak comprising more than 5% of the sample, and less than 6% for any peak comprising less than 5% of the sample. The fatty acids were identified on the basis of retention time and cochromatography with known standards, as well as from their behaviour on argentation TLC.

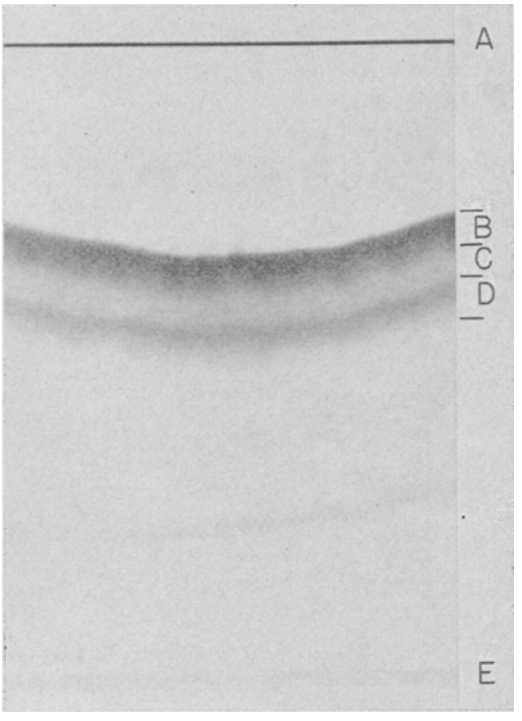

FIG 1. TLC of Bovine Milk Fat Triglycerides. *A*, solvent front; *B*, long chain triglycerides; *C*, medium chain triglycerides; *D*, short chain triglycerides; *E*, origin. Chromatography conditions as given in the text.

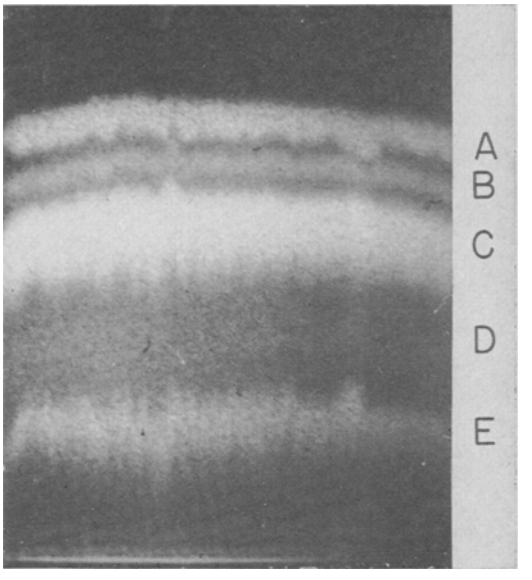

FIG. 3. $AgNO_3$-TLC of Short Chain Triglycerides. *A*, saturates; *B*, monoenes containing elaidic acid; *C*, monoenes containing oleic acid; *D*, dienes containing elaidic acid; *E*, dienes containing oleic acid. Chromatography conditions as given in text.

RESULTS AND DISCUSSION

Preliminary Resolution

Optimum resolution of triglycerides on thin-layer plates of silicic acid impregnated with silver nitrate can be obtained only with mixtures of unsaturated glycerides of comparable chain length and polarity. Since the triglycerides of bovine milk fat vary greatly in molecular weight and polarity, it was necessary to effect a preliminary segregation of the sample on the basis of chain length on conventional silica gel plates (see also Blank and Privett, ref. 5). In the present study three fractions (Fig. 1) were obtained and were designated

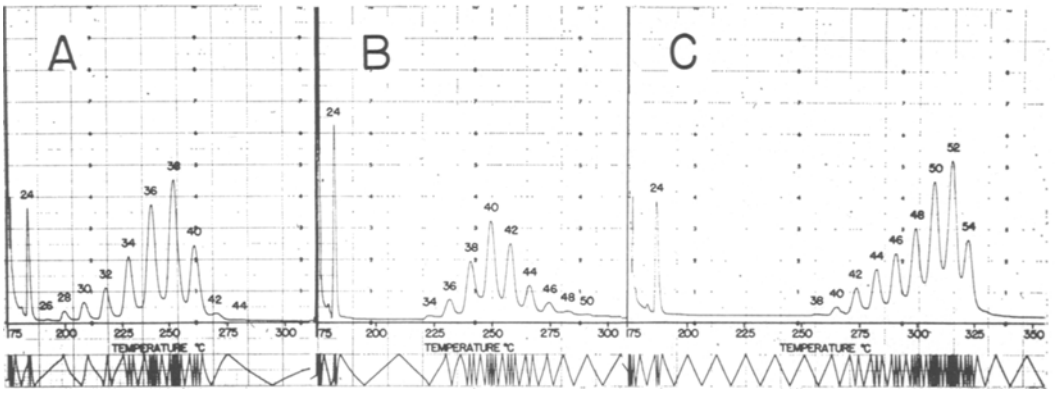

FIG. 2. GLC of Short, Medium and Long Chain Length Triglycerides of Bovine Milk Fat. *A*, short chain triglycerides; *B*, medium chain triglycerides; *C*, long chain triglycerides. Peak 24, trioctanoin used as internal standard. Temperature program as shown. Other operating conditions as stated in the text.

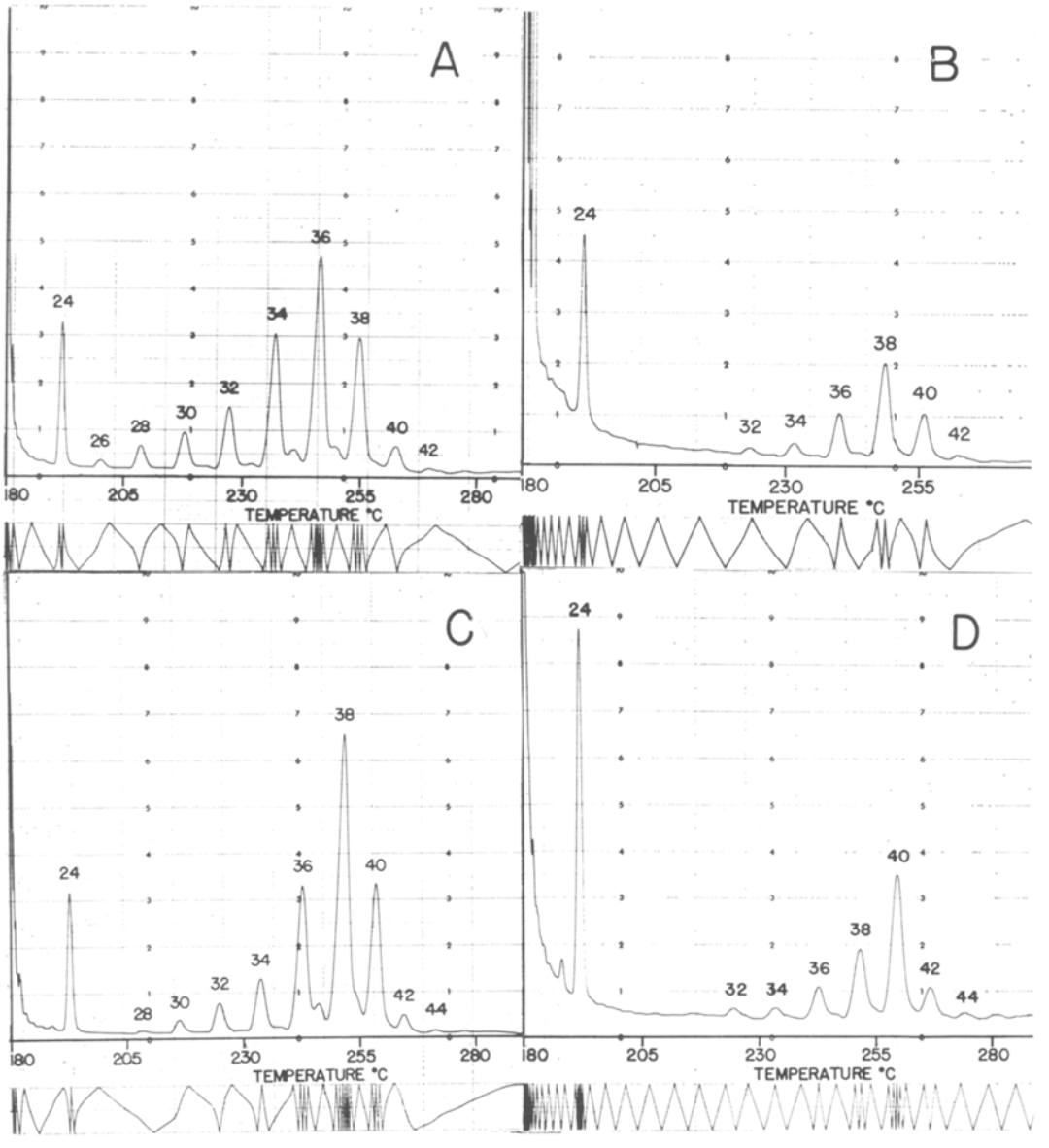

FIG. 4. GLC of Short Chain Triglycerides of Uniform Degree of Unsaturation. *A*, saturates; *B*, monoenes containing elaidic acid; *C*, monoenes containing oleic acid; *D*, dienes containing elaidic acid. Temperature program as shown. Other operating conditions as stated in text. Peak 24, trioctanoin used as internal standard.

short (SCT), medium (MCT) and long (LCT) chain triglycerides. On repetitive separations some variability was encountered but it did not impair the reproducibility of the method. The short chain triglycerides comprised 43.0-44.6% of the total triglycerides while the medium chain triglycerides accounted for only 16.9-17.3% of the original milk fat, the long chain triglycerides contributing the rest. Fig. 2 shows the molecular weight distribution of the three glyceride groups. The short and medium chain glycerides are considered in this paper while the long chain glycerides are discussed in a subsequent report (13).

Combined TLC-GLC Analysis of SCT

Fig. 3 is an example of the $AgNO_3$-TLC resolution of the short chain triglyceride frac-

TABLE I

Composition of Short Chain Triglycerides of Bovine Milk Fat

TG[a]	Saturates[b]		Monoenes[b]		Dienes[b]		Trienes[b]		Recovery[c]	Original[d]
	% band	% total	% band	% total	% band	% total	% band	% total	% total	% total
26	0.7	0.3	—	—	—	—	—	—	0.3	0.5
28	2.7	1.2	0.1	0.1	—	—	—	—	1.3	1.6
30	5.0	2.3	1.4	0.5	—	—	—	—	2.8	2.6
31	0.3	0.1	—	—	—	—	—	—	0.1	0.3
32	8.8	4.0	4.0	1.5	1.5	0.2	2.2	0.1	5.8	5.6
33	0.9	0.4	—	—	—	—	—	—	0.4	0.5
34	19.0	8.6	7.1	2.7	2.2	0.3	3.0	0.2	11.8	11.8
35	3.2	1.4	0.7	0.3	—	—	—	—	1.7	1.7
36	30.2	13.6	19.0	7.2	7.2	0.8	7.3	0.4	22.0	22.5
37	3.2	1.4	3.3	1.3	0.5	0.1	—	—	2.8	2.8
38	20.0	9.0	39.7	15.1	21.3	2.5	17.2	0.9	27.5	27.4
39	0.9	0.4	2.6	1.0	1.8	0.2	—	—	1.6	1.4
40	4.5	2.0	18.9	7.2	50.0	5.8	43.0	2.2	17.2	16.9
41	—	—	0.8	0.3	—	—	—	—	0.3	0.3
42	0.6	0.3	2.2	0.8	11.4	1.3	17.4	0.9	3.3	3.1
44	—	—	0.2	0.1	3.4	0.4	3.9	0.2	0.7	0.6
46	—	—	—	—	0.7	0.1	1.0	0.1	0.2	0.4
48	—	—	—	—	—	—	0.6	—	—	—
50	—	—	—	—	—	—	0.8	—	—	—
52	—	—	—	—	—	—	1.7	0.1	0.1	—
54	—	—	—	—	—	—	1.9	0.1	0.1	—
	100.0	45.0	100.0	38.1	100.0	11.7	100.0	5.2	100.0	100.0

[a] TG = Triglycerides identified by number of acyl carbon atoms per molecule.

[b] Saturates, monoenes, dienes, trienes = Triglyceride types containing 0, 1, 2, 3 double bonds per triglyceride molecule.

[c] Recovery = value obtained by proportional summation of the triglyceride types differing in degree of saturation.

[d] Original = value obtained for the sample before fractionation.

TABLE II

Fatty Acid Composition of Short Chain Triglycerides of Bovine Milk

FA[a]	Saturates[b]		Monoenes[b]		Dienes[b]		Trienes[b]		Recovery[c]	Original[d]
	% band	% total	% band	% total	% band	% total	% band	% total	% total	% total
4:0	18.9	8.5	20.0	7.7	16.8	2.0	15.0	0.7	18.9	19.1
6:0	7.1	3.2	6.3	2.4	7.4	0.9	7.2	0.4	6.9	6.2
8:0	2.6	1.1	1.9	0.7	1.3	0.2	1.4	0.1	2.1	2.0
10:0	4.4	2.0	2.4	0.9	1.2	0.1	1.6	0.1	3.1	2.9
12:0	5.0	2.3	3.0	1.1	1.3	0.2	2.2	0.1	3.7	3.4
14:0	14.7	6.6	7.7	2.9	3.4	0.4	4.9	0.3	10.2	10.2
14:1	—	—	—	—	0.3	0.0	1.7	0.1	0.1	0.2
15:0[e]	1.7	0.8	1.8	0.7	0.3	0.0	0.4	0.0	1.5	1.7
16:0	32.1	14.4	17.3	6.6	7.2	0.8	10.4	0.5	22.3	23.5
16:1	—	—	2.8	1.1	7.9	0.9	5.1	0.3	2.3	2.3
17:0[e]	1.0	0.5	0.8	0.3	0.8	0.1	—	—	0.9	1.2
18:0	12.5	5.6	4.8	1.8	2.4	0.3	2.8	0.1	7.8	9.1
18:1		—	31.2	11.9	34.4	4.0	22.7	1.2	17.1	16.3
18:2	—	—	—	—	15.3	1.8	15.2	0.8	2.6	1.6
18:3	—	—	—	—	—	—	9.4	0.5	0.5	0.3
	100.0	45.0	100.0	38.1	100.0	11.7	100.0	5.2	100.0	100.0

[a] FA = Fatty acids identified by the number of carbon atoms and double bonds in the fatty acid residue.

[b] Saturates, monoenes, dienes, trienes = Triglyceride types with 0, 1, 2, 3 double bonds per glyceride molecule.

[c] Recovery = Values obtained by proportional summation of the fatty acid composition of the triglyceride types differing in degree of saturation.

[d] Original = Values obtained for the fatty acid composition of the sample before fractionation.

[e] Consists of normal and iso-branched acids.

TABLE III
Composition of Medium Chain Triglycerides of Bovine Milk

TG[a]	Saturates[b]		Monoenes[b]		Dienes[b]		Trienes[b]		Recovery[c]	Original[d]
	% band	% total	% band	% total	% band	% total	% band	% total	% total	% total
34	1.3	0.5	—	—	—	—	—	—	0.5	0.4
36	6.8	2.6	1.7	0.7	—	—	—	—	3.3	3.8
37	1.3	0.5	—	—	—	—	—	—	0.5	0.4
38	22.1	8.5	9.2	3.5	2.8	0.4	—	—	12.4	13.3
39	2.9	1.1	1.8	0.7	0.3	0.0	—	—	1.8	1.0
40	30.1	11.7	28.3	10.8	9.8	1.4	3.3	0.3	24.2	25.0
41	3.8	1.4	3.3	1.3	0.6	0.1	—	—	2.8	2.0
42	19.2	7.4	27.1	10.4	35.0	5.0	16.4	1.4	24.2	23.0
43	1.3	0.5	1.8	0.6	1.0	0.2	—	—	1.3	1.6
44	7.8	3.0	15.1	5.8	25.2	3.6	22.8	2.0	14.4	13.3
45	0.7	0.3	0.8	0.3	0.4	0.1	—	—	0.7	0.8
46	2.2	0.8	6.2	2.4	12.6	1.8	22.3	2.0	7.0	7.4
47	0.0	0.0	0.5	0.1	0.1	0.0	—	—	0.2	0.5
48	0.5	0.2	2.7	1.1	6.4	0.9	14.7	1.3	3.5	3.1
49	—	—	0.2	0.1	0.1	0.0	—	—	0.1	0.2
50	—	—	0.9	0.3	3.1	0.5	11.5	1.0	1.8	2.2
52	—	—	0.4	0.2	2.1	0.3	6.4	0.5	1.0	1.6
54	—	—	—	—	0.4	0.1	2.6	0.2	0.3	0.5
	100.0		100.0		100.0		100.0			100.0
		38.5		38.3		14.4		8.7	100.0	

[a-d] Footnotes a-d as in Table I.

TABLE IV
Fatty Acid Composition of Medium Chain Triglycerides of Bovine Milk[a]

FA[a]	Saturates[b]		Monoenes[b]		Dienes[b]		Trienes[b]		Recovery[c]	Original[d]
	% band	% total	% band	% total	% band	% total	% band	% total	% total	% total
4:0	10.7	4.1	3.4	1.3	2.0	0.3	—	—	5.7	5.4
6:0	8.4	3.3	12.1	4.6	7.5	1.1	1.5	0.1	9.1	10.1
8:0	4.9	1.9	5.2	2.0	2.5	0.4	1.0	0.1	4.4	5.0
10:0	7.2	2.8	6.8	2.6	3.3	0.5	2.2	0.2	6.1	6.4
12:0	7.8	3.0	4.2	1.6	2.3	0.3	2.5	0.2	5.1	5.2
14:0	15.2	5.9	9.1	3.5	7.9	1.1	4.5	0.4	10.9	9.4
14:1	—	—	—	—	2.0	0.2	2.9	0.3	0.5	0.5
15:0[e]	1.5	0.6	2.5	0.9	0.2	0.0	—	—	1.5	1.3
16:0	30.8	11.9	17.7	6.8	13.8	2.0	20.6	1.8	22.5	22.9
16:1	—	—	3.6	1.4	5.7	0.8	2.6	0.2	2.4	2.5
16:2	—	—	—	—	3.1	0.5	1.3	0.1	0.6	0.5
17:0[e]	1.5	0.6	0.5	0.2	—	—	1.9	0.2	1.0	1.1
18:0	12:0	4.6	5.1	2.0	7.0	1.0	6.4	0.6	8.2	9.7
18:1	—	—	29.8	11.4	33.4	4.8	31.1	2.7	18.9	17.7
18:2	—	—	—	—	9.3	1.3	14.7	1.2	2.5	2.0
18:3	—	—	—	—	—	—	4.7	0.4	0.4	0.3
20:2	—	—	—	—	—	—	2.1	0.2	0.2	Trace
	100.0		100.0		100.0		100.0			100.0
		38.7		38.3		14.3		8.7	100.0	

[a-e] Footnotes a-e as in Table II.

tion. Two bands are obtained for both the monoenes and the dienes, which are due to the presence of small amounts of elaidic acid in these primarily oleic acid containing glycerides. Since oleic and elaidic acids were not readily resolved by the GLC of their butyl esters, the two monoene and the two diene bands were pooled for presentation of the data.

The GLC patterns of the triglycerides of the saturates, and the pooled monoenes and dienes are shown in Fig. 4. The segregation of the saturated and unsaturated triglycerides of same carbon number into different glyceride groups, greatly improves the GLC resolution of adjacent even and odd carbon number peaks, thereby allowing a much more accurate

estimate of the triglycerides of odd carbon number than is possible by GLC of total milk fat triglycerides.

The composition of the triglycerides and fatty acids of the various classes of saturation in the short chain fraction are presented in Tables I and II, respectively. The mole percent composition of the reconstituted triglycerides is very similar to the molar composition derived from a GLC examination of the total short chain fraction which indicates that no selective losses occurred during the silver nitrate TLC. Table II shows an equally complete reconstitution of the fatty acids.

In the saturates, which comprise 45.0% of the short chain fraction, the major fatty acids are butyric (18.9%), myristic (14.7%) and palmitic (32.1%), while the major triglyceride type is C_{36} (30.3%). This suggests that the mixed triglyceride containing butyric, myristic and palmitic acid is the major glyceride, however, large amounts of triglycerides containing one butyric acid in other combinations with myristic, palmitic and stearic acid also occurred. In the monoenes, which make up 38.1% of this fraction, the major acids are butyric (20.0%), palmitic (17.3%) and oleic (31.2%), while C_{36} (39.7%) is the major triglyceride type. Hence the major triglyceride must be made up of the butyric, palmitic and oleic acids, but the glycerides containing butyric and oleic acids in combination with myristic (C_{36}) or stearic (C_{40}) must also be significant. In the dienes, 11.7% of the total fraction, the major acids are butyric (16.8%), oleic (34.4%) and linoleic (15.3%) acids. Because of the requirement of two double bonds per triglyceride, the major triglycerides must be made up of two saturated fatty acids of a total of 20 acyl carbons and linoleic acid (C_{38}) and two oleic or one oleic and one palmitoleic acid in combination with butyric acid (C_{40}). The material remaining at the origin of the TLC plate accounted for only a minor proportion of the short chain fraction (5.2%). It contained 15.2% linoleic and 9.4% linolenic acids which must have occurred in combination with one unsaturated or two saturated fatty acids, respectively, to give major triglycerides with 36, 38 and 40 acyl carbon atoms. More exact identification of the triglycerides required the isolation of the glycerides of uniform degree of unsaturation and equal carbon number by preparative GLC.

Combined TLC-GLC Analysis of MCT

The medium chain length triglycerides were also resolved into four main fractions by means of TLC on silicic acid impregnated with silver nitrate. The mole percent composition of the glycerides of each TLC band is given in Table III, while the fatty acid data are presented in Table IV. The saturates, 38.5% of the total fraction, comprise the shorter chain glycerides of this fraction. The saturated components also contain the largest proportion of butyric acid (10.7%) of any of the triglyceride types in the medium chain length fraction. With an increase in unsaturation there is an increase in the chain length of the major triglycerides, as already noted for the short chain fraction. The monoenes (38.3%) were obtained in high purity, but the trienes (8.7%) were partly contaminated with dienes, which comprised a proportion (14.3%) intermediate between the monoenes and the trienes. The partial contamination of the trienes by dienes in this fraction was apparently due to the presence of butyric acid residues in these glycerides, which resulted in a somewhat stronger adsorption to the gel and a less effective differentiation between these unsaturates on the silver nitrate plates. Despite this slight imperfection in the separation, an excellent agreement was obtained between the values reconstituted from the separated components of the fractions and those obtained by the analysis of the total sample.

Preparative GLC Analysis

The combined TLC-GLC approach gives estimates for triglycerides of uniform molecular weight and degree of unsaturation. It does not permit the estimation of the amount of individual triglyceride types. This can be done by isolation of triglycerides of uniform molecular weight and identical degree of unsaturation by preparative GLC and the determination of the component fatty acids. Fig. 5 shows the preparative GLC resolution of the saturates and monoenes of the short chain triglyceride fraction. Triglycerides of carbon number greater than C_{42} could not be resolved with the present system. As shown by rechromatography on an analytical column, the peaks of the even carbon number were about 95% pure, but the odd carbon number peaks were only about 40% enriched. The latter triglycerides were collected from the effluent vented between the adjacent even carbon number peaks present in much higher concentration.

The fatty acid composition of the triglycerides recovered from preparative GLC is given in Table V. Table VI shows the empirical estimates for specific triglycerides calculated from the fatty acid data and the known nature

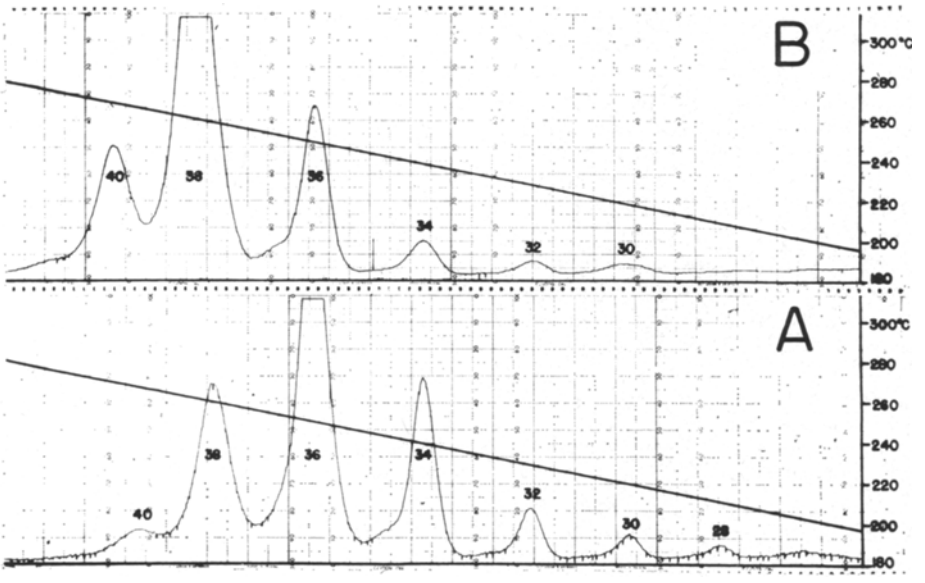

FIG. 5. Preparative GLC of Short Chain Triglycerides of Uniform Degree of Unsaturation. *A*, saturates; *B*, monoenes. Temperature program as shown. Instrument, Aerograph Autoprep 700 with flame ionization kit and splitter assembly. Column, 2 ft × 1/4 in. O.D. aluminum tube packed with 3% (w/w) JXR on silanized Chromosorb W (60-80 mesh). Nitrogen flow rate, 200 ml/min, 30 psi head pressure. Injector, 325C; detector, 330C. L & N 1-mv recorder with a 6 in. scale and signal switch to activate fraction collector. Sample, 50 μl of a 20% (w/v) solution of triglyceride in petroleum. Split ratio 5:1.

of segregation. The amounts of the individual odd carbon number triglycerides were obtained by subtracting the estimates for the even carbon number triglycerides present in the incompletely resolved mixture. The triglyceride types thus identified usually were still complex but the present techniques did not allow further resolution.

In the C_{34}-C_{40} triglycerides of the saturates and the C_{38}-C_{40} triglycerides of the monoenes, the major types of triglycerides consist of combinations of one short chain and two long chain acids. Thus, the major C_{54} component of the saturates is 16,14,4 (83%), of C_{56} it is 16,16,4 (77%), and of C_{58} it is 16,18,4 (70%) with considerable contribution from 16,16,6 (17%). In the monoenes, the C_{58} peak contains mostly 18:1,16,4 (87%), while

TABLE V

Fatty Acid Composition of Triglycerides of Uniform Molecular Weight and Degree of Saturation

	Saturates						Monoenes		
FA[a]	C32	C34	C35	C36	C37	C38	C37	C38	C40
4:0	22.1	31.6	29.0	29.5	26.4	23.4	27.6	29.9	21.3
6:0	8.5	2.3	3.5	1.2	3.8	5.7	3.0	2.0	10.0
8:0	3.0	—	0.4	0.4	—	3.6	—	0.7	0.6
10:0	14.0	—	—	—	—	0.5	1.6	0.1	0.7
12:0	11.7	5.0	1.1	0.6	1.6	0.5	1.4	0.6	0.7
14:0	16.0	26.0	12.1	7.2	8.4	3.9	3.7	2.1	2.1
15:0[b]	—	—	6.6	—	2.9	—	8.4	—	—
16:0	20.7	32.1	40.2	55.1	42.1	39.0	16.0	30.0	10.0
16:1	—	—	—	—	—	—	5.2	1.7	2.4
17:0[b]	—	—	2.8	—	3.2	—	2.9	—	—
18:0	4.0	3.0	4.3	6.0	11.6	23.4	2.7	1.7	21.3
18:1	—	—	—	—	—	—	27.5	31.2	30.9
	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

[a] FA = Number of carbon atoms and double bonds in the fatty acid residue.

[b] Consists of normal and iso-branched acids.

TABLE VI
Estimates of Specific Triglyceride Types[a]

Saturates (16.9% total)			
C32 (1.8% total)		C34 (3.8% total)	
18,10,4	12	18.12.4 =	10
16,12,4	30	16.14,4 =	83
16.10,6	25	16,12,6 =	7
16,8.8	55		100%
14,14,4	24		
10,10,12	3		
	100%		
C35 (0.6% total)		C36 (6.1% total)	
17,14,4	21	18.14.4	18
17,12,6	9	16.16,4	77
15,16,4	51	16,14,6	3
15,14.6	19	16,12,8	2
	100%		100%
C37 (0.6% total)		C38 (4.0% total)	
18,15,4	} 48	18,16,4	70
16,15.6		16.16,6	17
16.17.4	} 52	16.14,8	12
14,17.6		16,12,10	1
	100%		100%
Monoenes (10.6% total)			
C38 (6.8% total)		C37 (0.6% total)	
18:1,16.4	87	18:1 15.4	74
16:1,18.6	5	16:1.17,4	26
18:1,14,6	6	16:1.15.6	Trace
18:1,12,8	2		100%
	100%		
C40 (3.2% total)			
18:1,18,4	62		
18:1,16.6	26		
16:1,18,6	3		
18:1,14,8	3		
18:1,12,10	2		
16:1,14,10	2		
	100%		

[a]Experimental estimates derived from the fatty acid composition of the triglycerides which were recovered from preparative GLC. The order of the carbon numbers of the fatty acids in the triglycerides does not indicate a specific positional distribution.

the C_{40} peak contains 18:1,18,4 (62%), and 18:1,16,6 (26%). Of the minor peaks, of particular interest are the C_{38} triglycerides of the saturates, which contain large amounts of medium chain length fatty acids, C_{10}, C_{12}, and C_{14} (40.7%). It is seen that these triglycerides contain one short chain acid (C_4, C_6, or C_8) in combination with one medium chain length acid (C_{10}, C_{12}, C_{14}) and one long chain acid (C_{16} or C_{18}). This triglyceride peak also contained significant amounts (24%) of the 14,14,4 triglyceride.

The triglycerides of odd carbon number (C_{25}-C_{37}) were estimated to contain one long chain acid (C_{16} or C_{18}) and one short chain acid (C_4 or C_6) in combination with one odd carbon number fatty acid. In almost all of the isolated triglycerides of odd carbon number there were both pentadecanoic and heptadecanoic acids present. No even carbon number triglycerides containing two odd carbon number and one even carbon number fatty acids were found.

There are no data in the literature on the quantitative composition of such small groups of specific milk fat triglycerides as those analyzed in this study. Previous data from the GLC of the molecular distillates of butteroil indicated that some glycerides containing two short chain acids might occur but no dibutyryl glycerides would be possible (2, 3). On the basis of the proportion of the lowest molecular weight triglycerides detected by GLC (C_{26}-C_{30}) it can be estimated that no more than 0.5% of the total milk fat triglycerides could contain two C_4-C_8 fatty acids in the same triglyceride molecule. A random calculation would predict about ten times as much (6%). Trace amounts of short chain triglycerides containing two short chain fatty acids per glyceride have been isolated by liquid-liquid partition chromatography from milk serum (14).

The finding of significant amounts of the 16,16,4 triglyceride in the milk fat in the present study would appear to be in contradiction to the report of Pynadath and Kumar (15) who implied that such triglycerides might not occur. These authors claimed that the enzymes which govern the incorporation of short chain fatty acids into milk fat could not form this triglyceride to any detectable extent. The claim was based on the observation that butyryl CoA was not incorporated into 1,2-dipalmitin while palmitoyl CoA was readily taken up to form tripalmitin.

Mechanism of Biosynthesis

The observed characteristic association of the fatty acids in the milk fat triglycerides is significant in view of the proposal of Patton et al. (16) that glycerophosphatides might serve as intermediates in the biosynthesis of milk triglycerides. If indeed glyceryl phosphatides, such as lecithins, for example, do serve as the immediate precursors of milk fat triglycerides, then only one short or medium chain length fatty acid should occur per glyceride molecule, as demonstrated for the bulk of the short and medium chain length triglycerides in the present study, and these short chain acids should be confined to the 3 position of the glycerides as shown elsewhere (17, 18). Furthermore, a significant proportion of

the precursor glyceryl phosphatide must be disaturated, since about 40% of the short and medium chain triglycerides are trisaturated. Any monoenoic phosphatides serving as precursors should then contain a series of molecular species made up of the monoenoic acids in combination with saturated long chain acids in the proportion in which the latter occur in the short and medium chain triglycerides. The various species of these monoenoic glyceryl phosphatides must then have been combined with the short chain fatty acids in the proportion in which the latter occur in the analyzed triglyceride fractions. While the exact molecular species of any of the milk or mammary gland lecithins have not been determined, Nutter and Privett (19) have shown that disaturated lecithins of bovine milk serum comprise about 10% of the total lecithin, while the monounsaturated and diunsaturated species make up 44% and 27%, respectively. The proportions of these classes of lecithin would be expected to be about the same since the lecithins of the milk fat globule membrane possess a comparable composition of fatty acids (9). Assuming that the lecithins of the mammary gland, which participate in this hypothetical mechanism of milk fat biosynthesis, are not unlike those previously studied, it may be concluded that the results of the present study do not exclude the possibility that lecithins are the immediate precursors of the short and medium chain length triglycerides of milk fat.

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Sterol Metabolism. IV. Microbial Disposition of 5β-Cholestan-3β-ol

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ABSTRACT

Cholesterol and 5β-cholestan-3β-ol have been detected by thin-layer and gas-chromatographic means in hexane extracts of domestic sewage. Cholesterol was detected in the clear effluent after activated sludge treatment of domestic sewage, but 5β-cholestan-3β-ol was undetectable in treated sewage. Attempts to detect either sterol in hexane extracts of marine bay water have not been successful because of the interference of other lipid-like material.

INTRODUCTION

THE POSSIBILITIES of using saturated sterols, specifically 5β-cholestan-3β-ol, as a molecular marker of domestic sewage contamination of fresh water resources and of marine waters appealed to the authors. Although unsaturated sterols of diverse structure and 5α-stanols occur in tissue of multicellular life, 5β-stanols have not been found in tissues but appear to be formed exclusively by intestinal microfloral reduction of unsaturated sterols in mammalian metabolism.

Our earlier studies (1) and those of Jeffrey (2) suggest the presence of the unsaturated sterols, cholesterol, stigmasterol, and β-sitosterol, in sea water at levels close to the water solubility of cholesterol. The origin of these sterols and their role in the ecology of the sea are uncertain, though of interest for speculation and conjecture. However, no evidence is known which supports the presence of either 5α-stanols or 5β-stanols in natural waters.

Although the *trans* stanol 5α-cholestan-3β-ol is separated from parent cholesterol only with difficulty (silver nitrate-treated chromatoplates), the *cis*-isomer 5β-cholestan-3β-ol is readily resolved from cholesterol and other related unsaturated C_{27}-, C_{28}-, and C_{29}-sterols by simple thin-layer chromatographic means. These several considerations of the uniqueness of the 5β-stanols in nature, together with their ease of detection, prompted the present preliminary studies of 5β-cholestan-3β-ol metabolism.

EXPERIMENTAL PROCEDURES

All solvents used in this work were the manufacturer's analytical reagent grade and were redistilled shortly before use. All water samples were collected and handled in lipid-free glassware; there was no contact with plastic ware. Evaporation of solvents was done on an all-glass rotary evaporator unit under diminished pressure. Infrared absorption spectra were recorded on 1.5-mm potassium bromide disks containing sample by using a Perkin-Elmer Model 337 infrared spectrophotometer, equipped with a beam condenser. The reference sterols, cholesterol and 5β-cholestan-3β-ol, were commercial samples recrystallized to a state of high purity as evinced by melting-point, spectral, and chromatographic criteria.

Water Samples

Three different water-sample types were taken: a) raw sewage—samples (14 liters) were taken from the City of Galveston main plant and were subjected only to bar screening and grit removal prior to sampling; b) treated sewage—clear effluent samples (14 liters), rated as potable water, were taken after complete activated sludge treatment; c) bay water—samples (4 liters and 14 liters) were taken in the vicinity of the sewer plant outfall at distances of 50-2,000 m.

All water collections were made in lipid-free glass bottles over a period of six months. Ten separate collections were made for each of the three water-sample types. Each water sample was extracted directly by stirring vigorously with 4 liters (for 14-liter samples; for 4-liter bay-water samples, 2 liters of hexane were used) of redistilled hexane for 15 min. After phase separation a second and a third 4-liter hexane extract was made. Each hexane extract was evaporated under vacuum in an all-glass rotary evaporator unit, transferred to weighed vials with chloroform-methanol (7:1), and evaporated under nitrogen for sample weight. The lipid-extractable residues from each water sample were examined chromatographically and combined in 1-5 ml of chloroform-methanol (7:1) for deep-freeze storage awaiting further analysis.

Water-sample stirring was done with a 5-cm stainless steel propeller at a speed which gave thorough mixing of the liquids, with a deep vortex. Control extractions which were run as long as eight hours did not afford appreciable additional lipid-extractable material over that recovered after 15 min.

Thin-Layer Chromatography

Thin-layer chromatography (TLC) was conducted as previously described (3,4) on 5 × 20 and 20 × 20 cm chromatoplates of Silica Gel HF_{254} (E. Merck, Darmstadt) 0.25-mm thick, irrigated with benzene-ethyl acetate (2:1) principally; but the solvent systems acetone-heptane (1:1) and ethyl acetate-heptane (1:1) were also used as previously described (3). Developed chromatoplates were examined under ultraviolet (254 and 366 nm) light prior to visualization by spraying with 50% aqueous sulfuric acid. Full-color display was obtained by warming on an electric hot-plate, after which time the plate was charred to bring out any uncolored organic components.

Preparative TLC was conducted on 20 × 40 cm chromatoplates 1- and 2-mm thick of Silica Gel PF_{254}. Sterol sample solutions were applied by means of the Rodder Streaker (Rodder Instrument Company, Los Altos, Calif.). Sterol zones were detected by their absorption properties under ultraviolet light and by spraying a 1-cm end-portion of the chromatoplate with sulfuric acid and interpolation of zones from these findings.

Gas Chromatography

Gas chromatography was conducted on samples of the free sterols, their acetates, and their trimethylsilyl ethers by using Hewlett-Packard Corporation F and M Models 400 and 402 instruments, equipped with hydrogen flame detectors. Chromatography was performed on 1.83 m × 6 mm silanized glass U-tubes, packed with 3% SE-30 on 80-100 mesh Gas-Chrom Q and with 3% QF-1 on 100-120 mesh Gas-Chrom Q (both from Applied Science Laboratories, State College, Pa.). Injection port temperature was 260C, and column temperature was 230C. Nitrogen carrier gas was used at a flow rate of 20 ml/min.

Free sterols were analyzed as methylene chloride solutions directly. Steryl acetates were prepared by dissolving the sterol (100 μg) in 500 μl of dry pyridine and 300 μl of acetic anhydride. After standing overnight, methanol was added and the solvents were removed under vacuum. The residue was taken up in methanol-toluene several times for further vacuum evaporation, and the dried residue was taken up in methylene chloride for analysis. Trimethylsilyl ethers were prepared by using a commercial mixture of hexamethyldisilazane and trimethylchlorosilane in dimethylformamide (Tri-Sil DMF, Pierce Chemical Company, Rockford, Ill.). The sterol solution was held at room temperature until reaction was complete (5-60 min) and injected into the gas-chromatogram column for analysis.

Sterol Isolation

For purposes of detailed identification studies on the sterols detected in each individual raw and treated sewage sample, the 10 raw-sewage sample lipid-extract residues were combined, as were the lipids from the treated clear effluent extractions. The combined lipids from both raw and treated sewage were processed alike throughout the following chromatographic steps. The lipid sample was chromatographed on 70 g of silica gel, with elution by hexane (500 ml), 1% ethyl acetate in hexane (250 ml), 5% ethyl acetate in hexane (250 ml), 10% ethyl acetate in hexane (250 ml), 50% ethyl acetate in hexane (250 ml), and finally 50% ethyl acetate in methanol (250 ml). Each fraction was evaporated for TLC analysis, and those containing sterols (1-50% ethyl acetate in hexane) were combined and rechromatographed on 10 g of silica gel, taking 500 ml of hexane, 300 ml of 1% ethyl acetate in hexane, 300 ml of 5% ethyl acetate in hexane, 300 ml of 30% ethyl acetate in hexane, and 300 ml of 50% ethyl acetate in methanol cuts. The 1% ethyl acetate in hexane cuts containing 5β-cholestan-3β-ol were evaporated and chromatographed on a 2-mm chromatoplate with benzene-ethyl acetate (2:1). The saturated sterol zone was scraped off, packed in a small column, and eluted with 50% ethyl acetate in hexane, thus affording a saturated sterol fraction, the gas-chromatographic analysis of which as the free sterol, the steryl acetate, and the steryl trimethylsilyl ether established that 5β-cholestan-3β-ol was the major sterol present.

Other unidentified trace components were detected on gas chromatography, but none were of the same relative amount as was 5β-cholestan-3β-ol. Infrared spectra on the saturated sterol fraction were not of sufficient quality for identification purposes. Approximately 1 mg of the crude saturated sterol fraction was gas-chromatographed on a 6-mm 3% QF-1 column (1.83 m long) by using nitrogen carrier gas flow of 32 ml/min and temperature of flash heater, 250C; of oven, 230C; of detector, 240C; a stream splitter utilized approximately 2% of the effluent stream for flame detection. The unburnt 98% of the effluent stream was directed out of the oven through a silanized glass capillary tube 15-cm long. Sterol condensed in the capillary and was recovered with methylene chloride for infrared spectra. Spectra superimposable on that of authentic 5β-

cholestan-3β-ol were obtained. The gas-chromatographed 5β-cholestan-3β-ol sample could not be induced to crystallize.

Similar treatment of the 5% ethyl acetate in hexane cuts from the adsorption column afforded a purified unsaturated sterol fraction after thin-layer preparative chromatography. The sterol fraction was still impure, but cholesterol crystals, 10.7 mg, mp 121C (Kofler block), were obtained, the infrared absorption spectra of which supported their identification as cholesterol. Gas-chromatographic analysis of the crystalline sterol fraction established that cholesterol was the only sterol present, and gas-chromatographic retention data on the steryl acetate and trimethylsilyl ethers confirmed these findings. Preparative gas-chromatography of approximately 500 μg of the sterol on 6 mm 3% QF-1 columns under the same conditions as used for isolation of 5β-cholestan-3β-ol gave a crystalline cholesterol preparation, mp 138-142C, the infrared spectra of which were superimposable on those of authentic cholesterol. Repetition of the gas-chromatographic isolation gave the same results, and no further attempts were made to purify the cholesterol sample to a higher melting-point. Small amounts of other sterols or of 5β-stanols of C_{28}- and C_{29}-nature may have been present in the gas-chromatographed cholesterol, undetected because of the peak overlap on the 3% QF-1 columns but indicating their presence in the low melting-point of the major sterol cholesterol.

Column chromatography of combined hexane-extractables from bay-water samples, combined with TLC, failed to produce reliable evidence of the presence of either cholesterol or 5β-cholestan-3β-ol.

Control Experiments

Cholesterol was dissolved in water (36 μg in 3.6 liters, 4 μg in 4 liters), and extraction with a half-volume of hexane was made in the usual manner. TLC of the residue from the hexane extracts established that in both cases cholesterol was readily detected. Thus sterol detection at the 1-10 μg/liter concentration in water was reliable.

Hexane extraction of City of Galveston tap water failed to afford any evidences of sterol components, when examined chromatographically, although a weighable residue was obtained (1).

Solid Samples

One liter of recycled activated sludge from the domestic-sewage treatment plant was filtered to remove gross water. The water removed (800 ml) was extracted with hexane. The moist activated sludge was extracted as such with hexane for 40 hr in a Soxhlet extractor. The combined hexane extracts from the sludge and expressed water were evaporated under diminished pressure, and the odorous residue was analyzed on thin-layer chromatoplates (Fig. 1D).

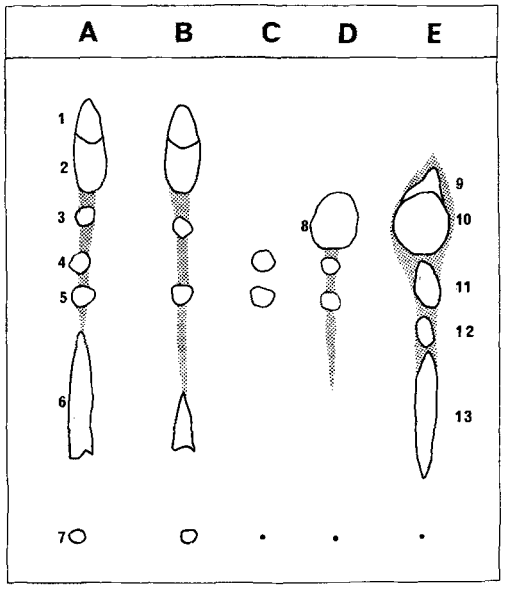

FIG. 1. Thin-layer chromatogram of lipid extractables from A, raw sewage, B, treated sewage; C, reference sterols; D, activated sludge; E, Galveston Bay. Components resolved are catalogued by number, color reaction, and identity: 1, very dark brown, unidentified; 2, brown, unidentified; 3, tan, unidentified; 4, tan, 5β-cholestan-3β-ol; 5, magenta, cholesterol; 6, tan, comet-shaped streak, unidentified; 7, dark brown, immobile components; 8, tan, unidentified; 9, brown, complex unidentified; 10-13, tan, unidentified.

Approximately 1 liter of sediment, dredged up from about 1 m in depth, 50 m from the domestic sewer outfall, was stirred extensively with 2 liters of hexane. The hexane extracts were evaporated under vacuum and analyzed on thin-layer chromatoplates. Many components were detected with 50% sulfuric acid, and no attempt was made to fractionate the sediment extract on the basis of its obvious complexity.

RESULTS

The total lipid-extractable matter from raw domestic sewage, from domestic sewage treated by the activated sludge process, and from bay waters is compared in Table I with similar find-

TABLE I
Total Lipid-Extractables from Waters

No.	Water sample	Total Lipid-Extractables, mg./liter water: Average[a]	Range
1	Raw sewage, Galveston	97.1	30–207
2	Treated sewage, Galveston	9.3	1.0–69
3	Galveston Bay	11.8	3.0–20
4	Gulf of Mexico[b]	21.8	11–32.6
5	Sea water[c]	3.0	0.40–9.10
6	Raw sewage, Topeka and Lawrence, Kans.[d]	122	94–150
7	Treated sewage, Topeka and Lawrence, Kans.[d]	51.5	18–91

[a] Average of 10 water samples reported on herein.

[b] From Matthews and Smith (5). These values are maximum values since some of the lipid weight can be accounted for by dialkyl phthalate esters leached from the plastic collection bottles.

[c] From Jeffrey (2).

[d] From Loehr and Higgins (6).

ings from prior work (5) and from the more extensive results of Jeffrey (2). Some related analyses of Topeka and Lawrence, Kans., sewage (6) are also included. From these gross analyses it may be concluded that domestic-sewage lipids are reduced substantially on treatment with activated sludge. In this work the reduction of total lipids was by a factor of 10.

More pointed work bore on the presence in sewage of the sterols, cholesterol and 5β-cholestan-3β-ol, both well recognized as major fecal sterols in the human body. Demonstration of the presence of cholesterol and 5β-cholestan-3β-ol in raw domestic sewage is based on thin-layer and gas-chromatographic evidence and on infrared spectral data in comparison with authentic sterols. Although analytically pure preparations of either sterol were not made, the combination of chromatographic and spectral data appears to be adequate to support the authors' conclusions. Gas-chromatographic retention data on the free sterols and on their acetates and trimethylsilyl ethers are presented in Table II.

A similar examination of treated-sewage clear effluent failed to give evidence of the presence of 5β-cholestan-3β-ol although the presence of cholesterol was demonstrated by chromatography. The amount of cholesterol detected in the clear effluent was substantially reduced from that of the raw sewage although quantitative data are not offered for this comparison. On the reasonable assumption of small losses of water because of evaporation during the process and of no dilution of sewage with water during treatment, a direct qualitative comparison of thin-layer and gas-chromatographic data on the 10 treated and untreated samples demonstrated uniformly the loss of 5β-cholestan-3β-ol and the reduction in amounts of cholesterol.

Because of the demonstrated loss of 5β-cholestan-3β-ol on activated sludge treatment of domestic sewage, an attempt was made to determine whether the 5β-stanol was removed physically with the activated sludge floc or whether the 5β-stanol was degraded to undetected metabolites during the treatment process. TLC examination of hexane extracts of activated sludge floc gave support to the physical

TABLE II
Chromatographic Mobility and Retention Data

No.	Sterol sample	Thin-Layer Mobility[a]: Sterol[b]	Steryl acetate[c]	Steryl TMS ether[d]	Gas-Chromatographic Relative Retention-Time[e]: Sterol 3% SE-30	Sterol 3% QF-1	Steryl acetate 3% SE-30	Steryl acetate 3% QF-1	Steryl Trimethylsilyl ether 3% SE-30	Steryl Trimethylsilyl ether 3% QF-1
1	Unsaturated sterol from raw sewage	1.00	1.46	1.56	1.01	1.01	1.46	1.59	1.26	0.68
2	Unsaturated sterol from clear effluent	1.00	.....	.....	1.00	1.00	.....	.....	.....	.....
3	Reference cholesterol	1.00	1.46	1.56	1.00	1.00	1.45	1.60	1.25	0.68
4	Saturated sterol from raw sewage	1.16[f]	1.46	1.54	0.90	0.89	1.28	1.45	0.99	0.55
5	Reference 5β-cholestan-3β-ol	1.16[f]	1.46	1.53	0.90	0.88	1.25	1.45	1.00	0.56

[a] Mobility in terms of cholesterol as unity, R_c values.

[b] Free sterols analyzed in benzene-ethyl acetate (2:1) system.

[c] Steryl acetates analyzed in ethyl acetate-hexane (1:1) system.

[d] Steryl trimethylsilyl ether derivatives analyzed in ethyl acetate-hexane (1:1) system.

[e] Relative retention times versus cholesterol as unity.

[f] Also analyzed as the free sterol in the acetone-heptane (1:1) system, R_c 1.06 for sewage sterol and reference 5β-cholestan-3β-ol.

removal of 5β-cholestan-3β-ol by the floc. In Fig. 1D is presented TLC evidence of the presence of both 5β-cholestan-3β-ol and cholesterol in the activated sludge floc. Although no attempt was made to isolate the sterols for a more rigorous identification, it is presently presumed that both fecal sterols are present in activated sludge floc. An active microbial metabolism of these fecal sterols during the treatment and digestion processes cannot be ruled out however.

Attention was next turned to the detection of 5β-cholestan-3β-ol in bay waters. From time to time about half of the domestic sewage load on the disposal plant is by-passed directly into Galveston Bay without any treatment other than bar screening and grit removal. Consequently bay waters adjacent to the sewer outfall must contain 5β-cholestan-3β-ol and cholesterol of fecal origin unless specific removal processes are at play in the bay waters.

Several factors interfere with or militate against detection of these fecal sterols in the relatively small water-samples taken in our studies. Massive dilution effects tend to dissipate fecal sterols to levels which escape present technical means of detection. Additionally luxuriant growth of algae and other microorganisms in the undisturbed areas near the sewer outfall, together with equally luxuriant black organic sediments on the bay bottom in this region, offer means for microbiological and physical removal of the fecal sterols along with other fecal components of raw sewage.

Examination of hexane extracts of bay-bottom muds and sediment from the sewer outfall vicinity failed to afford clear evidence for the presence or absence of the sought sterols. Very complex lipids were obtained from these extracts, and direct TLC analysis was inadequate to the task of definitive resolution of either 5β-cholestan-3β-ol or cholesterol. Similar hexane extracts of bay waters from the sewer outfall area were too complex for direct thin-layer chromatographic analysis. Much of the lipid-like complexity of the samples may be industrial wastes from nearby Texas City. Accordingly preliminary examination of bay muds and waters for fecal sterols is at present inconclusive.

DISCUSSION

As a result of earlier studies of sterols in marine waters (1,5) the authors became intrigued with the possibilities of using 5β-cholestan-3β-ol as a molecular marker of fecal contamination in natural waters. On examining the matter, it was obvious that no useful background to support the concept was available from the literature. Although the presence in domestic sewage of both cholesterol and 5β-cholestan-3β-ol as major fecal sterols of man could be assumed, almost no studies of this simple proposition have been made. The earlier thin-layer chromatographic work of Loehr and Higgins (6) suggested the presence of sterols among other classes of lipids in domestic sewage. However convincing evidence on the matter has not been published.

Demonstration of the presence of cholesterol and 5β-cholestan-3β-ol in domestic sewage enabled the authors to pursue the original thesis. The rationale for seeking to use 5β-cholestan-3β-ol specifically as a molecular marker of fecal contamination in natural waters required that the 5β-stanol, in fact, be present in domestic sewage and that it be removed from domestic sewage by adequate sewage treatment plants. Where these requirements are met, any demonstrated presence of 5β-cholestan-3β-ol in natural waters can be taken as unequivocal evidence of fecal contamination. Were 5β-cholestan-3β-ol to survive modern activated sludge or other treatment processes and pass into natural waters in the clear effluent, the presence of the 5β-stanol in natural waters would obviously not be significant.

The present work established the presence of the C_{27}-sterols cholesterol and 5β-cholestan-3β-ol in domestic sewage. However the recognized presence of C_{28}- and C_{29}-sterols, stigmasterol, campesterol, β-sitosterol, etc., of dietary source and their reduced 5β-stanol analogs in human feces suggests their possible presence in domestic sewage also. Minor components have been encountered in the partially purified, unsaturated sterol and 5β-stanol preparations, the recognition of which as C_{28}- and C_{29}-sterols is not unreasonable. However this point has not been examined for the moment since the more abundant 5β-cholestan-3β-ol serves adequately for the molecular marker purposes.

For reliance on the presence of 5β-stanols as indicators of fecal contamination the likely presence of other 5β-steroids not derived from fecal sources must be considered. The bile acids of higher animal life are obvious sources of 5β-steroids, but these bile acids, if present in water, must also derive from fecal contamination or from decomposing tissue. Other 5β-steroids of urinary excretion origin, while being *cis*-5β-steroids and also indicating sewage contamination, are of such diminished amounts and would require such intricate isolation and analysis methods as to make these 5β-steroids

unlikely of detection in simplified procedures. As a consequence, where detected, 5β-steroids of several types would argue for domestic sewage or offal contamination of natural waters. Were reliable means known for rapid recognition of the *cis*-5β-A/B ring fusion in steroid metabolites in extracts of waters, such means would be ideal for the recognition of domestic sewage contamination also.

ACKNOWLEDGMENTS

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Acetylenic Acid Biosynthesis in *Crepis rubra*

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ABSTRACT

Time studies of crepenynic acid synthesis in *Crepis rubra* show that this acid is not present in the seed for several days after flowering commences but builds up rapidly between the 14th and 28th days to become the major fatty acid of the seed oil.

Radioactive tracer studies clearly demonstrate that the acetylenic bond is introduced into the carbon chain of a preformed long-chain fatty acid rather than built in during formation of the carbon chain. The nearest precursor found is oleic acid. There is no conversion to crepenynic acid by seed preparations of *cis, cis*-linoleic acid, *cis,trans (trans,cis)*-linoleic acid, or *cis*-12,13-epoxy-oleic acid. Possible biosynthetic pathways to explain these results are suggested.

The crepenynic acid is chiefly, but not entirely, in triglycerides in the seed oil, and it has been shown to be esterified in the 2- and 3- positions of the triglyceride.

INTRODUCTION

NATURALLY OCURRING acetylenic fatty acids have been known for many years since the discovery of tariric (6-octadecynoic) acid by Arnaud in 1892 (1), but it is only in the last few years that studies on their biosynthesis have been undertaken. In 1957 Meade (2) was able to state "Nothing is known of the biogenesis of acetylenes, which are much higher energy compounds than the olefins, where the biogenetic routes are almost equally obscure. . . ."

From 1959 onwards, studies by Bu'Lock and his co-workers established that the carbon chains of several acetylenic compounds found in fungi could be derived from acetate, and subsequently the involvement of malonate was also demonstrated (3). This suggested a biosynthetic pathway closely related to that of normal fatty acids and led to the proposal that the triple bond was inserted during formation of the carbon chain. This was thought to occur by omission of the reduction steps of normal fatty acid synthesis, with enolization followed by a concerted decarboxylation and elimination.

The observations on acetate incorporation were extended to higher plants with studies on *Santalum acuminatum*, where ximenynic acid (*trans*-11-octadecen-9-ynoic) and several related acids were shown to be derived from acetate (4). The rates of incorporation of acetate into the various fatty acids suggested the prior synthesis of the normal fatty acids and their subsequent slow conversion to acetylenic acids, presumably by dehydrogenation. This pathway was supported by reports (5,6) that the fungus *Tricholoma grammopodium* was able to convert ^{14}C-oleic acid to ^{14}C-crepenynic acid (*cis*-9-octadecen-12-ynoic). The incorporation of oleate was stated to involve the conversion of an ethylenic bond to an acetylenic bond although this hypothesis was not supported by studies with radioactive linoleic acid.

For our own studies on acetylenic fatty acid biosynthesis the developing seed of *Crepis rubra* L. (Compositae) was chosen. Although the vegetative plant contains no acetylenic acid, the seed synthesizes a large amount (about 60% of total fatty acids at maturity) of only one acetylenic acid, crepenynic, thus providing a simple system for initial investigation. A preliminary account of some of our studies has already been published (7).

MATERIALS AND METHODS

Labeled fatty acids were purchased from the Radiochemical Centre, Amersham, Bucks., England. They were added to incubations as the sodium salt dissolved in distilled water with the aid of ultrasonic oscillation and a small amount of Tween 20.

1-^{14}C-Labeled geometric isomers of linoleic acid were prepared from 1-^{14}C-linoleate by partial isomerization with sodium nitrite and nitric acid (9). The mixed products were separated by argentation-TLC into three fractions: a) all *cis*, b) all *trans*, c) *cis,trans* and *trans,cis*. The latter fraction could not be resolved and, after hydrolysis and suspension in water, was used as such for incubation.

1-^{14}C-*cis*-12,13-Epoxy-*cis*-9-octadecenoic (vernolic) acid was prepared by a micro-modification of the method of Findley et al. (10). 1-^{14}C-Linoleic acid, with unlabeled methyl linoleate as a carrier, was epoxidized with peracetic acid. The carrier methyl esters were separated

from the free, radioactive acids by TLC, and the radioactive acids were then methylated with diazomethane and separated by TLC. Four separate bands were obtained: unchanged linoleate, 12,13-epoxyoleate, 9,10-epoxy-12-octadecenoate, and the di-epoxy derivative. The 12,13-epoxyoleate was eluted and saponified, and the epoxy acid was supended in water and used for incubation purposes.

Seeds of *Crepis rubra* L. were purchased from Thompson and Morgan, seed merchants, of Ipswich, England, and were grown in a greenhouse with cross-pollination of the flowers by hand. For the time studies of fatty acid accumulation in developing seeds, flower heads were labeled with the date on which they first began to open. Labeling was carried out three times a week for five weeks, at the end of which the first labeled seed heads were mature. All seed heads were then harvested at once, and the lipids were extracted and analyzed for fatty acid content and composition. Heptadecanoic acid was added in known amount as the internal standard to enable fatty acid compositions to be expressed in absolute terms as mg/100 seeds.

Incubations were carried out with seeds at different stages of development. The seeds were removed from the receptacle and finely chopped with scissors in the incubation medium. The flasks were plugged with cotton and shaken in a water bath at 25C in air and light. Incubation was terminated by the addition of 10-20 volumes of chloroform-methanol (2:1) and extraction of the lipids, which were transmethylated by refluxing with methanol-benzene-conc. H_2SO_4 (20:10:1) for 1 hr. Radioactive fatty acid esters were separated and assayed by radiochemical-GLC (8): nonradioactive samples were analyzed on a Pye model 104 gas chromatogram with flame ionization detector. Both machines were fitted with diethylene glycol adipate columns.

RESULTS

Time Study

In studies on the time sequence of total fatty acid synthesis in *Crepis* seeds, it was seen that a lag period up to 8-10 days after the opening of the flowers was followed by a rapid synthesis until about 28 days after flowering (Fig. 1), and the seed was fully ripe at 5 weeks. Crepenynic acid was not detectable at 4 days and increased only slowly between 7 and 11 days while the bulk of the synthesis occurred between the 14th and 28th days (Fig. 2). No crepenynate was produced in unfertilized seeds or in any vegetative part of the plant. The

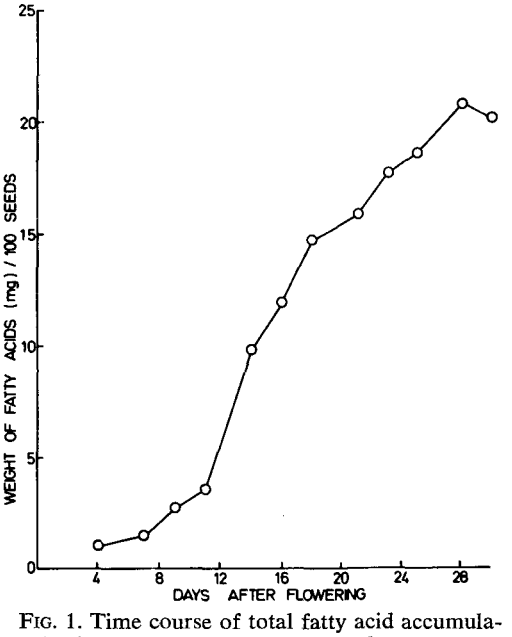

FIG. 1. Time course of total fatty acid accumulation in developing seeds of *Crepis rubra*.

major part of the increase in total acids prior to the crepenynate build-up was accounted for by linoleate, and the pattern of acids suggests a possible precursor-product relationship between linoleate and crepenynate.

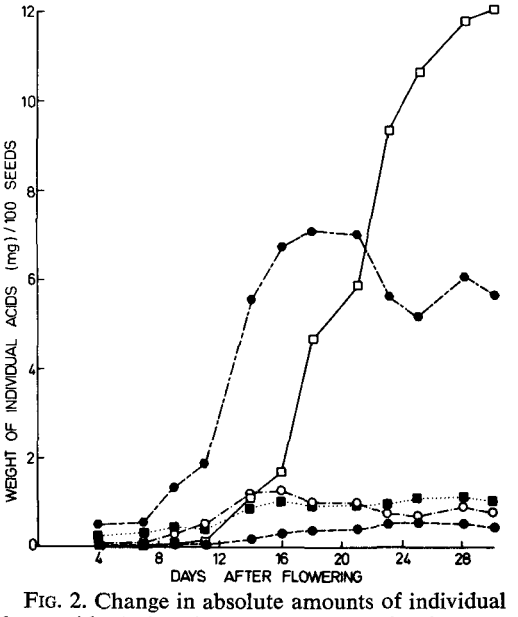

FIG. 2. Change in absolute amounts of individual fatty acids during development of seeds of *Crepis rubra:* □——□ crepenynate; ◆- - -◆ linoleate; ■.....■ palmitate; ○-.-.-○ oleate; ● - - ● stearate.

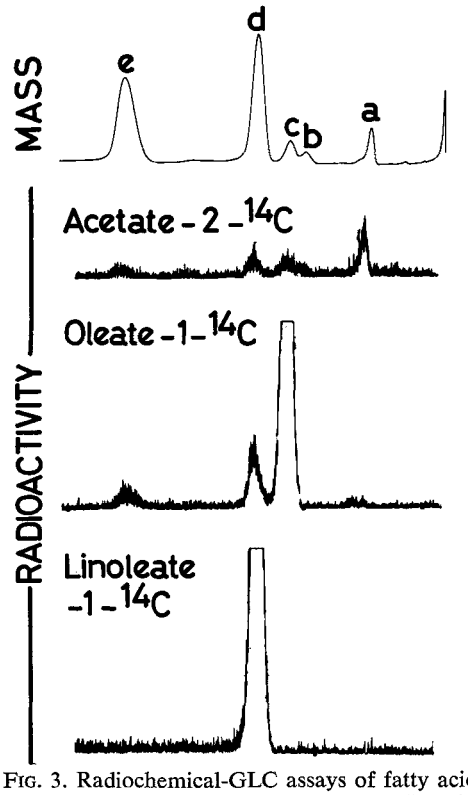

FIG. 3. Radiochemical-GLC assays of fatty acids of *Crepis* seeds after incubation with radioactive substrates. Mass peaks are: a, palmitic; b, stearic; c, oleic; d, linoleic; e, crepenynic.

Incubation Conditions

Incubations with radioactive fatty acids were carried out at two stages in development of the seeds: a) at the time of rapid synthesis of crepenynate (14-24 days) and b) when the seeds were virtually mature and synthesis was substantially complete (28 days onwards). Initial incubations in 0.2 M potassium phosphate buffer, pH 6.0, showed that mature seeds (time b) could incorporate radioactivity into crepenynate from 2-^{14}C- acetate and 1-^{14}C-oleate but not from 1-^{14}C-linoleate. The rapidly synthesizing seeds (time a), however, showed no incorporation of label into crepenynate from any of these exogenous substrates in chopped seed preparations. When the concentrated radioactive substrates were injected directly into seeds at this stage of growth and the seeds were left attached to the plant during incubation, a small amount of radioactivity was incorporated from acetate but none from oleate or linoleate.

A variety of buffers effective over the range pH 5-8 were tested as incubation media with both the young and older seeds, and distilled water and tap water were also used for comparison. The buffers were phosphate, arsenate, imidazole, collidine, veronal, triethanolamine, and Tris, all at pH 7.0. Surprisingly, the only medium in which both acetate and oleate were incorporated consistently into crepenynate, in both young and old seeds, was water; tap water was appreciably better than distilled water. With the use of tap water the levels of incorporation into crepenynate, as percentages of total radioactivity recovered, were: old seeds—from acetate, 20.6%, and from oleate, 10.1%; young seeds—from acetate, 11.5%, and from oleate, 5.7%. Tap water was used then as the medium for all further incubations.

Substrates

Under the above conditions there was again no incorporation of radioactivity into crepenynate from 1-^{14}C-linoleate in contrast to the ready incorporation of acetate and oleate (Fig. 3). 1-^{14}C-Oleyl-S-CoA, which was not converted by seed preparations in phosphate buffer, gave label in crepenynate when supplied to preparations in tap water. The level of incorporation however was no greater than that obtained with free oleic acid.

The identification of crepenynate in these cases was initially by GLC and was confirmed after isolation and purification by a combination of TLC, argentation-TLC, NMR spectroscopy, and hydrogenation. The purified, labeled crepenynate produced from 1-^{14}C-oleate was fully hydrogenated to stearic acid, which was subjected to chemical α-oxidation by the method of James and Hitchcock (8). The resulting homologous series of saturated acids was separated on radio-GLC, when it was seen that only 18:0 retained activity. Thus the crepenynate was labeled specifically in the carboxyl carbon atom and must have been derived directly from oleate by desaturation with no breakdown to acetate and resynthesis.

The 9-*cis*,12-*trans*-isomer of linoleic acid has been detected in small amount in the seed oil of *Crepis rubra* (11) and, in a preliminary report on part of this work (7), was suggested as a possible intermediate in the conversion of oleate to crepenynate. Incubations with 1-^{14}C-oleate gave rise to radioactivity in both *cis,cis*- and *cis,trans*-linoleate. When, however, 1-^{14}C-*cis,trans*-linoleate was incubated with either young or old *Crepis* seeds, no radioactivity was found in crepenynate.

Another minor component of the seed oil (12) which suggested itself as a possible intermediate was vernolic (12,13-epoxyoleic) acid. This also was synthesized with label in the carboxyl carbon atom and was incubated

with *Crepis* preparations at both stages of development; again, no conversion to crepenynic acid was observed, nor has radioactivity yet been found in vernolic acid after incubation with oleic acid.

Cofactors and Inhibitors

The conversion of oleate to crepenynate requires oxygen but is independent of light although the latter has a slight stimulatory effect, presumably indirect. The conversion system is very thermolabile and is completely inhibited at 32C, also by freezing and thawing the intact seeds or whole seed heads. Aging in water for 4 hr before the addition of substrate also causes complete inhibition of oleate desaturation.

Attempts to study cofactor requirements were hindered by failure to obtain an active cell-free system. In chopped seed preparations however, the addition of 10^{-3} M ATP caused 60% inhibition of the conversion (Table I); CTP, GTP, and ITP at the same concentration had no effect. p-Chloromercuribenzoate (pcmb) at 10^{-5} M caused 50% inhibition and at 10^{-4} M completely inhibited crepenynate synthesis, suggesting the participation of sulfhydryl enzyme. Addition of mercaptoethanol (10^{-3} M) did not reverse the inhibition but had the opposite effect in that crepenynate synthesis was completely eliminated and linoleate synthesis, little affected by pcmb, was severely reduced.

Sterculic acid, a known inhibitor of stearate to oleate desaturation in animals (13) and plants (14), at 10^{-3} M inhibited crepenynate synthesis strongly but had little effect on the 18:1 → 18:2 desaturation. This pattern of inhibition of crepenynate synthesis with little or no effect on linoleate synthesis was also shown by hydrogen peroxide, potassium ethyl xanthate, sodium pyrophosphate, and Tween 20. The Tween is normally used to emulsify the fatty acid substrates but then has a final concentration of 0.024 mg/ml in the incubation medium, at which concentration no inhibitory effect is detectable.

The beneficial effects of tap water as an incubation medium suggested stimulation by metal ions, possibly Ca^{++} or Mg^{++}, or Cu^{++} solubilized from copper water pipes. Mg^{++} at 10^{-2} M gave slight stimulation, but Ca^{++} (10^{-2} M) completely inhibited desaturation. When 10^{-3} M EDTA was added to the tap water, this also stimulated conversion of oleate, suggesting that its effect might be owing to preferential chelation of the Ca^{++} normally present in this water, allowing the uptake of Mg^{++}. Cu^{++} at 10^{-2} M caused complete inhibition but appeared to stimulate slightly when used at 10^{-4} M or 10^{-5} M. To check this, incubations were carried out in tap water with 10^{-3} M potassium ethyl xanthate, which chelates As, Co, Cu, Mo, and Pb, and with 10^{-2} M sodium pyrophosphate, which binds Na and Cu. Because Cu^{++} is the common factor between these two chelating agents and both inhibited crepenynate formation without affecting linoleate synthesis, it seems likely that copper is involved in at least one step of the oleate→ crepenynate sequence.

Crepenynic acid, the end-product, when added to the medium in a concentration of 2×10^{-2} M, completely inhibited desaturation of oleate to crepenynate and caused more than 95% inhibition of linoleate synthesis. This may be due to end-product inhibition of the enzyme system, but perhaps more likely is the reduced availability to the enzyme of the substrate oleate due to the large dilution with another $\triangle^9$-unsaturated fatty acid.

TABLE I

Compounds Inhibiting the Conversion of Oleic Acid to Crepenynic Acid in Chopped Seed Preparations

Inhibitor	Concentration	% Inhibition
ATP	10^{-3} M	60
pcmb	10^{-5} M	50
Sterculic acid	10^{-3} M	79
Hydrogen peroxide	10^{-3} M	100
Potassium ethyl xanthate	10^{-3} M	72
Sodium pyrophosphate	10^{-2} M	81
Crepenynic acid	2×10^{-2} M	100
Tween 20	6 mg/ml	100
Ca^{++}	10^{-2} M	50

TABLE II

Percentage Fatty Acid Composition of Ripe *Crepis* Seed Lipids
(Only acids constituting 1% or over in at least one fraction are listed. Vernolic acid is not included as it is lost under the acid transmethylation conditions necessary to preserve crepenynate.)

Fraction	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	Crep.	Unknown
Total lipids	+	+	5.2	+	1.9	3.7	32.1	+	+	54.7	+
Triglycerides	+	+	4.4	+	1.8	3.8	29.2	+	+	58.8	+
Polar lipids	+	3.4	19.5	+	3.6	4.3	61.8	3.3	1.2	1.7	+
Sterol esters	5.2	10.9	12.5	+	4.9	4.8	46.5	1.0	1.6	1.3	11.1
Mono- + diglycerides	3.3	12.8	27.5	4.3	8.3	7.2	23.4	3.6	3.0	6.2	+

TABLE III

Radioactivity in Crepenynate and Oleate from Lipid Fractions of Young *Crepis* Seeds After 12 Hours of Incubation with 1-^{14}C-Oleate

Fraction	Specific Activity[a]		Mass crep./ Oleate
	Crepenynate	Oleate	
Sterol esters	0.05	0.3	0.85
Triglycerides	0.95	19.9	0.46
Free fatty acids	0.00	0.6	0.15
Mono- + diglycerides	6.50	2.5	0.13
Polar lipids	0.00	1.4	0.37

[a] Specific activities are in arbitrary units and are calculated from the radio-GLC traces as area of radioactive peak/area of mass peak; the sensitivity settings are kept constant throughout.

Crepenynate Metabolism

Crepenynate is the major fatty acid in the oil of mature *Crepis* seeds, comprising over 50% of the total. The bulk of the seed oil is triglyceride, which contains a high proportion of the crepenynate (Table II). This acid is not, however, confined to triglycerides; small amounts are present as sterol esters and in polar lipids and rather more in the mono- plus diglyceride fraction.

Analyses were carried out on lipid fractions from young developing seeds after incubation with 1-^{14}C-oleate in order to study the distribution of the newly synthesized crepenynic acid (Table III). Points of interest in these results are the high level of radioactivity in crepenynate in the mono- plus diglyceride fraction, and the lack of label in crepenynate in the polar lipids. Oleate, by contrast, has a low specific activity in the partial glycerides, and most of the exogenous oleate appears to be incorporated in the final acylation, which gives triglyceride.

The triglycerides of mature seeds were isolated by TLC and subjected to partial hydrolysis with pancreatic lipase (15). A comparison of the fatty acid composition of the starting material with that of residual triglyceride isolated after lipolysis shows (Table IV) that there was no significant preferential cleavage of individual glyceride species. The presence of 81% crepenynate in the monoglyceride recovered indicates a large incorporation of this acid in the 2-position. As the triglyceride contains 60% crepenynate or almost two residues per triglyceride molecule, it would be expected that one of the outer positions would be completely occupied by this acid. This distribution was supported when it was shown, by a modification of the procedure of Morris (16), that a major triglyceride species contained crepenynic acid in positions 2 and 3. Full details of this method will be published separately.

TABLE IV

Fatty Acid Composition of Products of Incomplete Cleavage of *Crepis* Triglycerides by Pancreatic Lipase (Only acids comprising 1% or more in one fraction are shown.)

	16:0	18:0	18:1	18:2	20:0	Crep.
Initial TG	4.3	1.7	3.7	28.8	<1	60.11
Residual TG	4.5	1.8	4.1	28.3	<1	58.6
Monoglyceride	<1	—	1.8	15.8	—	80.9
Fatty acids	7.1	2.9	5.5	36.1	1.1	46.3

DISCUSSION

These studies establish that the acetylenic bond of crepenynic acid is formed by desaturation of a preformed long-chain fatty acid, and it is reasonable to assume a similar origin for other acetylenic fatty acids. The long-chain acid converted to crepenynate by this seed system is oleic acid or its derivative oleyl-S-CoA. The observation that oleyl-S-CoA is converted only to the same extent as the free acid is open to at least two interpretations: if the CoA derivative is an intermediate in the conversion, then its formation is not rate-limiting for the over-all reaction sequence; alternatively it may be cleaved by the tissue to the free acid, which is then metabolized. The latter explanation is most likely in the case of this chopped preparation as the CoA esters of long-chain fatty acids are believed not to penetrate intact cells.

Linoleic acid seems theoretically the most likely intermediate between oleate and crepenynate; however, neither the *cis,cis-* nor a mixture of the *cis,trans-* and *trans,cis-*isomers was further desaturated under conditions which were favorable for conversion of acetate, oleate, and oleyl-S-CoA. A possibly analogous case is seen in the formation of ricinoleic acid in the castor bean (*Ricinus communis*). Here also linoleic acid might be expected to be a precursor, but it has been shown repeatedly that, while oleate was converted to ricinoleate, linoleate was not (17-19). It has been demonstrated recently that the conversion of oleate to ricinoleate occurs, not via linoleic acid, but by either direct hydroxyl substitution or direct insertion of oxygen into oleate (20). It is possible that, in *Crepis*, oleate is converted by a similar mechanism to an oxy-acid-enzyme complex, with cyclization to form epoxy-oleate-enzyme, which may then be hydrated and doubly dehydrated to yield the acetylenic bond. It is assumed that exogenous epoxyoleate could not exchange with the endogenous enzyme-bound intermediate. This explanation would

fit with all our results. Alternatively, if enzyme-bound intermediates are involved, oleate could equally well be converted to crepenynate via bound linoleate. Again no intermediates need be detectable, and exogenous linoleate need not exchange with the bound intermediate.

The action of pcmb at 10^{-5} M suggests the participation of an SH-enzyme in oleate to crepenynate conversion but not in the oleate to linoleate conversion. At 10^{-4} M inhibition is complete, but the inhibitory action may not be specific for SH groups at this higher concentration. The increased inhibition cannot, however, be due to reaction of pcmb with carboxyl groups of the substrate as this would also inhibit linoleate synthesis. The failure of mercaptoethanol to reverse the inhibition caused by 10^{-5} M pcmb, but rather to intensify it, is unexplained at present, but it may be noted that this compound has been observed strongly to inhibit fatty acid desaturation in *Chlorella* cell-free systems (21).

Mg^{++} appears to be necessary to one of the enzymes involved, probably in the activation of the oleate. If a nucleotide triphosphate is involved in the activation, it is not ATP. Further studies on the precise mechanism and cofactor requirements of the system have been hampered by our inability to obtain an active cell-free system.

Lipid analyses of labeled material and the localization of crepenynate in the 2- and 3-positions of triglyceride suggest that 1-monoglyceride is the primary acceptor of newly synthesized crepenynate. This, coupled with the lack of label in the crepenynate of polar lipids, argues against the operation of the Kennedy pathway in triglyceride synthesis in this tissue.

Lipase hydrolysis yielded free fatty acids much enriched in oleate (5.5%) compared with the monoglycerides produced (1.3%), a finding which, together with the labeling data, suggests that exogenous oleate acylates position 3 in minor species of triglyceride. The heavy preponderance of the saturated acids in the free fatty acids after lipolysis demonstrates their localization on the outer positions of triglyceride, and as the major acid component, crepenynic acid, is predominantly esterified to the 2- and 3-positions, the location of saturated acids specifically on the 1-position is thereby implied.

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The 9-Hexadecenoic and 11-Octadecenoic Acid Content of Natural Fats and Oils

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ABSTRACT

The monoenoic methyl esters from numerous fats and oils which contained appreciable *cis*-9-hexadecenoic acid (*cis*-9-16:1) were isolated by liquid-solid chromatography on silver nitrate-silica gel. Analysis of the monoenes by packed and capillary column gas-liquid chromatography showed that significant amounts of *cis*-11-octadecenoic acid (*cis*-11-18:1) were present in all samples. The amount of *cis*-11-18:1 found in the monoenoic methyl esters increased proportionally to logarithmic increases in the *cis*-9-16:1 level. Most analyses reported in the literature also show this proportionality. This mathematical relationship suggests that chain elongation of *cis*-9-16:1 to *cis*-11-18:1 is a biosynthetic pathway operative in a wide variety of species.

INTRODUCTION

THE CHAIN ELONGATION OF *cis*-9-16:1 to form *cis*-11-18:1 is a biosynthetic pathway that has been well-documented in the case of fatty acid synthesis by bacteria (1-4). Several investigators have reported that the fatty acids of rat lipids contain both *cis*-9-16:1 and *cis*-11-18:1 (1,5,6) and it is well-established that many common vegetable oils which contain low levels of *cis*-9-16:1 also contain small amounts of *cis*-11-18:1 (7-9). We became interested in determining if these two fatty acids are always present together in natural fats and oils and, if so, whether there is any relationship between their respective levels. Recent articles on the fatty acid composition of a spore oil (10) and two seed oils (11,12) which are rich in *cis*-9-16:1 have reported the presence of correspondingly high levels of *cis*-11-18:1. Brockerhoff and Ackman (13), in work published after completion of the present study, reported significant amounts of *cis*-9-16:1 and *cis*-11-18:1 in numerous animal fats and marine oils. In the present investigation a variety of animal fats, marine oils and plant oils containing relatively high levels of *cis*-9-16:1 were analyzed for their *cis*-11-18:1 content and a mathematical relationship was shown to exist between the levels of these two fatty acids for a majority of the samples. The literature data were also shown to follow the same relationship.

EXPERIMENTAL

Source of Samples

The avocado (California), lard (Hormel), Macadamia nut and one of the crude menhaden oil (Humko Products) samples were recent purchases from commercial sources. All of the other marine oils were crude oils rendered sometime in 1963 and stored under nitrogen at 1C since receipt. The camel, hippopotamus and beef fats were available from an earlier study (14) and had been stored under similar conditions. The *Asclepia syriaca* and *Doxantha unguis-cati* seeds were kindly supplied by C. Y. Hopkins, National Research Council of Canada, Ottawa, Canada. The exact source of the *Lycopodium* spore fat, obtained several years ago, is unknown. The dried nut, fruit and seed samples were ground in a Wiley mill (avocado pulp excepted) and extracted with petroleum ether to isolate the crude oil.

Methyl Ester Preparation

Methyl esters were prepared by refluxing 1.0 g of oil with 20 ml of 0.5 N HCl (anhydrous) in methanol for 4.5 hr. The esters were extracted into chloroform, water-washed and dried using standard techniques (15). The purity of the methyl esters was checked by thin-layer chromatography on Silica Gel G, using a 100 μg sample, developing with a solvent mixture of hexane-ethyl ether-acetic acid (80:20:1, v/v/v), spraying with 25% sulfuric acid and charring at 260C. No free fatty acid or residual triglyceride was detected, but several of the ester samples, particularly those derived from marine oils, showed some barely visible components at or near the origin. These impurities, because of their low level and high polarity, did not interfere with the isolation of the monoenoic esters by liquid-solid chromatography.

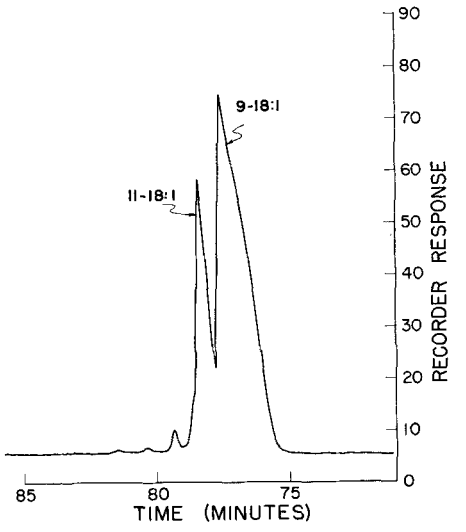

FIG. 1. Capillary GLC of methyl octadecenoates from antarctic whale oil.

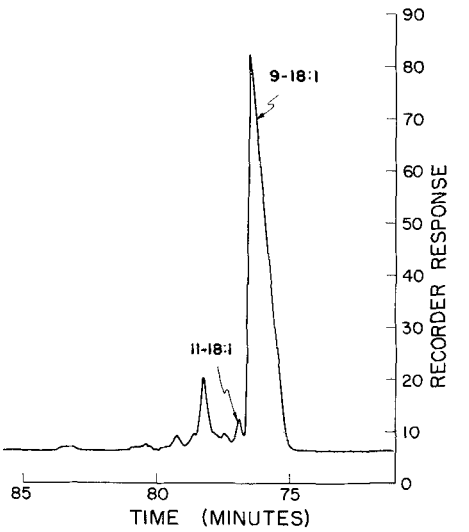

FIG. 2. Capillary GLC of methyl octadecenoates from beef tallow.

Liquid-Solid Chromatography

The monoenoic methyl esters were separated from the saturated and polyenoic ester components by chromatography on silver nitrate-silica gel as previously described (16). Columns containing 60-65 g of adsorbent-Celite 545 (9:1) were used for 1.0 g of methyl esters. Saturated esters were eluted with 350-400 ml of petroleum ether-benzene (80:20), monoenoic esters with 700-800 ml of petroleum ether-benzene (60:40). The saturate-monoene and monoene-diene separations were clear-cut in all cases except the *Asclepia syriaca* methyl esters, where the high diene content caused co-elution of diene with monoene and a second chromatographic separation of the impure monoene fraction was necessary.

GLC Analysis

The chain length distribution of the monoenoic methyl ester samples was obtained on an F&M Scientific Model 720. A 10 ft × 0.25 in. O.D. stainless steel column was packed with 15% EGSS-X on 60/80 mesh Gas-Chrom P. The operating temperature was 190C and the helium flow rate 70 ml/min. Peak areas were determined by multiplying the peak height times the width at half height. Differences in response were corrected by applying a factor consisting of the square root of the molecular weight of the respective ester. These factors have been shown to give adequate agreement with weight per cent for synthetic ester mixtures.

The monoene isomer distributions were obtained by GLC on a polyphenyl ether coated capillary column using essentially the conditions described previously (16). One exception, the temperature program of 140-180C at 0.5C, was made so that the isomers would be eluted in less than 90 min. Peak areas were determined as above. It was assumed that no correction factors were required within a given chain length and degree of unsaturation.

The actual level of the monoene isomers was calculated by proportioning the weight per cent (as determined by packed column GLC) on the basis of the isomer distribution (as determined by capillary GLC).

Oxidative Cleavage

All of the plant and several of the marine oil monoene fractions were oxidatively cleaved with periodate-permanganate as previously described (17). For the five plant oils, the *cis*-11-18:1 content was calculated from the undecanedioic acid cleavage fragment after correction for the *cis*-11-16:1 (calculated from the pentanoic acid fragment) and the 20:1 (assumed to be *cis*-11-20:1) determined by GLC of the original monoenes on a packed column.

RESULTS

Figs. 1 and 2 illustrate typical separations obtained by capillary GLC on the methyl octadecenoates for two different *cis*-9-18:1/*cis*-11-18:1 ratios. There was no difficulty in determining the amount of *cis*-9-16:1 in the methyl hexadecenoates, particularly since it was the major component in most cases, and hence the hexadecenoate region is not shown.

TABLE I

Isomer Distribution in Monoenoic Esters from Various Fats and Oils

Sample	Monoene ester fraction, wt %	Composition of monoenes[a] by GLC, wt %				
		Cis-9-16:1	Other 16:1	*Cis*-9-18:1	*Cis*-11-18:1	Other 18:1
Lycopodium	53.8	43.6	0.3	40.8	14.3	....
Macadamia nut	79.6	22.9	1.0	67.2	3.7	0.9
Doxantha unguis-cati	69.8	69.0	2.9	4.7	21.0	1.7
Asclepias syriaca	38.6	27.2	1.3	20.2	44.9	1.5
Avocado	70.8	6.3	0.2	85.8	7.2	....
Seal (Alaskan)	49.0	32.7	3.0	34.7	11.1	1.9
Menhaden (Atlantic)	25.6	37.5	2.9	33.3	11.5	4.2
Menhaden (Humko)	25.0	47.9	2.1	24.0	13.6	1.2
Whale (Antarctic)	44.7	20.8	2.4	45.0	15.4	2.1
Whale (Icelandic)	53.4	13.5	1.4	39.2	9.5	1.9
Herring (Icelandic)	46.6	8.9	2.0	17.6	3.2	1.7
Pilchard (South Africa)	26.6	33.0	1.7	24.0	13.8	1.0
Camel	31.4	4.0	3.4	70.6	4.3	12.6[b]
Hippopotamus	40.6	3.5	1.3	57.2	4.1	27.4[b]
Lard (Hormel)	46.7	4.2	0.6	83.0	6.1	2.3
Beef	33.4	2.9	0.9	76.1	2.6	13.7[b]

[a]20:1 in the plant oils and animal fats and 20:1 and 22:1 in the marine oils are the other major components.

[b]*Trans*-monoenes are the major contributors here.

Table I gives data obtained on the isolated monoenoic ester fractions by a combination of capillary and packed column GLC. All of the samples contained significant amounts of *cis*-11-18:1 as expected because of their high *cis*-9-16:1 levels. Both samples of menhaden oil contained appreciable *cis*-11-18:1 whereas a previous detailed study of this oil (18) reported *cis*-9-18:1 to be the only 18:1 isomer present.

The *cis*-11-18:1 contents of the plant oil monoene fractions calculated from the oxidative cleavage data were 14.0, 3.0, 20.6, 44.7 and 6.6%, respectively, for the *Lycopodium,*

TABLE II

Literature Data on Monoene Isomer Distributions in Various Fats and Oils

Sample	Monoene ester fraction, wt %[b]	Composition of monoenes[a], wt %				
		Cis-9-16:1	Other 16:1	*Cis*-9-18:1	*Cis*-11-18:1	Other 18:1
Cottonseed (7)	18.2	2[c]	....	93[c]	5	....
Lycopodium (10)	65.9	49[c]	....	36[c]	14	....
Doxantha unquis-cati (12)	(83)	77[c]	....	5[c]	18	....
Asclepias syriaca (11)	(40)	25[c]	....	38[c]	37	....
Herring (22)	(49)	11	3	32	9	3
Cod Liver (23)	(54)	18	1	30	10	<1
Cod (13)	(47)	14	2	25	10	4
Mackerel (13)	(37)	10	3	24	7	2
Lobster (13)	(43)	15	2	29	17	1
Seal (13)	(53)	33	1	22	9	8
Rat (5)	(35)	7	2	80	9	2
Rat (13)	(39)	9	1	81	9	....
Duck (13)	(55)	10	1	82	7	....
Dog (13)	(47)	9	1	82	8	....
Pig (13)	(54)	4	1	83	11	....

[a]20:1 in the plant oils and animal fats and 20:1 and 22:1 in the marine oils are the other major components.

[b]In those cases where a single monoenoic ester fraction (containing all the monoenes) was not isolated, the total monoenoic methyl ester level as determined by GLC is given in parentheses.

[c]Small amounts of other isomers are probably included in these figures.

Macadamia nut, *Doxantha unguis-cati, Asclepias syriaca* and avocado samples. These are in reasonable agreement with the results in Table I considering the assumptions involved in the cleavage data calculations and the resolution problem in GLC for low levels of *cis*-11-18:1. The *cis*-11-18:1 content of the marine oil monoenes could not be calculated from the cleavage data because of the many different chain lengths and isomers present. However, the samples were cleaved to check for the absence of other 18:1 isomers which would interfere with the *cis*-9-18:1/*cis*-11-18:1 ratio determined by capillary GLC. Only trace amounts ($< 0.2\%$) of decanedioic and low levels ($< 1\%$ total) of octanedioic and heptanedioic acid fragments were detected in the cleavage fragments, thus precluding the presence of much *cis*-10-, 8- and 7-18:1, respectively.

Reliable data from the literature on the *cis*-9-16:1 and *cis*-11-18:1 contents of various fats and oils are given in Table II. The analyses were not all obtained by the same combination of methods. Brockerhoff and Ackman (13) obtained their data by capillary GLC of the original methyl esters without any prior separations. The remainder of the references are about equally divided between the use of capillary GLC and oxidative cleavage on various monoene-rich fractions. Several reports in the literature on the composition of marine oil monoenes (19-21) were noted included in the Table because the monoene fraction isolated contained only a portion of the total available monoene and the methods of isolation used could lead to selective enrichment of some of the isomers.

Fig. 3 is a semi-log plot of the *cis*-9-16:1 content vs. the *cis*-11-18:1 content of the monoenes from both Tables I and II. A majority of the results approximate the relationship indicated by the straight line, regardless of whether the data are from an animal fat, marine oil or plant oil. The *Asclepias syriaca* oil samples are the most obvious exceptions. Macadamia nut oil also deviates considerably from the plot but in the opposite direction in that a high *cis*-9-16:1 content is accompanied by a rather low *cis*-11-18:1 level.

The data in Tables I and II and Fig. 3 suggest that chain elongation of *cis*-9-16:1 to *cis*-11-18:1 is a biosynthetic pathway operative in a wide variety of species.

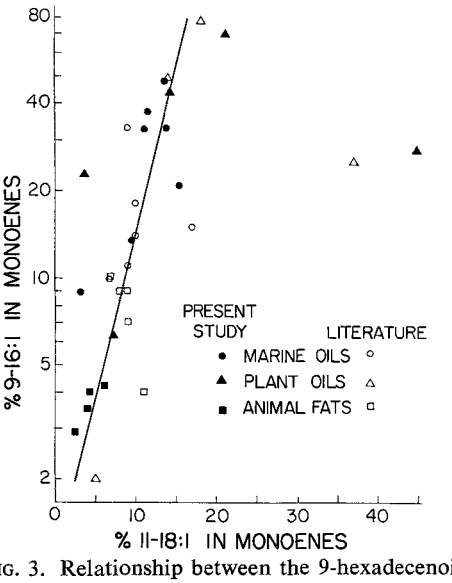

FIG. 3. Relationship between the 9-hexadecenoic and 11-octadecenoic acid content of the fats and oils in Tables I and II.

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Metabolism of Chimyl Alcohol and Phosphatidyl Ethanolamine in the Rat Brain[1]

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ABSTRACT

Following intracerebral injection of ^{14}C-phosphatidylethanolamine and ^{3}H-chimyl alcohol into 18 day old rats, the ethanolamine phosphoglycerides were isolated and analyzed. The ^{14}C and ^{3}H activities in the dimethyl acetals derived from alkenyl acyl ethanolamine phosphoglycerides and in the glyceryl ethers derived from the alkyl acyl ethanolamine phosphoglycerides were measured. The absence of ^{14}C in the dimethyl acetals indicates that phosphatidyl ethanolamine is not transformed into phosphatidal ethanolamine under these circumstances. The increase with time of the ^{3}H content of the glyceryl ethers and dimethyl acetals indicates that chimyl alcohol was a precursor of both types of phospholipids.

INTRODUCTION

ALTHOUGH DERIVATIVES of alkyl and alkenyl glyceryl ethers have been shown to be widespread components of both neutral and phospholipids (2,3,16), the biogenesis of these ether linkages is still obscure. It has been considered possible that phospholipids containing these groupings may be formed by reduction of the corresponding diacyl compounds (7,17) and equally possible that the alkenyl derivatives could be formed by dehydrogenation of the alkyl ethers (18). However, the mechanism of formation of the latter is not understood nor can it be surmised whether the transformations of one class to another might take place as the glyceryl ethers or as the phospholipids.

Experiments in this laboratory (1) in which ^{14}C-labeled palmitaldehyde was injected directly into the brains of 18 day rats provided evidence that the aldehyde did not react directly with some glycerol derivative to give an alkenyl ether. Rather, it was first oxidized to palmitic acid, which was then incorporated into the ethanolamine phosphoglycerides. On the other hand, Thompson (15) has found that in the slug, *Arion ater,* the alkyl, acyl GPE[1] appears to be the precursor of the alkenyl, acyl GPE. Malins (8) has reached the same conclusion for the dogfish and Horrocks and Ansell (4) have suggested that the same pathway exists in the rat brain.

The present study represents an attempt to clarify the interrelationships among these compounds.

PROCEDURES

Materials

Uniformly-labeled ^{14}C phosphatidylethanolamine, obtained from Applied Science Laboratories, Inc., was purified by chromatography on a DEAE cellulose column before use. Chimyl alcohol, uniformly-labeled in the alkyl moieties with tritium was a gift from G. A. Thompson, Department of Biochemistry, University of Washington and was purified by thin-layer chromatography (see below) before use. All solvents were ACS reagent grade and were distilled prior to use. Ethanol (1%) was added to the chloroform as a preservative.

Methods

Treatment of Animals. Three groups of 18 day old male Sprague-Dawley rats were obtained from Berkeley Pacific Laboratories together with their lactating mothers. Rats of this age were used since the synthetic reactions leading to myelin formation are at a maximum at this time (9). Each rat was anesthetized lightly with ether and was given an intracerebral injection of 0.375 ml of an emulsion consisting of: 20 mg, 6 μc chimyl alcohol-^{3}H, 7.3 mg, 25 μc PE-^{14}C and tween 20 (36 mg/ml). Groups A, B and C were sacrificed after 4, 8 and 16 hr, respectively.

The animals were sacrificed by CO_2 asphyxiation and the brains removed and immediately frozen on dry ice. The pooled brains from each group were extracted with chloroform: methanol (2:1) under nitrogen as described by Rouser *et al.* (11). Evaporation of the extract in a rotary flash evaporator at 30C followed by drying in vacuo over KOH gave the total lipid.

[1] Abbreviations used; CA, Chimyl alcohol; GLC, gas liquid chromatography; GPE, glyceryl-3-phosphorylethanolamine; PE, phosphatidylethanolamine; TLC, thin-layer chromatography; TFA, trifluoroacetyl.

TABLE I
Radioactivity Recovered in Total Lipid Extract after Intercerebral Injection of ^{14}C PE and ^{3}H Chimyl Alcohol into 18-Day-Old Rat Brains

Group of Rats	A	B	C
Number of rats/group	5	5	4
Activity of ^{14}C PE inject/rat (cpm x 10^{-6})	1.36	1.36	1.36
Activity of ^{3}H chimyl alc inject/rat (cpm x 10^{-6})	0.34	0.34	0.40
Exposure time to substrates (hr)	4	8	16
Pooled weights of brains (g)	7.3	8.2	6.5
Extracted lipid (% wet wt of brain)	6.7	8.5	7.1
Activity in total lipids			
– ^{14}C (cpm x 10^{-6})	3.46	3.45	3.12
– ^{3}H (cpm x 10^{-6})	0.65	0.66	0.51
Per cent of injected activity recovered in total lipid			
– ^{14}C PE	51	50	50
– ^{3}H chimyl alc	38	32	32

Separation of the Lipid Components. The total lipids were fractionated on a DEAE cellulose column as described by Rouser et al. (11,12). The eluting solvents and components of each eluate were as follows: chloroform-methanol (9:1) — cholesterol, ceramide, cerebroside, choline phosphoglycerides and sphingomyelin (front fraction); chloroform-methanol (7:3) — ethanolamine phosphoglycerides; methanol — water-soluble non-lipid material; glacial acetic acid — free fatty acids, serine phosphoglycerides, gangliosides; methanol to wash out the acetic acid; chloroform-methanol: 28% ammonium hydroxide (4:1:-0.2) — inositol phosphoglycerides and other acidic lipids. The eluates from the column were monitored continuously by TLC using the solvent system chloroform-methanol-28% ammonium hydroxide (50:49:1) and standards obtained from Applied Science Laboratories.

The fatty acid and aldehyde moieties of the ethanolamine phosphoglycerides were obtained by subjecting the isolated fraction to methanolysis according to the method of Morrison and Smith (10). The resulting mixtures of methyl esters and dimethyl acetals were analyzed directly by GLC or the dimethyl acetals were isolated by saponification of the mixture with 5% methanolic KOH at 60C for 1 hr. The dimethyl acetals were extracted into petroleum ether (br 30-60C) and the aqueous phase was acidified and the fatty acids extracted with petroleum ether and re-esterified with diazomethane. The conditions for GLC and subsequent collection of the methyl esters and dimethyl acetals were as described previously (1).

The ethanolamine phosphoglyceride fraction was analyzed for glyceryl ether components by the acetolysis procedure recommended by Thompson (14). The glyceryl ethers thus obtained were converted to the TFA derivatives by the method of Wood and Snyder (19) and were analyzed by GLC on an ethylene glycol succinate column. The identity of the glyceryl ethers was checked by TLC with the solvent system petroleum ether-ether-glacial acetic acid (40:60:1).

The front fraction isolated from the DEAE-cellulose column was analyzed for saturated and α,β-unsaturated glyceryl ethers as follows: The fraction was first subjected to TLC with the system chloroform-methanol (98:2). A dividing line was drawn below the cholesterol band and the rest of the plate was developed with chloroform-methanol (95.5). Bands corresponding to (a) choline phosphoglycerides, sphingomyelins, cerebroside, (b) monoglyceride ceramide, glyceryl ether, (c) cholesterol, (d) diglyceride, (e) aldehyde, cholesterol esters, triglycerides were separated, scraped off the plate and extracted with appropriate solvents. The several components were subjected to methanolysis and analyzed for dimethyl acetals as described. The glyceryl ethers were freed by refluxing the neutral lipids with 2 N ethanolic KOH (14) and were identified by GLC of their TFA derivatives.

Counting was performed in a Packard Tri-Card Scintillation Spectrometer with samples dissolved in Toluene containing 5 g PPO and 0.3 g dimethyl POPOP per liter. Efficiencies were 15% for ^{3}H and 62% for ^{14}C.

RESULTS

Each rat received approximately 1.6 μc ^{14}C-PE and 0.4 μc ^{3}H-chimyl alcohol intracerebrally. The weights and radioactivities of the brain lipids of the 3 groups of rats are shown in Table I. It is evident that label from both substrates was incorporated into the brain lipids of the rats. The weights and radioactivities of the chromatographically separated lipid fractions are recorded in Table II. As expected, there were no significant differences in the per cent composition of the brain lipids of the different groups. A major portion of the ^{14}C activity (25-35%) appears in the front fraction, which contains cholesterol, ceramide, cerebroside, choline phosphoglycerides, sphingomyelin and lyso phosphatidyl choline. In addition, there seem to be no significant differences in distribution of ^{14}C (from PE) with time. The ^{3}H (from chimyl alcohol), on the other hand, appears to decrease

TABLE II

Weights and Radioactivity of Lipids in Rat Brains after Injection of ^{14}C Phosphatidyl Ethanolamine and ^{3}H-Chimyl Alcohol

Total lipid chromatographed	Group A (2 hr)			Group B (4 hr)			Group C (16 hr)		
	Wt. 487.4	^{14}C-PE $3.5x10^6$	^{3}H-CA $0.65x10^6$	Wt. 695.3	^{14}C-PE $3.5x10^6$	^{3}H-CA $0.66x10^6$	Wt. 462	^{14}C-PE $3.1x10^6$	^{3}H-CA $0.51x10^6$
Per cent distribution[a]									
Front fraction	50.4	31.5	78.1	55.6	26.9	74.9	47.9	35.2	66.9
Phosphatidyl ethanolamine fraction	20.9	26.8	9.6	19.3	33.9	14.0	21.3	26.8	18.4
Non-lipid fraction	8.9	2.7	1.2	7.8	2.7	1.00	9.4	2.1	1.4
Phosphatidyl serine fraction	10.8	15.4	5.2	11.2	14.2	4.2	12.4	12.5	4.8
Inositide fraction	9.1	26.0	7.0	6.3	22.4	6.1	9.0	23.3	8.6
Per cent recovery	99.0	82.0	108.0	93.2	86.0	98.4	97.5	76.1	106.0

[a] All values are expressed as a per cent of the total weight of radioactivity obtained from the column (see under methods for description of chromatographic separation).

in the front fraction and increase in the ethanolamine phosphoglycerides during the times studied, indicating possible incorporation of chimyl alcohol into PE or closely related compounds. When this fraction was separated, following methanolysis, into methyl esters, dimethyl acetals and TFA derivatives of chimyl alcohol, the results shown in Table III were obtained. It was found that ethanolamine phosphoglycerides of both alkyl ether and alkenyl ether types contained ^{3}H from the chimyl alcohol, but had incorporated no ^{14}C from the injected PE.

The front fraction from group B was separated into its components by TLC, as described above, giving the distribution of weight and radioactivity shown in Table IV. When these fractions (other than cholesterol) were subjected to methanolysis to obtain the methyl esters and acetals or to hydrolysis (14) to obtain the glyceryl ethers, it was found that the ^{14}C activity of all fractions was in the methyl esters. Most of the ^{3}H activity of the phospholipid fraction was also in the methyl esters but in the fraction migrating with monoglyceride, the ^{3}H activity was associated entirely with the glyceryl ethers. The diglyceride fraction, following hydrolysis, had 12% of the ^{3}H activity in the methyl esters and 50-60% in the glyceryl ethers. The distribution of activity in the triglyceride fraction was about the same.

TABLE III

Relative Specific Activities[a] of the Methyl Esters, Dimethyl Acetals and TFA Derivatives from Rat Brains after Injection of PE and Chimyl Alcohol

	Group A (4 hr)		Group B (8 hr)		Group C (16 hr)	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Methyl esters						
16:0	41.8	....	27.5	....	28.4	....
18:0	4.2	....	1.6	....	1.2	....
18:1	14.2	....	10.4	....	9.8	....
Dimethyl acetals						
16:0	....	2.8	....	3.0	....	10.4
18:0	....	....	....	....	....	....
18:1	....	....	....	....	....	....
TFA glyceryl ethers						
16:0	....	30.7	....	38.5	....	65.2
18:0	....	....	....	....	....	....
18:1	....	....	....	....	....	....

[a] Average from three determinations. Relative specific activities taken as ratios of cpm in a collected fraction to the peak area of the fraction.

TABLE IV

Percent Distribution of Activity in the Front Fraction from DEAE Cellulose Separation of Lipids from Brains of Group B Rats

Fraction	Wt	^{3}H	^{14}C
Phospholipid phosphatidyl choline sphingomyelin cerebroside	55.9	13.2	69.0
Monoglyceride Fraction (containing glyceryl ethers)	8.5	50.9	11.5
Cholesterol	21.3	3.2	2.3
Diglyceride Fraction (containing glyceryl ethers)	5.5	16.3	3.5
Triglyceride	8.5	16.2	13.5

DISCUSSION

The previous study (1) revealed that palmitaldehyde is not incorporated directly into the alkenyl ether linkage of phosphatidal ethanolamine and indicated that this type of compound arises by reduction of an acyl ester linkage. It was not possible from those results, however, to state at what stage such a reduction might take place.

The present experiments indicate that it does not take place in the phospholipid stage since there was no incorporation of ^{14}C from the injected PE into the dimethyl acetals or glyceryl ethers of the extracted ethanol phosphoglyceride fraction. Therefore, any reduction of the acyl group to the alkenyl must take place as the diglyceride or phosphatidic acid. Kiyasu and Kennedy (6) have shown that such a diglyceride (α-alkenyl-β-acyl-) could be converted to ethanolamine plasmalogen by a particulate fraction from rat liver.

On the other hand, the present results also provide evidence that the glyceryl ethers may serve not only as precursors of the alkyl-acyl phosphoglycerides but also of the alkenyl acyl compounds since ^{3}H from the injected chimyl alcohol does appear in both the dimethyl acetals and the glyceryl ethers derived from this fraction. Thus, an oxidation of the ether linkage of the type proposed by Thompson (15) is implicated. Such an oxidation has been found in part by Tietz et al. (18) although the product was not the alkenyl ether. The evidence available at the present time supports both a reductive and an oxidative origin for the alkenyl ether linkage of the phosphatidal ethanolamines but does not decide between them. It is possible that both pathways exist in the rat brain. The stage at which such reactions take place is also open to question although the present evidence supports the idea that it is at the "diglyceride" stage. These questions await the results of additional experiments.

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The Lipids of Human Pancreas with Special Reference to the Presence of Fatty Acid Methyl Esters

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ABSTRACT

Total lipids were extracted from human pancreas with chloroform-methanol, chloroform-methanol following acidification, and benzene. A similar proportional amount of total lipid was obtained by each procedure. Regardless of the method of extraction (i.e., whether or not methanol was present), a small proportion (about 1%) of the total lipid was found to consist of fatty acid methyl esters. Triglycerides constituted the major fraction (about 80%) of the pancreatic lipids; in addition to methyl esters, the remaining lipids comprised free fatty acids, phospholipids, cholesterol esters, and traces of free cholesterol. In general, each class of lipid had a similar over-all fatty acid composition with palmitic and oleic acids as predominant components. The methyl esters had a relatively high content of linolenic acid, and the free fatty acids contained a notably high proportion of palmitic acid, in each case accompanied by a corresponding decrease in the proportion of oleic acid present.

INTRODUCTION

THOUGH METHYL ESTERS of fatty acids can readily arise as artifacts during the extraction or storage of lipids in the presence of methanol (1), trace amounts of such esters have been detected in lipids extracted from guinea pig liver (2), mouse liver (3), and human liver (4) with solvents other than methanol. When the chloroform-methanol extraction procedure of Folch et al. (5) was used by Saladin and Napier (6) to extract lipids from various materials (rat and dog tissues, monkey pancreas, human serum, rat chow, corn oil, and olive oil) only traces of methyl esters were detected of which, as these investigators recognized, part or all could have arisen as artifacts.

In contradistinction to the reports of the presence of traces of fatty acid methyl esters in material of biological origin, Leikola et al. (7) claimed that they were a major component of human pancreatic lipids; of 305 mg of lipids extracted with chloroform-methanol, 55.6 mg (about 18%) consisted of methyl esters. Saladin and Napier (6) noted that this finding was in marked contrast to their observation that, following extraction with chloroform-methanol, the lipids of dog, rat, and monkey pancreas contained less than 0.6% of methyl esters. Since free fatty acids apparently formed a considerable part of the total lipids isolated by Leikola et al. (7), Saladin and Napier (6) suggested that the presence of methyl esters in relatively large amount could have been associated with autolysis of the tissue lipids. Esterification of free fatty acids or transesterification of bound acids with methanol of the extraction solvent mixture could have been catalyzed by carbonate arising from pancreatic bicarbonate (1) and free fatty acids might also have been esterified by an enzymic process (8).

We have therefore carefully reexamined human pancreatic lipids for the presence of fatty acid methyl esters and, at the same time, have taken the opportunity to isolate the major classes of lipid and record their fatty acid composition.

EXPERIMENTAL

Four specimens of human pancreas were obtained at autopsy as soon as possible after death and, when necessary, frozen at –20C until they could be analyzed.

In preliminary experiments the methods as described by Leikola et al. (7) were used to extract and identify lipids from frozen sections of about 0.5 g of pancreas. In subsequent investigations lyophilized pancreatic tissue was used; this was prepared from a homogenate of 75 g of pancreas (pooled tissue from three individuals) and 135 ml of ice water. Of the resulting dry powder (27 g), 5-g portions were extracted by three different methods: a) according to the two-stage process of Bligh and Dyer (9) in which a mixture of chloroform, methanol, and water was employed; b) again using the method of Bligh and Dyer (9), except that the pH of the aqueous slurry was adjusted to 2.5 with HCl (to neutralize any bicarbonate present) before chloroform and methanol were added; and c) with benzene

TABLE I
Proportion of Fatty Acid Methyl Esters in Lipids Extracted by Different Procedures from Human Pancreas

Solvent used for extraction	Method of assay of methyl esters	Methyl esters as % of total lipid
Chloroform-methanol	Direct weighing of esters isolated by TLC	0.97
Chloroform-methanol, after acidification	Direct weighing of esters isolated by TLC	0.75
Benzene	(i) By GLC of total lipid plus methyl heptadecanoate	0.93
	(ii) By GLC of the cholesterol ester fraction (isolated by TLC) plus methyl heptadecanoate	1.13

under reflux for 1 hr. Each extraction procedure yielded approximately 2.5 g of crude lipid, accounting for about 50% of the weight of dry tissue or about 18% on a wet-weight basis.

The crude lipid (2.0 g) from each extraction was separated into its component classes (cholesterol esters, triglycerides, free fatty acids, and phospholipids) by chromatography on silicic acid as described by Garton et al. (10); fatty acid methyl esters, if present, accompany the cholesterol esters.

The cholesterol ester fractions from extraction procedures a) and b) were separately subjected to thin-layer chromatography (TLC) as described by Lough et al. (1) to separate from cholesterol esters the material migrating with the same R_f as authentic methyl esters. The TLC bands corresponding to cholesterol esters and fatty acid methyl esters were scraped off the plates, and each lipid class was recovered from the adsorbent by extraction with diethyl ether. After the ether was removed and the lipids were weighed, the fraction corresponding to methyl esters was subjected to gas-liquid chromatographic analysis (GLC) as outlined below.

The methyl ester content of the cholesterol ester fraction of the total lipids extracted into benzene by procedure c) was determined by GLC, following the addition of methyl heptadecanoate (6.0 mg) to 60.0 mg of the fraction, thus providing an internal standard for calculation of the proportion of methyl esters which were initially present. In addition, samples of the total lipids extracted by benzene were similarly submitted to GLC, again after the addition of a known amount (1.5 mg) of methyl heptadecanoate to 33.0 mg of total lipids.

Before and after hydrogenation, methyl esters of the fatty acid constituents of the various classes of lipid were prepared and analyzed by GLC on two liquid phases, namely, polymerized ethylene glycol succinate (PEGS) and Apiezon L, as previously described by Duncan and Garton (11,12). The fatty acid methyl esters present initially in the total lipids were similarly analyzed, except that they were chromatographed on only one liquid phase (PEGS) and were not subjected to hydrogenation.

RESULTS AND DISCUSSION

Presence of Fatty Acid Methyl Esters

In the preliminary experiments in which the methods of Leikola et al. (7) were used, examination of the total lipids by TLC revealed the presence of a constituent, present apparently in traces, the R_f of which corresponded to that of fatty acid methyl esters. The systematic investigation which followed showed (Table I) that, by using lyophilized pancreas as starting material, fatty acid methyl esters were present in the lipids extracted by chloroform-methanol, by chloroform-methanol following acidification (to eliminate bicarbonate), and by benzene. Methyl esters were not detected (i) in the extraction solvents themselves when appropriate volumes of chloroform-methanol and of benzene were carefully distilled almost to dryness, and (ii) following the passage through a silicic acid column of this residual solvent plus a volume of the mixture of diethyl ether and light petroleum corresponding to that used to elute the cholesterol ester fraction from the pancreatic lipids.

Regardless of the method of extraction the same amount of total lipid containing a similar small proportion (about 1%) of methyl esters was obtained, and so it must be concluded that these had not arisen as artifacts ascribable to the presence of methanol in the extracting solvent. However, as pointed out by Fischer et al. (3), the presence, in the pituitary of several mammals, of an enzyme (13) which can hydrolyze S-adenosylmethionine to yield free methanol leaves open the possibility that small amounts of methanol might arise endogenously in other tissues and participate in the formation of methyl esters either in vivo or post mortem. Nevertheless, as mentioned in the Introduction, it would appear that the high content of methyl esters in human pancreatic lipids reported by Leikola et al. (7) may well have resulted from the use of methanol to extract autolyzed tissue.

TABLE II
Composition of Human Pancreatic Lipids

Lipid	% of total lipid
Triglycerides	79.90
Free fatty acids	11.50
Phospholipids	5.60
Cholesterol esters	2.25
Fatty acid methyl esters	0.75
Free cholesterol	Trace

Composition of Pancreatic Lipids

Though the crude lipid obtained by each of the three extraction procedures was fractionated and the fatty acid composition of each fraction was determined, the results were almost identical and so only one set, that deriving from extraction procedure (b), is presented.

As Table II shows, triglycerides constituted by far the greatest proportion (about 80%) of the total lipids; the remainder consisted of free fatty acids, phospholipids, and cholesterol esters together with small amounts of methyl esters and traces of free cholesterol. A similar predominance of triglycerides has been reported in the pancreatic lipids of the ox and the guinea pig by Prottey and Hawthorne (14). With respect to human pancreatic lipids, our observations differ from those of Leikola et al. (7), who found triglycerides in amount corresponding to only 12.2% of the total lipids, the major lipid fraction (about 36%) consisting principally of free fatty acids.

In common with the lipids of human adipose tissue (15) the major components of all the pancreatic lipids were palmitic and oleic acids (Table III). In general, each class of lipid had a similar over-all fatty acid composition, though noteworthy features were the relatively high content of linolenic acid in the methyl esters and of palmitic acid in the free fatty acids. In each case there was a corresponding decrease in the proportion of oleic acid present.

TABLE III
Fatty Acid Composition of Human Pancreatic Lipids
(Relative Weight Percentages)

Fatty acid	Triglycerides	Free fatty acids	Phospholipids	Cholesterol esters	Fatty acid methyl esters
14:0	3.9	3.2	5.5	2.6	4.0
14:1	0.9	—	—	0.8	0.5
16:0	22.0	40.9	26.0	24.0	25.2
16:1	6.9	4.6	8.3	6.8	9.2
18:0	5.3	6.4	6.9	5.0	6.9
18:1	54.0	37.6	42.8	47.7	32.4
18:2	6.4	4.0	9.3	8.6	7.4
18:3	—	2.7	1.2	3.6	11.6
Others[a]	0.6	0.6	—	0.9	2.8

[a]Traces of 12:0, together with acids of chain-length > 18:0

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The Distribution of ^{14}C-Labeled Cholesterol in the Dog: Effect of Long-Term Epinephrine Administration

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ABSTRACT

A time course study of ^{14}C-cholesterol distribution in dogs was performed after the intravenous administration of 4-^{14}C-cholesterol. Liver cholesterol attained isotopic equilibrium with plasma cholesterol within 24 hr after the administration of the labeled cholesterol. The tissue cholesterol pools of most organs attained isotopic equilibrium with plasma cholesterol between the second and ninth day after 4-^{14}C-cholesterol had been given. Thoracic aorta cholesterol equilibrated most slowly with plasma cholesterol. Thirty-seven to 75 days after the ^{14}C-cholesterol had been given, the specific radioactivity of thoracic aorta cholesterol was 2 to 5 times greater than that of plasma cholesterol and 1.73 and 1.65 times greater than that of the abdominal and terminal aortic segments respectively. Adrenal gland cholesterol attained specific radioactivities greater than that of plasma cholesterol between the fourth and ninth day after 4-^{14}C-cholesterol had been given, and this relationship was maintained over the 75-day period of observation. The specific radioactivity of bile cholesterol was less than that of plasma cholesterol at all time periods.

The daily administration of epinephrine in oil over a period of four to seven weeks was accompanied by a more equal distribution of ^{14}C-cholesterol throughout the length of the aorta. An increase in the specific radioactivity of kidney cortex cholesterol, relative to that of plasma and kidney medullary cholesterol, also was observed in epinephrine-treated dogs.

INTRODUCTION

TIME COURSE STUDIES have shown some striking species differences in the pattern of distribution of ^{14}C-labeled cholesterol between plasma and tissue cholesterol components. Avigan et al. (1) reported that in several tissues of the rat, notably kidney and muscle, the specific activity of the tissue cholesterol was greater than the specific activity of plasma cholesterol 14 days after the administration of 4-^{14}C-cholesterol, and this divergence was considerably increased at later time periods. Clinical studies in man indicated a different pattern of ^{14}C-labeled cholesterol distribution; the specific radioactivities of plasma cholesterol were the same as those of tissue cholesterol after equilibration had taken place between plasma and tissue cholesterol components and exogenously administered 4-^{14}C-cholesterol. This distribution was maintained for many months (2).

Preliminary experiments in this laboratory suggested that the distribution of ^{14}C-labeled cholesterol in dogs after the administration of 4-^{14}C-cholesterol may be essentially similar to that reported for man with two exceptions: the distribution between plasma and aorta cholesterol and between plasma and adrenal cholesterol. The unusual distribution of ^{14}C-cholesterol, particularly with regard to the aorta, became evident at later time periods after the administration of 4-^{14}C-cholesterol. This report describes the pattern of distribution of ^{14}C-cholesterol in dogs at periods of one to 75 days after injecting 4-^{14}C-cholesterol.

The broad spectrum of physiologic and metabolic effects of epinephrine includes well-defined changes in cardiovascular function. The positive inotropic and vasopressor responses to epinephrine are most prominent (3), but changes in the composition of plasma lipid components (4,5) may presage overt alterations in arterial or arteriolar morphology. For example, the administration of a long-acting preparation of epinephrine is accompanied by increases in plasma cholesterol concentrations (5), which, if sustained for long periods of time, might be reflected in increased cholesterol concentrations in arterial or arteriolar tissues (6). An attempt therefore was made to determine the effects of chronic administration of long-acting epinephrine on the distribution of ^{14}C-cholesterol in dogs.

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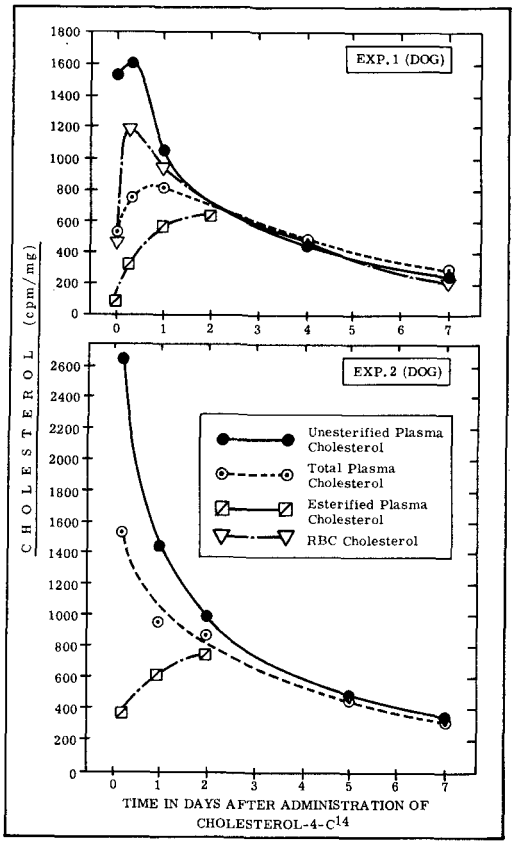

FIG. 1. Distribution of ^{14}C-cholesterol in blood cholesterol fractions after the intravenous administration of 4-^{14}C-cholesterol to dogs. The time course of ^{14}C-cholesterol distribution, illustrated in the top graph, was most frequently observed. Highest specific activities of plasma unesterified and erythrocyte cholesterol occurred 6 hr after the labeled sterol had been given, and total plasma cholesterol attained highest specific activity 24 hr after the injection of 4-^{14}C-cholesterol. In three experiments, as illustrated in the lower graph, total plasma cholesterol attained peak specific activity within 6 hr after the administration of 4-^{14}C-cholesterol.

EXPERIMENTAL PROCEDURES

Adult Beagle dogs were maintained in the research animal quarters for at least three weeks prior to being included in an experiment. The dogs were given water and dry dog food ad libitum (Canine Checkers, Ralston-Purina Company, St. Louis, Mo.). A supplement of ¼ lb of beef was given to each dog six days per week, beginning at the time of the administration of 4-^{14}C-cholesterol.

Eight adult Beagle dogs, five male and three female, were utilized for the first series of experiments. Each of seven dogs was given 4-^{14}C-cholesterol (4-^{14}C-Cholesterol, 58.2 millicuries/millimole, was obtained from New England Nuclear Corporation, Boston, Mass.) intravenously in a dose of 2 μc/kg body weight; the dose for the eighth dog was 3 μc/kg body weight. Blood samples were taken, after the administration of the labeled cholesterol, at several hourly and daily intervals.

The 4-^{14}C-cholesterol was administered to each dog according to the following method. The labeled sterol, when received from the commercial source, was dissolved in benzene. The benzene was evaporated, and the residue was dissolved in ethanol to give a solution containing 10 or 20 μc/ml. A requisite amount of the ethanolic solution was drawn into a syringe and quickly mixed with 6 to 8 ml of blood taken from the jugular vein. The contents of the syringe were injected into a jugular vein, and the syringe was "rinsed" four times with 6 to 8 ml of blood, which was returned to the dog via the jugular vein. No attempt was made to ascertain the radiochemical purity of the labeled cholesterol. In order to minimize degradation of the 4-^{14}C-cholesterol, the product was administered as soon as possible after its receipt. In the first series of experiments, the labeled cholesterol was administered within seven days of its receipt, and during the interval the ethanolic solution of 4-^{14}C-cholesterol was stored at –19C.

The utility of this method for administering ^{14}C-labeled cholesterol is shown in Figure 1. The time course of distribution of ^{14}C-cholesterol into the various fractions of dog blood is not completely comparable with that reported by Porte and Havel (7), who administered dog serum lipoproteins labeled in vitro with 4-^{14}C-cholesterol. However, ^{14}C was rapidly incorporated into blood cholesterol fractions, and the pattern of equilibration of ^{14}C-cholesterol between the blood cholesterol fractions is similar to that reported by Porte and Havel (7).

The experiments were terminated according to the following schedule: two at 24 hours, one at two days, one at four days, two at nine days, one at 26 days, and one at 37 days after the administration of 4-^{14}-cholesterol. Each dog was anesthetized with pentobarbital sodium and exsanguinated by severing the jugular veins and carotid arteries. Gall bladder bile and segments of some or all of the following organs were taken for cholesterol extraction: heart, kidney, aorta, adrenal gland, skeletal muscle, lung, liver, and pancreas.

TABLE I

Distribution Ratios of ^{14}C-Cholesterol Between Plasma, Bile, and Tissue After Administration of 4-^{14}C-Cholesterol to Dogs[a,b]

Experiment Number	Time after 4-^{14}C cholesterol (days)	Bile	Liver	KC	KM	HV	HA	ADR[c]	LU	Ao
160	1	0.87	1.06	0.30	0.45	0.57	0.28	0.35	0.67	0.04
161	1	0.40	0.96	0.19	0.24	0.38	0.10	0.32	0.61	0.04
162	2	0.98	1.04	0.72	0.91	1.00	0.98	0.70	1.32	0.14
182	4	0.70	0.72	0.58	0.84	1.00	0.22	0.90	0.83	0.10
181	9	0.74	0.95	1.02	1.10	1.40	0.85	1.70	1.06	0.18
187	9	0.64	0.87	1.13	1.34	1.28	0.82	1.23	1.13	0.32
1008	26	0.62	0.93	1.08	1.24	1.06	1.00	2.50	1.14	0.95
1111	37	0.82	0.98	0.96	0.92	1.00	0.79	1.48		2.00

[a] Expressed as cpm/mg bile or tissue cholesterol/cpm/mg plasma cholesterol.

[b] KC—kidney cortex; KM—kidney medulla; HV—ventricular myocardium; HA—atrial myocardium; ADR—adrenal gland; LU—lung; Ao—thoracic aorta.

[c] The entire adrenal gland of the dog was used; no attempt was made to separate cortex from medullary tissue. Brain cholesterol was isolated in two experiments (No. 1008 and 1111) and was devoid of radioactivity.

A second series of experiments utilized eight adult male Beagle dogs. Each dog was given 4-^{14}C-cholesterol, 4 μc/kg; and the dogs were randomly paired. The 4-^{14}C-cholesterol was administered in this series of experiments within 48 hr after its receipt from the commercial source. At intervals of 21 to 32 days, after the administration of 4-^{14}C-cholesterol, one dog in each pair was given epinephrine in oil (Adrenalin^{R} in oil, Parke-Davis and Company, Detroit, Mich.), 0.8 mg/kg body weight/day subcutaneously for 12 days. The control dog in each pair received subcutaneous injections of peanut oil. The dogs were given a two-day respite from treatment, and daily injections were given five days a week over a period of one to three weeks. A final series of injections was administered for 12 days, and the experiment was then terminated. The schedules of treatment and total duration of the experiments are summarized in Table II.

Blood samples were taken at several daily intervals prior to and during the period of epinephrine or vehicle administration. Final blood samples were taken at the conclusion of the experiment. At various intervals during the experiments, blood samples were taken at 2, 4, and 6 hr after the administration of epinephrine or the vehicle for the determination of glucose concentrations in plasma. Plasma glucose concentrations in excess of 250 mg/100 ml for a period of at least 4 hr after the subcutaneous injection of epinephrine in oil were taken as evidence of satisfactory epinephrine absorption (8). It is also assumed that doses of epinephrine capable of producing this degree of hyperglycemia would also result in both increased force of contraction of the heart and increased arterial blood pressure for a period of several hours after the administration of the catecholamine.

The experiments were terminated as described, and segments of tissue were taken for estimation of cholesterol concentrations and specific radioactivity. Also, the heart and liver were removed from each dog, trimmed of accessory tissue, and weighed.

Methods for the determination of glucose concentrations in plasma and of total cholesterol concentrations in plasma and tissues and for the radio assay of ^{14}C-cholesterol were the same as those described (8). In all experiments, cholesterol was isolated as the digitonide, and radioactivity was determined by plating the digitonide on planchets and counting in a thin-window gas-flow counter.

TABLE II

Protocols for Long-Term Experiments with Epinephrine in Oil

Dog Number	Duration of experiment[a] (days)	Time at which treatment was started[b] (days)	Duration of treatment[c] (days)
489-490	60	28	32
483-484	67	21	46
501-502	70	30	40
506-508	75	32	43

[a] From day in which 4-^{14}C-cholesterol was given to the termination of the experiment.

[b] Number of days after the administration of 4-^{14}C-cholesterol when treatment was started.

[c] Total period during which epinephrine in oil or peanut oil was administered.

TABLE III
Changes in Plasma Cholesterol Concentrations in Dogs Treated with Peanut Oil (Control) and with Epinephrine in Oil

Group	Plasma Cholesterol—mg/100 ml				
	Start[a]	Day No. 11	Start[b]	Day No. 7	Day No. 11
Control (N = 4)	126 ± 22.3	130 ± 13.5	127 ± 14.0	135 ± 2.6	118 ± 10.4
Epinephrine (N = 4)	111 ± 7.6	185 ± 18.0	129 ± 10.3	181 ± 7.5	168 ± 6.8
p(t)[c]	> 0.10	< 0.05	> 0.50	< .01	< .01

[a] Plasma cholesterol concentrations prior to the beginning of the first period of treatment.

[b] Plasma cholesterol concentrations prior to the beginning of the last period of treatment; a two-day interval separated this period of treatment from a prior 12-day or five-day period in which the catecholamine or the vehicle had been given.

[c] Significance of the difference between groups.

RESULTS

The distribution of ^{14}C-cholesterol between plasma and bile or tissues has been expressed as the ratio cpm/mg bile or tissue cholesterol/cpm/mg plasma cholesterol. An arbitrary ratio of 1.4 or less (tissue cholesterol specific radioactivity not more than 40% greater than that of plasma cholesterol) has been selected as the point at which plasma and tissue cholesterol specific radioactivities are considered to be essentially the same.

The time course of ^{14}C-cholesterol distribution in the first group of dogs is summarized in Table I. The specific radioactivity of liver cholesterol was the same as total plasma cholesterol specific radioactivity at all time periods recorded. Cholesterol from kidney, heart, and lung attained specific radioactivities comparable with plasma cholesterol at variable time periods between the second and ninth day after 4-^{14}C-cholesterol had been given. The specific radioactivity of adrenal cholesterol was greater than that of plasma cholesterol at 9, 26, and 37 days after injection of the labeled sterol. Cholesterol of the thoracic aorta attained isotopic equilibrium with plasma cholesterol between the 9th and 26th day; the specific radioactivity of the thoracic aorta cholesterol was twice that of plasma cholesterol 37 days after 4-^{14}C-cholesterol had been given.

TABLE IV
Specific Radioactivity of Plasma Cholesterol in Dogs Treated with Peanut Oil (Control) and with Epinephrine in Oil

Group	cpm/mg Plasma Cholesterol		
	14th Day[a]	60th-75th Day[a]	Percentage Decrease
Control (N = 4)	670 ± 47.5	57 ± 8.7	91.3 ± 1.74
Epinephrine (N = 4)	655 ± 29.2	48 ± 7.1	92.6 ± 0.75

[a] Time in days after administration of cholesterol. Treatment with peanut oil or with epinephrine in oil was begun on the 21st day after 4-^{14}C-cholesterol had been given to one pair of dogs. The administration of epinephrine in oil or the vehicle was begun on the 28th-32nd day after 4-^{14}C-cholesterol had been given in three pairs of dogs.

The difference between the specific radioactivities of thoracic aorta cholesterol and plasma cholesterol was increased at later time periods, i.e., 60–75 days after the administration of 4-^{14}C-cholesterol (Table VI). Similar but smaller differences were observed when the specific radioactivity of cholesterol from segments of abdominal and terminal aorta were compared with plasma cholesterol specific radioactivities (Table VI). Adrenal cholesterol specific radioactivity was almost twice that of plasma at these later periods (Table V). The specific radioactivity of all other tissues studied was either equal to or not more than 40% greater than the specific activity of plasma cholesterol. Cholesterol obtained from gall bladder bile had specific radioactivities which were less than that of plasma cholesterol at all time periods (Tables I and V).

Epinephrine administration was accompanied by significant increases in plasma cholesterol concentrations (Table III). During the two-day interval between treatment periods, plasma cholesterol concentrations decreased toward control levels (Table III). Heart weight in epinephrine-treated dogs was 90.5 + 4.4 g/10 kg body weight and in the control dogs 73.0 + 4.0 g/10 kg body weight; the difference was significant below the 5% level.

The specific radioactivity of plasma cholesterol, 14 days after the administration of 4-^{14}C-cholesterol, was comparable in both groups of dogs (Table IV). A decline of more than 90% of the specific radioactivity of plasma cholesterol, recorded on the 14th day after the labeled sterol had been given, occurred during the subsequent 46 to 61 days of the experi-

TABLE V

Distribution Ratios of ^{14}C-Cholesterol Between Plasma, Bile, and Tissues in Dogs Given Peanut Oil (Control) or Epinephrine in Oil[a,b]

Treatment	Bile	L	SM	HA	HV	KC	KM	ADR	LU	PAN
Control (N = 4)	0.81±0.03	1.12±0.044	1.22±0.09	0.84±0.11	1.15±0.10	1.12±0.05[c]	1.12±0.07	1.89±0.21	1.37±0.23	1.33[d]
Epinephrine (N = 4)	0.91±0.06	1.13±0.04	1.38±0.067	1.01±0.25	1.06±0.27	1.31±0.05[c]	1.16±0.05	1.58±0.06	1.28±0.02	1.33±0.2

[a] Expressed as cpm/mg bile or tissue cholesterol/cpm/mg plasma cholesterol.

[b] L—liver; SM—skeletal muscle; HA—cardiac atrial muscle; HV—cardiac ventricular muscle; KC—kidney cortex; KM—kidney medulla; ADR—adrenal gland; LU—lung; PAN—pancreas.

[c] Significance of difference between groups: $p(t) < 0.02$.

[d] Only three observations were made, and no standard errors have been calculated.

ment. Differences in the specific radioactivity of plasma cholesterol in control and epinephrine-treated dogs and in the percentage decrease in radioactivity at the end of the experimental periods were not significant.

The distribution of ^{14}C-cholesterol between plasma, bile, and tissues in control and epinephrine-treated dogs is summarized in Tables V, VI, and VII. An increase in the specific radioactivity ratio for kidney cortex cholesterol (cpm/mg kidney cortex cholesterol/cpm/mg plasma cholesterol) was observed in epinephrine-treated dogs (Table V), and this change was reflected in a relative increase in kidney cortex cholesterol specific radioactivity when compared with the radioactivity of medullary cholesterol (Table VI).

The distribution of cholesterol specific radioactivity within the aorta appeared to be modified in epinephrine-treated dogs (Tables VI and VII). A decrease in the increment between the specific radioactivity of thoracic aorta cholesterol and the abdominal and terminal segments was observed in epinephrine-treated dogs, and this change was statistically significant when the cholesterol radioactivity of thoracic and terminal aortic segments were compared (Table VII).

No other significant changes in the distribution of ^{14}C-cholesterol were observed in epinephrine-treated dogs (Table V). The increase in the specific radioactivity ratio for skeletal muscle and the decrease in the distribution ratio for the adrenal gland were consistent changes in epinephrine-treated dogs and may attain statistical significance in larger samples.

No differences were observed in the concentrations of tissue cholesterol in control and epinephrine-treated dogs, and these negative results have not been included.

TABLE VI

^{14}C-Cholesterol Distribution Between Plasma and Aortic Segments in Dogs Given Peanut Oil (Control) or Epinephrine in Oil[a]

Experiment Number	Duration[c]	Distribution Ratios[b] Th Ao	Ab Ao	Ter Ao
		Control		
490	60 Days	2.63	1.79	1.84
484	67 Days	3.04	1.32	1.59
501	70 Days	4.34	2.95	2.72
506	75 Days	5.08	3.16	3.17
	Average	3.77 ± 0.65	2.31 ± 0.50	2.33 ± 0.36
		Epinephrine		
489	60 Days	3.83	2.60	2.64
483	67 Days	2.40	2.11	2.78
502	70 Days	2.33	1.90	2.34
508	75 Days	3.77	3.60	3.47
	Average	3.08 ± 0.42	2.55 ± 0.38	2.81 ± 0.24

[a] Expressed as cpm/mg bile or tissue cholesterol/cpm/mg plasma cholesterol.

[b] Th Ao—thoracic aorta; Ab Ao—abdominal aorta; Ter Ao—terminal aorta; cholesterol concentrations in both control and treatment groups were: thoracic aorta, 1.5-1.7 mg/gm wet weight; abdominal aorta, 1.4-1.7 mg/gm wet weight; and terminal aorta, 1.5-1.9 mg/gm wet weight.

[c] Indicates duration of experiment in days.

DISCUSSION

That the liver is the primary site of cholesterol synthesis in the dog has been demonstrated both by in vitro (9) and in vivo (10)

TABLE VII

Distribution of ^{14}C-Cholesterol Within Aortic Segments and Within the Kidneys of Dogs Treated with Peanut Oil (Control) or Epinephrine in Oil

Group	ThAo/AbAo[a]	ThAo/TerAo[b]	KC/KM[c]
Control (N = 4)	1.73 ± 0.212	1.65 ± 0.122	0.99 ± .023
Epinephrine (N = 4)	1.23 ± 0.093	1.10 ± 0.123	1.12 ± .03
p(t)	> 0.05	< 0.02	< 0.02

[a] Cpm/mg thoracic aortic cholesterol/cpm/mg abdominal aorta cholesterol.

[b] Cpm/mg thoracic arotic cholesterol/cpm/mg terminal aorta cholesterol.

[c] Cpm/mg kidney cortex cholesterol/cpm/mg kidney medulla cholesterol.

experiments. The patterns of distribution of ^{14}C-cholesterol in dogs therefore would be compatible with the attainment of equilibrium between plasma and nonneural extrahepatic tissue cholesterol stores other than those of the aorta and adrenal gland. The small standard errors for the distribution ratios (Table V), despite the extended differences in the lengths of the experiments, indicate that the distribution of ^{14}C-cholesterol between plasma and tissues remains constant over long periods of time. Chobanian and Hollander (2) have reported a similar distribution of ^{14}C-cholesterol for most tissues in man. The significant differences in ^{14}C-cholesterol distribution, when the studies in man are compared with those in the dog, involve the aorta and adrenal gland; in man, these tissues maintain cholesterol specific radioactivities comparable with that of plasma for many months (2). At time periods of 60 to 75 days after 4-^{14}C-cholesterol was given, there was a small increment in the specific radioactivity of cholesterol from all tissue studied except atrial myocardium. This increase over the specific radioactivity of plasma cholesterol may indicate the incorporation of ^{14}C-cholesterol into cellular elements which assume a structural role with a somewhat slower turnover rate.

Rosenfeld and Hellman (11) have shown that bile cholesterol is in equilibrium with the plasma-liver cholesterol pool in patients with total biliary drainage. In the experiments discussed here the specific radioactivity of bile cholesterol was consistently less than that of plasma or liver cholesterol. The divergence in radioactivity between bile and liver cholesterol suggests some heterogeneity in the mixing of unlabeled sterol from exogenous sources or newly synthesized with the larger liver-plasma pool. Unlabeled cholesterol, either from exogenous sources or newly synthesized, could dilute the smaller biliary cholesterol pool to a greater extent than the much larger cholesterol compartments in liver and in plasma. The differences between the distribution of ^{14}C-cholesterol into bile in these experiments in the dog and in the experiments reported by Rosenfeld and Hellman (11) may result from a) species differences or b) the diversion of bile flow to the exterior with markedly altered gastrointestinal function as the normal enterohepatic circulation of bile salts and cholesterol was abolished.

Unesterified cholesterol in the adrenal cortex and in plasma, in the dog, may be maintained at comparable specific radioactivities by continued feeding of 4-^{14}C-cholesterol (12). However the unesterified cholesterol fraction in the dog adrenal gland accounts for not more than 10% of the total adrenal cholesterol concentration (13). The relatively high specific radioactivities of total adrenal cholesterol at later time intervals after a single injection of ^{14}C-cholesterol may represent the presence of two cholesterol pools with different turnover rates. The adrenal cortex may contain a small unesterified cholesterol pool in equilibrium with plasma cholesterol, which is the direct precursor of adrenal cortical steroids (11) and a larger esterified sterol compartment with a much slower turnover.

The cholesterol pool of the thoracic aorta in dogs apparently interchanges at slower rates with plasma cholesterol than do the cholesterol pools of other nonneural organs. The relatively high specific activity of thoracic aorta cholesterol at later time intervals is compatible with both a slow turnover rate and absence of in-situ synthesis of cholesterol. Eckles et al. (10), using ^{14}C-labeled acetate in vivo, have shown that in-situ cholesterol synthesis is negligible in the aorta of dogs. The lower segments of the aorta show a pattern similar to that of the thoracic aorta, but retention of ^{14}C-cholesterol with time is greatest in the thoracic aorta.

Long-term epinephrine administration apparently resulted in a more equal distribution of ^{14}C-cholesterol throughout the length of the aorta (Table VIII). The larger variations in specific radioactivity of aorta cholesterol (Table VI) may have reflected individual variation among the dogs as well as the differences in duration of the experiments. Whatever their sources, these large variations preclude any interpretation of the processes involved in producing the changes in the distribution of ^{14}C cholesterol in the aortas of epinephrine-treated dogs. Further evidence that long-term epinephrine may have altered the dynamics of cholesterol distribution derives from the changes in the distribution of renal ^{14}C-cholesterol in epinephrine-treated dogs. The relatively small increase in the ratio cpm/mg kidney cortex cholesterol/cpm/mg plasma cholesterol in epinephrine-treated dogs may have resulted from a change of considerable magnitude in the distribution of ^{14}C-cholesterol into the arterial walls of the renal cortical vasculature since cholesterol in the arterial walls would be diluted by the larger mass of cholesterol in the parenchymal cells of the kidney. Confirmation of these results in a larger series of animals and extension of the data to other arterial systems,

such as cerebral and coronary, may have relevance in experimental analyses of the pathogenesis of arterial and arteriolar disease.

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Comparison of Antioxidant Activities of Tocol and Its Methyl Derivatives

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ABSTRACT

In tests with purified menhaden oil at 37C and with squalene at 37C and 50C, unsubstituted tocol, 7,8-dimethyl tocol (γ-tocol), and 8-methyl tocol (δ-tocol) were superior antioxidants to 5,7-dimethyl tocol and 5,7,8-trimethyl tocol (α-tocol). In contrast to previous reports, there were no differences in the order of the activities of the tocopherols at different temperatures.

INTRODUCTION

In 1937 OLCOTT AND EMERSON (1) observed that the effectiveness of α-(5,7,8-trimethyl)-, β-(5,8-dimethyl)-, and γ-(7,8-dimethyl)-tocols as in vitro antioxidants did not parallel their effectiveness in overcoming the sterility of the vitamin E-deficient female rat. γ-Tocol was the most effective antioxidant in lard or oleo oil at 75C; α-tocol was superior as the vitamin. Hove and Hove (2) later suggested that the relative in vitro antioxidant effectiveness of α- and γ-tocols depended upon the temperature of the tests, and that α-tocol was in fact a superior antioxidant to γ-tocol at body temperature (37C). Lea (3) then compared these and other tocols in several substrates and at several temperatures and reported that the relative activities differed depending upon these variables. For example, α-tocol was reported to be a more effective antioxidant than the others in a highly unsaturated fat and at low temperature. However, the substrates used in all of these studies contained peroxides.

If vitamin E functions in vivo primarily as an antioxidant, an hypothesis that is currently supported by some investigators (see Ref. 5), but questioned by others (6-9), it is obviously important to have more complete information. We report here a reinvestigation, but in purified substrates, of the antioxidant activities of α-, γ-, and δ-tocols, and of unsubstituted tocol and 5,7-dimethyl tocol. Lea (3) had reported that tocol was not as effective as γ-tocol under any of his conditions; a synthetic preparation of 5,7-dimethyl tocol was also included in his studies.

EXPERIMENTAL

Materials and Methods

The tocols were Distillation Products, Hoffman-La Roche, or Pierce Chemical Company products and were used without further purification. The substrates for antioxidant assays were squalene (Eastman) and a menhaden oil (a highly unsaturated oil) that had been molecularly distilled (Bureau of Commercial Fisheries, Seattle, Wash.). Each was further purified by silicic acid chromatography as follows: 50 g of silicic acid was slurried in 30-60 petroleum ether (redistilled) and packed in a 2.5 cm × 40 cm column under 2.75 psi N_2 pressure to a column height of 25 cm. The substrate (25 g) in 5 times its volume of petroleum ether, was applied to the column and then eluted with additional petroleum ether. The leading edge of the substrate (about 0.5-1 g) was discarded. A 500 ml fraction was then collected (approximately 60% of the substrate), evaporated and diluted with solvent to give a final concentration of 0.2 g/ml. This material was used only when peroxide and TBA tests were negative. Antioxidant activity was measured by a weight-gain method (10) as follows: Covered beakers containing 200 mg of substrate with and without additive were incubated in constant temperature draft ovens. Once or twice daily they were removed from the oven, tested for rancidity by odor, cooled to room temperature, weighed and returned to the oven. The end of the induction period was indicated by a sharp gain in weight which usually coincided with the development of the odor of rancidity. Antioxidant concentrations were chosen so as to give induction periods of convenient range (1 to 30 days).

Peroxides were determined by slight modification of the AOCS Method Cd8-53. Samples were held in a nitrogen atmosphere during the one-minute reaction period with potassium iodide. Iodine indicator (Paragon, Eastern Chemical Corp.) was used to detect the end point of the thiosulfate titration. The TBA test was carried out as described by Kwon and Watts (11).

RESULTS

The antioxidant activities of the five tocols were compared at 37C in purified squalene and

TABLE I
Antioxidant Activity of Tocols in Menhaden Oil at 37C

Tocol added	Induction Period (Days)[a] Concentration (μm/200 mg of substrate)						
	0.25	0.5	1.0	2.0	2.5	5.0	7.5
Run 1							
Tocol	2;2	3;3					
α	1.5;2.5	3;3	5;5				
γ	3;3	5;5					
s	2;2	4;4					
Run 2							
Tocol	2;2	2.5;2.5	3;3	3;3			
α	2;2	1;1	3;3	3;5	7;7		
γ	2;2	3;3	4;5	6;6			
s	2;2	2;2	3;3	4;4			
Run 3							
Tocol			5;6		7;7	11;11	13;13
α			6;6		7;7	7;7	7;7
γ			8;8		10;10	12;12	13;15
s			6;6		9;9	12;12	14;18
Run 4							
5,7-Dimethyl					6;6		6;6
α					6;6		6;6
γ					13;13		13;13

[a] Induction periods without additive, 1 day. Duplicate runs are separated by semicolons.

menhaden oil and at 50C in squalene. Several runs are reported (Tables I-III). Duplicates within a single run usually showed the same induction periods, but the results in separate runs were occasionally variable. α-Tocol was an inferior antioxidant compared to the others under all conditions except at low concentrations in menhaden oil at 37C (Table I), in which case there was no apparent advantage. Initial weight gains occurred at approximately the same time but the oil protected by γ-tocol did not continue to gain weight as rapidly (Fig. 1). Fig. 1 also illustrates the increased rate of chain initiation typical of higher concentrations of α-tocol (12, 13). The initial weight gain at concentration of 0.2 μm/200 mg was faster than that at 0.05 μm/200 mg, but the "break" to more rapid weight gain occurred after a longer incubation period.

The relative antioxidant activities of the tocols in squalene were generally the same as in menhaden oil. Squalene has advantages over other lipid substrates in that it is available commercially, is readily purified, and has a sharp break at the end of the induction period. In squalene the tocols showed the same relative order of antioxidant activity as in menhaden oil, but the data were more reproducible. Hence, relative antioxidant effectiveness did not appear to be modified by the ethylene-interrupted double bond system. Higher concentrations of α-tocol (2-4 μm/200 mg) were required to show the initial prooxidant phenomenon in squalene than in menhaden oil (0.2 μm/200 mg) (data not shown).

DISCUSSION

Both Hove and Hove (2) and Lea (3) reported that α-tocol was equal to or better than γ-tocol as antioxidant at 37C, but at higher temperatures γ-tocol was superior. Peroxides were present in their systems. Under our initially peroxide-free conditions, α-tocol was always a poorer antioxidant except that at a

TABLE II
Antioxidant Activity of Tocols in Squalene at 37C[a]

Tocol added	Induction period (days) Concentration (μm/200 mg substrate)			
	0.05	0.125	0.15	0.25
Run 1				
Tocol	2.5;2.5		10;11	
α-	0.5;0.5		1;1	
γ-	2.5;2.5		9;9	
s-	1.5;1.5		6;7	
Run 2				
Tocol		10;16		16;21
α-		2;2		3;3
γ-		10;12		15;16
s-		7;7		13;17
Run 3				
5,7-Dimethyl		10;10		14;14
α-		10;10		15;16
γ-		>35		>35

[a] Induction period without additive, 0.5 days.

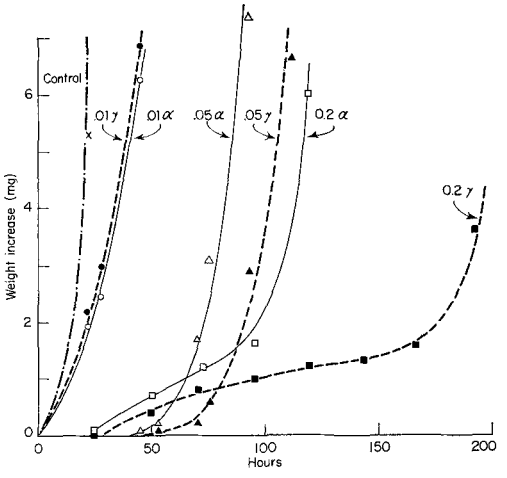

FIG. 1. Comparative antioxidants effectiveness of 5,7,8 (trimethyl)- and 7,8 (dimethyl)-tocols in menhaden oil at 37C. Concentration of tocols shown as μm/200 mg substrate.

relatively low concentration the activities of the two were approximately equal (Fig. 1).

The 5-methyl substituent present in α-tocol is absent in γ- or δ-tocols or in unsubstituted tocol. Other 5-methyl substituted tocols are β-(5,8-dimethyl)tocol, ε-(5,8-dimethyl)tocotrienol and ζ-(5,7,8-trimethyl)tocotrienol. In preliminary studies not reported here these compounds also were found to be considerably less effective antioxidants than γ-, δ-, or unsubstituted tocol. Hence a free hydrogen at the 5 position of the tocol ring gives maximum antioxidant activity (cf. Lea and Ward, Ref. 14). In a parallel case, Horswill and Ingold (15) compared the products of oxidation of 2,4-di-t-butyl phenol with those of 2,4,6-tri-t-butyl phenol. Those of the former, with a hydrogen ortho to the phenolic group, gave a more complex series of reaction products, some with orthoquinones as intermediates. Similarly, in the tocol series, those members containing hydrogen at position 5 could conceivably yield orthoquinones having antioxidant properties; substantiating evidence is being sought.

Within the limits of the observations reported in this paper, 5,7-dimethyl tocol had the same antioxidant activity as α-tocol. The 8-methyl substituent therefore did not measurably change the antioxidant activity. This is also indicated by the approximately equal antioxidant effectiveness of tocol and 8-methyl tocol (δ-tocol). The *vitamin E* activity of 5,7-dimethyl tocol has not yet been reported. It might be predicted that 5,7-dimethyl tocol would be more effective in vitamin E activity than γ-tocol, since it is already known that β-tocol (5,8-dimethyltocol) is more effective than γ-tocol (7,8-dimethyltocol) (15).

Since our data show that, even with highly unsaturated substrates and at body temperature, α-tocol is inferior to γ-tocol as an antioxidant, its superior vitamin-E activity (16) must depend on factors other than uncomplicated antioxidant activity. Differences in rates of absorption, cell permeability and excretion have been previously suggested (2, 17) as important contributing factors. Other difficulties in accepting the concept that vitamin E activity resides solely in the antioxidant properties of tocols have recently been discussed by Green et al. (6-9).

TABLE III

Antioxidant Activity of Tocols in Squalene at 50C[a]

Tocol added	Induction period (days) Concentration (μm/200 mg substrate)					
	0.05	0.15	0.2	0.25	0.4	0.6
Run 1						
Tocol	2;2	5;6		10;11		
α-	0.5;0.5	0.5;0.5				
γ-	2;3	7;9		11;12		
s-	1;1	2;5				
Run 2						
Tocol			6;7		12;14	11;14
α-			1;1		2;2	3;3
γ-			7;8		12;14	16;17
s-			5;6		11;11	14;14
Run 3						
5,7-Dimethyl			6;6		8;9	9;9
α-			6;6		8;9	9;9
γ-			23;22		24;28	22;28

[a] Induction period without additive, 0.5-1 day.

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Isolation and Characterization of Cholesterol-5β,6β-Oxide from an Aerated Aqueous Dispersion of Cholesterol

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ABSTRACT

An unknown autoxidation product in an aerated cholesterol sol was isolated by preparative thin layer chromatography. This compound was identified as cholesterol-5β,6β-oxide by gas liquid chromatography along with infrared and mass spectrometry.

INTRODUCTION

NUMEROUS STUDIES HAVE SHOWN that cholesterol can be easily autoxidized in the colloidally-dispersed state. Lifschütz (1, 2) introduced the term "oxycholesterol" to denote the air-oxidation product of cholesterol, but more recent studies have indicated that numerous compounds are present in aerated cholesterol sols (3). Bergstrom and Wintersteiner (4), using an aerated aqueous dispersion of cholesterol at 37C and 85C, identified the major oxidation product as 7-ketocholesterol along with 7α-hydroxycholesterol, 7β-hydroxycholesterol and 3,5-cholestadiene-7-one. In addition to the above-mentioned compounds, Mosbach et al. (5) isolated cholestane-3β,5α,6β-triol from air-oxidized cholesterol as a minor reaction product.

During the study of air-oxidation products of colloidally-dispersed cholesterol, a relatively large spot of an unidentifiable oxidation product (compound X) was noted on an analytical thin-layer chromatoplate. The compound X spot overlapped slightly on the 7-ketocholesterol spot. The object of this study was to separate compound X from other steroids by preparative thin layer chromatography and to characterize the purified compound by gas-liquid chromatography (GLC) as well as by infrared and mass spectrometry.

EXPERIMENTAL

Materials

Cholesterol, purchased from Eastman Kodak, Rochester, N.Y., was recrystallized from methanol. Cholestane-3β,5α,6β-triol as well as the triacetate and cholesterol-5α,6α-oxide were obtained from Steraloids, Inc., Pawling, N.Y.

Cholesterol-5β,6β-oxide was prepared by a method similar to that of Davis and Petrow (6). About 50 mg of cholestane-3β,5α,6β-triol triacetate and 125 mg KOH were dissolved in 4 ml of absolute ethanol. The solution was refluxed for 2 hr and then diluted with distilled water to a volume of 50 ml. The mixture was extracted with 50 and 25 ml successive portions of ethyl ether. The combined ether extract was washed with distilled water to neutrality and dried with anhydrous Na_2SO_4. Ethyl ether was evaporated under reduced pressure. The β-oxide was crystallized from methanol. The melting point was 132C; yield, 58%.

Autoxidation of Cholesterol in the Dispersed State

The autoxidation of cholesterol was carried out by a method similar to that of Mosbach et al. (5). Prior to air-oxidation, cholesterol was dispersed in an aqueous continuous phase containing stearate as a surfactant. The continuous phase was prepared by dissolving 250 mg of stearic acid and 300 mg of trisodium phosphate in 37% ethanol solution. About 80 ml of this solution was diluted to 500 ml with distilled water and placed in a 1 liter wide-mouthed bottle. The pH of the solution was 8.5. After dissolving 1 g of recrystallized cholesterol in 75 ml of boiling absolute ethanol, the cholesterol solution was gradually added to the stirred stearate solution. The resulting dispersion was aerated by rapid stirring for 4.5 hr at 70C and 1.5 hr at 83C. Upon cooling to 25C, the dispersion was acidified to a pH of 6.0.

Steroids were extracted from the aqueous dispersion with three 200-ml portions of ethyl ether. The combined extracts were washed four times with 150 ml of 2% KOH solution to remove stearic acid and then washed with water to remove KOH. The ethyl ether in the extract was evaporated under reduced pressure to obtain a steroid residue.

Alkaline Treatment of Autoxidation Products of Cholesterol

The mixture of autoxidation products was treated with a hot alkaline solution to decompose 7-ketocholesterol, which overlapped with compound X. The autoxidation product residue (from above) was dissolved in 120 ml of

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warm absolute ethanol. After adding an alkaline solution (8 g KOH in 12 ml of distilled water) to the steroid solution, the mixture was refluxed for 2 hr, cooled and diluted with 250 ml portions of ethyl ether. The combined ethyl ether extracts were washed with 2% KOH solution and distilled water. Ethyl ether in the extracts was removed under reduced pressure. The residue was dissolved in 40 ml of chloroform-methanol (95:5, v/v).

Separation of Compound X from Other Steroids by Preparative TLC

The steroids in the chloroform-methanol (from above) were separated by preparative TLC on layers of silica gel without a binder (Adsorbosil 2, Applied Science Laboratories, Inc.) with a thickness of 1 mm.

One part of silica gel powder was slurried with 1.5 parts of water for spreading on 200 × 200 mm glass plates with a Desaga/Brinkmann adjustable applicator. Each plate was activated at about 110C for approximately 1 hr. Application of a large amount (about 30 mg per plate) of autoxidized cholesterol to preparative TLC plates was facilitated by a capillary tube applicator consisting of a series of 40 permanently-spaced melting-point tubes. The plates were first developed with a solvent system of ethyl ether-cyclohexane (90:10). After the plates were dried at 25C, they were further developed with ethyl ether. When the plates were sprayed with 0.2% dichlorofluorescein in ethanol, the compound X zone, as well as other steroid zones, were visualized under ultraviolet light.

The compound X zone on each plate was swept into a sintered-glass filter tube. The steroid was eluted from silica gel with chloroform-methanol (50:50) and the eluate was concentrated under reduced pressure. The concentrated steroid solution was applied to silica gel plates (1 mm thickness) for further purification. With ethyl ether as the developing solvent, the dye remained at the origin and compound X moved toward the center of the plate. The position of the steroid band was detected by applying 50% H_2SO_4 to a confined center channel and warming slightly in a 50C oven. The steroid in the H_2SO_4-treated zone became fluorescent under U V light. Elution of the steroid from silica gel was carried out as mentioned before. The steroid was crystallized out from methanol.

Analytical TLC

A 0.25 mm layer of silica gel (Adsorbosil 2) slurry was applied to 200 × 200 mm glass plates for analytical determinations. The plates were activated for about 1 hr at approximately 110C. Ethyl ether was used to develop the plates. Visualization of spots was attained by spraying with 50% H_2SO_4 and heating the plates in an oven at 110C. To tentatively identify steroid spots, R_c values were calculated. R_c is defined as the distance of the sample spot from the origin/distance of the cholesterol spot from the origin.

Gas-Liquid Chromatography

Silyl ether derivatives of steroids were prepared according to the method of Vandenheuvel et al. (7). Gas-liquid chromatography was carried out with Barber-Colman Model 10 gas chromatograph with a hydrogen flame ionization detector. Pyrex glass columns (U-shaped, 6 ft long, 4 mm I.D.) were packed with 1% SE-30, 1% QF-1 and 1% NGS on Gas-Chrom-Q (silane-treated support), 80/100 mesh. The operating conditions were as follows: nitrogen carrier gas flow rate, 95 ml/min for SE-30 and 120 ml/min for QF-1 and NGS; column temperature, 212C; flash evaporator temperature, 280C. A 10 ul Hamilton syringe was used to inject a suitable volume (usually 2 ul) of steroid solution. Retention times were calculated relative to cholestane.

Infrared Spectroscopy

Infrared spectra of steroids were obtained with Beckman IR-4 infrared spectrophotometer. The steroids were dissolved in carbon tetrachloride to prepare approximately 1% solutions.

Mass Spectroscopy

The mass spectra of steroids were obtained with a Consolidated Electrodynamics Corp. spectrometer, Model 21-103 C. The sample size was approximately 1 mg for each determination. The ionization potential was 70 ev and the ionizing current was 50 uA.

RESULTS AND DISCUSSION

When an aerated cholesterol sol was examined by analytical TLC, nine distinct spots were detected on a H_2SO_4-charred chromatoplate (Fig. 1, A). The following steroid spots in chromatogram A of Fig. 1 were identified by spot position (R_c values) and spot colorations: cholesterol (spot 2), 7-ketocholesterol (spot 6), 7β-hydroxycholesterol (spot 7), 7α-hydroxycholesterol (spot 8) and cholestane-3β, 5α,6β-triol (spot 9). The major spot above the 7-ketocholesterol was designated as compound X (spot 5). Upon heating the TLC plates at 60C for 30 min, the compound X spot turned yellow. Further heating caused

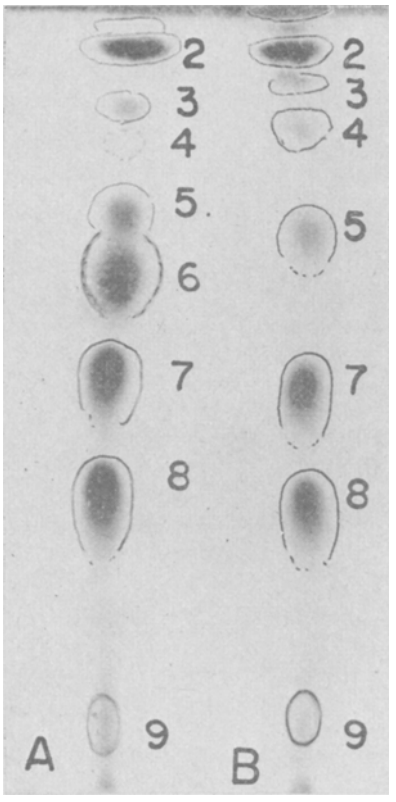

FIG. 1. Thin-layer chromatograms of steroids in aerated cholesterol sol. *A*, No alkaline treatment. *B*, Hot alkaline treatment.

spot darkening with no hue alteration. Smith et al. (8), using two-dimensional TLC, reported that 15 autoxidation products were formed during aeration of cholesterol dispersions at 85C. Only four steroid products, 7α- and 7β-hydroxycholesterols, 7-ketocholesterol and cholestane-3β,5α,6β-triol, were identified by these investigators.

Preparative TLC was selected for the separation of compound X from other steroids because of the excellent resolving power. However, the complete avoidance of overlapping of compound X and 7-ketocholesterol bands on TLC plates could not be accomplished by solvent combinations or double development procedures. Thus, degradation of 7-ketocholesterol, without the destruction of compound X, was considered essential for the isolation of purified compound X. When the autoxidized cholesterol mixture was treated with a hot alkaline solution, 7-ketocholesterol was the only major steroid to be decomposed (Fig. 1, B). Numerous decomposition products, including 3,5-cholestadiene-7-one as the major product,

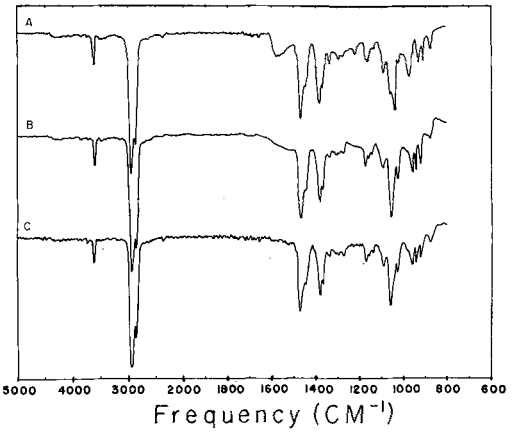

FIG. 2. Infrared spectra of cholesterol-5,6-oxides and compound X. *A*, α-oxide. *B*, β-oxide. *C*, Compound X.

were formed. All of the products of 7-ketocholesterol degradation had R_c values much higher than that of compound X (R_c = 7.4). Bergstrom and Wintersteiner (9) mentioned that 7-ketocholesterol was decomposed in hot alkaline medium, and suggested 3,5-cholestadiene-7-one as the reaction product.

Compound X, eluted from adsorbent bands and crystallized from methanol, was considered pure since only one spot was visualized on a developed chromatogram and one peak on the gas chromatogram. The compound was subjected to infrared spectroscopic analysis for elucidation of the chemical structure. The infrared spectrum for 1% solution of compound X in CCl_4 is presented in Fig. 2. The strong absorption bands in the functional group region between 5,000 and 1,350 cm^{-1} were located at 3,600, 2,900, 1,470 and 1,384 cm^{-1}. The presence of a hydroxyl group in the compound was indicated by the hydroxyl stretching vibration at 3,600 cm^{-1}. Judging from the limited size of the band at 3,600 cm^{-1}, a monohydroxy sterol was considered as a possibility. The absence of a strong band in the neighborhood of 1,700 cm^{-1} provided evidence that a carbonyl group was not present in the compound. Infrared spectrum of cholesterol, with a double bond at C_5,C_6 in the B ring, has a weak stretching band in the region of 1,680 cm^{-1}. With no absorption band in the vicinity of 1,680 cm^{-1}, compound X apparently does not have a 5,6 double bond structure. Introduction of oxygen at the 5,6 position of cholesterol during autoxidation to form cholesterol oxide was considered to be a reasonable explanation for the lack of double

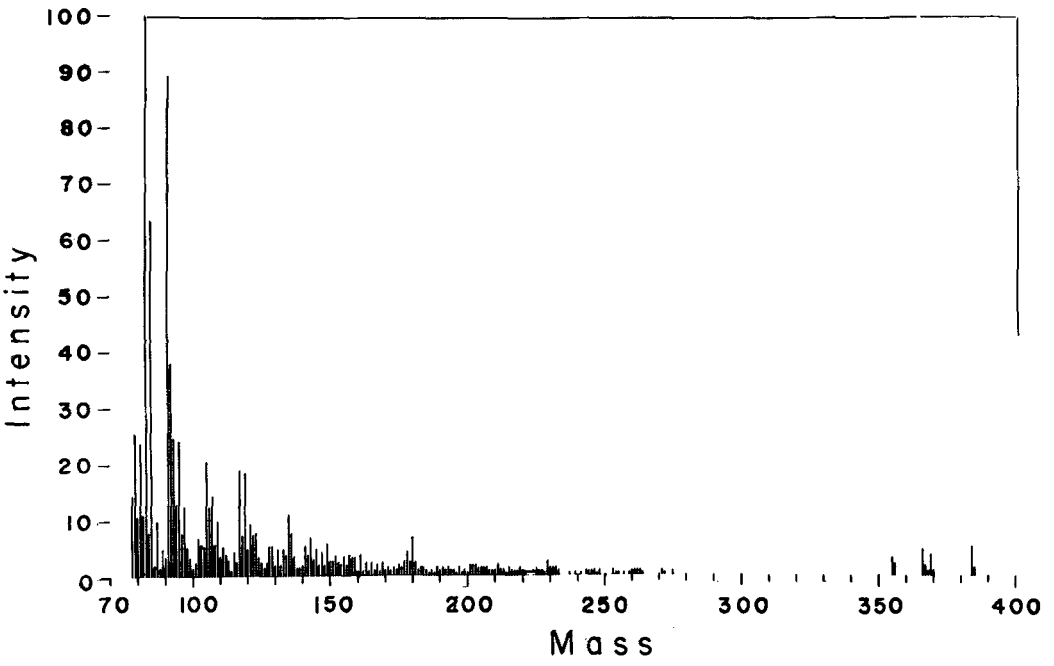

FIG. 3. Mass spectrum of compound X.

bonding in compound X. Cholesterol oxide, with asymmetric carbons at positions 5 and 6, can exist in epimeric forms, namely α- and β-oxides. Recently, Fioriti and Sims (10) reported the presence of cholesterol α-epoxide in crystalline cholesterol previously heated at 175C. Further, Bergstrom and Samuelsson (3) implied that cholesterol oxide is an intermediate in the reaction scheme proposed for the autoxidation of cholesterol. On the presumption that compound X was a cholesterol oxide, the purified compound suspended in a small amount of water was heated in a sealed melting point tube at 115C for 4 hr to fracture the epoxide ring, if present. Submission of the heated compound to TLC indicated that the reaction product was cholestane-3β,5α,6β-triol. According to Westphalen (11), cholesterol α-oxide, heated under the above-mentioned conditions, was converted to the trans-triol (3β, 5α,6β-triol). More recently, Plattner et al. (12) found that when the acetate of either α- or β-oxide of cholesterol was heated with water at 160C, the product was the trans-triol monoacetate.

Elemental analysis of compound X provided the following percentage composition: C = 80.8, H = 11.1 and O = 8.8 (determined separately). If compound X is regarded as cholesterol oxide, then the percentage composition of $C_{27}H_{46}O_2$ is C = 80.4%, H = 11.4% and O = 8.2%. From a comparison of the calculated and experimental percentages, it may be concluded that the composition of compound X is similar to that of cholesterol oxide.

Synthetic cholesterol-α-oxide and cholesterol-β-oxide were used as standards to confirm the structure of compound X as cholesterol oxide. Each of the crystallized synthetic oxide isomers did not contain nonoxide impurities since one spot on the developed analytical TLC plate and one peak on the gas chromatogram were obtained. Using ether as the developing solvent for TLC, the α- and β-oxides, as well as compound X, had similar R_c values (about 7.4) and yellow spot coloration (H_2SO_4 spray). Even with a variety of other TLC solvent systems, the α- and β-isomers could not be sep-

TABLE I

Gas Chromatographic Data on Cholesterol-5,6-oxides

Steroid	Relative retention time[a]			Steroid number
	SE-30	QF-1	NGS	SE-30
Cholesterol-5α,6α-oxide	3.00	7.10	19.2	30.40
Cholesterol-5β,6β-oxide	3.00	7.00	19.6	30.40
Cholesterol-5α,6α-oxide (Silyl ether derivative)	3.73			
Cholesterol-5β,6β-oxide (Silyl ether derivative)	3.58			

[a] Relative to cholestane.

arated. The α- and β-oxides were examined by gas-liquid chromatography with selective (QF-1 and NGS) and nonselective (SE-30) liquid phases. The relative retention times (RRT) and steroid numbers, presented in Table I, were similar for both of the oxide isomers. Although the RRT of silyl ether derivatives of the oxides were slightly different, mixed isomers did not separate into two distinct peaks on SE-30. The RRT and steroid number of compound X were similar to those for the α- and β-oxides.

Infrared and mass spectroscopic analyses were carried out to establish conclusively the structural nature of compound X. The infrared spectra of α-oxide, β-oxide and compound X are presented in Fig. 2. Identical absorption spectra in the functional group region (5,000-1,350 cm^{-1}) were obtained for α-oxide, β-oxide and compound X. However, in the fingerprint region (1,350 to 600 cm^{-1}), the positions and intensities of the bands were considerably different for α-oxide and β-oxide. In this frequency region, each of the isomers had one major band with maximum absorption at 1,030 cm^{-1} for the α-oxide and 1,060 cm^{-1} for the β-oxide. The entire spectrum of compound X was identical with that of authentic β-oxide. Further proof of the structural identification of compound X as cholesterol-5β,6β-oxide was gained from mass spectroscopic analysis. The spectrum of compound X (Fig. 3) was the same as that for cholesterol-β-oxide. The parent peak was not present in the mass spectrum of compound X, but the peak at m/e 384 may be attributable to a dehydration product. Thus, the addition of 18 (H_2O) to 384 is equivalent to the expected molecular weight of 402 for cholesterol-5,6-oxide.

When compound X was mixed with authentic cholesterol-5β,6β-oxide, no melting point depression of the β-oxide (mp 132C) was noted. The melting point of the authentic α-oxide was 142C.

In summary, the experimental data of this study supports the assumption that compound X is indeed cholesterol-5β,6β-oxide.

ACKNOWLEDGMENT

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Circum-Annual Changes in Triglyceride Fatty Acids of Bat Brown Adipose Tissue

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ABSTRACT

A circum-annual study of the fatty acids of brown adipose tissue triglycerides of *Eptesicus fuscus* has demonstrated a rhythmic pattern of change. This is seen as a reciprocal shift of the levels of oleic and linoleic acids. Oleic acid levels are lower during the summer months and higher in the winter months. Levels of palmitic and linoleic acids reach maximal values in midsummer and fall significantly during the winter.

Homogenates of brown adipose tissue produce more $^{14}CO_2$ from 1-^{14}C-palmitic acid than from 1-^{14}C-oleic acid when incubated at temperatures below 20C. The formation of $^{14}CO_2$ from either substrate was maximal in the neighborhood of 30C, and the temperature effect was enhanced by stimulation with DL-carnitine.

It is proposed that the rhythmic change in brown adipose tissue triglyceride composition is a reflection of the different rates of fatty acid oxidation and the absence of normal food intake for extended periods of time.

INTRODUCTION

CONSIDERABLE EVIDENCE now exists that the brown adipose tissue of hibernating animals serves as a thermogenic source during the process of arousal (1-3). In the most recent published review of the subject Joel (3) advanced 12 significant areas of biochemical and physiological evidence in support of this view.

Ball and Jungas (4), Dawkins and Hull (5) have proposed that the energy of thermogenesis arises from lipolysis and reesterification of the triglycerides of brown adipose tissue. The energy for reesterification supposedly comes from combustion of a part of the released fatty acids. Reesterification requires adenosine triphosphate (ATP), and Ball's mechanism can therefore be described as a special ATP'ase system. The triglycerides formed by reesterification trap approximately one-half of the energy resulting from ATP hydrolysis, and the remainder is available as heat.

Chaffee et al. (6) proposed an alternative thermogenic mechanism, depending on a shuttle of material via the oxidation-reduction pair, α-glycerophosphate and dihydroxyacetone phosphate, between the intramitochondrial and extramitochondrial pyridine nucleotides. Cytoplasmic NADH + H^+ would transfer an electron pair to cytoplasmic dihydroxyacetone phosphate, and part of the α-glycerophosphate so formed could carry the electron pair into the mitochondria. The α-glycerophosphate is then oxidized by a flavoprotein which, in the proposed mechanism, is not coupled with a phosphorylating step. The yield of ATP produced by this mechanism would be less than that produced by intramitochondrially oxidized NADH; the lowered yield of ATP would be therefore associated with the liberation of more energy as heat. Chaffee et al. further suggest that brown adipose tissue during cold acclimation has an enhanced capacity for fat oxidation.

It was noted earlier (7) that the level of ATP in brown adipose tissue of the bat (*Eptesicus fuscus*) is highest during the dormant phase of hibernation and that it falls markedly during the arousal process. It was also demonstrated that placing summer-captured animals into the cold (5C) induced a significant increase in ATP levels of the brown adipose tissue. This seemed to imply a specific effect of the thermal environment, not necessarily related to season, and further implied that entrance into the dormant phase automatically invoked some physiologically imperative preparation for arousal. Observations on the change of ATP levels are consistent with either of the proposals cited above.

It is known that the respiratory quotient of hibernating animals indicates a dependence on stored fats (R.Q. = 0.7). Thus it seemed worthwhile to explore changes in brown adipose tissue triglycerides which might also be temperature-dependent and which might relate to thermogenesis. Since such effects might be induced by seasonal changes, we have made a circum-annual study of the fatty acids of brown adipose tissue triglycerides. Contrary to an-

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other report (8), we have found distinct and regular changes in fatty acid composition over the course of a year. In addition, we have found a specific difference in the in-vitro oxidation of individual fatty acids by homogenates of brown adipose tissue.

MATERIALS AND METHODS

Animals

Male and female specimens of *Eptesicus fuscus* were captured in the eastern counties of Iowa. Summer animals were trapped in barns and other farm buildings; these were transferred to small screen-wire cages and kept at refrigerator temperatures (5C) for at least 48 hr before sacrifice. This insured, in keeping with earlier observations on ATP levels, that the summer animals were studied under the same thermal conditions as the winter animals. Winter animals were trapped in caves; these animals were kept in a large artificial cave (15 ft × 20 ft × 40 ft), the front of which was enclosed by a tightly screened flyway (15 ft × 20 ft × 30 ft).

It had previously been established that the prevailing temperature of our artificial cave closely approximated temperatures of the natural caves in which the bats had been trapped. A high relative humidity and adequate drinking water were insured by a constant flow of water across the floor of the cave. Other than the few mosquitoes which normally winter over in the artificial cave, no food supply was provided, and the mosquitoes had seemingly disappeared by early January. In view of the bat population in the hibernaculum (150-250 animals) the insect supply was considered to be an insignificant food source.

Frequent observations indicated that the captured animals associated in clusters much as in the wild. By marking captured individuals with dyes, we observed that the population was fairly mobile within the hibernaculum, as has previously been reported for wild animals (9). For these reasons we feel that the conditions established were a fair approximation of the normal habitat of hibernating bats. Winter animals were brought from the hibernaculum to the laboratory as needed in small screen-wire cages and kept at refrigerator temperatures for at least 48 hr before sacrifice.

Preparation of Lipid Extracts

Animals were sacrificed by decapitation. The interscapular lobes of brown adipose tissue were excised, freed from extraneous tissue, and immediately homogenized in ice-cold methanol (6.7 volumes). Chloroform (6.7 volumes) was added, and the mixture was rehomogenized. A second aliquot of chloroform was added, and the homogenization was repeated. The stepwise addition of chloroform was found to give a smoother homogenate. The tissue debris was filtered off, and the extract was washed according to Folch et al. (10). The extracted lipids were evaporated to dryness under nitrogen, then taken up in 0.5 ml of chloroform-methanol (2:1).

The triglycerides were separated from the extracted lipids by thin-layer chromatography (TLC) on 0.375-mm layers of Merck Silica Gel G. (Brinkmann Instrument Company, Long Island, N. Y.), which had been activated at 110C for 1 hr. Samples were applied to the plates according to Achaval and Ellefson (11). The plates were developed by ascending chromatography in a solvent system composed of n-heptane, ether, and acetic acid (80:20:1, v/v). The bands were visualized by light spraying with 2,7-dichlorofluorescein and observation in ultraviolet light. The separated triglycerides were transesterified by the method of Morrison and Smith (12).

The fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC) by using a Barber-Colman Model 5000 chromatograph, equipped with a flame detector and a ball and disk integrator (Barber-Colman Instrument Company, Rockford, Ill.). Samples were applied in spectroanalyzed n-heptane (Fisher Scientific Company, Pittsburgh, Pa.) to columns containing either 10% ethylene glycol adipate or 15% ethylene glycol succinate on 100-110 mesh Anakrom ABS (Analabs, Hamden, Conn.). Columns were operated at 185C, at which temperature the separation of $C_{18:0}$ and $C_{18:1}$ peaks was virtually complete. Quantitative standardization of the chromatograph was based on analysis of standard methyl ester mixtures (Hormel Institute, Austin, Minn.); the correction factors departed from unity by less than ±2% over the range $C_{14:0}$ to $C_{20:0}$.

Preparation of Homogenates

The preparation of brown adipose tissue homogenates and the incubation media for the study of temperature-dependent fatty acid oxidation were exactly as described by Fritz (13) for rat heart homogenates except that in some experiments succinate (1.2×10^{-4}M final concentration) was added. Tissue from three or four animals was pooled, and the homogenates were adjusted in volume so that the protein

content by the Folin-Ciocalteu method (14) was approximately 1.0 mg per ml.

Preparation of Albumin-Fatty Acid Complex

Crystalline bovine serum albumin (Pentex, Kankakee, Ill.) was used as obtained. A single lot of this material was used for all the experiments to be described. No further analysis for fatty acids was made since Chen (15) had previously shown the fatty acid content of similar preparations to be not greater than 1.05 moles per mole of protein. The 1-^{14}C-palmitic acid and 1-^{14}C-oleic acids were obtained from Tracerlab (Boston, Mass.) or New England Nuclear (Boston, Mass.) and appropriately diluted with pure unlabeled material (Hormel Institute, Austin, Minn.) so that the final albumin-fatty acid complex, when measured in a Packard Tri-Carb liquid scintillation spectrometer, gave 300,000 cpm when a 0.5-ml aliquot was added to 15 ml of Bray's (16) scintillation fluid. The requisite quantity of fatty acid was dissolved in a slight excess of potassium hydroxide, to which was added sufficient albumin to give a molar ratio of 1:8 (albumin-fatty acid). Fritz et al. (17) had indicated that this was an optimal ratio for fatty acid oxidation in heart muscle homogenates. The pH of the solution was finally adjusted to 7.5 with dilute hydrochloric acid.

Radiopurity of the fatty acid methyl esters from the albumin-fatty acid complex was determined by silver-ion TLC according to Morris (18). The fatty acids were extracted from the complex and transesterified as described above for brown adipose tissue. The thin-layer plates showed that the radioactivity was confined to a single spot and further indicated that the labeled complex preparations were 95-97% pure with respect to the fatty acid under study.

Incubation of Tissue Homogenates

Incubations were performed under air in Kontes vessels, fitted with double rubber septa and polypropylene center wells (Kontes Glass Company, Vineland, N. J.) containing hyamine hydroxide (Packard Instrument Company, LaGrange, Ill.) on filter paper as the CO_2 trapping agent. The vessels were incubated in Eberbach metabolic shakers (Eberbach Corporation, Ann Arbor, Mich.), thermostatted to $\pm 0.5°$.

Aliquots of the fatty acid complex (0.5 ml) were pre-equilibrated with incubation medium (1.0 ml), and the reaction was started by the addition of tissue homogenate (1.0 ml). The incubations lasted 60 min although control experiments indicated that the $^{14}CO_2$ production was a linear function of time up to 90 min. At the end of the experimental period the reaction was stopped by the addition of 1.0 ml of 30% phosphoric acid, and the flasks were shaken for another 30 min. At the end of this period the polypropylene center wells were clipped from their supports, and the well plus the contents were transferred to 15 ml of toluene-PPO-POPOP solution in scintillator vials. The radioactivity was determined to 1% confidence limits.

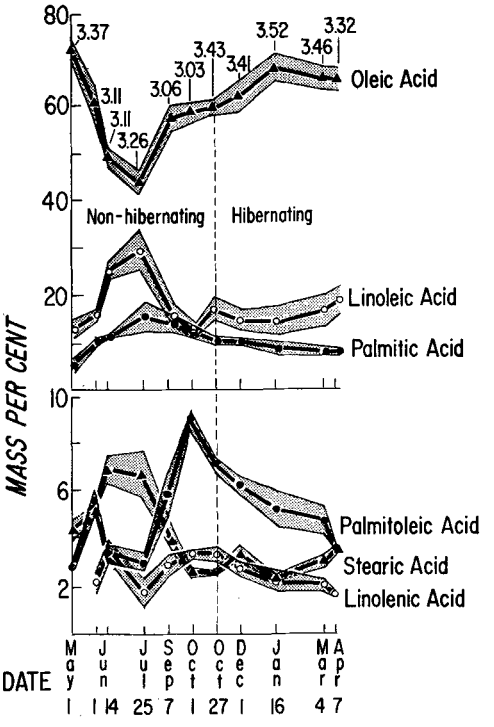

FIG. 1. Fatty acid composition of brown adipose tissue triglycerides as a function of time. The points are the means of the determinations; the widths of the shaded bands represent the standard deviations. The numbers at the top of the figure represent the double-bond content per mole of triglyceride, theoretically derived from the determined fatty acid composition. The dormant phase of hibernation for the year (1966-67) extended approximately from October 27 to April 7.

RESULTS

Circum-Annual Pattern of Fatty Acids

The results of gas-liquid chromatographic analysis of the fatty acid distributions of the brown adipose triglycerides are shown in Fig. 1. The solid lines represent the means of from four to six independent analyses on separate

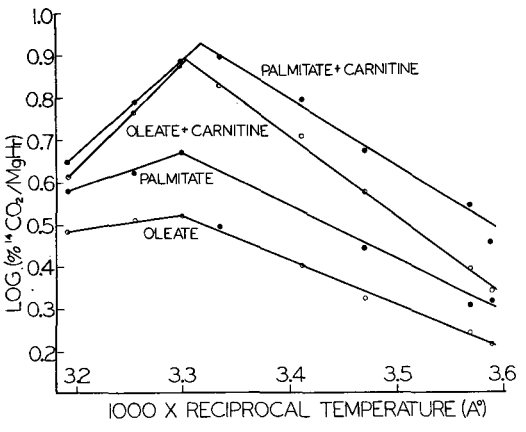

FIG. 2. Production of $^{14}CO_2$ from 1-^{14}C-labeled palmitic and oleic acids by homogenates of brown adipose tissue as a function of temperature. The points are the log averages of duplicate determinations, uncorrected for differences in carbon numbers of the two acids.

tissue samples; the widths of the shaded bands represent the standard deviations of the means. There is a shift in the composition of triglyceride fatty acids, and a striking reciprocity of change in the mass percentages of oleic and linoleic acids, for which the correlation coefficient was 0.72. During hibernation the value for linoleic acid was surprisingly constant whereas the level of oleic acid increased significantly ($P < 0.01$). Palmitic and palmitoleic acids show significant increases ($P < 0.01$) prior to the onset of hibernation and a significant decline thereafter. Unlike the brown adipose tissue of the mouse (19), the rat (20), or one other species of bat (8), only traces ($<1\%$) of fatty acids shorter than C_{16} or longer than C_{18} were encountered in the chromatograms, and these were ignored. The level of stearic acid is unusually low compared with the rat.

If the mass percentages of the individual fatty acids are converted to mole fractions and if due account is taken of the degree of unsaturation, it appears that the total number of double bonds per mole of possible triglycerides is significantly higher during the hibernating period than during the rest of the year. These values are indicated by the numbers along the top of Fig. 1. If the value for January is compared with the (nonhibernating) value for early October, a ratio of 1.16 is obtained; and even if the March value is employed, the ratio is still 1.14. These ratios are higher than those calculated from the iodine titration data of Fawcett and Lyman (21) for hamster brown adipose tissue, where the ratio was only 1.09. While our data, as well as those of Fawcett and Lyman, may reflect dietary influence to an indeterminate extent, both sets of values support the previously recorded impression that brown adipose tissue triglycerides are more unsaturated during the dormant phase (22). The bat, which hibernates more readily than the hamster, shows a greater degree of unsaturation during the dormant phase. Furthermore, when the bat arouses during the hibernating period, he probably will find no food. This is not true for the hamster.

For the rat, a nonhibernator, the situation seems to be different since Fawcett and Lyman state that fed rats show no change in unsaturation whether maintained at 23C or at 5C. Chalvardjian (20) presented detailed analytical data from which we have calculated that the brown adipose tissue of fed rats, presumably at room temperature, contains 2.77 double bonds per mole of triglyceride, or distinctly less than the bat. This may reflect a difference in diet, a species difference, or both. He also presented data which we cannot quantitatively assess but which supports the argument that starvation of the rat by itself induces an increase in the unsaturation of brown adipose triglycerides. Thus it is not yet clear whether starvation, cold exposure, or both account for the increase in unsaturation.

Oxidation Rate as a Function of Temperature

The oxidation of 1-^{14}C-palmitic and 1-^{14}C-oleic acids by homogenates of brown adipose tissue was examined at different temperatures by measurement of the $^{14}CO_2$ produced per milligram of homogenate protein per hour. Duplicate incubations were performed in the presence and absence of DL-carnitine. A considerable day-to-day variation was observed in the capacity of brown adipose homogenates to oxidize fatty acids, similar to that observed by Fritz (13) in working with rat heart homogenates. The experimental values were corrected in terms of the $^{14}CO_2$ produced under the standard conditions but were not corrected for the difference in carbon numbers of the fatty acids. The results of these experiments are shown in Fig. 2, in which the log of the average $^{14}CO_2$ production is plotted against the reciprocal of the absolute temperature. The maximum production of $^{14}CO_2$ at or near 30C is evident for either fatty acid in the presence of DL-carnitine stimulation.

Temperature-Dependent Oxidation Ratios

The hypothesis of a temperature-dependent difference in the capacity of brown adipose

homogenates to oxidize palmitic acid more rapidly than oleic acid was also explored. To avoid the day-to-day variations mentioned above, triplicate samples of homogenate were simultaneously incubated at all temperatures and under all conditions. Stimulation by DL-carnitine and by succinate (1.2×10^{-4}M), together or individually, was examined. A complete set of flasks was incubated simultaneously for each 1-^{14}C-labeled fatty acid, and the entire experimental program was repeated twice. Correction for the carbon number difference was made by multiplying the counts from oleic acid by 1.125. The ratio of corrected oleic acid counts to palmitic acid counts was determined at each temperature for all conditions of stimulation. The appropriately grouped ratios were then examined by the Student t-test for significance at the 5% level.

The results of these experiments are summarized in Table I. With but two exceptions, the data for temperatures of 5C, 10C, and 15C support the hypothesis that lower temperatures favor the oxidation of palmitic acid over oleic acid by homogenates of bat brown adipose tissue. Above this temperature there is no longer substantial evidence for the hypothesis. Stoffel and Schiefer (23) have observed a substrate difference between palmitic and oleic acids when oxidized by rat liver mitochondria in the absence of carnitine stimulation although the difference disappeared in the presence of carnitine. Fritz et al. (24) have also shown that rat heart muscle homogenate produced $^{14}CO_2$ more rapidly from 1-^{14}C-palmitic acid than from 1-^{14}C-oleic acid at 37C. Although these tissues show a preferential oxidation of palmitic acid over oleic acid at 37C, brown adipose tissue demonstrates this difference consistently only at lower temperatures. This may represent a specific adaptation of the tissue to the metabolism of fatty acids at lower temperatures.

TABLE I

Oxidation Ratios. C18:1/C16:0, by Bat Brown Adipose Tissue Homogenates

Incubation temperature	Carnitine + Succinate +	+ -	- -	- +
5	0.74±.04[b] (n=6)	0.83±.07[b] (n=6)	0.84±.04[b] (n=6)	0.76±.04[b] (n=6)
10	0.72±.05[b] (n=6)	0.86±.06[b] (n=6)	0.81±.06[b] (n=6)	0.84±.15 (n=4)[a]
15	0.74±.07[b] (n=6)	0.98±.15 (n=6)	0.88±.01[b] (n=6)	0.85±.08[b] (n=6)
20	0.79±.05[b] (n=5)[a]	1.04±.09 (n=4)[a]	1.04±.13 (n=5)[a]	0.90±.04[b] (n=4)[a]
25	0.93±.12 (n=5)[a]	1.12±.18 (n=3)	0.90±.18 (n=5)	0.94±.05 (n=3)[a]
30	1.11±.12 (n=6)	1.01±.15 (n=6)	0.78±.08[b] (n=6)	0.90±.18 (n=6)
35	1.03±.11 (n=5)[a]	0.97±.12 (n=6)	0.88±.08 (n=3)[a]	0.98±.18 (n=6)

[a] Lost experimental flasks.

[b] Ratio differs from unity significantly at the 5% level.

Succinate and carnitine, when present, at initial concentrations of 1.2×10^{-4}M and 5×10^{-4}M respectively. For other details see text.

Ratio of oxidation of oleic acid to palmitic acid by homogenates of brown adipose tissue as a function of temperature. At temperatures below 20C the ratio shows a significant difference at the 5% level of confidence. Values are corrected for carbon number difference.

DISCUSSION

Hibernation has been defined by Kayser (25) as a "state of slowed life that occurs during the winter months of the northern hemisphere." The dormant phase of hibernation is associated with a lowered temperature which is a complex function of body mass, body surface, and ambient temperature (26-28). *Eptesicus fuscus* is a true hibernator, and it has been demonstrated that its hiberating core temperature may be as low as 5C (28-30).

During the dormant phase white adipose stores of triglycerides virtually disappear while brown adipose triglycerides decrease much less (25,31). During the arousal phase, brown adipose triglyceride consumption shows a sharp rise (3). Under conditions prevailing in the natural state, the dietary intake of hibernating bats is probably very small, primarily because of the lack of flying insects during the late fall and winter months. For this reason the hibernating bat may be regarded as a nearly closed metabolic system, depending almost entirely on the energy of stored substrates.

Little information concerning the fatty acid composition of the insect diet consumed by bats is available, and it is therefore impossible at present to determine the extent to which diet may influence the summer changes which were observed. However the effects which might be attributed to diet seem to have disappeared by early October, before the onset of the dormant phase of hibernation. Regardless of the effects of diet, the collected circum-annual analyses illustrate still another rhythmic pattern associated with natural hibernation (24). The rhythmicity of the data also explains the earlier observation of Wells et al. (8), who reported no difference between the "hibernating" and "nonhibernating" states in the fatty acids of brown adipose triglycerides. Their observations at single points might easily be matched by picking selected points from the authors' own data for which no test of significant dif-

ference could be obtained, yet systematic observation clearly supports the idea of a temporal variation related to external environment. Since the animals were not kept under constant conditions, the possibility of a true seasonal change will not be discussed.

If only that portion of the data shown in Fig. 1 which relates to the dormant phase of hibernation is considered, there is still a slight but significant ($P < 0.01$) decrease with time in the level of palmitic and palmitoleic acids and a significant increase in the level of oleic acid. In view of the difference in the capacity of brown adipose homogenates to oxidize palmitic and oleic acids, it is postulated that palmitic acid, and perhaps palmitoleic acid, represent preferred substrates for this tissue as for others discussed previously. The observed increase in oleic acid represents gradual accumulation during the dormant period when the lower core temperature and the lack of normal food intake combine to make this hypothesis most feasible.

The circum-annual rhythm is explained on the basis of the interacting factors described above. On account of the very low metabolic rate existing during the protracted dormant period, the changes which were recorded proceed on a long-time base compared with corresponding changes recorded for starved rats.

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Dietary Fat Effect on Incorporation and Release of Lipids and Cholesterol by Rat Intestinal Slices

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ABSTRACT

The effect of saturated and unsaturated fats on in vitro formation and release of lipids and cholesterol from ^{14}C acetate by rat intestinal tissue was investigated. The rats were fed a basal diet enriched with either 25% corn oil or lard and then sacrificed after a 10- or 25-day feeding period. It was observed that a similar ^{14}C lipid content but a greater ^{14}C cholesterol content was found in the intestinal tissue of rats fed corn oil than in rats fed lard for 10 days. After a longer period of feeding of 25 days, the intestinal tissue ^{14}C cholesterol level was decreased in the corn oil fed rats without any significant effect on other lipids. These data suggest that corn oil in some way influences cholesterol biosynthesis depending upon its degree of unsaturation and the period of time for which it is fed. The decrease at the later time might involve some mechanism which aids in getting rid of accumulated tissue cholesterol. Less ^{14}C lipid and ^{14}C cholesterol were released by the intestinal tissue of rats fed the unsaturated fat as compared with those fed the saturated fat, suggesting a possible role in vivo in reducing blood lipids and blood cholesterol levels.

INTRODUCTION

THE INTERRELATIONSHIP of polyunsaturated fatty acids (PUFA) and cholesterol metabolism has been a subject of wide interest during recent years. The liver appears to be the main endogenous site of origin of serum cholesterol (1). High cholesterol intakes have been shown to inhibit endogenous cholesterol formation in the liver by a feedback mechanism (2). Dietary PUFA has been shown to increase the cholesterol content of the liver (3). Increasing intake of PUFA by increasing liver cholesterol content may aid in lowering blood cholesterol levels by inhibiting cholesterol synthesis by the liver. However, extrahepatic tissues like the intestine have been reported to synthesize cholesterol (4, 5) but there appears to be no feedback mechanism involved and consequently cholesterol synthesis by intestinal tissue continues in the cholesterol fed rats (2,6). The recent studies of Lindsey and Wilson (7) and Dietschy and Siperstein (8) have amply demonstrated that cholesterol is synthesized in the intestine. Results in an earlier study (9) suggested that the intestine might play some role in lowering blood cholesterol and lipid levels in rats when fed diets enriched with large amounts of PUFA. Thus the possibility that a high intake of PUFA may also have an effect upon cholesterol synthesis and release by intestinal tissue seems worthy of further study. The present investigation was planned to investigate this possibility.

EXPERIMENTAL

Sixteen male albino rats from Holtzman were maintained on commercial rat pellets prior to initiation of the experiment. Rats weighing 155-160 g were divided into two groups and fed on diets of ground rat pellets[2] enriched with 25% corn oil, or with 25% lard. Rats were fed thus over either a 10- or 25-day period. Average final weights of the latter group were 214 and 211 g, respectively, for lard and corn oil fed rats. At end of feeding period the rats were fasted 24 hr and refed ad libitum for 24 hr prior to sacrificing. The intestinal tract was removed immediately and flushed well with 0.9% cold saline. Only the lower half of well-washed intestine was used for preparing the slices. After slices were again washed with ice-cold Krebs-bicarbonate buffer, the excess moisture was removed with dry filter paper. About 0.5 g samples were weighed and placed in incubation flasks containing 5.0 ml of Krebs Ringer-bicarbonate buffer at pH 7.4 with about 1.0/ml μc of ^{14}C acetate and a total acetate concentration of 0.2 mg/ml. The flasks were incubated in a Dubnoff metabolic shaker at 37C for 2 hr in an atmosphere of 95% O_2 and 5% CO_2. After incubation, the contents were centrifuged and supernatants were decanted. Both supernatant and tissues were digested with excess alcoholic KOH and after acidification, the lipids were

[1] Postdoctoral Fellow, Robert A. Welch Foundation.

[2] Uncle Johnny's BSD Laboratory Animal Diet contains not less than 26.0% protein, 5% fat prepared by Uncle Johnny Mills, Houston, Texas.

TABLE I

^{14}C Acetate Utilization and Lipid Formation and Release by Intestinal Tissue

Diet[a] and difference[b]	Acetate[c] utilization cpm/g x 10^{-3}	Total lipid activities		Cholesterol activities		
		Supernatant cpm/g	Tissue cpm/g x 10^{-2}	Supernatant cpm/g	Tissue cpm/g	Tissue (25 da)[d] cpm/g
La	3425 ± 220	4622 ± 300	1017 ± 143	243 ± 41	3361 ± 474	2171 ± 95
CO	4151 ± 259	3604 ± 256	1177 ± 127	197 ± 25	4787 ± 595	1424 ± 65
%	+21.3	-22.0	+15.7	-30.4	+42.4	-34.4

[a] La and CO for diets containing lard and corn oil, respectively.

[b] Difference % = CO-La/La x 100.

[c] All values given as cpm/g tissue ± SE.

[d] Only these values listed for 25 day period since others not significantly different from 10 day period.

extracted with chloroform. The solution was filtered twice through dry filter papers and the solvent evaporated under nitrogen. The total lipids were weighed, then dissolved in chloroform and aliquots were taken for total lipid activity.

Labeled cholesterol was separated from the lipids by thin-layer chromatography (TLC) and its radioactivity measured by liquid scintillation counting. Plates were prepared according to the method of Brown and Johnston (10) and developed for 45 min using the solvent system hexane-diethyl ether-glacial acetic acid (80:16:4). The dried chromatogram was viewed under ultraviolet light and spots were outlined, scraped and transferred into a vial. Fifteen milliliters of a naphthalene-dioxane liquid scintillator (11) was then added to the vial. The radioactivity of the sample was determined in a Beckman liquid scintillation counter. The samples were checked for quenching and no quenching was observed when the naphthalene-dioxane scintillation system was used.

RESULTS AND DISCUSSION

Acetate utilization as measured by its removal from the media during incubation with the intestinal tissue of the rats fed on the PUFA diet was greater than that of the rats fed on the saturated fat diet (Table I). The tissue total lipid activity was not significantly different in the two groups of animals while the tissue cholesterol activity was much higher in case of the rats fed corn oil diet for 10 days. At first corn oil appears to be associated with higher and then later with a lower cholesterol level by some alteration in the cholesterol metabolism. PUFA (corn oil) and the more saturated fat (lard) thus appear to have a different metabolic influence on cholesterol metabolism. This different metabolic influence on cholesterol metabolism seems related in some way to the degree of unsaturation of the dietary fat.

An increase in tissue cholesterol after corn oil feedings has been reported earlier by Swell et al. (12) and Hill et al. (13). However, it is interesting that intestinal tissue cholesterol level in case of corn oil fed rats was decreased after a 25-day feeding period. This may result from either a decreased formation with passage of time and/or to some other unknown mechanism involved in getting rid of excess accumulating tissue cholesterol in order to correct an unphysiological condition. This finding is in line with a similar to be reported change in liver cholesterol content when studied over several time periods.

The supernatant lipid activity gains appeared to be less in case of corn oil fed rats than in lard fed rats after both time periods. Both supernatant total lipid and cholesterol were less in corn oil fed rats indicating that less lipid and cholesterol were released from this tissue although its early formation was higher in the latter case as compared to lard fed rats. If the decreased cholesterol formation and release after unsaturated fat diets should play a still more effective role in vivo as compared to present vitro study, it might aid in reducing blood lipid and blood cholesterol levels. The quantitative significance of these findings awaits further experimentation.

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In Vitro Incorporation of Acetate-1-^{14}C into the Phospholipids of Rabbit and Human Endometria[1]

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ABSTRACT

Endometria from nonpregnant and 6-day pregnant rabbits and from humans in the proliferative and secretory phases were incubated with 1-^{14}C-acetate. $^{14}CO_2$ was collected, and subsequently the amounts, specific radioactivities, and in some cases the fatty acid compositions of the isolated phospholipids were determined. Phosphatidyl choline was the phospholipid present in highest amount in endometria from both nonpregnant and pregnant rabbits, and in human endometria; this phospholipid also showed the highest degree of incorporation of ^{14}C-acetate. Pregnancy in the rabbit seemed to decrease the incorporation of ^{14}C-acetate into most of the endometrial phospholipid classes. In humans, the incorporation of acetate into phosphatidyl choline and phosphatidyl ethanolamine was lower in the secretory than the proliferative endometria.

Of the fatty acids, linoleic acid in phosphatidyl choline and phosphatidyl ethanolamine of the rabbit endometria showed a significant relative increase during pregnancy and palmitoleic acid showed a decrease.

INTRODUCTION

CYCLIC ALTERATIONS in endometrial lipids have been demonstrated histochemically in guinea pigs (1), ferrets (2), monkeys (3), and humans (4). Increased amounts of endometrial osmophilic fat have been found during early pregnancy in the rat (5,6), cat (7), and human (4).

Quantitative chemical studies by Okey et al. (8) of the pig endometrium indicated that the concentrations of cholesterol and lecithin were highest during the luteal phase of the cycle. In the rat uterus, however, the period of maximal phospholipid content was found to occur during the follicular phase (9). Goswami et al (10) found that during estrus and in ovariectomized animals given estrogens that the relative amounts of triglycerides were lower, and phospholipids were higher than in the diestrus period.

In a chemical study of the rabbit endometrium by Ray and Morin (11), the endometrium of the implantation site in the 8-day pregnant rabbit showed lower percentages of total lipids than either the nongravid or the gravid interimplantation endometrium. The glycerides of the interimplantation areas were higher than those of the nongravid endometrium, and also showed higher proportions of linoleic and arachidonic acids. The implantation sites contained less glyceride and essential fatty acids than the interimplantation areas.

Hormonal alterations occurring during pregnancy may possibly influence endometrial phospholipid metabolism. In the uterus of castrated rats, Davis and Alden found that estrogen treatment increased the concentration of phospholipids, whereas progesterone had no effect (9). Estradiol has been found to increase ^{32}P incorporation into the total phospholipids of subcellular particles of whole rat uterus (12), and also in another study to increase the synthesis of choline, ethanolamine and inositol containing phospholipids from ^{32}P-phosphate and ^{14}C-acetate precusors (13).

The objectives of the present experiments were to determine the content of specific phospholipids and phospholipid fatty acids in the endometrium and to study the alterations in concentration and metabolism of these phospholipids occurring during pregnancy in the rabbit and during the menstrual cycle in humans.

EXPERIMENTAL

Twenty-four female New Zealand white rabbits, 4 months old, and fed a stock commercial diet (Purina) since weaning were divided into one group of 6 and another group of 18. Six rabbits were mated to induce pregnancy. After 6 days the rabbits were anesthetized by intravenous injection of Sodium Pentobarbital. Uteri of the mated rabbits were examined and all found to be gravid. Endometrial samples from one pregnant rabbit and endometrium of approximately equal weight from three nonpregnant rabbits were each placed in separate flasks

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[2]Ford Foundation Postdoctoral Fellow.

with 10 ml of Tyrode's solution (14). (A total of six incubations with gravid endometria and six with nongravid endometria were done.) After addition of 50 μc sodium 1-^{14}C-acetate (Nuclear-Chicago, Specific Activity 29.0 mc/mM) to each, the flasks were incubated for 4 hr in an air atmosphere at 37.5C in a Dubnoff metabolic shaking incubator. Carbon dioxide was collected in a center well containing 20% NaOH. The flasks were then placed on ice, and after cooling to 0C the contents were centrifuged, washed twice with 20 ml 0.9% NaCl for 20 min each time and recentrifuged. The tissues were then refrigerated overnight in 50 ml of 0.155 M unlabeled sodium acetate. The next day, after one additional washing with 0.9% NaCl and centrifugation, the total endometrial lipids were extracted in a VirTis 45 Homogenizer with two successive 50 ml portions of methylal/methanol (4:1). The combined extracts were evaporated to dryness in a rotary vacuum evaporator and under N_2, redissolved in chloroform, filtered, and evaporated down to 0.5 ml. Silica Gel H (Brinkmann) plates 0.5 mm thick were prepared by the method of Skipski (15). After drying and heating at 110C for 1 hr, the plates were placed in the developing mixture and the solvents allowed to rise to the top of the plates, in order to remove impurities. The plates were then reactivated for 1 hr at 110C just prior to use. The total endometrial lipids were applied as a band across the plate and the phospholipids separated using an ascending developing mixture of chloroform-methanol-acetic acid-water (25:15:4:2). The lysophosphatidyl choline, sphingomyelin, phosphatidyl choline, phosphatidyl inositol, phosphatidyl serine, and phosphatidyl ethanolamine bands were collected with a vacuum zone extractor. Elution of the phospholipids from the silica gel was accomplished as previously described (14). Aliquots of the eluates from each fraction were analyzed for phosphorous content (16). Other aliquots were assayed for radioactivity using a PPO-POPOP in toluene scintillator and a Packard Model 3314 liquid scintillation spectrometer. Quenching was monitored by the channels ratio technique, and all counts corrected to 100% efficiency.

The remainder of the fractions were then hydrolyzed and methylated (17) and the fatty acid composition of each phosphatidyl choline and phosphatidyl ethanolamine fraction was determined by gas-liquid chromatography in a Barber-Colman Model 10 Gas Chromatograph using diethylene glycol succinate on Gas-Chrom P, 70-80 mesh, at 187C with an argon pressure of 24 lb. The fatty acids were identified by comparison of retention times to those of standards (obtained from the National Institutes of Health and Calbiochem) and by graphic representation of retention times. The areas under the peaks were estimated by triangulation. Only the major fatty acids (16:0, 16:1, 18, 18:1, 18:2, and 20:4) have been included in the tabulations. Other fatty acids, identified in amounts either too small or having too long a retention time to measure accurately, were 14, 18:3, 20:1, 20:3 (8,11,14)[3], 22:4, 22:5, and 22:6.

TABLE I
Amounts of Individual Phospholipids in Rabbit Endometrium[a]

Rabbit group	Nongravid	Gravid
Lysophosphatidyl choline	2.0±1.3	2.6±1.8
Sphingomyelin	5.8±2.4	4.4±1.1
Phosphatidyl choline	19.0±4.6	15.7±5.2
Phosphatidyl inositol	5.3±2.9	6.1±1.2
Phosphatidyl serine	11.7±4.2	9.6±2.7
Phosphatidyl ethanolamine	14.3±3.2	12.4±4.5

[a]Milligrams phospholipid/g dry weight endometrium (means of determinations from 6 incubations per group ± standard deviations).

The radioactive CO_2 evolved was determined by precipitating an aliquot of NaOH Center well solution with 10% $BaCl_2$. The light precipitate of $BaCO_3$ which formed was trapped by filtration as a very thin layer on a disc of Whatman No. 42 filter paper and then the paper with the precipitate counted in the PPO-POPOP scintillator as described above.

In a second series of experiments, samples of human endometria were obtained immediately after hysterectomy for fibroids in women 30-40 yr of age. Four samples were obtained in approximately the midproliferative phase and four in the midsecretory phase of the menstrual cycle as determined both clinically and histologically. The samples were incubated with 50μC 1-^{14}C-acetate (Specific Activity 29.0 mC/mM) in Tyrode's solution for 4 hr under the same conditions described above for the rabbit endometrial samples. Carbon dioxide collection and counting, washing of aortas, extraction, thin-layer chromatography, phosphorus analysis and liquid scintillation counting were conducted the same way as in the rabbit experiments.

The probabilities (p) that apparent differences in the data were due to chance was calculated by the student's t test, and the P values are given in the tables and text.

[3]Eicosatrienoic acid with double bonds in the 8-9, 11-12 and 14-15 positions as counted from the carboxyl end of the molecule.

TABLE II

Specific Radioactivities of Individual Phospholipids of Rabbit Endometrium after Incubation with 1-^{14}C-Acetate[a]

Rabbit group	Nongravid	Gravid	*P*
Lysophosphatidyl choline	479±109	260± 45	<0.01
Sphingomyelin	350± 88	178± 64	<0.01
Phosphatidyl choline	1,360±221	742±147	<0.01
Phosphatidyl inositol	427± 76	315± 70	<0.05
Phosphatidyl serine	794±332	672±283	<0.6
Phosphatidyl ethanolamine	1,120±245	656±207	<0.01

[a]DPM/mg. phospholipid (means of 6 incubations per group ± standard deviations).

TABLE III

Amounts of 1-^{14}C-Acetate Incorporated into Individual Phospholipids of Rabbit Endometria[a]

Rabbit group	Nongravid	Gravid	*P*
Lysophosphatidyl choline	14± 3	11± 2	<0.1
Sphingomyelin	32± 8	12± 4	<0.01
Phosphatidyl choline	403±65	181±36	<0.01
Phosphatidyl inositol	35± 6	30± 7	<0.3
Phosphatidyl serine	144±60	100±42	<0.2
Phosphatidyl ethanolamine	250±54	126±40	<0.01

[a]$\mu\mu$ moles 1-^{14}C-acetate incorporated/g dry weight of endometrium (means of 6 incubations per group ± standard deviations).

RESULTS

The amounts of the individual phospholipid fractions in the non-gravid and gravid endometria are indicated in Table I. The order of concentration of phospholipids was phosphatidyl choline > phosphatidyl ethanolamine > phosphatidyl serine > sphingomyelin ≅ phosphatidyl inositol > lysophosphatidyl choline. There were no significant differences in amounts of phospholipids in endometria of nonpregnant as compared to pregnant rabbits.

During the incubation with 1-^{14}C-acetate the $^{14}CO_2$ evolved was 120,400 ± 13,500 DPM/mg dry tissue from the nongravid endometria and 125,000 ± 10,650 DPM/mg from the gravid endometria.

The specific activities of the individual rabbit endometrial phospholipids after incubation with ^{14}C-acetate are indicated in Table II. Phosphatidyl choline showed the highest specific activity and sphingomyelin and lysophosphatidyl choline, the lowest. Pregnancy seemed to significantly decrease the specific activity of phosphatidyl choline and phosphatidyl ethanolamine and also the other phospholipids to a lesser extent (all differences were significant to *P* values of < 0.05 except for phosphatidyl serine). The amount of 1-^{14}C-acetate in $\mu\mu$ moles incorporated into each phospholipid are shown in Table III. The results in general paralleled those for specific activity.

The fatty acid compositions of the two highest concentration phospholipids, phosphatidyl choline and phosphatidyl ethanolamine, are indicated in Table IV. The percentages of linoleic acid in both phosphatidyl choline and phosphatidyl ethanolamine were significantly higher in the pregnant than in the nonpregnant state ($P < 0.01$). In both phospholipids, palmitoleic acid was lower in gravid endometria ($P < 0.01$).

The amounts of individual phospholipids in human endometria in the proliferative and secretory phases are indicated in Table V. The order of concentration of the phospholipids was phosphatidyl choline > phosphatidyl ethanolamine > phosphatidyl serine > sphingomyelin > lysophosphatidyl choline > phosphatidyl inositol. The endometria of the proliferative phase showed a higher concentration of phosphatidyl choline than did the secretory phase ($P < 0.05$).

In Table VI are indicated the specific activities of the individual human endometrial phospholipids after incubation with ^{14}C-acetate and in Table VII the number of $\mu\mu$ moles of acetate incorporated/g dry weight of tissue. Of the phospholipids, phosphatidyl choline incorporated the ^{14}C-acetate most actively, in both the proliferative and the secretory endometria. The specific activity and amount of acetate incorporated into phosphatidyl choline was lower in

TABLE IV

Percentages of Fatty Acid Methyl Esters in Phosphatidyl Choline and Phosphatidyl Ethanolamine of Rabbit Endometrium[a]

	Phosphatidyl choline		Phosphatidyl ethanolamine	
Rabbit group	Nongravid	Gravid	Nongravid	Gravid
Fatty acid				
16:0	16.0±2.1	13.5±3.7	10.9±4.6	10.3±2.0
16:1	10.6±1.7	5.8±1.5	11.5±2.3	6.8±1.1
18:0	21.2±5.0	15.6±4.4	23.6±3.0	17.7±5.8
18:1	24.2±2.4	29.1±4.9	25.1±4.7	28.2±2.5
18:2	12.5±0.8	19.8±2.7	10.0±1.9	17.6±2.3
20:4	15.4±3.5	16.2±2.6	18.6±2.1	19.5±3.0

[a]Percentage of each fatty acid methyl ester (means of 6 determinations per group ± standard deviations).

TABLE V

Amounts of Individual Phospholipids in Human Endometria[a]

Phase	Proliferative	Secretory
Lysophosphatidyl choline	2.6±0.9	1.9±0.5
Sphingomyelin	3.1±0.9	2.5±1.1
Phosphatidyl choline	20.5±2.3	15.0±2.7
Phosphatidyl inositol	2.0±0.7	2.5±0.9
Phosphatidyl serine	7.2±2.1	5.6±1.8
Phosphatidyl ethanolamine	8.6±1.9	9.1±2.5

[a] Milligram phospholipid/g dry weight endometrium (means of determinations from 4 incubations for each phase ± standard deviations).

TABLE VII

Amounts of 1-^{14}C-Acetate Incorporated into Individual Phospholipids of Human Endometrium[a]

Phase	Proliferative	Secretory	*P*
Lysophosphatidyl choline	31± 7	17± 4	<0.02
Sphingomyelin	29± 5	31± 7	<0.7
Phosphatidyl choline	1,280±198	665±104	<0.01
Phosphatidyl inositol	20± 4	27± 5	<0.1
Phosphatidyl serine	255± 39	181± 28	<0.05
Phosphatidyl ethanolamine	292± 66	180± 41	<0.05

[a] Micromicromoles 1-^{14}C-acetate incorporated/g dry weight of endometrium (means of 4 incubations from each phase ± standard deviations).

the secretory than in the proliferative phase. The activity in phosphatidyl ethanolamine in the secretory phase showed a lesser but also significant decrease.

The proliferative endometria evolved more $^{14}CO_2$ (2,230 ± 460 DPM/mg dry tissue) than did the secretory endometria (1,270 ± 355).

DISCUSSION

In the rabbit and human endometrium, as has been found in most other mammalian organs (18), phosphatidyl choline was the predominant class of phospholipid. In the present experiments on the female rabbit endometrium in vitro, incorporation of ^{14}C-acetate was highest into phosphatidyl choline, similar to the high in vivo incorporation of ^{14}C-acetate into phosphatidyl choline of rat liver (19) and in vitro into rabbit testes (20) and aorta (21).

The decreased incorporation of acetate into the phospholipid classes of the gravid rabbit endometria was not due to increased oxidation and resulting decreased availability of substrate, as indicated by the similar amount of $^{14}CO_2$ produced by endometria from the nonpregnant and pregnant groups. An increased oxidation of formed fatty acids in the gravid endometria also cannot explain the observed differences, since this would have produced more $^{14}CO_2$.

TABLE VI

Specific Radioactivities of Individual Phospholipids of Human Endometrium after Incubation with 1-^{14}C-Acetate[a]

Phase	Proliferative	Secretory	*P*
Lysophosphatidyl choline	770±162	564±133	<0.1
Sphingomyelin	608±109	793±190	<0.2
Phosphatidyl choline	4,020±623	2,850±448	<0.05
Phosphatidyl inositol	568±153	689±133	<0.3
Phosphatidyl serine	2,280±349	2,080±323	<0.5
Phosphatidyl ethanolamine	2,180±496	1,270±290	<0.02

[a] DPM/mg phospholipid (means of 4 incubations of each phase ± standard deviations).

Estrogens have been demonstrated to increase total phospholipid synthesis in the liver and uterus (12,22). In the whole uterus, estrogens were found to increase the synthesis of choline, ethanolamine and inositol containing phospholipids (11). Some studies have indicated that estrogens may increase the hepatic synthesis of particular species of lecithins (23). The present in vitro experiments with the rabbit endometrium, however, have shown that during pregnancy, when there is an increase in circulating estrogens, the predominant effect is a decrease in the incorporation of ^{14}C-acetate into most phospholipid classes. This probably represents a decreased net synthesis, although factors such as permeability changes and variation in intermediate pool size of substrate have not been ruled out by the present experiments (these factors were also not ruled out in the above cited studies by other investigators). The difference between the present results and those of the former studies may be due to the high levels of progesterone or to the increased gonadotropic hormones during pregnancy. Concurrent administration of estradiol and progesterone to ovariectomized female rats seems to produce a lesser effect on uterine subcellular phospholipid distribution than can be produced by the estrogen alone (24). In the mouse uterus, progesterone appears to antagonize the increased concentration of phospholipids produced by estrogens (12).

The predominant effect of pregnancy on the phospholipid fatty acids in the rabbit was an increased proportion of linoleic acid in phosphatidyl choline and phosphatidyl ethanolamine. A similar increase was also found in a previous study in the total glycerides of the inter-implantation areas of the 4 and 8 day gravid rabbit endometrium. Since linoleic acid is an essential fatty acid, and cannot be synthesized by the animal, it is possible that the increased proportion of linoleic acid in the glycerides and phospholipids of the endome-

trium facilitates the acquisition of this fatty acid by the embryo.

In one previous study (25), human endometrium was found to incorporate less ^{14}C-acetate into the total phospholipids during the secretory than during the proliferative phase of the menstrual cycle. The present experiments have confirmed this finding and indicate in addition that the lesser incorporation into phosphatidyl choline, and also to some extent into phosphatidyl ethanolamine is where the decrease occurs. The secretory endometrium was also found to oxidize ^{14}C-acetate at a slower rate than the proliferative endometrium.

In the human, as in the pregnant rabbit, the decreased incorporation of ^{14}C-acetate into the endometrial phospholipids that was observed in this study during the secretory phase of the cycle may represent an antagonism by progesterone of the prior estrogenic stimulation of phospholipid synthesis during the proliferative phase.

The possibilities also exist as in the rabbit that a decreased permeability of the endometrial cell membranes to acetate or an increased intermediate pool size of acetate in the endometrium during the secretory phase in humans may have been responsible for the observed decreased incorporation of ^{14}C-acetate into the phospholipids. Further experimentation will be required to exclude these possibilities.

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Fatty Acid Metabolism in the Chloroplast Lipids of Green and Blue-Green Algae

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ABSTRACT

The pattern of uptake of radioactivity into chloroplast lipids when a green alga *(Chlorella vulgaris)* was incubated with sodium 2-^{14}C-acetate differed appreciably from that obtained when two blue-green algae *(Anabaena cylindrica* and *Anacystis nidulans)* were incubated under similar conditions.

The fatty acids of the digalactosyl diglyceride and sulphoquinovosyl diglyceride fractions from the blue-green algae were labeled more rapidly than were those of the corresponding fractions from *C. vulgaris,* whereas the activity in the acids of the phosphatidyl glycerol fraction from *A. cylindrica* and *A. nidulans* was relatively lower than that in the green alga. The results indicate that the metabolic behavior of chloroplast lipids may vary considerably according to the class of alga concerned.

In all three alga, the evidence points to an intermediary function for the chloroplast lipids in fatty acid synthesis.

Only limited exchange of acyl groups between the different chloroplast lipids seemed to occur during photoautotrophic growth.

INTRODUCTION

IN A PREVIOUS COMMUNICATION (1) we described the metabolism of 2-^{14}C-acetate by dark-grown cells of *Chlorella vulgaris.* From the results of these studies, we suggested that some of the cellular lipids have an intermediary function in fatty acid metabolism while others appear less active metabolically and are possibly more important as structural units.

In order to obtain additional and more specific information regarding lipid metabolism associated with photosynthesis, we have compared the incorporation of sodium 2-^{14}C-acetate into the lipids of two purely photoautotrophic organisms, namely, the two blue-green algae, *Anacystis nidulans* and *Anabaena cylindrica.* with that in a photoautotrophically cultured green alga, *Chlorella vulgaris.* To date, little information regarding the lipid metabolism of blue-green algae has been reported, despite the fact that these organisms differ markedly from the higher algae as regards morphology and lipid composition (2-4).

We discuss in this paper those results which relate particularly to the chloroplast lipids, namely the galactosyl diglycerides, sulphoquinovosyl diglyceride (sulfolipid) and phosphatidyl glycerol.

EXPERIMENTAL

Algae

Chlorella vulgaris, strain no. 211/11h from the Cambridge Collection of Algae and Protozoa, was grown photoautotrophically in a medium containing KNO_3 (1000 mg), K_2HPO_4 (200 mg), KH_2PO_4 (200 mg), $MgSO_4$, $7H_2O$ (200 mg), "Metals 45" (5) (20 mg), $CaCl_2$ (100 mg) and Na_2EDTA (20 mg) in 1 liter of aqueous medium brought to pH 6.9 with HCl. *Anacystis nidulans,* strain no. 1405/1 from the Cambridge Collection of Algae and Protozoa, was grown on the inorganic medium of Kratz and Myers (6) and *Anabaena cylindrica* was cultured on the standard medium for nitrogen-fixing algae (7).

Incubations with Metabolite

All three algae were incubated with 2-^{14}C-acetate in essentially the same manner. Cells were harvested by centrifugation and were then resuspended in the culture medium to give thick suspensions which contained 1.2-2.0 g wet weight of cells in 10 ml. To these suspensions 2-^{14}C-acetate was added at a concentration of 40 μC (8.8 mg) per 60 ml of suspension. The mixtures were incubated by placing the suspensions (usually 60-100 ml) in 250 ml conical flasks loosely stoppered with glass wool, and shaking the flasks in the light at 30C. The light-source consisted of four 40W strip-lamps suspended about 25 cm above the incubation flasks. Fifteen-Milliliter samples were removed from the flasks at desired intervals.

Under the conditions employed, cell division of the algae occurred not more than once every 24 hr, and appreciable net synthesis of

lipid probably did not occur during the periods of incubation employed.

Isolation and Fractionation of Lipids

Incubations were stopped at the appropriate time by the addition of propan-2-ol (20 vol) to the reaction mixture, which was then shaken and filtered and the residue reextracted with chloroform-methanol (2:1 v/v). The combined filtrates were concentrated in vacuo and washed with 0.9% NaCl to remove water-soluble impurities (8).

The lipid extracts from each incubation were fractionated by a combination of column chromatography on DEAE-cellulose (acetate form) and by preparative TLC on silica gel (9).

Radioactivity in Lipid Extracts

Prior to fractionation, an aliquot from each lipid extract was dried on a planchet and the ^{14}C-activity in the sample counted on a standard end-window counter. The proportion of activity in each lipid class was obtained by applying a standard quantity of extract to a thin-layer chromatogram of silica gel and developing the chromatogram with chloroform-methanol - acetic acid - water (85:25:10:3.7 v/v). The chromatogram was then scanned for ^{14}C-activity using the apparatus described by Ravenhill and James (10), and the relative activities in each fraction were calculated by a comparison of the areas of the peaks produced on the radiochromatogram. Because it is sometimes difficult to achieve a satisfactory resolution of all the lipid classes by the thin-layer system described above, a preliminary separation of acidic lipids (free fatty acid, phosphatidyl glycerol, sulphoquinovosyl diglyceride and phosphatidyl inositol) from the other lipid classes was obtained by passing a solution of the lipid extract in chloroform-methanol (2:1 v/v) over a column of DEAE cellulose (acetate form). Only the acidic lipids were retained by the column and were subsequently recovered by eluting the column with chloroform-methanol (2:1 v/v) saturated with concentrated NH_4OH. Equivalent quantities from each subfraction were then chromatographed separately and the activity in each component determined as described above.

Radioactivity of Individual Fatty Acids

Purified lipids were refluxed with methanol-benzene-concentrated H_2SO_4 (20:10:1 v/v) for 90 min. The methyl esters of the component fatty acids were extracted with light petroleum (bp 60-80C), dried, and analyzed on a radiochemical gas chromatograph employing diethylene glycol adipate as stationary phase. Comparative specific activities are expressed as ratios of radioactivity peak area to mass peak area.

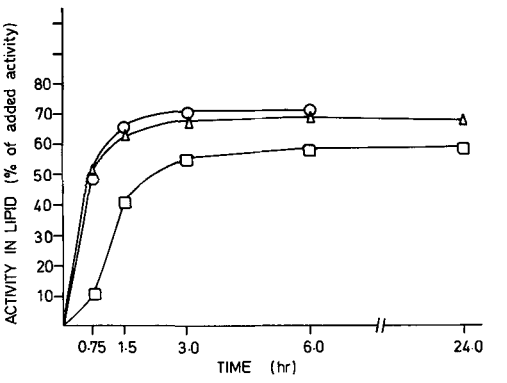

FIG. 1. Uptake of radioactivity into lipids of algae when incubated with Na-2-^{14}C-acetate. ○, *Chlorella vulgaris;* △, *Anacystis nidulans;* □, *Anabaena cylindrica.*

RESULTS

Lipid Composition

Phosphatidyl choline, phosphatidyl inositol and phosphatidyl ethanolamine were absent from the extracts of both blue-green algae, in accordance with previous observations (2, 3).

Uptake of Label into Lipids

In *C. vulgaris* and *A. nidulans* the amount of label in lipid increased rapidly for the first 90 min and thereafter rose only slightly (Fig. 1). In both algae approximately 70% of the added acetate was eventually incorporated into lipid. Incorporation of label into the lipids of *A. cylindrica* increased steadily for about 3 hr and then only slightly for the remaining 21 hr. About 60% of the added acetate was converted into lipid by this alga.

In all algae, about 90% of the label incorporated into lipid was located in the acyl groups.

Distribution of Label among Lipid Classes

The distribution of ^{14}C-activity among the lipid classes of each alga at different times during incubation is presented in Fig. 2-4. One notable feature of these results is the comparatively slight variation with time in the proportion of counts in the individual lipid classes. On the other hand, each alga differed

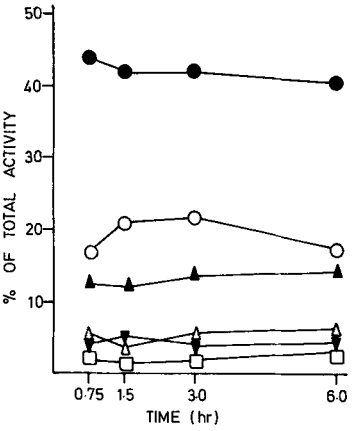

FIG. 2. Distribution of radioactivity among the acyl lipids of *C. vulgaris* during incubation. ○, monogalactosyl diglyceride; △, digalactosyl diglyceride; □, sulphoquinovosyl diglyceride; ●, phosphatidyl glycerol; ▲, phosphatidyl choline; ▼, phosphatidyl ethanolamine. Triglyceride and pigment fractions account for the remaining activity.

significantly from the others in the relative distribution of counts among the different classes of chloroplast lipid. The distribution of label among the lipid classes in photoautotrophic *C. vulgaris* (Fig. 2) after 3 hr incubation was generally quite similar to that found in the heterotrophically grown alga when incubated under similar conditions (1) except that in the latter culture the phosphatidyl choline fraction contained a higher proportion of the total counts. In *A. cylindrica* (Fig. 3), the digalactosyl diglyceride and sulphoquinovosyl diglyceride fractions contained a much higher proportion of the total counts than was observed in *C. vulgaris*, and the proportion of counts in the phosphatidyl glycerol

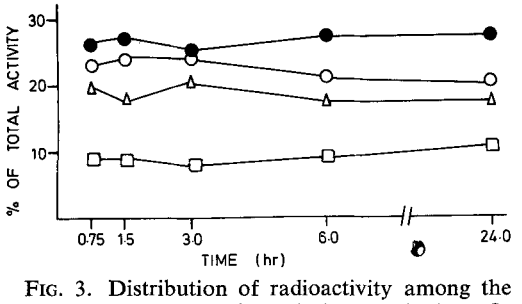

FIG. 3. Distribution of radioactivity among the acyl lipids of *A. cylindrica* during incubation. ○, monogalactosyl diglyceride; △, digalactosyl diglyceride; □, sulphoquinovosyl diglyceride; ●, phosphatidyl glycerol. Triglyceride, pigment and an uncharacterized glycolipid (which contained no acyl groups) account for the remaining radioactivity.

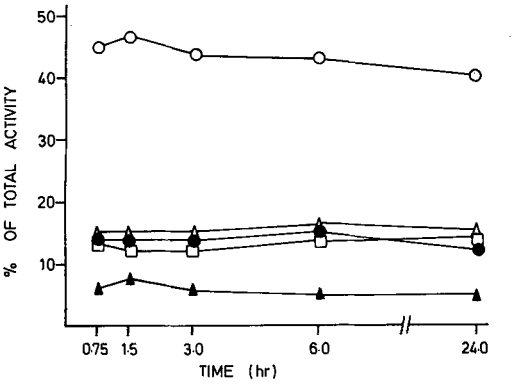

FIG. 4. Distribution of radioactivity among the acyl lipids of *A. nidulans* during incubation. ○, monogalactosyl diglyceride; △, digalactosyl diglyceride; □, sulphoquinovosyl diglyceride; ●, phosphatidyl glycerol; ▲, unidentified acyl lipid. Triglyceride and pigment account for the balance of activity.

fraction was considerably depressed. The monogalactosyl diglyceride fraction of *A. nidulans* (Fig. 4) contained over 40% of the total counts incorporated into the lipids of this alga, and the other three chloroplast lipids each contained roughly equal proportions of the remaining radioactivity. A small quantity of the lipid-bound ^{14}C-activity in *Anacystis* was found in an uncharacterized lipid previously detected in this alga by Nichols et al. (2).

Fatty Acids

The fatty acid compositions of the algal lipids were similar to those already reported for these organisms (2,3,11) and did not vary during the periods of incubation.

Tables I-VI present some of the data obtained regarding the levels of ^{14}C-activity in the fatty acids of the individual lipid classes during the periods of incubation.

The changes in relative specific activities of the C_{14} and C_{16} saturated acids in the chloroplast lipids from the three algae are given in Tables I and II, and these figures are generally consistent with the orders of labeling observed with the intact lipids (Fig. 2-4). For example, the specific activities of the component acids from the phosphatidyl glycerol fraction of *C. vulgaris* were generally higher than those of the monogalactosyl diglyceride fraction, and the acids from the two other chloroplast lipids contained much less label than either of these. In the blue-green algae, the acids in the phosphatidyl glycerol fractions were considerably less active relative to those of the other chloroplast lipids than was observed with *C. vulgaris*.

TABLE I

Changes in the Relative Specific Activities of Palmitic Acid in the Chloroplast Lipids of Three Algae During Incubation with Na-^{14}C-Acetate

	Alga											
	C. vulgaris				A. cylindrica				A. nidulans			
Lipid	0.75 hr	1.5 hr	3.0 hr	6.0 hr	0.75 hr	1.5 hr	3.0 hr	6.0 hr	0.75 hr	1.5 hr	3.0 hr	6.0 hr
Monogalactosyl diglyceride	290	123	71	74	31	65	65	87	173	204	147	163
Digalactosyl diglyceride	16	26	24	42	5	14	19	41	73	70	87	78
Sulphoquinovosyl diglyceride	52	66	62	87	51	71	81	80	102	107	117	103
Phosphatidyl glycerol	430	574	400	750	30	113	132	152	91	80	77	61

TABLE II

Changes in the Relative Specific Activities of the $C_{14:0}$ Saturated Acid in the Chloroplast Lipids of Three Algae During Incubation with Na-^{14}C-Acetate

	Alga											
	C. vulgaris				A. cylindrica				A. nidulans			
Lipid	0.75 hr	1.5 hr	3.0 hr	6.0 hr	0.75 hr	1.5 hr	3.0 hr	6.0 hr	0.75 hr	1.5 hr	3.0 hr	6.0 hr
Monogalactosyl diglyceride	150	233	154	230	19	62	50	50	2875	3825	2500	1520
Digalactosyl diglyceride	6	17	20	66	10	22	40	60	1722	2280	1337	1200
Sulphoquinovosyl diglyceride	20	40	31	116	20	60	60	14	2333	2440	2750	2500
Phosphatidyl glycerol	200	550	140	633	20	60	100	62	1800	1866	2000	1360

Discussion

In a previous paper (1), we described the incorporation of label into lipids when dark-grown *C. vulgaris* was incubated in the light with 2-^{14}C-acetate. In the studies described here, the cells employed had been cultured photoautotrophically, and a comparison of the two series of results over comparable periods reveals certain differences in the pattern of labeling. In particular, the phosphatidyl choline fraction exhibited a higher relative uptake and turnover of label during the earlier studies than was observed in the present work (Fig. 2). In addition, there were greater fluctuations in the relative activities in the different lipid fractions from the studies involving dark-grown cells than were obtained with the photoautotrophic culture. These differences are probably due to the profound morphological and metabolic changes which take place when dark-grown heterotrophic cells are forced to take on a purely photoautotrophic kind of metabolism, and which involve the development of chloroplasts and the onset of general photosynthetic metabolism. During such a period, one might expect to find greater variations in lipid metabolism than were observed in the present studies in which the only change in environment was the temporary one due to the added acetate. Some indication of the radical changes which took place when the dark-grown cells were incubated photoautotrophically are provided by consideration of the major variations in fatty acid composition which occurred in some of the lipids during the period of incubation (1). In the present studies, no significant changes in fatty acid composition was observed in any of the algae. Not being a major lipid of chloroplasts, phosphatidyl choline is presumed to be mainly located in other cell organelles, such as mitochondria (12). Consequently the comparatively high proportion of label incorporated into this fraction when dark-grown *C. vulgaris* was incubated with 2-^{14}C-acetate may be attributed to residual heterotrophic metabolism to which mitochondria and other cell organelles make a greater contribution than they do during photo-

synthesis, when chloroplast metabolism is predominant.

The general pattern of labeling of fatty acids in the lipids of light-grown *C. vulgaris* was generally similar to that observed with dark-grown cells. In both series of experiments, the drop or leveling in uptake of label between 90 min and 3 hr was followed by a period in which some of the specific activities (particularly that of C_{18} monoenoic acid) rose again, and we have already suggested that this may be an effect of the added acetate (1).

The fatty acids of the sulphoquinovosyl diglyceride and digalactosyl diglyceride fractions from *A. cylindrica* obtained far higher specific activities (relative to those of the same acids in the other fractions) than were observed in the corresponding fractions from *C. vulgaris* (Tables I and II). As in the green alga, the fatty acids of the digalactosyl diglyceride fraction accumulated label at a slower and steadier rate than did those of the other lipids.

The patterns of labeling of fatty acids of the lipids from *A. nidulans* differed markedly from those observed in the other algae, and the fatty acids of the digalactosyl diglyceride and sulfoquinovosyl diglyceride fractions possessed relative metabolic activities greater than those observed in *C. vulgaris* (Tables I and II).

In the previous communication (1), we classified the lipids of *C. vulgaris* into two broad groups according to their relative uptake of label from ^{14}C-acetate. One group comprised those lipids which were initially more highly and rapidly labeled and in which some fatty acids showed rapid rates of synthesis and turnover. We suggested that the chloroplast lipids from this group (monogalactosyl diglyceride and phosphatidyl glycerol) might be involved as intermediates in the fatty acid metabolism of the chloroplast, and that the lipids of the less rapidly labeled group (including sulphoquinovosyl diglyceride and digalactosyl diglyceride) might be more important in structural roles. The results presented in this paper suggest that similar conclusions may be valid for *C. vulgaris* growing photoautotrophically, but the data obtained from the parallel studies involving the two blue-green algae indicate that these general conclusions cannot be extended to the photosynthetic apparatus of all algae. In particular, the fatty acids of the sulphoquinovosyl diglyceride and digalactosyl diglyceride fractions of the two blue-green algae are far more active metabolically than in *C. vulgaris*, and those of the phosphatidyl glycerol fraction showed a relatively low activity. From these data, it is clear that lipid metabolism in chloroplasts may differ widely according to the class of organism studied, at least from the point of view of turnover of fatty acids in the individual lipids. It would be interesting to establish whether carbohydrate turnover in the chloroplast glycolipids also differs according to the class of alga or whether the major differences are only in fatty acid metabolism.

Despite these specific differences in lipid metabolism certain general features are common to the results from all three algae. Thus although some 90% of the total radioactivity incorporated into fatty acids had become lipid-bound by 90 min (in the case of *C. vulgaris* and *A. nidulans*) or 3 hr (*A. variabilis*), considerable changes in the proportion of label in each class of fatty acid occurred subsequently. Consequently changes in the structure of fatty acids must occur after incorporation into lipid. Evidently in all three algae the lipids are not mere acceptors of the end-products of fatty acid synthesizing systems but are in some way intermediates in the system. This intermediate function could either be that of a true substrate for the reaction producing the change in structure, or alternatively the lipid could act as a transport agent moving between the enzyme sites. In the latter case, the fatty acid would be split from the lipid on arrival at the enzyme site and then returned (presumably to the corresponding lyso- compound) after the structural modification. The first suggested function could presumably operate only for desaturase systems since chain elongation appears to require the fatty acid in the form of a suitable thiol ester (to CoA or acyl-carrier protein) (13,14).

On the basis of our earlier studies, we concluded that the function of transport agent or fatty acid donor was the most likely for these lipids, and suggested that transference of fatty acids from one lipid class to another was possible at any stage, and particularly among those lipids showing similar rates of labeling (1). A general feature of our present work is that in all three algae there were only slight variations in the proportion of activity in each lipid class throughout the course of each incubation (Fig. 2-4). This suggests that transfer of fatty acid from one lipid class to another did not occur to any large extent, because the specific activities of the fatty acids in different lipid classes at any moment are sufficiently different that substantial acyl transfer would

TABLE III

Changes in the Distribution of Radioactivity Among the Fatty Acids of the Monogalactosyl Diglyceride Fraction of *Anacystis nidulans*

Time (hr)	^{14}C activity in fatty acid (percentage of that in all fatty acids)				
	$C_{14:0}$ acid	$C_{14:1}$ acid	$C_{16:0}$ acid	$C_{16:1}$ acid	$C_{18:1}$ acid
0.75	34	0	66	0	0
1.5	29	4	63	4	0
3.0	25	7	56	13	0
6.0	19	10	51	20	0
24.0	3	22	43	30	2

result in marked differences in the total activity in the lipid. Assuming no significant degree of acyl transfer between lipid classes, then a distinct and isolated series of fatty acid transformations must occur within each lipid class. This would require that within each lipid class the passage of label through the constituent fatty acids should be consistent with the established pathways of fatty acid synthesis in algae, and an examination of our results shows this requirement to be fulfilled in the majority of cases.

For example, the changes in distribution of activity among each fatty acid of the monogalactosyl diglyceride fraction of *A. nidulans* is given in Table III. These figures are consistent with a steady desaturation of the C_{14} and C_{16} saturated acids to the corresponding monoenoic acids. The increase of total activity in the C_{16} acids is balanced by that lost from the C_{14} acids and would represent a certain degree of chain elongation converting C_{14} saturated acid to C_{16} saturated acid. In the sulphoquinovosyl diglyceride fraction of *A. nidulans* (Table IV), the desaturation of the C_{14} saturated acid occurs more slowly than in the monogalactosyl diglyceride. This pathway of activity is consistent with a steady desaturation of the C_{14} and C_{16} saturated acids coupled with a certain degree of conversion of C_{14} saturated acid to C_{16} saturated acid. In both

TABLE IV

Changes in the Distribution of Radioactivity Among the Fatty Acids of the Sulphoquinovosyl Diglyceride Fraction of *Anacystis nidulans*.

Time (hr)	^{14}C activity in fatty acid (percentage of that in all fatty acids)				
	$C_{14:0}$ acid	$C_{14:1}$ acid	$C_{16:0}$ acid	$C_{16:1}$ acid	$C_{18:1}$ acid
0.75	32	3	62	3	0
1.5	30	5	55	10	0
3.0	27	5	58	10	0
6.0	26	5	56	12	1
24.0	21	8	44	25	2

TABLE V

Changes in the Distribution of Radioactivity Among the Fatty Acids of the Sulphoquinovosyl Diglyceride Fraction of *Anabaena cylindrica*

Time (hr)	^{14}C Activity in fatty acid (percentage of that in all fatty acids)					
	$C_{14:0}$ acid	$C_{16:0}$ acid	$C_{16:1}$ acid	$C_{18:0}$ acid	$C_{18:1}$ acid	$C_{18:2}$ acid
0.75	0	60	6	18	15	0
1.5	1	57	8	15	20	0
3.0	2	55	7	12	24	0
6.0	2	55	7	8	23	5
24.0	2	43	6	3	34	12

TABLE VI

Changes in the Distribution of Radioactivity Among the Fatty Acids of the Monogalactosyl Diglyceride Fraction of *Chlorella vulgaris*

Time (hr)	^{14}C Activity in fatty acids (percentage of total in all fatty acids)							
	$C_{14:0}$ acid	$C_{16:0}$ acid	$C_{16:1}$ acid	$C_{16:2}$ acid	$C_{18:0}$ acid	$C_{18:1}$ acid	$C_{18:2}$ acid	$C_{18:3}$ acid
0.75	3	25	17	3	2	41	9	0
1.5	2	11	24	7	0	37	19	0
3.0	2	7	20	14	0	24	32	0
6.0	2	5	15	18	0	22	39	1

these examples the small quantity of activity eventually found in C_{18} monoenoic acid would result from chain elongation of C_{16} saturated acid to C_{18} saturated acid. C_{18} saturated acid seldom accumulates in algae, and is very rapidly desaturated to the monoenoic acid.

Similar results were obtained with most of the individual lipid classes of *A. cylindrica* and *C. vulgaris,* for which the results from the sulphoquinovosyl diglyceride and monogalactosyl diglyceride fractions, respectively, are given in Tables V and VI. Both sets of results are compatible with progressive desaturation of the C_{16} and C_{18} acids coupled with a certain amount of conversion of C_{16} saturated acid to C_{18} saturated acid.

Alterations of fatty acid structures within separate lipid classes coupled with only a minor degree of acyl transfer between lipid classes, could account, at least partially, for the established tendencies of certain fatty acids to be associated with specific classes of lipid.

The conclusions drawn from the results presented in this paper may be summarized as follows:

Firstly, a comparison of the uptake of radioactivity by the individual lipid classes of the three algae indicates that the metabolic behavior of individual chloroplast lipids may vary considerably according to the class of alga concerned.

Secondly, it is probable that the chloroplast lipids of all three algae have some intermediary function in fatty acid synthesis. We obtained little evidence for the exchange of acyl residues between chloroplast lipids during photoautotrophic growth, although our results do not preclude this occuring to a very limited extent.

Thirdly, the differences observed between the results reported here for photoautotrophic *C. vulgaris* and those described previously for dark-grown cells of this alga, can be accounted for by residual heterotrophic metabolism in the latter cultures.

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Liver Lipids During Development

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ABSTRACT

The fatty acid composition of the major liver microsomal phospholipids has been studied during pre- and postnatal development of the rabbit. The fatty acid composition of the total lipids, phosphatidyl choline, and phosphatidyl ethanolamine from animals –6, –3, 0, +3, +6, +9, +16, and +112 days of age was determined. Fatty acid composition is similar in phosphatidyl choline and phosphatidyl ethanolamine for oleic acid at +3, +6, +9, and +16 day old animals; palmitoleic acid at +9 day old animals and linoleic acid at –6, –3, and 0 day old animals.

Palmitoleic acid demonstrated a uniform decrease during early development in the total lipids and in both phosphatidyl choline and phosphatidyl ethanolamine; however, in the 112 day animal, the amount was just slightly lower than that observed for the earliest prenatal animal studied. Oleic acid decreased considerably during early postnatal development in the total lipids, phosphatidyl choline and phosphatidyl ethanolamine, but an increase in the 112 day animal was observed. Linoleic acid fluctuated considerably throughout postnatal development in the total lipids as well as in the two major phosphatides.

Lecithin biosynthesis has been studied by two pathways during development of rabbit liver from –6 days to +110 days. The two pathways of lecithin biosynthesis were evaluated by assaying the activities of the liver enzymes choline phosphotransferase and phosphatidylmethyltransferase at different time intervals during development. The greater enzymatic activity was observed in the cholinephosphotransferase during development.

INTRODUCTION

VERY LITTLE BIOCHEMICAL information is available in the area of development of the membranes of mammalian tissues, particularly in the microsomes. Lathe and Flint (1,2) have studied the development of rabbit liver microsomal membranes through the assessment of the composition of protein and nonprotein components as well as by the study of the activities of various microsomal enzymes during development. Dallner, et al. (3) have studied the synthesis of rat liver microsomal membrane-bound enzymes during development. They reported that an extensive synthesis of microsomal membranes occurs near the end of the gestation period. Miller and Cornatzer (4) have shown a progressive increase in concentration (μg phospholipid phosphorus/mg protein) of microsomal phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol during development (–12, –9, 0, +2, +9, and +14 days of age). However, the concentration of the individual phospholipids of mitochondria does not change during development.

Dallner et al. (5) has investigated the fatty acid composition of the rat liver microsomal total lipids for 0 day, +5 day and +90 day animals. He reported large deviations among the three ages studied. Dobiasova et al. (6) have reported the fatty acid composition for various whole tissues of the rat during postnatal development. A more comprehensive study of the fatty acid composition of the microsomes during development has, however, been lacking.

In this communication we are reporting data for the fatty acid composition of the total lipids, phosphatidyl choline, and phosphatidyl ethanolamine isolated from developing rabbit liver microsomes. Lecithin biosynthesis, by two pathways, has been assayed during embryological development by determining the activities of choline phosphotransferase and phosphatidylmethyltransferase.

MATERIALS AND METHODS

New Zealand white rabbits maintained on a constant diet consisting of rabbit chow mixed with whole wheat (State Mill, Grand Forks, North Dakota) (1:1, w/w) and supplemented with alfalfa were used throughout the experiments. The animals were housed in steel wire-bottom cages and were allowed free access to food and water.

Pregnant females at predetermined gestation

periods were sacrificed by a blow on the head. The young rabbits were sacrificed by decapitation on the specified days. The fetal rabbits were not separated as to sex, since the sex organs do not become active until after puberty. The livers were rapidly excised, weighed, and the gallbladders removed as soon as possible. They were then homogenized in ice cold 0.25 M sucrose in a Potter-Elvehjem homogenizer with the aid of a close fitting Teflon pestle. The resulting homogenates were diluted with 0.25 M sucrose so as to achieve a 10-fold dilution of sucrose to liver, i.e., 1 g of liver to 10 ml of sucrose.

The nuclei, cell wall debris and mitochondria were sedimented by centrifugation in a refrigerated centrifuge at 10,000 × *g* for 10 min. The resulting pellet was washed two times with 0.25 M sucrose and the supernatant saved for preparation of the microsomes. The microsomes were prepared by centrifugation at 78,500 × *g* for 45 min using a No. 30 rotor in a Model-L Spinco preparatory ultracentrifuge.

Lipids were extracted with chloroform-methanol (2:1, v/v) and were washed with 0.04% $CaCl_2$. The procedure of Folch, et al. (7) was used for this preparation. In situations where the lipids had to be stored, they were dissolved in chloroform and stored at 0C under an atmosphere of nitrogen. Storage was never for more than 48 hr. Phosphatidyl choline and phosphatidyl ethanolamine were separated by chromatography on silicic acid-impregnated glass fiber as described by Cornatzer, et al. (8). Identification of the phospholipids was made using highly purified standards and comparing the R_f's of these to the unknown phospholipids and chemical spot tests as previously reported (8). The recovery values of individual phospholipids were similar to those reported (8). After development, which was accomplished in 4-6 hr, the chromatograms were hung in a fume hood (fan not running) and allowed to dry for approximately 1 hr. Duplicate chromatograms dried for 1 hr, compared with samples not dried, gave arachidonic values (25.6%, 29.5% and 25.4%, 24.6%) respectively. Similar data were observed for linoleic acid (9.1%, 10.0%, and 9.7%, 10.1%). The chromatograms were then sprayed with an aqueous solution of Rhodamine 6G (10 mg/liter). While the chromatograms were still wet, they were visualized under an ultraviolet light in a darkened room. The separated phosphatide areas were then outlined and cut from the paper.

Methyl esters of the fatty acyl portions of the phospholipids were prepared by transmethylation without removing the phosphatides from the chromatogram in accordance with methods described by Morgan, et al. (9).

When the fatty acid composition of the microsomal total lipids was desired, the total lipid preparation, which comprised mainly phospholipid with small amounts of neutral lipid, was taken to dryness and refluxed in 5% sulfuric acid in methanol (10).

Gas Chromatography

Chromatography of the methyl esters of the fatty acids was performed on a Model-10 Barber-Colman gas chromatograph (Barber-Colman Company, Rockford, Ill.). The machine was equipped with an argon ionization detector utilizing a ^{90}Sr source. Two U-shaped glass columns measuring 6 ft. by 4 mm (I.D.) containing 1% ethylene glycol adipate on Chromosorb W 60/80 mesh (Analytical Engineering Laboratories, Inc., Hamden, Conn.) were used. The flash heater, detector, and column temperatures were maintained at 275C, 225C, and 195C, respectively. Argon with an inlet pressure of 14 lb/in.^{2} was employed as the carrier gas.

Methyl ester derivatives of the fatty acids isolated from the phospholipids was identified by comparing the retention ratios (relative to methyl palmitate) to those obtained from standards (Applied Science Laboratories, Inc., State College, Penn.). The linearity of the detector response was verified by quantitating a mixture of fatty acid methyl esters of known composition. Quantitative results with National Heart Institute fatty acid standard mixture D (Applied Science, State College, Penn.), agreed with the stated composition data with a relative error less than 5%. Peak areas were calculated as the product of the peak height and the width at half peak height, and percentage distributions are given in terms of peak areas (11).

The lipids in the laboratory chow were extracted using the method described by Folch et al. (7) and the methyl esters of the fatty acids were obtained by refluxing the lipids with 5% sulfuric acid in methanol. Results for this determination are given in Table I.

Lecithin Biosynthesis

The materials used were: ^{14}C methyl-S-adenosyl methionine (New England Nuclear Corp., Boston, Mass.); unlabeled S-adenosyl methionine (Calbiochem, Los Angeles, Calif.); distearoyl N,N-dimethyl phosphatidyl ethanolamine (Erich Baer, University of Toronto and D. Shapiro, Weizmann Institute, Reho-

vath, Israel); 1,2-^{14}C-cytidine diphosphocholine (E. P. Kennedy, Harvard University); tris, deoxycholic acid and Tween-20 (Sigma Chemical Company, St. Louis, Mo.). Diglycerides were prepared from egg lecithin by the method of Hanahan (12). The preparation used gave only one spot when subjected to thin-layer chromatography (TLC) (13).

Freshly prepared whole liver homogenates were used in all of the assays. Protein was measured by the method of Lowry (14) using bovine serum albumin as the standard. Approximately 10 mg of protein was used in each assay. The assays were carried out in a shaking water bath at 30C. The distearoyl N,N-dimethyl phosphatidyl ethanolamine preparation was checked for purity and gave only one spot when chromatogrammed on silica gel by the method described by Artom (15). The N,N-dimethyl phosphatidyl ethanolamine was emulsified in 0.2 M Tris-HCl containing 0.4% deoxycholic acid as described by Rehbinder and Greenberg (16). Use of a Potter-Elvehjem homogenizer and heating the resulting emulsion in a boiling water bath produced a dispersion that was stable as demonstrated by TLC when stored at 4C under an atmosphere of nitrogen. Suspensions of diglycerides were prepared in a manner similar to that described for the emulsification of the ethanolamine phosphatides. The diglycerides were prepared from egg lecithin by the method of Hanahan et al. (12) and were stored in chloroform under an atmosphere of nitrogen at –10C. The purity of the diglycerides was checked by TLC. Just prior to use, small amounts of the chloroform solution were evaporated to dryness under a stream of nitrogen. Tris-HCl (0.2 M), containing 0.1% Tween-20, was used to emulsify the diglycerides. Suitable emulsions could be prepared by this method.

A modification in the method described by Rehbinder and Greenberg (16) was used to assay the phosphatidylmethyltransferase reaction, which involved the following changes: The reaction was stopped by the addition of 0.1 ml of concentrated HCl; 1.7 ml of water was then added and the contents thoroughly mixed. This was followed by the addition of 1.7 ml of n-butanol. The tubes were shaken again and allowed to stand for 30 min. They were mixed a third time and centrifuged. Aliquots of the upper butanol-lecithin phase were removed and subjected to TLC, in accordance with the procedure described by Parker and Peterson (17). Nonlabeled phosphatidyl choline was chromatogrammed with the labeled phosphatidyl choline to aide in the visualization of the chromatogram. The chromatograms were visualized by exposure to iodine vapors. The lecithin spot was outlined and after sublimation of the iodine, was scraped into vials containing a toluene scintillating-thixotropic gel suspension.

TABLE I

Fatty Acid Composition of the Diet
Fatty Acids Listed as Number of Carbon Atoms: No. of Double Bonds

Fatty acid	Per cent composition by weight
12	t[b]
14:1[a]	t
16	17.3
16:ISO[a]	t
16:1	t
18	t
18:1	29.2
18:2	47.4
18:3	3.9

[a]Tentative identification.
[b]Trace quantity, less than 1%.

There is no physical exchange between the ^{14}C methyl-S-adenosyl methionine and N,N-dimethyl phosphatidyl ethanolamine or lecithin. The radioactive lecithin isolated represents newly made lipid. Similarly, there is no exchange between 1,2-^{14}C cytidine diphosphocholine, and lecithin and the radioactive lipids isolated represents newly made phosphatidyl choline. The choline-phosphotransferase reaction was followed using the method described by Kennedy (18). Aliquots of the final chloroform-lecithin extract were placed into vials and evaporated to dryness prior to the addition of the scintillation solvent.

Assays were made in which the lipid substrate was omitted from the reaction mixture. The activities of both enzymes are expressed as mμmoles of lecithin synthesized. These values were obtained by subtracting the amount of lecithin synthesized in the controls from the amount of lecithin produced in the experiments, containing added lipid substrate. The activity was calculated by division of the number of counts found in the lecithin by the specific activity of the adenosyl methionine and cytidine diphosphocholine. The specific activities of the substrates used were 170,000 cpm per μmole and 66,000 cpm per μmole for the adenosyl methionine and cytidine diphosphocholine, respectively.

RESULTS

The results obtained for the determinations of the fatty acid compositions of the microsomal total lipids, phosphatidyl choline and

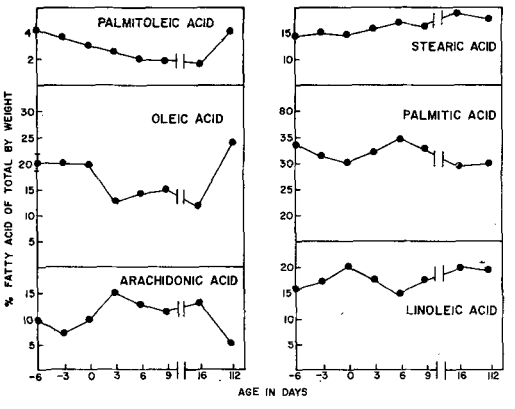

FIG. 1. Fatty acid composition of rabbit liver microsomal total lipids during pre- and postnatal development. Each point on the graph represents from 4 to 7 determinations. Several livers were pooled in younger animals for each sample. The vertical bars on the graphs represent the standard deviation whenever it was found to be larger than the symbol used to identify it.

phosphatidyl ethanolamine for the various ages studied are given in Fig. 1 and 2. Results are given for the 6 major fatty acids: palmitic, palmitoleic, stearic, oleic, linoleic and arachidonic acid. The others found (lauric, tridecanoic, myristic, isopalmitic, hexadecatrienoic, and heptadecanoic) were in concentrations of less than 1%.

Fig. 1 shows the various concentrations of

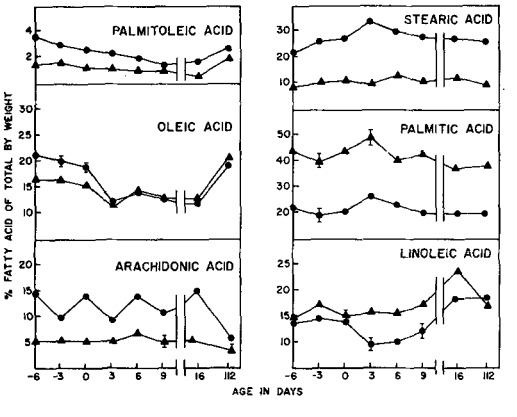

FIG. 2. Fatty acid composition of rabbit liver microsomal phosphatidyl choline (▲) and phosphatidyl ethanolamine (●). Each point on the graph represents from 4 to 7 determinations. Several livers were pooled in younger animals for each sample. The vertical bars on the graph represent the standard deviation whenever it was found to be larger than the symbol used to identify it.

three major fatty acids found in the total lipids. As can be seen, palmitic acid shows a slight decrease as gestation progresses; however, an increase was observed during early postnatal development with a level in the matured animal about the same as that seen in the newborn animals. Palmitoleic acid declined throughout pre- and postnatal development, increasing however in the 112 day animal. Stearic acid showed a progressive increase throughout the period studied. Oleic acid diminished considerably during postnatal development, although showing an increase in the oldest animal. The level of linoleic acid fluctuated during development. The longest fatty acid studied, arachidonic acid, increased immediately following birth but substantially decreased in the maturing animal. The changes found in the microsomal total lipids were reflected in phosphatidyl choline and phosphatidyl ethanolamine. This was anticipated, since these two phosphatides represent about 70% of the total phospholipids found in the rabbit liver microsomes (4).

Fig. 2 illustrates the fatty acid composition of phosphatidyl choline and phosphatidyl ethanolamine over the same period studied for the total lipids. Palmitic acid shows remarkably similar trends in both phosphatidyl choline and phosphatidyl ethanolamine, increasing slightly during early postnatal development but showing a slight decrease in the 112 day animal as opposed to the −6 day fetus. Palmitoleic decreased substantially throughout pre- and postnatal development in phosphatidyl choline. The same trend was found in phosphatidyl ethanolamine but not to such a great extent. It is of interest to note the large increase in palmitoleic acid found in the 112 day animal over the 16 day rabbit. Stearic acid remained relatively constant in concentration throughout the entire period studied in both phosphatidyl choline and phosphatidyl ethanolamine, increasing only 4% in both lipids. Both phosphatidyl choline and phosphatidyl ethanolamine (Fig. 2) exhibit similarities in their concentrations of oleic acid during the period studied. Oleic acid gradually decreased during pre- and postnatal development, showing a rise in the oldest animal as did palmitoleic acid. Linoleic acid concentration decreased in phosphatidyl ethanolamine during early postnatal development. The lowest concentration was observed at +3 days of age and was followed by an increase in concentration until +16 days of age. The linoleic acid concentration in phosphatidyl choline did not change during development

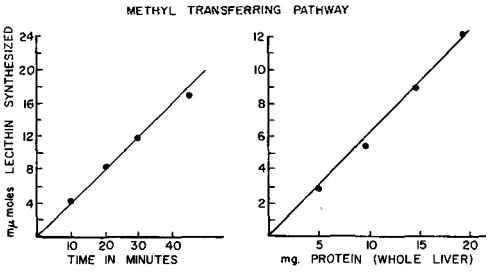

FIG. 3. Effect of incubation time and enzyme concentration on the phosphatidylmethyltransferase reaction for the formation of lecithin. Each reaction mixture contained 1 μmole of distearoyl N,N-dimethylphosphatidylethanolamine emulsified in 1 ml of 0.2 M tris-HCl pH 8.5 containing 0.4% deoxycholic acid, 1 μmole of adenosyl ^{14}C-methylmethionine and enzyme in a final volume of 1.7 ml.

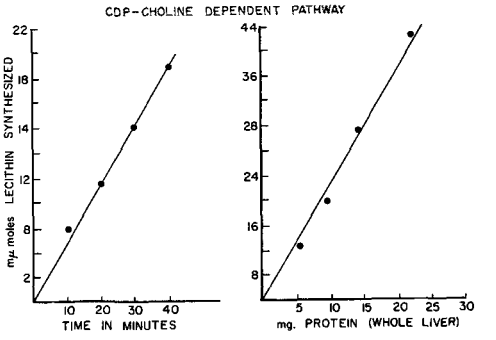

FIG. 4. Effect of incubation time and enzyme concentration on the cholinephosphotransferase reaction for the formation of lecithin. Each reaction mixture contained 1 mg of diglyceride emulsified in 1 ml of 0.2 M tris-HCl pH 8.5 containing 0.1% Tween-20, 10 μmoles of $MgCl_2$, 0.5 μmoles of 1,2^{14}C-cytidine diphosphocholine and enzyme. The final volume of the reaction mixtures was 1.7 ml.

except for an increase in concentration at +9 and +16 days of age. Arachidonic acid fluctuated considerably in phosphatidyl choline, decreasing about 50% in concentration in the oldest animals when compared to the –6 day fetuses. This fluctuation was not observed to such an extent in phosphatidyl ethanolamine.

Lecithin Biosynthesis

Two basic parameters were evaluated by both systems under investigation. It was the intent of these studies to determine conditions whereby the two pathways being studied were linear with respect to time and enzyme concentration. As can be seen in Fig. 3 and 4, both systems were linear with respect to both parameters. These studies were made using the 110 day old rabbit as a source of enzyme. From the results of these studies it was decided to conduct the assay during development for 30 min using approximately 10 mg of protein. Fig. 5 and 6 illustrate the activity of both reactions during pre- and postnatal development.

DISCUSSION

The results shown in Fig. 1 for the concentrations of the monounsaturated fatty acid, namely palmitoleic acid and oleic acid, show a progressive decrease during early development. Only in the 112 day animal are the concentrations of these acids as high as they were in the early fetuses. The higher concentrations of the unsaturated fatty acids found in the 112 day animals may be due to a change in diet, since the young do not start eating solids until the 12th or 13th day after birth. The corresponding saturated fatty acids, palmitic and stearic showed a slight increase or remained constant during the neonatal period. The liver cells may be supplied with different fatty acids in utero as compared to suckling and when on a solid diet. These differences in fatty acids would be reflected in phosphatidyl choline and phosphatidyl ethanolamine. The composition of the fatty acid in the diet fed to the mother and consumed by the 112 day old rabbits is shown in Table I. The composition is oleic acid, 29.2%; linoleic, 47.4%; linolenic, 3.9%; palmitoleic acid, trace only. The decreased concentration of oleic acid and palmitoleic acid in phosphatidyl choline and phosphatidyl ethanolamine may be due to the change in diet.

Mammalian tissues have long been known to be capable of synthesizing unsaturated fatty acids from saturated fatty acid precursors as shown by the classic experiments of Schoenheimer and Rittenberg (19). More recently, Bloomfield and Bloch (20), using in vitro techniques, have demonstrated yeast preparations capable of carrying out this monoenoic process with palmitic acid. Bernard et al. (21) have shown that the system responsible for the conversion of stearic acid to its unsaturated analogue is located in the microsomes of rat liver. It is possible that the female hormones in utero might influence the biosynthesis of phosphatidyl choline and phosphatidyl ethanolamine since Bjornstad and Bremer (22) have demonstrated that the incorporation of 1,2-^{14}C-ethanolamine and CH_3-^{3}H methionine into phosphatidyl choline is greater in the female than in the male ani-

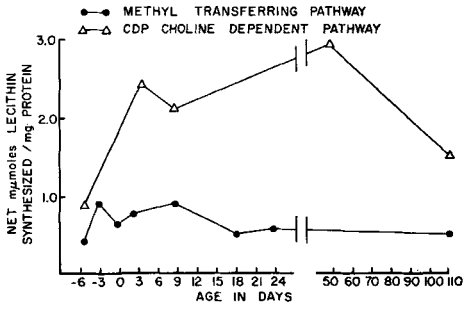

FIG. 5. The activity of the liver choline phosphotransferase and phosphatidylmethyltransferase reaction during pre- and postnatal development as a function of age. The activity is expressed on a per milligram protein basis. Incubation was for 30 min and approximately 10 mg protein was used in each assay. Other conditions were as described in the legends for Fig. 3 and 4. Each point represents single animals.

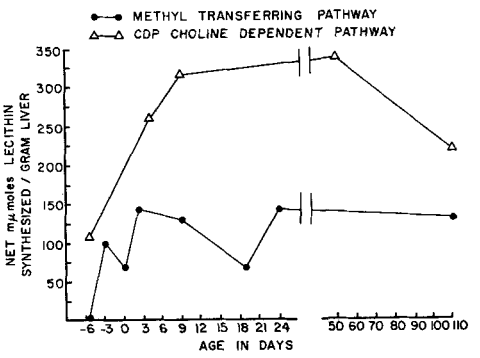

FIG. 6. The activity of the liver choline phosphotransferase and phosphatidyl methyltransferase during pre- and postnatal development as a function of age. The activity is expressed on a per gram of liver basis. Incubation was for 30 min. and approximately 10 mg protein was used in each assay. Other conditions were as described in the legends for Fig. 3 and 4. Each point represents single animals.

mal. Estrogen should have its major effect before birth since the fetus liver would be exposed to the highest concentrations at this time. However, the lowest activity of the methyl transferring pathway was observed in –6 day old animals (Fig. 5 and Fig. 6). In view of the fact that this pathway and the CDP choline pathway were at low levels of activity before birth, this may be a reflection of "immaturity" of the enzyme system in the liver at this stage of development, rather than a specific hormonal effect.

Phosphatidyl choline in adult mammals is known to be synthesized by two different pathways. Kennedy (23) has demonstrated a pathway by which cytidine diphosphocholine reacts with an α,β-diglyceride to form lecithin. The other system discovered by Bremer and Greenberg (24), which involves the transfer of methyl groups from adenosyl methionine to intact phospholipids, presumably begins with phosphatidyl ethanolamine as an acceptor to produce phosphatidyl choline. Phosphatidyl ethanolamine is presently known to be synthesized by one pathway (23), analogous to the interaction of cytidine diphosphocholine and the diglyceride where cytidine diphosphoethanolamine participates in the reaction rather than cytidine diphosphocholine. The fatty acid composition of phosphatidyl choline and phosphatidyl ethanolamine are different in the adult liver. Phosphatidyl ethanolamine contains larger amounts of the longer polyunsaturated fatty acids than does phosphatidyl choline. Weiss et al. (25) has hypothesized that this may be due to a preferential selectivity of the enzymes to the diglyceride substrates in both reactions.

Balint et al. (26) has recently shown in adult rats that ^{3}H-methyl-choline is preferentially incorporated into linoleoyl lecithin whereas methylation of phosphatidyl ethanolamine leads to formation of arachidonoyl lecithins. This observation has been further extended by Rytter et al. (27), who observed the specificity for the incorporation of 1,2-^{14}C-choline and 1,2-^{14}C-ethanolamine into four different microsomal lecithin fractions, separated in degree of fatty acid unsaturation. Choline was primarily incorporated into fraction 4 which contained the lecithin with two, or less than two, double bonds. Administration of ethanolamine produced a lecithin enriched in polyunsaturated fatty acids with the peak of incorporation of the isotope occurring in fraction 1 (containing 44.6% docosahexaenoic acid). The results in Fig. 5 and 6 show that for the CDP-choline dependent pathway to be more active during embryological development than methyl transferring pathways, the lecithin produced at this time should be low in concentration of unsaturated fatty acids. The increase in activity of this pathway may be the explanation for the increased amount of linoleate in the lecithin, or possibly, the availability of an increased amount of linoleoyl diglyceride may account for the increased activity of the pathway.

ACKNOWLEDGMENTS

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The Fatty Acid Composition of Some Entomophthoraceae. II. The Occurrence of Branched-Chain Fatty Acids in *Conidiobolus denaesporus* Drechsl.[1]

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ABSTRACT

Lipids extracted from *Conidiobolus denaesporus* Drechsl. were found to contain three branched-chain fatty acids, which together comprised about 35% of the total fatty acids of the fungus. The branched-chain acids were identified by gas-liquid chromatography, infrared and mass spectroscopy as 12-methyl tridecanoic, 12-methyl tetradecanoic, and 14-methyl pentadecanoic acids respectively.

The *n*-saturated acids comprise C12, C13, C14, C16, and C18. The *n* C16, *n* C18, and *n* C20 unsaturated acids were also found. The occurrence of 15.2% of myristic acid and of 8.9% of eicosatetraenoic acid provides a further distinctive feature of the lipids of *Conidiobolus denaesporus.*

INTRODUCTION

BRANCHED-CHAIN FATTY ACIDS are of fairly common occurrence in nature, being found principally in bacterial and animal lipids (1,2). Although studies on fungal lipids are still somewhat limited (3) however, branched-chain fatty acids have so far been detected in the lipids of only three fungi, where they were present at low levels, 1% or less of the total fatty acids (4,5). Apparently both the *iso* and *anteiso* series were represented. (Terms *iso* and *anteiso* denote a methyl group on the ω-1 and ω-2 C-atom respectively of the fatty acid.)

This paper describes the isolation and characterization of three branched-chain fatty acids from *Conidiobolus denaesporus*, which together comprise approximately 35% of the total fatty acids of this fungus.

MATERIALS AND METHODS

The organism used in this work was obtained from the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands. It was cultured at 23C on a rotary shaker in a glucose-peptone-yeast extract-potassium dihydrogen phosphate medium, as previously described (6). The mycelium was harvested after two to four days of incubation and was washed three times with distilled water. The purity of the culture was checked regularly throughout the work by microscopic examination and by subculturing on solid media.

The techniques used for lipid extraction and purification have previously been described (6). Lipid samples were stored in chloroform solution at –20C until used.

Fatty Acid Analysis

Lipid samples were transesterified by the method of Morgan et al. (7) or by the official AOCS method (8). The two methods gave similar results.

Methyl esters were analyzed by gas-liquid chromatography (GLC) on a 5-ft × ¼-in. O.D. stainless steel column, packed with 15% diethylene glycol succinate (DEGS), on a 100/110 mesh Anakrom AB support in an Aerograph 204, equipped with a flame ionization detector. The esters were provisionally identified by comparing their retention times with those of standards or by comparing logarithmic plots of retention ratios.

Semipreparative GLC was carried out with the same column, or with a 10-ft × ¼-in. stainless steel column, packed with 15% Apiezon L on the same support. A stream splitter (ratio 10:1) was installed between the column exit and the detector. Samples were collected manually by inserting a 180-mm × 2-mm I.D. Pyrex tube, fitted with a neoprene gasket in the collection port of the stream splitter during elution of the compound, as monitored by the detector. The carrier gas was nitrogen, and the instrument was operated at 155-200C with the DEGS column and at 235-240C with the Apiezon L column.

Quantitative estimates of the various esters were obtained from the areas under the peaks, which were measured with the aid of a Disc integrator. Quantitative results with National Heart Institute Fatty Acid Standard D agreed with the standard composition data with a relative error of less than 5% for major components (> 10% of the total mixture) and less

[1] Contribution No. 94.

TABLE I
The Fatty Acid Composition of *Conidiobolus denaesporus*

Acid	Percentage by Weight	
	Before hydrogenation	After hydrogenation
12:0	1.1	1.0
13:0	0.6	0.7
U1	19.7	19.4
14:0	15.2	14.9
U2[a]	13.4	12.6
U3	1.8	1.8
16:0	10.4	15.2
16:1	5.4	—
18.0	2.5	22.4
18.1	15.2	—
18:2	1.8	—
18:3	2.1	—
20:0	—	12.0
20:1	1.0	—
20:2	0.4	—
20:3	0.5	—
20:4	8.9	—

[a] Includes 1.5% pentadecanoic acid.

than 10% for minor components (<10% of the total mixture).

Prior to preparative GLC the unsaturated fatty acids were eliminated from the mixed esters by column chromatography on silver nitrate-impregnated Florisil (9). Hexane containing 0.1% diethyl ether eluted the saturated esters, together with the unknowns, while the unsaturated esters remained on the column.

Hydrogenation of the methyl esters was carried out over a platinum oxide catalyst by the method of Farquhar et al. (10).

Mass spectra of the isolated methyl esters were obtained on a Hitachi Perkin-Elmer RMU-6D single focusing instrument using a liquid sample inlet system at 135-150C and a pressure in the analyzer of about 1×10^{-6} torr.

Infrared spectra were obtained with a Perkin-Elmer Model 337 spectrophotometer. Lipid samples were prepared either as potassium bromide discs or as thin films on potassium bromide plates. The isolated methyl esters were converted to the free acid form by saponification with methanolic potassium hydroxide before infrared spectroscopy.

RESULTS

Gas-Liquid Chromatography

GLC of the fatty acid methyl esters from *C. denaesporus* gave the composition shown in Table I; U1, U2, and U3 could not be identified by the usual procedures. They had carbon numbers of 13.55, 14.75, and 15.55 respectively on DEGS, and these values were not altered by GLC on Apiezon L or by hydrogenation of the sample prior to GLC. These values are in close agreement with those reported by Kaneda (11) for *iso* tetradecanoic, *anteiso* pentadecanoic, and *iso* hexadecanoic acids respectively. Therefore U1, U2, and U3 may be provisionally assigned these identities.

Hydrogenation did not affect the proportion of these acids in the mixture (Table I), and further confirmation of the fully saturated nature of these acids was obtained when they were eluted from a silver nitrate-impregnated Florisil column with hexane containing 0.1% diethyl ether.

Mass Spectroscopy

Mass spectra of U1, U2, and U3 are shown in Fig. 1-3 respectively. These indicate a molecular weight of 242 for U1, 256 for U2, and 270 for U3. All spectra have a base peak of m/e = 74, characteristic of saturated fatty acid methyl esters, and a peak owing to the acylium ion at m/e = M-31.

Although *iso* fatty acid esters give mass spectra similar to those of normal straight-chain esters, the small peak at m/e = M-65 in the spectrum of U1 and U3 is characteristic of the *iso* configuration (12). Thus the identity of U1 is indicated as the methyl ester of *iso* tetradecanoic acid, and U3 as the methyl ester of *iso* hexadecanoic acid.

Similarly U2 may be identified as the methyl ester of *anteiso* pentadecanoic acid since the peak at m/e = M-29 is higher than the peak at m/e = M-31, characteristic of branched-chain esters with the *anteiso* structure (12).

Known samples of these acids were not available for comparison, but mass spectra of methyl esters of analogous higher-molecular-weight *iso* and *anteiso* branched-chain acids, together with the straight-chain acids tetradecanoic and pentadecanoic, were used for comparison and assessment of the mass spectra.

Infrared Spectroscopy

Further confirmation of the structure of U1, U2, and U3 was obtained from their infrared spectra, where the terminal carbon atoms show characteristic absorption in the region 1380 to 1360 cm^{-1}.

The spectra of both U1 and U3 show a doublet, with absorption bands of nearly equal intensity in this region, which is directly attributable to the *iso*propyl $[(CH_3)_2 CH–]$ configuration of the terminal carbon atoms (13). Although *neo* acids also show a doublet in this region owing to their *t*-butyl $[(CH_3)_3 C–]$ end-group, the peak at 1360 cm^{-1} is of much great-

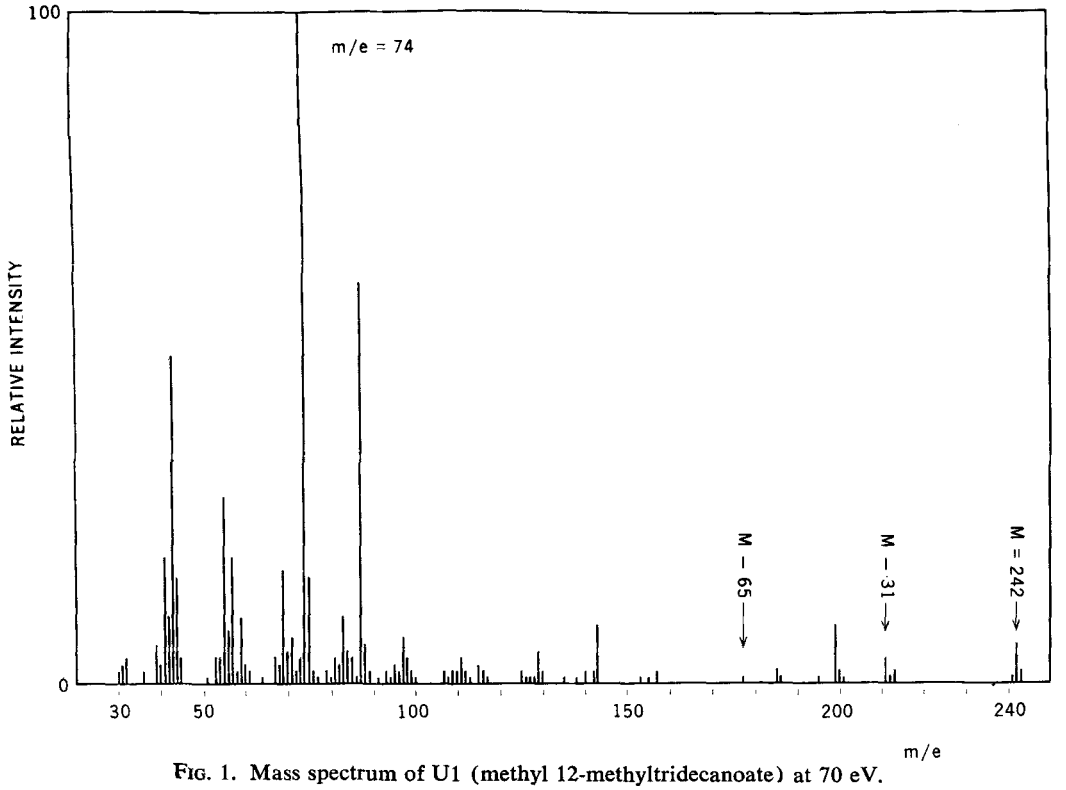

FIG. 1. Mass spectrum of U1 (methyl 12-methyltridecanoate) at 70 eV.

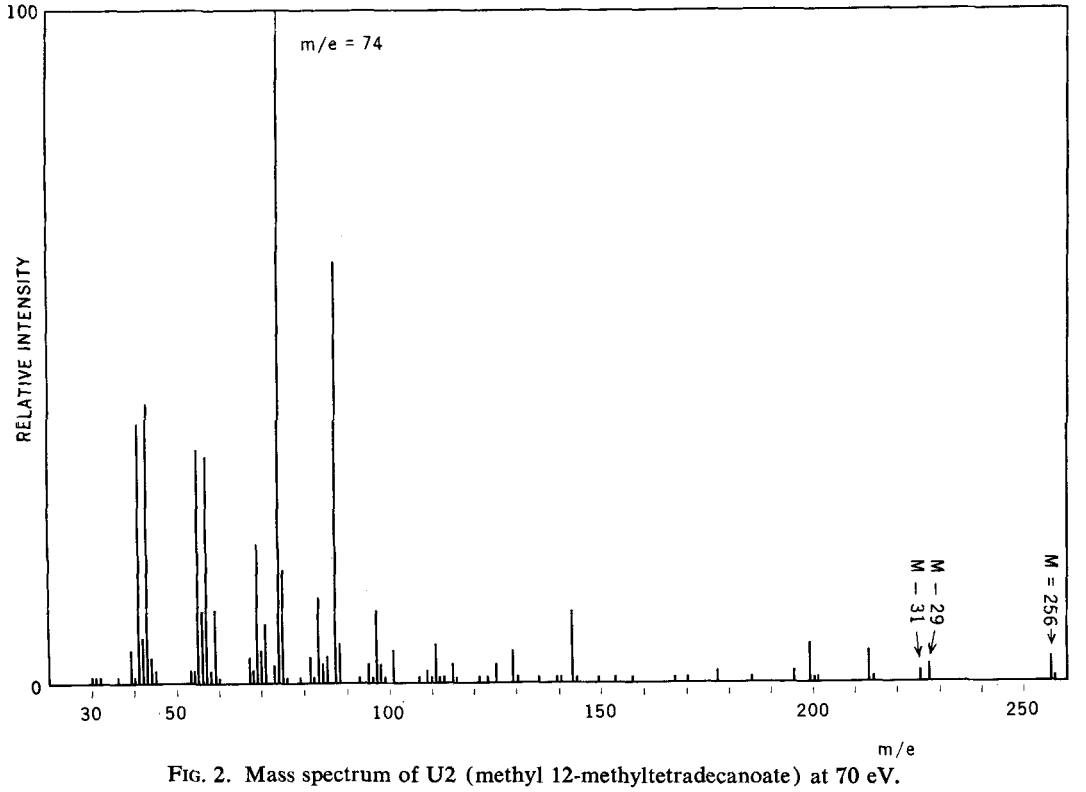

FIG. 2. Mass spectrum of U2 (methyl 12-methyltetradecanoate) at 70 eV.

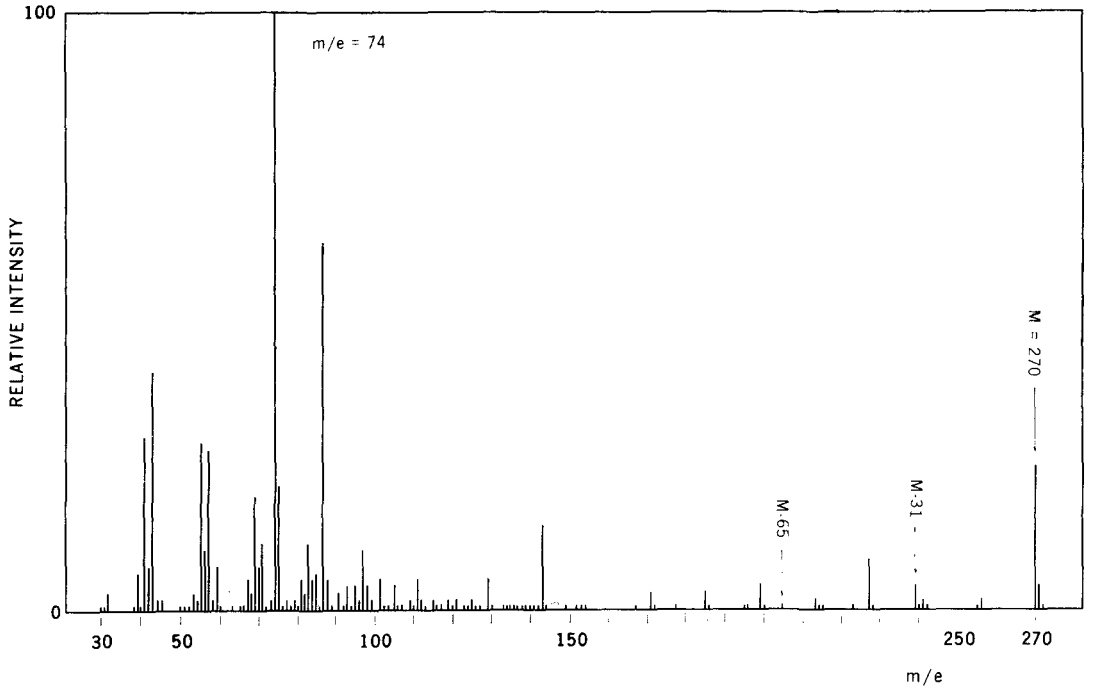

FIG. 3. Mass spectrum of U3 (methyl 14-methylhexadecanoate) at 70 eV.

er intensity than that at 1380 cm^{-1}. The intensity of the progression bands in the region 1350 to 1180 cm^{-1}, attributable to the methylene [$-CH_2-$] groups of the fatty acid, increases towards lower frequencies for U1 and U3, in agreement with the findings of Kaneda (11) for these acids.

The spectrum of U2 shows a single absorption peak at 1380 cm^{-1} characteristic of the *s*-butyl end-group [$CH_3\,CH_2\,CH(CH_3)-$] of the *anteiso* configuration. Straight-chain fatty acids also give a peak at this frequency because of their methyl end-group but of lower intensity than that of the *anteiso* acids (13).

DISCUSSION

Previous reports of branched-chain fatty acids in fungal lipids are confined to the observation of small amounts (1% or less) of these acids in the three imperfect fungi, *Pithomyces chartarum*, *Cylindrocarpon radicicola*, and *Stemphylium dendriticum*, on the basis of GLC data (4, 5). The *P. chartarum* mycelium contained the branched-chain acids, undecanoic to heptadecanoic inclusive, of apparently both the *iso* and *anteiso* configuration (4); *C. radicicola*, a branched-chain heptadecanoic acid; and *S. dendriticum*, a branched-chain tridecanoic acid (5). Shaw (14) however did not detect any branched-chain fatty acids in the lipid of *C. radicicola*.

Branched-chain fatty acids have now been shown to comprise approximately 35% of the total fatty acids of *Conidiobolus denaesporus*, a phycomycete of the family Entomophthoraceae.

Although biological systems are known to be capable of synthesizing *iso* branched-chain acids containing both an even and an odd number of carbon atoms, *C. denaesporus* lipid contains only those with an even number of carbon atoms. This is similar to the situation found in human (15) and sheep lipids (16) but differs from that found in bacteria, where both even and odd carbon number *iso* acids occur, with the odd carbon number acids usually predominating (11,17). The *anteiso* acid from *C. denaesporus* has an odd number of carbon atoms, consistent with the observation that naturally occurring *anteiso* acids have an odd number of carbon atoms.

About 65% of the total fatty acids of *C. denaesporus* are saturated (Table I). This finding is in sharp contrast to the fatty acid compositions of *C. osmoides* and *C. thromboides*, which each contain about 30% saturated acids (6). Apart from the branched-chain fatty acids, the main difference in fatty acid compositions between *C. denaesporus* and

the other two *Conidiobolus* species is the much lower content of the monounsaturated acids oleic and palmitoleic in *C. denaesporus*. The *C. denaesporus* lipid also contains γ-linolenic, eicosatrienoic, and eicosatetraenoic acids, also a relatively high level of myristic acid, which, although uncommon in most fungi, are present in the other two *Conidiobolus* species and some closely related *Entomophthora* species (6). The polyunsaturated acids have previously been demonstrated in the lipid of several other phycomycetes (14, 18, 19).

Preliminary experiments have shown that the branched-chain acids are located mainly in the neutral lipid fraction and thus appear to substitute for the monounsaturated acids in this fraction.

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SHORT COMMUNICATIONS

Fatty Acid Composition of Polar Lipids of Cotton Buds

A PREVIOUS STUDY (1) of the fatty acids derived from the lipid extract of the bud (square) of the cotton plant (Gossypium sp.) disclosed the presence of 17 acids, with 74% of these appearing in the neutral lipids. Iskhakov and Vereshchagin (2) found 15 different fatty acids in the total lipids from roots, stems, leaves, bolls, and flowers of the cotton plant. This report covers a more detailed fatty acid characterization of the polar lipids from the cotton bud.

Lipids were extracted by the method of Folch et al. (3) from freshly picked Deltapine Smoothleaf cotton buds (squares) containing the sepal, calyx and petal. After they were washed with 0.88% (w/v) KCl, the extracts were placed in the freezer at –20C for 24 hr. Ice was removed by filtration through a cotton plug under nitrogen.

The total lipid sample (3.2 g) was applied to a silicic acid column (50 g, 2.5 × 14 cm) in chloroform and the triglycerides, sterol esters, fatty acids and nonpolar pigments eluted with this solvent. The polar lipids were eluted from the column with absolute methanol, reduced to dryness, and then dissolved in chloroform-methanol (9:1) for chromatography on DEAE-cellulose. DEAE-cellulose (capacity 0.87 meg/g) was converted to acetate form prior to use (4), and a 5.5 (I.D.) × 20 cm column prepared. A load of 590 mg of polar lipids was added to the top of the column and elution begun. The elution solvents were those of Rouser et al. (4).

Final component separation was performed by thin-layer chromatography (TLC) on standard 20 × 20 cm plates, coated with 250-micron layers of silica gel-calcium sulfate (9:1, w/w). The solvent systems used in TLC were those of Rouser et al. (5) as used by Lepage (6) for plant lipids.

Methyl esters were prepared by the modified procedure of Mason et al. (7). The samples of lipid or free fatty acid were dissolved in a 10 fold (w/v) excess of absolute methanol containing 1% sulfuric acid and 1%, 2,2-dimethoxypropane, sealed in a culture tube and heated at 65C for 3 hr. The reaction mixture was extracted with pentane, washed with water, dried over anhydrous sodium sulfate, and made to volume. Qualitative and quantitative determinations of the methyl esters were made by GLC equipped with flame ionization detector on a 10-ft, ⅛-in O.D. column packed with 10% DEGS on 60/80 mesh HMDS-treated chromosorb-w. A nonpolar column of SE-52 of the same dimensions was used to confirm the fatty acid analysis. The injector, column, and detector temperatures were 210C, 185C, and 185C respectively. The instrument was calibrated with authentic standard mixtures of fatty acid methyl esters.

Lyophilized venom of the Eastern Diamondback Rattlesnake, *Cortalus adamanius,* was made up to a concentration of 2.0 μg of venom per microliter of buffer, as outlined by Menzel et al. (8). The lecithins, purified by TLC, were dissolved in peroxide-free ether to give a final concentration of 30 micromoles per milliliter of phosphorus. To each ml of ethereal solution, 10 ml of enzyme solution was added. The reaction mixture was shaken for 2 hr at 37C, the ether evaporated under N_2, and vacuum dried at room temperature. The residue was taken up in 2 ml of 1% methanol in chloroform, the solution placed on a silicic acid column (5 g, 1.5 × 12 cm), and the fatty acids eluted by adding 50 ml of the same solvent. The unreacted lecithins were eluted with 25% methanol in chloroform (50 ml) and the lysolecithins with 85% methanol in chloroform (100 ml).

Nitrogen was determined by the micro-Kjeldahl procedure with cupric sulfate. After digestion was complete the solution was made basic with 40% NaOH and the ammonia swept into 0.02 N sulfuric acid for 45 min with nitrogen. Nessler reagent (5 ml) was added, the solution made to 20 ml and color intensity determined at 480 mμ.

TABLE I

The Fatty Acid Composition of Cotton Bud Polar Lipids.

Lipid[a]	Distribution of lipid %[b]	Distribution of fatty acids % 16:0	18:0	18:1	18:2	18:3
PC	30.4	20.4	5.2	10.7	30.4	33.3
DGD	20.5	18.8	....	4.7	7.9	68.6
SG	3.8	29.2	....	18.7	....	52.1
MGD	24.2	6.6	....	2.3	5.5	85.6
PE	2.6	26.1	....	....	47.4	26.5
PI	11.5	37.3	6.3	9.0	8.5	38.9

[a] PC—phosphatidyl choline; DGD—Digalactosyl Diglyceride; SG—Sterol Glycoside; MGD—Monogalactosyl Diglyceride; PE—Phosphatidyl Ethanolamine; PI—Phosphatidyl Inositol.

[b] Percentages based on ferric hydroxamate complex.

TABLE II

The Composition (Molar Percentages) of Fatty Acids Occupying the α and β Position of the Lecithins from Cotton Buds[a]

Position	Fatty acids and standard deviations Molar percentage			
	16:0	18:1	18:2	18:3
α	23.7 ± 0.07	19.3 ± 0.11	32.7 ± 0.22	24.5 ± 0.07
β	4.0 ± 0.10	20.3 ± 0.21	38.8 ± 0.29	38.8 ± 0.33

[a] Composition was calculated by comparison of peak heights on gas chromatograms. Each result was based on at least 5 determinations. The mean values along with standard errors are given.

Phosphorus was determined by the procedure of Chen et al. (9) as modified by Mitlin (10), amino nitrogen by the method of Lea and Rhodes (11), sugar by the method of Dubois et al. (12), and fatty acid ester groups by the method of Rapport and Alonzo (13).

A recovery of 93% of the polar lipids from DEAE-cellulose and thin layer chromatography as measured by the ferric hydroxamate complex (Table I). The percentage of individual fatty acids in Table I and molar percentage in Table II were determined from GLC data. The glycolipids and phospholipids contain mostly unsaturated fatty acids, with linolenic acid (18:3) predominating. Linoleic acid (18:2) is the major fatty acid in phosphatidyl ethanolamine. Palmitic acid (16:0) was higher in concentration in phosphatidyl inositol than in the other components.

Findings regarding the positional specificity of venom phospholipase A (14, 15), showed that the α position of egg yolk lecithins is occupied predominately by saturated fatty acids. In cotton bud lecithins both the α position (65%) and β position (96%) contained predominately unsaturated fatty acids. The nitrogen to phosphorus ratio of the lecithins was 1.01. Quantitative recovery of phosphorous and nitrogen was made in the lysolecithins.

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[Received Feb. 2, 1967]

Selection and Evaluation of Gas Chromatographic Stationary Phases for Qualitative Separation of Components of Lipid Mixtures

THE CONSTANT DEVELOPMENT of new column materials has been a major factor in the advancement of gas chromatography as an analytical tool. At the same time, the prolification of new columns has made it difficult for the experienced chromatographer to make a proper choice; for a novice the selection can be overwhelming. Very often one may prepare a column for a specific separation to find out later that an existing column may have done the job, possibly not as well, but at least satisfactorily. Much time is lost in trying columns which have no chance in succeeding and in trying columns which are practically duplicates of columns previously examined.

Rohrschneider (1, 2) has developed a system for classifying columns according to polarity. This system is used here to illustrate the effect of change in polarity of several new silicone stationary phases on separations of fatty acid esters, estrogens and 17-ketosteroids.

The new stationary phases are a series of silicones containing from 0-65% phenyl groups based on substitution of methyl groups in a

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The new stationary phases are a series of silicones containing from 0-65% phenyl groups based on substitution of methyl groups in a

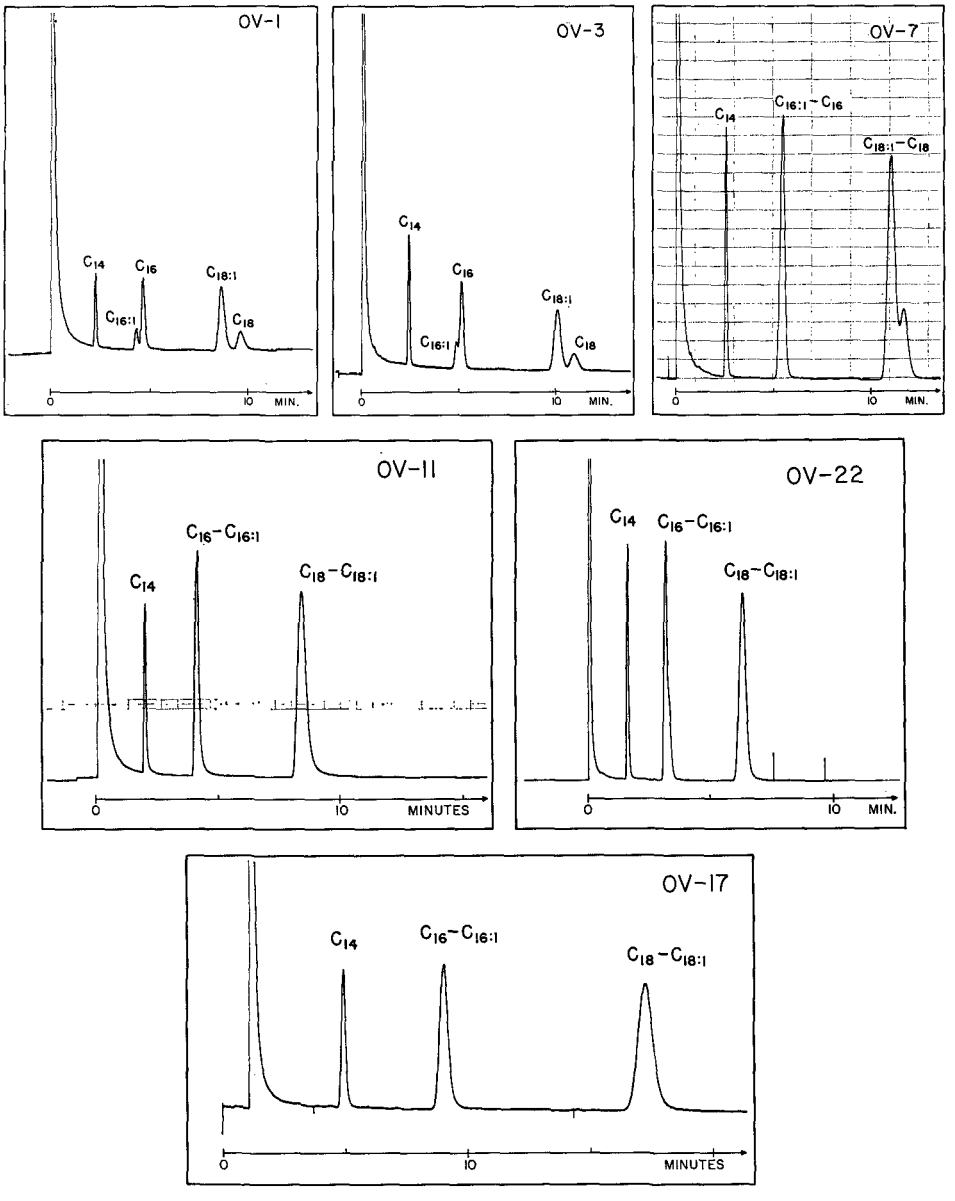

FIG. 1. Fatty acid methyl esters.

dimethylpolysiloxane polymer. These are described in Table I. These phases were characterized by determination of the Rohrschneider constants (1) using 10 ft × ⅛ in. O.D. aluminum columns packed with 20% of the individual silicones plus 0.1% Alkaterge T on 60/80 Acid Washed Chromosorb W. A Varian Model 204B Chromatograph was used. Columns were operated at 100C with a flow rate of 27 ml/min.

Analyses of fatty acid esters, estrogens and 17-ketosteroids were performed in a Barber-Colman Model 5000 Chromatograph. The columns were glass U-tubes, 6 ft × 4 mm I.D. packed with 3% of the individual silicones on 80/100 Chromosorb W HP. Analyses were performed at 200C for the fatty acid esters, and 250C for estrogens and 17-ketosteroids. Flow rates were approximately 60 ml/min.

The 6 new silicone stationary phases are of particular interest because of their exceptional thermal stability. With the advent of coupled

TABLE I
Rohrschneider Constants

Phases % Phenyl	Silicone	X	Y	Z	U	S
OV-1 / OV-101 (liquid)	0	0.16	0.20	0.50	0.85	0.48
OV-3	10	0.42	0.81	0.85	1.52	0.89
OV-7	20	0.70	1.12	1.19	1.98	1.34
OV-11	35	1.13	1.57	1.69	2.60	1.95
OV-17	50	1.30	1.66	1.79	2.83	2.47
OV-22	65	1.58	1.80	2.04	3.27	2.59
QF-1	....	1.09	1.86	3.00	3.94	2.41
XE-60	....	2.08	3.85	3.62	5.13	3.45
DEGS	....	4.93	7.58	6.14	9.50	8.37

gas chromatography-mass spectrometer detection systems the need for phases with low bleed rate has been emphasized. The purpose of this work was to determine the separating characteristics of these phases, particularly as applied to lipids. In addition, it was desirable to present these data in such a form as to permit comparison of these new stationary phases with materials already in use. The latter step was made possible by the use of the Rohrschneider (1) method for classification. According to this method, the polarity of a stationary phase is dependent upon the type of sample being analyzed. Columns are characterized by determining the Kovats (2) indices, I_p, for benzene, ethanol, methyl ethyl ketone nitromethane and pyridine. The indices for these compounds are then compared to indices, I_u for the same compounds determined on a nonpolar column (squalene). The Rohrschneider constants, X, Y, Z, U and S, are equal to the difference between the Kovats Indices found on the polar column and those found on the nonpolar column, and divided by 100:

$X = (I_p\text{-}I_u)/100$ for benzene. This method of characterizing columns and predicting column performance is treated thoroughly in the references cited.

In Table I are the Rohrschneider constants for the silicone phases. Also included are constants for several other commonly used phases. To illustrate the utility of the Table in selection of stationary phases for specific applications, a study was made of fatty acid methyl esters: myristate, palmitate, palmitoleate, stearate and oleate. A nonpolar column would be expected to elute the unsaturates before the saturates while for a highly polar column the reverse would be true. Fig. 1 shows the shift of the unsaturates relative to saturates.

Note that, even for OV-22 the most polar of the silicones, complete separations are not obtained as they might be on a polyester column such as DEGS. This is to be expected since the constants for OV-22 are considerably smaller than those for DEGS; the X value on OV-22 is only 1.58 whereas for DEGS the X value is 4.93. In fact, the moderately polar neopentyl glycol succinate polyester has an X value of 2.68 and is known to give only a very slight indication of elution of unsaturates after the saturates. Therefore, for separation of the fatty acid methyl esters it will be necessary to select a column with a much higher value for X.

The TMS derivatives for estrone, estradiol and estriol were analyzed on the same 6 columns. Estrone and estradiol differ in that the former contains a keto group in the 17 position, while the latter has a hydroxyl at the same point; both have hydroxyl groups in the 3 position. As the phenyl content of the stationary phases is increased, the separation between estrone and estradiol decreases. Note from Table I that the increase in the Z term is greater than the X or Y term and consequently the keto group is more affected by the increase in phenyl content. A commonly used column for this separation is the QF-1 silicone. Note in Table I that the Z value is considerably higher than X or Y. This illustrates the point that the polarity of a column is dependent on sample type and that the fluorosilicone QF-1 is highly polar, or selective for ketones. These separations are shown in Fig. 2.

The six phases were also used to separate the trimethylsilyl derivatives of a mixture of steroids: androsterone (AND), etiocholanolone (ETIO), dehydroepiandrosterone (DHEA), 11-ketoetiocholanolone (11-keto) and 11-β-hydroxyetiocholanolone (11-βOH). As the phenyl content of silicone increases the androsterone and etiocholanolone separation improves. The major change however is with the 11-ketoetiocholanolone and 11-β-hydroxyetiocholanolone. The difference between these last two components is in the 11 position. When silyl derivatives of both compounds are prepared, the keto group will remain unchanged, while the OH group will be converted to a TMS ether. On a nonpolar column the latter compound would be eluted later since it is higher boiling. As polarity of a column increases, the 11-keto should move toward the 11-BOH. Fig. 3 shows this to be the case. In fact, with OV-22 the 11-keto moves beyond the 11-βOH which has moved closer to the DHEA. This column also gives the best separation between androsterone and etiocholanolone.

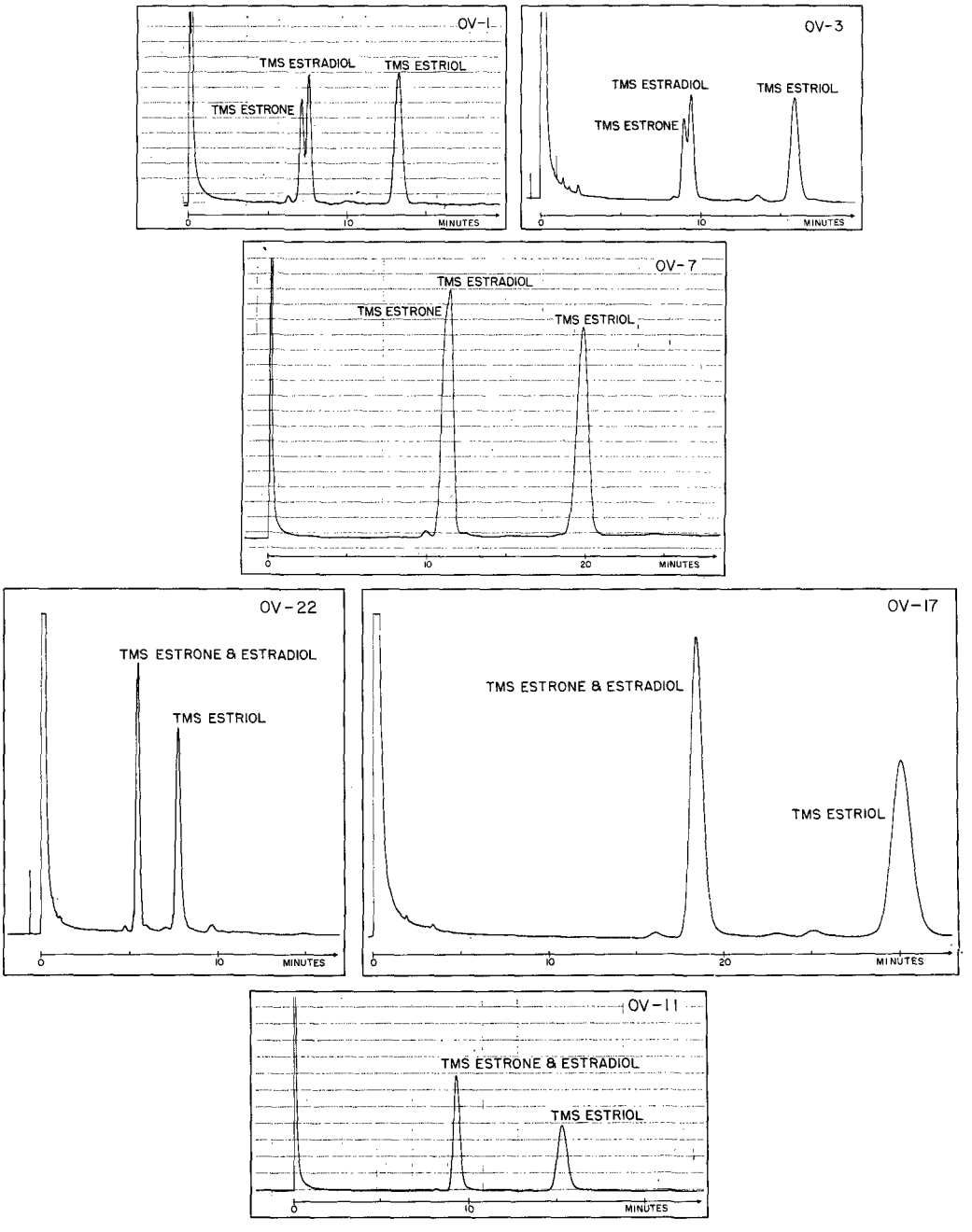

FIG. 2. Estrogens.

If the trimethyl silyl ethers are prepared using bis N, O trimethylsilylacetamide (BSA), the 11-βOH position is not affected. A similar series of chromatograms were obtained and the 11-BOH was found to elute at a point beyond that of the completely derivatized 11-βOH. The former contained one TMS ether and a free hydroxyl at the eleven position, while the latter contained two TMS ether groups. When the TMS derivatives are prepared with BSA, complete separation of DHEA, 11-βOH and 11-keto is obtained with all of the phenyl sub-

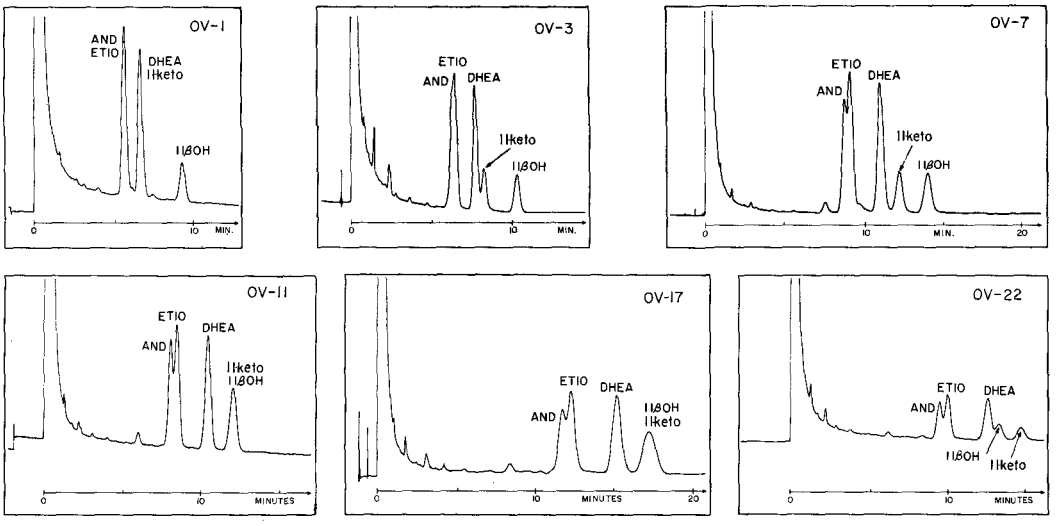

FIG. 3. 17-Ketosteroids.

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The new series of phenyl silicones provides a means of having a wide variety of thermally stable columns all of similar chemical nature.

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UDP-Galactose: N-Acetylgalactosaminyl-(N-Acetylneuraminyl) Galactosyl-Glucosyl-Ceramide Transferase Activity in Adult Frog Brain

THE UDPgal:galNAc-(neuNAc)gal-glu-cer galactosyl transferase which catalizes the following reaction galNAc-(neu NAc) gal-glucer + UDP gal ⟶ gal-galNAc-(neuNAc)gal-glu-cer + UDP, has previously been found only in the brains of embryonic or very young animals (1,2). This report demonstrates that a similar activity is present in adult frog brain.

Brains from mature *Rana pipiens* (J. M. Hazen and Co., Alberg, Vt.) were homogenized in 0.25 M sucrose – 0.1% mercaptoethanol and added to the reaction mixture (Table I). The reaction was stopped after 4 hr by the addition of chloroform-methanol (2:1). After the radioactivity remaining at the origin in the borate chromatography system was determined, the gangliosides were extracted with chloroform-methanol (2:1) and spotted on Silica Gel G thin-layer plates. The gangliosides were separated on the plates with chloroform-methanol-water (61:32:7) (3). The radioactivity of separated galNAc-(neuNAc) gal-glu-cer, gal-galNAc-(neuNAc)gal-glu-cer and the combined polyneuraminylgangliosides was determined (Table II).

From Table I it can be seen that addition of galNAc-(neuNAc)gal-glu-cer increases incorporation over six-fold in the frog brain

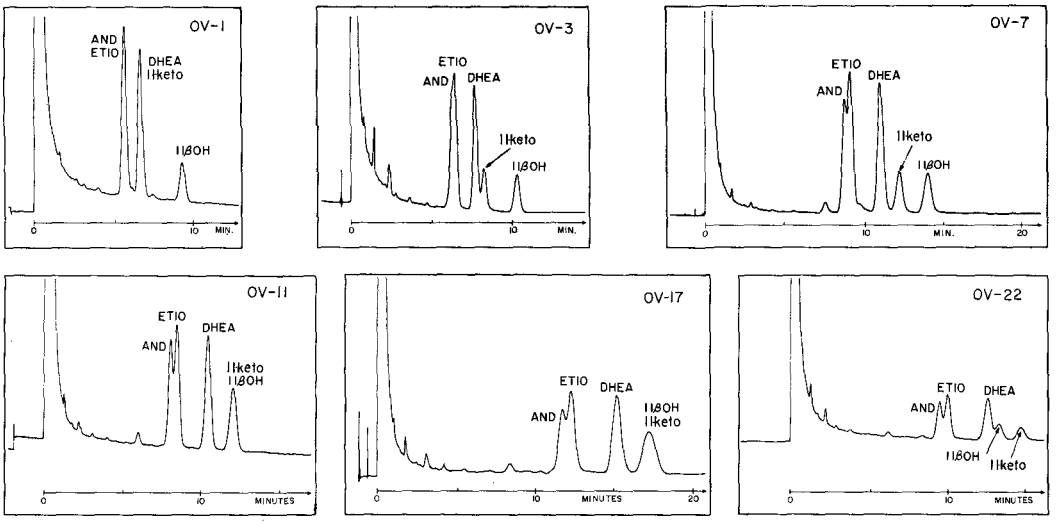

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TABLE I[a]

UDP-gal: galNAc-(neuNAc)-gal-glu-cer Galactosyl Transferase Activities in the Brain of Frog and Rat

Incubation mixtures (1,2) contained the following components in a total volume of 0.1 ml: Tween 80. 100 μg; CF-54, 200 μg; acceptor, 100 mμmoles; UDP-($U^{14}C$) gal (International Chemical and Nuclear Corp.), 50 mμmoles containing 129,000 c.p.m.; particulate enzymes preparation from frog brains or rat brains, 0.7 mg of protein. After 4 hr at 25C, the mixtures were assayed by ascending paper chromatography in 1% $Na2B_4O_7$ for 6-7 hr. The substrate and its degradation products migrated near the solvent front while the product remained at the origin. The origin plus one inch was counted in the toluene liquid scintillation system.[b]

	Acceptor	
	None	GalNAc-(neuNAc)gal-glu-cer
Adult frog	473 (2)	2980 (2)
Adult rat	714	724

[a] Values of this table given in c.p.m.

[b] PPO and POPOP (Nuclear Chicago Corp.)

TABLE II[a]

Product Analysis of Frog Brain UDPgal: galNAc-(neuNAc)gal-glu-cer Galactosyl Transferase

After having been counted in the toluene scintillation system, the chromatography papers from Table I, were removed and washed several times with toluene, allowed to dry and extracted with chloroform-methanol (2:1). No attempt was made to quantitatively extract the gangliosides. The extracts were taken to dryness and spotted on a thin layer Silica Gel G plate in a total of 30μl and the gangliosides separated with chloroform-methanol-water (61:32:7). The spots corresponding to galNAc-(neuNAc)gal-glu-cer, gal-galNAc-(neuNAc) gal-glu cer and the combined polyneuraminyl gangliosides scraped off the plates and counted in the toluene liquid scintillation system.[b] These values represent incorporation with galNAc-(neuNAc)gal-glu-cer, as an acceptor. Radioactivity from endogenous acceptor is not readily soluble in chloroform-methanol (2:1).

Product	Gangliosides	
GalNAc-(neuNAc)gal-glu-cer	24	39
Gal-galNAc-(neuNAc)gal-glu	985	832
Combined polyneuraminyl gangliosides	157	135

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homogenate whereas it has no effect on incorporation in the rat brain homogenate. Table II illustrates that 84% of the product recovered was chromatographically indistinguishable from the desired gal-galNAc-(neuNAc)gal-glu-cer product.

The result reported here lead us to conclude that during evolutionary development, mammals have acquired the ability to shut off the UDP-gal: galNAc-(neuNAc) gal-glu-cer galactosyl transferase activity while frogs have not acquired this mechanism.

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Determination of Double Bond Position in Mono-Unsaturated Fatty Acids Using Combination Gas Chromatography Mass Spectrometry

THE APPLICATION OF mass spectrometry to the determination of double bond position has received limited use in the past since isomeric unsaturated compounds generally give very similar spectra. Recently, McCloskey and McClelland (1) have applied combined gas liquid chromatography and mass spectrometry (GLC-MS) to the O-isopropylidine derivatives of the dihydroxy fatty acid esters which undergo characteristic cleavage to indicate the double bond position in the original unsaturated ester. Niehaus and Ryhage (2) have reported the determination of double bond position in fatty acids containing from 1-4 double bonds using GLC-MS of polymethoxy fatty acid esters.

A facile method for the location of double bonds in monounsaturated fatty acids has been developed, and the results obtained from several monoenoic acids are presented in this study. Hydroxy acid derivatives of unsaturated fatty acid methyl esters were prepared by oxidation with OsO_4 using a modification of the method reported by McCloskey and McClelland (1). The corresponding trimethylsilyl

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TABLE II[a]

Product Analysis of Frog Brain UDPgal: galNAc-(neuNAc)gal-glu-cer Galactosyl Transferase

After having been counted in the toluene scintillation system, the chromatography papers from Table I, were removed and washed several times with toluene, allowed to dry and extracted with chloroform-methanol (2:1). No attempt was made to quantitatively extract the gangliosides. The extracts were taken to dryness and spotted on a thin layer Silica Gel G plate in a total of 30μl and the gangliosides separated with chloroform-methanol-water (61:32:7). The spots corresponding to galNAc-(neuNAc)gal-glu-cer, gal-galNAc-(neuNAc) gal-glu cer and the combined polyneuraminyl gangliosides scraped off the plates and counted in the toluene liquid scintillation system.[b] These values represent incorporation with galNAc-(neuNAc)gal-glu-cer, as an acceptor. Radioactivity from endogenous acceptor is not readily soluble in chloroform-methanol (2:1).

Product	Gangliosides	
GalNAc-(neuNAc)gal-glu-cer	24	39
Gal-galNAc-(neuNAc)gal-glu	985	832
Combined polyneuraminyl gangliosides	157	135

[a] Values of this table are given in cpm.

[b] PPO and POPOP (Nuclear Chicago Corp.)

homogenate whereas it has no effect on incorporation in the rat brain homogenate. Table II illustrates that 84% of the product recovered was chromatographically indistinguishable from the desired gal-galNAc-(neuNAc)gal-glu-cer product.

The result reported here lead us to conclude that during evolutionary development, mammals have acquired the ability to shut off the UDP-gal: galNAc-(neuNAc) gal-glu-cer galactosyl transferase activity while frogs have not acquired this mechanism.

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S. Basu and S. Roseman provided helpful suggestions and gifts of galNAc-(neuNAc) gal-glu-cer and gal-galNAc-(neuNAc)gal-glu-cer. This investigation was supported in part by Public Health Service Research Grant No. NB-05104 from the National Institute of Neurological Diseases and Blindness.

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Determination of Double Bond Position in Mono-Unsaturated Fatty Acids Using Combination Gas Chromatography Mass Spectrometry

THE APPLICATION OF mass spectrometry to the determination of double bond position has received limited use in the past since isomeric unsaturated compounds generally give very similar spectra. Recently, McCloskey and McClelland (1) have applied combined gas liquid chromatography and mass spectrometry (GLC-MS) to the O-isopropylidine derivatives of the dihydroxy fatty acid esters which undergo characteristic cleavage to indicate the double bond position in the original unsaturated ester. Niehaus and Ryhage (2) have reported the determination of double bond position in fatty acids containing from 1-4 double bonds using GLC-MS of polymethoxy fatty acid esters.

A facile method for the location of double bonds in monounsaturated fatty acids has been developed, and the results obtained from several monoenoic acids are presented in this study. Hydroxy acid derivatives of unsaturated fatty acid methyl esters were prepared by oxidation with OsO_4 using a modification of the method reported by McCloskey and McClelland (1). The corresponding trimethylsilyl

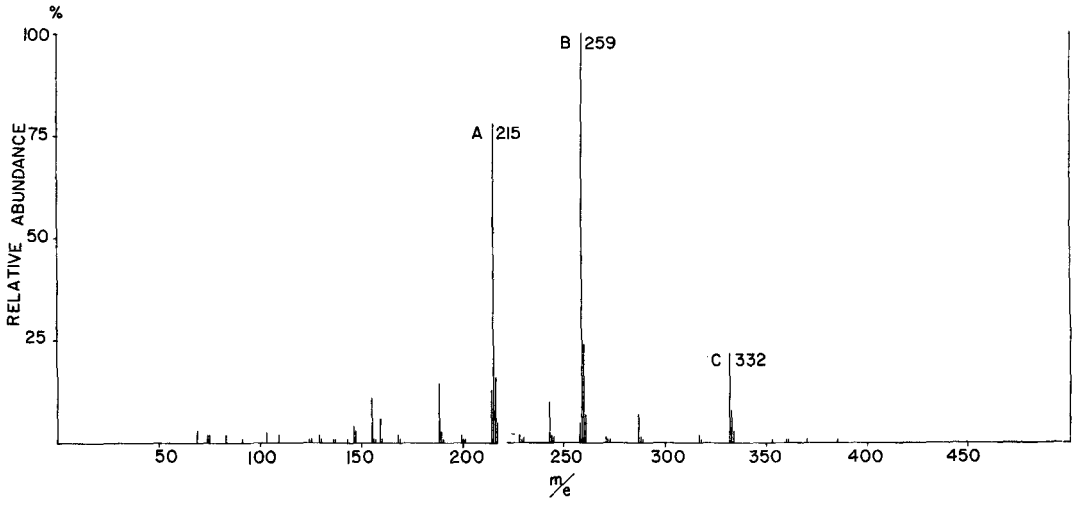


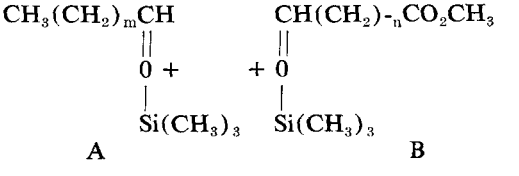
FIG. 1. Mass spectrum (20 ev) of the trimethylsilyl ether derivative of hydroxylated methyl elaidate.

ether derivatives were prepared and subsequently analyzed by combined GLC-MS. High purity methyl esters were either prepared in our laboratory or obtained commercially (Supelco Inc.). Samples of eicos-5-enoic acid and methyl 12- and 15-octadecenoates were furnished through the courtesy of Dr. H. J. Dutton, NRRL, USDA.

Methyl esters of unsaturated acids (< 1 mg) were oxidized using OsO_4 (1) and the reaction mixture was directly extracted with 5 ml of $CHCl_3$ after the addition of 2 ml of water. The $CHCl_3$ extract was dried over Na_2SO_4 and the solvent removed with a stream of nitrogen. To the hydroxy ester thus obtained was added 0.5 ml of bis trimethylsilyl acetamide (BSA) (or 0.5 ml hexamethyldisilazine + a drop of trimethylsilyl chloride), and the mixture was heated under reflux for 1 hr. The material was evaporated *in vacuo* with slight warming and the resultant trimethylsilyl ether derivative was dissolved in a small amount of isooctane (50 μl) prior to analysis by GLC-MS.

Mass spectra were determined with a Perkin Elmer Hitachi RMU6E single focusing mass spectrometer equipped with a gas chromatographic inlet system. The helium separator was maintained at 290C as was the ion source and the heated transfer line from the GLC. The ionization potential was 20 e.v. and ionizing current was 55 μa. Spectra were recorded in 4-10 sec to m/e 600 at the apex of the GLC peak (1-50 μg of material) as determined by the continuous record produced by the total ionization monitor. The gas chromatograph employed a s.s. column 2 ft x 1/8 in. packed with 5% SE-30 coated on 60-80 mesh Chromosorb W(aw), with a flow rate of 25 ml/min. Approximately 80-85% of the effluent was diverted to the mass spectrometer. Elution of individual compounds was achieved by programming the column temperature from 175C to 270C at 8°/min. Injector temperature was 280C, and detector at 300C. Optimum performance was obtained when both the GLC column, the separator and heated inlet line were silanized with "Silyl 8" column conditioning agent. Gas chromatographic methods for the determination of hydroxy fatty acids as their trimethylsilyl ether derivatives using other column types have been previously reported by Wood et al. (3).

The mass spectral fragmentation patterns of 11 methyl esters of varying chain length and position of unsaturation contained two major ions of the general structure A and B, in all cases, representing cleavage between the carbon atoms bearing the trimethyl silyl ether



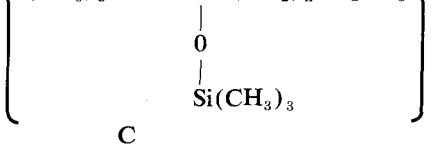
groups corresponding to the position of the double bond in the original molecule (Table 1). However, in the case of ricinoleate, which yields the same two ions as oleate (m/e 259,-332), an additional ion at m/e 189 was found as the base peak, corresponding to cleavage at the carbon atom containing the TMS group on the 12-hydroxyl group. The same three ions were also found in the spectrum of the

PRINCIPAL ION FRAGMENTS

Tri methylsilyl ether derivative of the corresponding hydroxylated methyl ester.	Fragment[a] m/e	Relative Abundance %
cis-9-hexadecenoate	187	78
	259	100
	332	21
cis-6-octadecenoate	257	88
	217	100
	290	11
cis-9-octadecenoate	215	100
	259	98
	332	22
trans-9-octadecenoate	215	81
	259	100
	332	16
cis-12-octadecenoate	173	78
	301	100
	374	14
cis-15-octadecenoate	131	30
	343	100
	416	5
12-hydroxy-cis-9-octadecenoate	187	100
	259	80
	332	22
cis-5-eicosenoate	299	95
	203	100
	276	27
cis-11-eicosenoate	215	67
	287	100
	360	16
cis-13-docosenoate	215	93
	315	100
	388	8
cis-15-tetracosenoate	215	72
	343	100
	416	10

[a] Ions presented in the order A,B,C, as indicated in the text.

palmitoleate derivative. However, the tri-TMS-derivatives may be easily separated from the di-TMS derivative formed from oleate and palmitoleate via GLC. The mass spectrum obtained for the di-TMS derivative of hyeroxylated methyl elaidate as illustrated in Figure 1. In all cases, a third major ion, C, (relative intensity 8-27%) was present which appears to be a rearrangement ion of the general structure:



C

No qualitative differences were found in the mass spectra of the derivatives corresponding to methyl oleate and elaidate. In all of the spectra, ions were found at m/e 73, 75, 146, and 157, which corresponded to fragments arising from the trimethyl silyl moiety, and at m/e M-15 and M (relative intensity 0.09%). Studies of the applicability of this method to compounds having up to 4 double bonds are currently in progress.

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[Received March 25, 1968]

Effect of Hypercholesteremia on the Activity of Serum Lecithin-Cholesterol Acyltransferase

IN 1935, SPERRY (1) reported evidence of the presence in human serum of an enzyme capable of esterifying free cholesterol. In recent years this enzymic activity has been re-investigated by Glomset (2). Serum free cholesterol esterifying activity has been described in a number of species including man (2-6), rat (5, 7), monkey (5), baboon (6) and chicken (8). This enzyme has tentatively been named lecithin-cholesterol acyltransferase (LCA).

To ascertain whether there was LCA activity in rabbit serum, we initially investigated serum from normal rabbits and from rabbits rendered hypercholesteremic by cholesterol feeding, and extended this study to include the serum of the chick and the rat.

Dutch belted rabbits were maintained for 2

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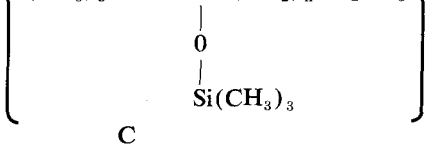

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To ascertain whether there was LCA activity in rabbit serum, we initially investigated serum from normal rabbits and from rabbits rendered hypercholesteremic by cholesterol feeding, and extended this study to include the serum of the chick and the rat.

Dutch belted rabbits were maintained for 2

TABLE I

Effect of Hypercholesteremia on the Activity of the Serum Lecithin-Cholesterol Acyltransferase Enzyme of Various Laboratory Animals

Experiment	Species	Serum	Serum cholesterol (mg/100 ml) Before incubation[a] Free	Before incubation[a] esterified	After incubation[a] Free	After incubation[a] esterified	Esterification rate[c]
1	Rabbit	Normal (4)[b]	14.7	12.8	6.58	20.0	8.12 ± 0.53
		Hypercholesteremic (4)	558.0	1750	606.0	1778	0.00
2	Chicken	Normal (15)	26.6	176.0	15.24	187.1	11.3 ± 1.12
		Hypercholesteremic (15)	194.9	583.5	193.4	583.5	1.50 ± 3.0
3	Rat	Normal, male (7)	13.5	37.7	6.72	46.3	6.82 ± 0.31
		Hypercholesteremic, male (6)	30.8	133.2	28.0	136.8	2.81 ± 1.60
		Hypercholesteremic, female (6)	30.0	119.0	24.8	124.7	5.21 ± 0.00
4	Rat	Normal, male (4)	14.7	38.3	8.10	44.7	6.58 ± 1.31
		Normal, female (4)	20.5	49.6	8.92	60.6	11.6 ± 0.65
		Triton-treated, male (3)	521.8	264.2	521.5	264.5	0.30 ± 0.00
		Triton-treated, female (3)	657.8	284.5	649.3	288.5	8.50 ± 0.00

[a] All samples assayed in duplicate.

[b] Number of individual normal or hypercholesteremic samples which were pooled; 1 ml of each serum sample was pooled.

[c] Milligrams free cholesterol esterified/100 ml serum/24 hr.

months on a laboratory chow diet augmented with 2% cholesterol and 6% corn oil. Chickens (white leghorn) were made hypercholesteremic by feeding a semisynthetic diet containing 2% cholesterol and 5% corn oil for 1 month, while rats (Wistar) were made hypercholesteremic either by dietary means (2% cholesterol, 0.5% cholic acid, 5% corn oil for 21 days), or by intraperitoneal injection of a 50% solution of Triton WR-1339 (1 ml/200 g) (9,10). At the end of each of the respective experimental periods, both normal and hypercholesteremic animals were bled by venipuncture, and the serum was immediately separated. Sera from normal or from hypercholesteremic animals of each species were then pooled for assay of cholesterol.

The free and esterified cholesterol concentration of all samples was determined by the method of Sperry and Webb (11). Aliquots (1-2 ml) of each serum pool were incubated in 25 ml or 50 ml volumetric flasks in a 37C water bath for 24 hr, at which time free and ester cholesterol concentrations were again determined. The decrease in serum free cholesterol concentration observed after incubation was taken as the measure of extent of esterification, and hence of LCA activity. LCA activity then was expressed as milligrams of free cholesterol esterified/100 ml serum/24 hr. Solvents and reagents of analytical grade purity were used in this study. Sterile glassware was used throughout.

Experiment 1 (Table I) shows that the LCA activity of normal rabbit serum is in the range reported for normal sera of other species. The complete absence of LCA activity in the hypercholesteremic sera was unexpected, although Aftergood and Alfin-Slater (12) have reported a diminution of LCA activity in the sera of hyperlipemic rats.

Experiment 2 shows that a 3- to 4-fold increase in the serum cholesterol level of chickens results in a 7-fold reduction in LCA activity. In rats (Experiment 3), a moderate dietary cholesteremia (three- to four-times normal) causes a 2.4-fold reduction of LCA activity in males and a 1.3-fold reduction in females. In severely hypercholesteremic rats (Experiment 4), LCA activity is reduced 22-fold in the male and 1.4-fold in the female.

Because of the relatively small number of rats used, no definite conclusion can be drawn about the rate difference between males and females.

Preliminary studies with fresh human serum suggest that, when the average serum total cholesterol is below 300 mg/100 ml the esterification rate is 29 mg/100 ml/24 hr, but when the cholesterol concentration is above 350 mg/100 ml the esterification rate is 18 mg/100 ml/24 hr.

The Michaelis constant (K_m) for the LCA enzyme of normal and hypercholesteremic rabbit, chick or rat sera have not been determined. If this serum enzyme has a low turnover number, and if the assay system is not adequately sensitive to detect small changes in free cholesterol concentration in serum which contain large amounts of free cholesterol, then a large error could have been made in the comparison of esterification rates of normal and hypercholesteremic serum. The standard deviation of a replicate analysis of

each sample was so small that this possibility seems unlikely. Studies are also being made to determine if this rate difference exists in other species, and whether the extent of this effect may be related to susceptibility to cholesterol-induced atherosclerosis.

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[Received Jan. 25, 1968]

In Vitro Effect of Prostaglandin (PGE_1) on the Release of Glycerol and the Metabolism of Palmitic Acid in Rat Adipose Tissue

BERGSTRÖM ET AL (1-3) discovered and characterized a number of prostaglandins which inhibit hormonally induced lipolysis in rat and human adipose tissue in vitro. Prostaglandin (PGE_1) inhibts the lipolysis both in fasted and fed rats (4, 5). Triglyceride in epididymal fat pads is continuously metabolized and resynthesized which potentially may involve alterations in the rate of release of glycerol and fatty acids. This study is a report on the effects of PGE_1 on release of glycerol and fatty acids from adipose tissue when no hormones are added to medium and the tissue is pretreated in the presence and absence of PGE_1. Also the effect of PGE_1 on metabolism of 1-^{14}C-palmitic acid during incubation with adipose tissue was investigated.

Table I shows the response of PGE_1 on the release of glycerol and free fatty acids. A similar response with PGE_1 was observed regarding glycerol and fatty acid release whether tissue was pretreated with PGE_1 prior to incubation or only incubated in medium containing PGE_1. The ratio of fatty acids to glycerol freed found to be less than 3 might be explained by the free fatty acids being further re-esterified or oxidised. As previously reported, the determination of glycerol seems

TABLE I

Response to PGE_1 on Release of Free Fatty Acids, Glycerol and CO_2 from Rat Adipose Tissue

Tissue treatment	FFA released	Glycerol released	CO_2[a] liberated
	μeq/g/hr	μmoles/g/hr	cpm/0.1g
Incubated with PGE_1	4.20 ± 0.28	2.46 ± 0.27	1905
Incubated (control)	5.16 ± 0.39	4.27 ± 0.75	1747
Pretreated with PGE_1	4.65 ± 0.30	2.33 ± 0.29	1226
Pretreated (control)	6.35 ± 0.45	3.67 ± 0.16	1322

From epididymal fat pads of 5 normally fed rats, 0.3 g tissue was incubated in duplicate for 2 hr at 37C in presence of 95% O_2 and 5% CO_2 in 2 ml of Krebs-Ringer bicarbonate medium (pH 7.4) that contained 3% bovine serum albumin and 1-^{14}C-palmitic acid with and without PGE_1 (0.2μg/ml). The FFA released were determined according to Dole's procedure (7) and glycerol by Korn's method (8). Similar tissues were pretreated with PGE_1 for 15 min, washed well and incubated as above for another 2 hr. Values are means ± standard error of mean. $P \leqq 0.02$ in all cases.

[a] This incubation was carried out at 37C for 1 hr in presence of CO_2-free air, and CO_2 liberated was collected in 1 N NaOH and activity measured in Beckman scintillation counter. Values are averages of 2 experiments in duplicate.

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From epididymal fat pads of 5 normally fed rats, 0.3 g tissue was incubated in duplicate for 2 hr at 37C in presence of 95% O_2 and 5% CO_2 in 2 ml of Krebs-Ringer bicarbonate medium (pH 7.4) that contained 3% bovine serum albumin and 1-^{14}C-palmitic acid with and without PGE_1 (0.2μg/ml). The FFA released were determined according to Dole's procedure (7) and glycerol by Korn's method (8). Similar tissues were pretreated with PGE_1 for 15 min, washed well and incubated as above for another 2 hr. Values are means ± standard error of mean. $P \leqq 0.02$ in all cases.

[a] This incubation was carried out at 37C for 1 hr in presence of CO_2-free air, and CO_2 liberated was collected in 1 N NaOH and activity measured in Beckman scintillation counter. Values are averages of 2 experiments in duplicate.

TABLE II
PGE_1 Effect on Esterification of Palmitic Acid

Tissue treatment	Total lipid activity	Triglyceride	Lower glycerides
Incubated with PGE_1	14618 ± 95	13063 ± 459	989
Incubated (control)	13073 ± 75	9088 ± 265	1110
Pretreated with PGE_1	11862 ± 112	9318 ± 248	909
Pretreated (control)	9090 ± 82	6038 ± 86	803

Incubation media and conditions were as described in Table I. Tissues were removed, well washed, and lipid was extracted by Folch's method (9). One aliquot was taken to determine the total lipid activity while another was used for separation of glycerides by TLC (10). Radioactivity was similarly measured. Values expressed in cpm/0.1 g wet tissue and were corrected for unincubated controls (195 ± 20) with SEM.

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The results also suggest an effect of PGE_1 on metabolism of 1-^{14}C-palmitic acid. Similar activities in the CO_2 released were obtained when the tissue was pretreated with or without PGE_1 which suggests that PGE_1 might not have an appreciable effect on $^{14}CO_2$ formation under these conditions. However, a slightly higher incorporation into CO_2 was observed when tissue was incubated in the presence of PGE_1, but its effect, if any, is slight.

Incorporation of 1-^{14}C-palmitic acid into tissue lipids and glycerides is shown in Table II. There was more incorporation of 1-^{14}C-palmitic acid into tissue lipids when tissue was pretreated or incubated with PGE_1 as compared to results in its absence; slightly less when pretreated may be due to its availability for a shorter time period. Most of the activity was found in triglyceride fraction with much less in lower glycerides.

One is tempted to speculate that, when available to the tissue, PGE_1 might be playing an important role in triglyceride breakdown and resynthesis in rat adipose tissue. Further studies are in progress.

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[Received March 21, 1968]

LETTERS TO THE EDITOR

Differentiation of Nitrogenous Phospholipids by Infrared Spectroscopy

Sir: In a recent publication, Nelson (Nelson, Lipids *3*, 104, 1968) reported a method for the differentiation of nitrogenous phospholipids by infrared absorption in the region of 9-11 μ. A similar method, making use, however, of the region from 5.5-7.0 μ, was reported by Baer and Blackwell 5 years ago (Baer and Blackwell, J. Biol. Chem. *238*, 3591-3594, 1963). Apparently, it has escaped general notice, possibly because it was not reported independently, but as part of a paper describing the synthesis of a phospholipid. We would like to draw attention to it, since the two methods are complementary. That of Baer and Blackwell is based on the observation that a stepwise replacement of the 2 hydrogen atoms of the amino group by methyl-groups leads to a successive disappearance of

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the absorption bands at λ 6.50 μ and 6.15 μ. At the same time the broad region of absorption above the peaks at λ 3.45 μ and 3.55 μ which is characteristic for phosphatidylethanolamines, changes to a narrow band with the introduction of the second methyl group. The spectra of phosphatidyl-N,N-dimethylethanolamines thus resemble much more closely those of lecithins, except that the latter in addition have a strong absorption band at λ 10.35 μ. This band is not shown by either cephalins, N-methylcephalins or N,N-dimethylcephalins. The differences in the infrared spectra of phosphatidylethanolamines and their N-alkyl-substituted derivatives in the region from 5.5-7.0 μ and at λ 10.35 μ thus are sufficiently characteristic to permit a rapid differentiation and unambiguous identification of their nitrogenous moieties by infrared spectroscopy.

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[Received April 4, 1968]

Relationship Between Linoleic Acid and Other ω6 Fatty Acids of Human Red Cell Total Phospholipid

Sir: In a previous study of the fatty acid distribution of the red cell total phospholipid of patients with abetalipoproteinemia (Phillips, and Dodge, J. Lab. Clin. Med. *71,* 629, 1968), we found that the relative amount of linoleic acid (18:2ω6[1]) was strikingly decreased, presumably on the basis of essential fatty acid depletion secondary to malabsorption, but the relative amounts of most of the longer chain ω6 fatty acids were increased. This finding was unexpected because the longer-chain ω6 fatty acids not obtained from the diet are apparently synthesized from 18:2ω6 (Steinberg, et al, J. Biol. Chem. *220,* 257, 1956; Klenk, Advan. Lipid Res. *3,* 1, 1965). Indeed, most studies on essential fatty acid deficiency of lower animals show a decrease in the level of red cell arachidonic acid (20:4ω6), the major longer-chain ω6 fatty acid, as well as of 18:2ω6 (Greenberg and Moon, Arch. Biochem. Biophys. *94,* 405, 1961; Mohrhauer and Holman, J. Lipid Res. *4,* 346, 1963; Walker and Kummerow, J. Nutr. *82,* 329, 1964).

To investigate the relationship between 18:2ω6 and other ω6 fatty acids, the data from a previous study (Dodge and Phillips, J. Lipid Res. *8,* 667, 1967) of the fatty acid and phospholipid distribution of red cell total phospholipid from 10 normal subjects were analyzed. In that study (Dodge and Phillips, J. Lipid Res. *8,* 667, 1967) the total red cell phospholipid was isolated by silicic acid thin-layer chromatography (TLC), and the fatty acids were separated into groups according to the degree of unsaturation by TLC of the mercuric acetate adducts prior to gas-liquid chromatography. The relationship between the relative amount in mole % of 18:2ω6 and the sum of the other ω6 fatty acids comprising individually greater than 0.5% of the total phospholipid fatty acid, i.e., 20:4ω6, 22:4ω6, 20:3ω6, 22:5ω6, is shown in Fig. 1. The com-

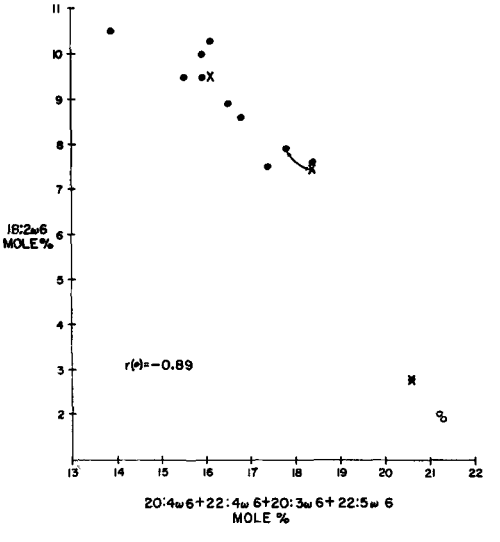

FIG. 1. Relationship between relative amount of linoleic acid (18:2ω6) and longer chain ω6 fatty acids of human red cell total phospholipid. ●, normal subjects; ○, patients with abetalipoproteinemia; ×, normal subjects, mercuric acetate adducts not made; ×, patient with abetalipoproteinemia, mercuric acetate adducts not made; arrows indicate measurements on same subject done four months apart; r(●), correlation coefficient of 10 normal subjects in whom mercuric acetate adducts were made.

[1]In this abbreviation of the fatty acids, the first two digits state the number of carbon atoms, the third digit states the number of double bonds, and the digit after the omega states the end-carbon chain-length, which is the number of carbon atoms from the methyl end of the acyl chain to the middle of the terminal double bond.

the absorption bands at λ 6.50 μ and 6.15 μ. At the same time the broad region of absorption above the peaks at λ 3.45 μ and 3.55 μ which is characteristic for phosphatidylethanolamines, changes to a narrow band with the introduction of the second methyl group. The spectra of phosphatidyl-N,N-dimethylethanolamines thus resemble much more closely those of lecithins, except that the latter in addition have a strong absorption band at λ 10.35 μ. This band is not shown by either cephalins, N-methylcephalins or N,N-dimethylcephalins. The differences in the infrared spectra of phosphatidylethanolamines and their N-alkyl-substituted derivatives in the region from 5.5-7.0 μ and at λ 10.35 μ thus are sufficiently characteristic to permit a rapid differentiation and unambiguous identification of their nitrogenous moieties by infrared spectroscopy.

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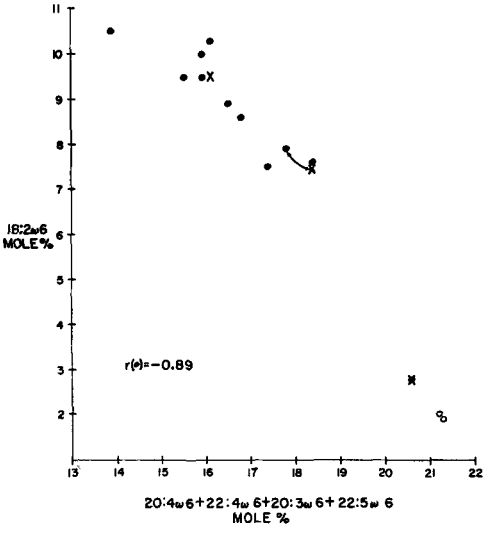

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parable values for red cell samples from three patients with abetalipoproteinemia and for two additional samples from normal subjects are also plotted. In the sample from one of the patients with abetalipoproteinemia and in the two additional samples of normal subjects, the mercuric acetate adducts were not made (Fig. 1); the results, however, were similar with or without use of the adduct method.

An inverse relationship between the relative amount of 18:2ω6 and the sum of the four major longer-chain ω6 fatty acids of the red cell total phospholipid of the normal subjects is apparent (Fig. 1); the correlation coefficient (r) of the values for the 10 normal subjects, in whom the adduct method was used, was –0.89. When 18:2ω6 was plotted against 20:4ω6 + 22:4ω6 + 20:3ω6, r was equal to –0.90; against 20:4ω6, it was –0.60. No correlation was found between the sum of the major longer-chain ω6 fatty acids and palmitic acid (r = +0.12), suggesting that the relationship with 18:2ω6 was not due to a dilutional effect as the values are reported in relative amounts. The values for the patients with abetalipoproteinemia (Fig. 1) appear to agree with this correlation; thus, the ω6 fatty acid changes in the red cells of these patients with abetalipoproteinemia could be explained on the basis of malabsorption of 18:2ω6 without having to invoke other abnormal underlying mechanisms.

Since most of the red cell 18:2ω6 resides in lecithin while the longer-chain ω6 fatty acids are predominantly in phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS) (Dodge and Phillips, J. Lipid Res. *8*, 667, 1967) the possibility arises that this fatty acid relationship reflects an inverse relationship between lecithin and PE and PS. Such a relationship is suggested between the red cell lecithin and PS in these 10 normal subjects by the r of –0.72. However, the linoleic acid level of the red cell lecithin in patients with abetalipoproteinemia has been reported to be considerably reduced (Ways, Reed and Hanahan, J. Clin. Invest. *42*, 1248, 1963). Furthermore, the relative amount of 22:6ω3, which, like the longer-chain ω6 fatty acids, accurs largely in red cell PE and PS (Dodge and Philips, J. Lipid Res. *8*, 667, 1967), was reduced in the red cells of the patients with abetalipoproteinemia (Phillips and Dodge, J. Lab. Clin Med. *71*, 629, 1968); 22:6ω3 and 18:2ω6 showed no inverse relationship in the red cells of the 10 normal subjects (r = +0.54). Finally, changes in phospholipid distribution in the patients with abetalipoproteinemia (Phillips and Dodge, J. Lab. Clin. Med. *71*, 629, 1968) did not appear to be great enough to account for these fatty acid changes.

Plasma levels of 18:2ω6 and 20:4ω6 in the 10 normal subjects appeared to reflect red cell levels (Phillips and Dodge, J. Lipid Res. *8*, 676 1967), and a suggestive inverse relationship between these two fatty acids (r = –0.66) was found in the plasma; yet PS comprised less than 2% of the plasma phospholipid and PE less than 4% (Phillips and Dodge, J. Lipid Res. *8*, 676, 1967). Variations in the distribution of these phospholipids could not account for the fatty acid differences which were observed. Significant correlation between 18:2ω6 and lecithin in red cells or plasma was not found in this small series.

The inverse relationship between 18:2ω6 and the longer-chain ω6 fatty acids in normal human red cells, therefore, does not appear to be a consequence of a similar relationship between individual phospholipids; yet the phospholipid distribution may reflect to some extent the fatty acid distribution. The mechanism responsible for this fatty acid relationship is unclear but may involve competition at absorptive or biosynthetic sites.

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[Received Jan. 12, 1968]

Chromatography of Lipids on Polystyrene Gel Columns

Sir: Tipton et al. (Tipton, Paulis, and Pierson, J. Chromatog. *14*, 486-489, 1964) have reported on the use of columns of divinyl cross-linked polystyrene beads for the fractionation of beef lung lipids using benzene as the eluting solvent. The system described offered the advantage that the less stable polar lipids were eluted first followed by the non-polar lipids. Since large amounts of relatively insoluble neutral lipids are encountered in extracts of peripheral nerve (Berry, Cevallos, and Wade, JAOCS *42*, 492-500, 1965), this method appeared to

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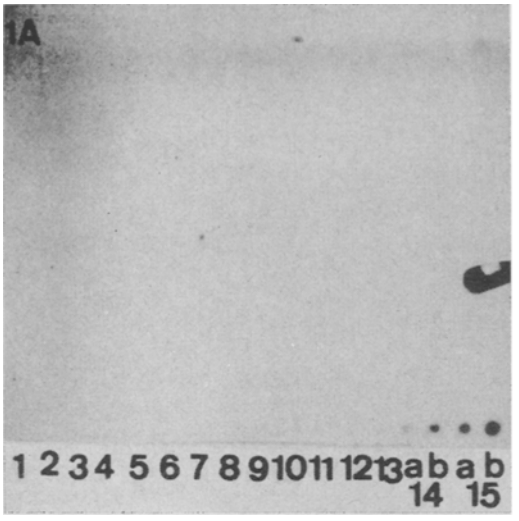

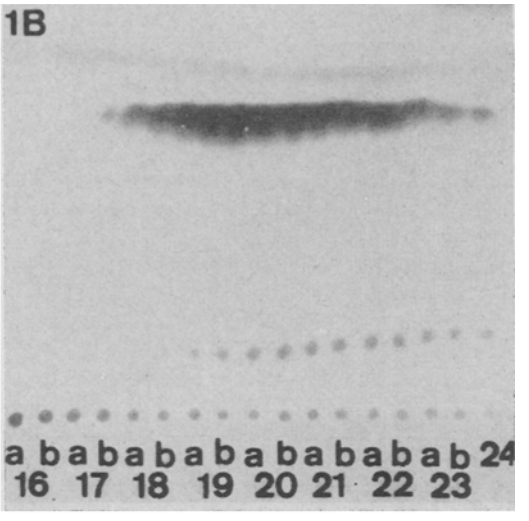

Fig. 1A and B. Benzene eluates from a 2.5 × 23 cm column of 10^3-sized polystyrene beads, 19-37μ, with 2% divinylbenzene cross-linking. 5 ml fractions 1-13. 12.5 ml fractions 14-24. 396 mg of beef sciatic nerve lipids eluted from Sephadex with chloroform-methanol 19:1 saturated with water. 10μl of each fraction applied to plate. Silica gel-Mg silicate (9:1, w/w). Solvent: hexane/ether/HAc (80:20:1).

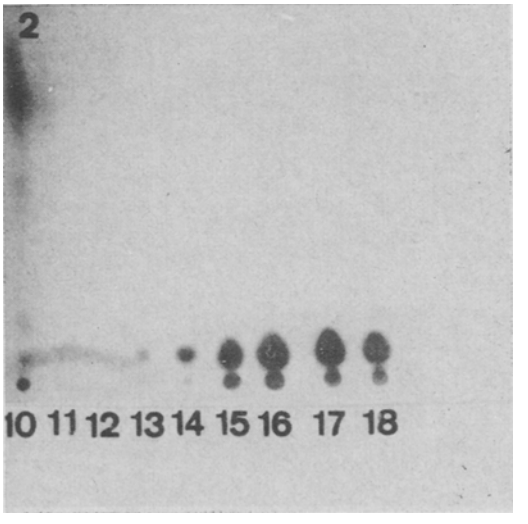

Fig. 2. 5 ml fractions from column described in Fig. 1A and B. 79 mg cholesterol and 14 mg lecithin. 10μl of each fraction applied to plate. Solvent hexane/ether/HAc (80:20:1).

have an advantage over silicic acid as a preliminary step for separating the relatively polar and non-polar lipids of nervous tissue without prolonged contact of the late-eluting polar lipids with silicic acid.

Thin layer chromatography (TLC) of column effluents was carried out as described by Rouser et al. (Rouser, Galli, Lieber, Blank, and Privett, JAOCS *41*, 836-840, 1964) using silica gel-magnesium silicate (9:1) plates and chloroform-methanol-water (65:25:4) or hexane/ether/HAc (80:20:1) as developing solvents.

A lipid extract of beef sciatic nerve was fractionated on a Sephadex column as described by Siakotos and Rouser (Siakotos and Rouser, JAOCS *42*, 913-919, 1965). The fraction (396 mg) eluting with chloroform-methanol (19:1) saturated with water was reduced to minimum volume and applied to a 2.5 x 23 cm column of polystyrene beads with 2% divinylbenzene cross linking. No resolution was obtained using 10 ml fractions and eluting with benzene at 2 ml/min. The recovery of added lipid was only 78.5%. Most of the lipid was eluted in the first 100 ml. The recovered material was re-applied to the same column and 5 ml fractions were collected. Fig. 1A and B shows that elution of polar lipids began after 130 ml; elution of neutral lipids began after 305 ml. However, polar lipids continued to be eluted throughout all neutral lipid fractions. The weight recovery was 222% which indicates elution of material from the column in addition to the lipid was applied. No solid material was eluted from the column with the initial solvent washes prior to application of sample. Infrared spectra did not reveal the presence of polystyrene in the eluates.

Fig. 2 shows that on a 23 cm column, 2.5 cm I.D., of polystyrene beads with 10^3 Å permeability range (Waters Associates, Inc.,

Framingham, Mass.) 19-37 μ, with 2% divinylbenzene cross-linking and using benzene as the eluting solvent marked overlap was observed between cholesterol and lecithin (Applied Science Labs, Inc., State College, Pa.) when a mixture of 79 mg of cholesterol and 14 mg lecithin was applied. This separation was not improved by changes in lipid load, solvent flow rate, or fraction volumes.

The apparent separations achieved by Tiption et al. (Tipton, Paulis, and Pierson, J. Chromatog. *14*, 486-489, 1964) may have resulted from use of small sample sizes for analysis with the result that overlapping of fractions was present but not detected. Radin et al. (Radin, Lavin, and Brown, J. Biol. Chem., *217*, 789-796, 1965) used polystyrene bead columns to separate cerebroside from cholesterol and cholesterol esters.

These attempts indicate that at present, polystyrene gel columns are of little or no value for the preliminary separation of polar and non-polar lipids in quantitative analysis.

ACKNOWLEDGEMENTS

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Separation of Lipids Containing Phytanic Acid by Thin-Layer Chromatography

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ABSTRACT

Cholesteryl phytanate and triglycerides containing phytanic acid were separated from their normal fatty acid analogs by thin-layer chromatography. The presence of the branched-chain fatty acid makes the lipid less polar, and this effect becomes more pronounced as the number of phytanic acid residues in the triglycerides is increased. Phytanic acid was prepared from commercial phytol by catalytic hydrogenation, followed by catalytic oxidation. It contained 5% pristanic acid.

INTRODUCTION

During studies of Refsum's syndrome, a neurological disease with accumulation of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) in the tissue lipids, it was found that thin-layer chromatography (TLC) of plasma lipids could be used as a sensitive diagnostic method (1). The triglycerides separated into three, and the cholesterol esters into two, fractions apparently because of the presence of phytanic acid. However no structural characterization of the fractions was made. Cholesteryl phytanate and triglycerides containing phytanic acid were therefore synthesized, and their thin-layer chromatographic behavior was studied.

EXPERIMENTAL PROCEDURES

Preparation of Phytanic Acid. Commercial phytol (Merck, for biochemical purposes) was hydrogenated with Raney nickel as the catalyst (2). Dihydrophytol was purified on aluminum oxide and catalytically oxidized (3) to phytanic acid in the following manner. One gram of PtO_2 (Adams Catalyst) in 100 ml of *n*-heptane was reduced with hydrogen for 15 min in a 500-ml reaction vessel with magnetic stirring. The hydrogen was replaced by nitrogen, and 6.0 g of dihydrophytol (0.02 moles) in 150 ml of *n*-heptane were added, together with 2 g of granulated silica gel (to absorb water). Oxygen at atmospheric pressure was led in under rigorous stirring for 36 hr at 50C. After filtration the solvent was evaporated, and the oily residue was taken up in 50 ml of 5% ethanolic KOH. The solution was diluted with water almost to precipitation, and unsaponifiable material was removed by extraction with light petroleum (bp 40-60C). The solution of potassium phytanate was finally acidified with 2M sulfuric acid, and the free phytanic acid was extracted with light petroleum. After washing with water, drying, and evaporation the yield was 5.3 g (84%).

Part of the sample was esterified with methanol and subjected to gas chromatography in combination with mass spectrometry (1). The methyl phytanate had a 5% contaminant, identified as methyl pristanate (methyl ester of 2,6,10,14-tetramethylpentadecanoic acid). This preparation was used without further purification for the syntheses described below.

Preparation of Cholesteryl Phytanate and Triglycerides Containing Phytanic Acid. Glycerol was esterified with fatty acids by using the trifluoroacetic anhydride method (4). Phytanic, oleic, eurcic acids and an equimolar mixture of oleic and phytanic acids were used to prepare the corresponding triglycerides. Acylation of 1-monopalmitin (5) and 2-monopalmitin (6) with the use of the acid chloride of phytanic acid in the presence of pyridine (7) gave the corresponding triglycerides with phytanic acid in positions 2,3 and 1,3 respectively. All reaction mixtures were filtered through aluminum oxide and finally purified by chromatography on silicic acid by using diethyl ether in *n*-hexane as eluant.

Thin-Layer Chromatography. Silica Gel G, Merck (batch 5143750 d), or Silica Gel G, Fluka (batch 99726 k), and a spreader (Desaga), adjusted to 0.25 mm, were used. The layer thickness was estimated to 0.15 mm. The plates (20 × 20 cm) were dried for 30 min at 120C immediately before use. The tanks were lined with filter paper for solvent saturation. The *n*-hexane-diethyl ether-acetic acid (90:10:1,v/v/v and 90:1:0.25, v/v/v) was used to separate the triglycerides (Fig. 1a) and esters of cholesterol (Fig. 1b) respectively. Spots were made visible by iodine vapor.

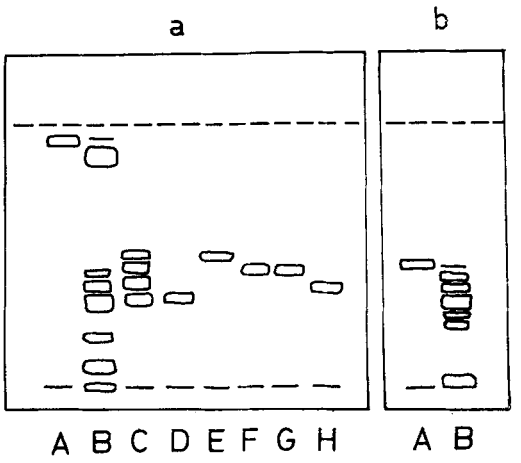

FIG. 1. Thin-layer chromatograms of lipids containing phytanic acid on Silica Gel G. A: cholesteryl phytanate; B: plasma lipids of a case with Refsum's syndrome; C: mixture of triglycerides containing phytanic and oleic acid; D: triolein; E: glyceryl triphytanate; F: triglyceride with one palmitic acid residue in position 1 and phytanic acid residues in positions 2 and 3; G: triglyceride with one palmitic acid in position 2 and phytanic acid residues in positions 1 and 3; H: trierucin. Plates a and b were developed in *n*-hexane-diethyl-ether-acetic acid (90:10:1, v/v/v and 90:1:0.25. v/v/v) respectively. Spots were made visible with iodine vapor.

RESULTS AND DISCUSSION

Figure 1 is a drawing of two thin-layer chromatograms with synthetic lipids and plasma lipids from a case with Refsum's syndrome. Fig. 1a shows the separation of the mixed triglycerides containing phytanic and oleic acids (C) into four fractions, three of which move identically with the triglycerides of the abnormal plasma. By comparison with known triglycerides (D-G) the four fractions (C) from top to bottom were identified as the glyceryl triphytanate, the glycerides containing one oleic and two phytanic acid residues, and two oleic acid and one phytanic acid residues respectively, and finally triolein.

The positional isomers (F, G) of the triglyceride containing one palmitic acid and two phytanic acid residues do not separate. Cholesteryl phytanate separates under the same conditions from a mixture of normal saturated and unsaturated cholesterol esters. With a less polar solvent (Fig. 1b), where normal cholesterol esters separate according to the degree of unsaturation (8), cholesteryl phytanate moves faster than the saturated normal esters.

The data given in this paper present further evidence that the earlier described (1) plasma lipid fractions containing phytanic acid from cases of Refsum's disease are triglycerides and a cholesterol ester. Branched hydrocarbon chains thus increase the thin-layer chromatographic mobility of the lipid. It should be remembered however that a similar effect is produced by a simple lengthening of the hydrocarbon chain, as demonstrated for trierucin (H) and shown previously for, e.g., sphingolipids (9).

Concerning the detection of Refsum's disease, the following procedure is recommended. TLC of plasma lipids, by using a control reference sample (e.g., C in Fig. 1), allows a rapid screening. For the final chemical diagnosis in positive cases, gas chromatography, preferably in combination with mass spectrometry (1), should be used. The preparation of methyl esters and column conditions for combined gas chromatography and mass spectrometry were described in the first paper (1).

The remarkable influence of branched hydrocarbon chains on the physicochemical properties of lipids may have a corresponding expression in tissues, especially in partially ordered lipid systems, such as membranes. In fact, the symptoms of Refsum's syndrome originate in nerves (polymembrane structures), where a decreased conductivity may be owing to the presence of phytanic acid (10,11,1).

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Characterization of Sterols by Gas Chromatography-Mass Spectrometry of the Trimethylsilyl Ethers

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ABSTRACT

The utility of combined gas chromatography-mass spectrometry in the analysis and characterization of sterols has been explored. Methylene unit (MU) values and principal mass spectrometric data are presented for trimethylsilyl ethers of 28 sterols, including the major natural sterols. The diagnostic value of the fragmentation of trimethylsilyl ethers of Δ^5-3β-hydroxysteroids has been confirmed. Characteristic fragmentations of Δ^4-3β-trimethylsilyloxysteroids, and of $\Delta^{5,7}$-3β-trimethylsilyloxysteroids were also found. Location of side-chain hydroxyl groups is facilitated by the α-cleavages typical of the trimethylsilyl ethers. Fragmentations of saturated sterols, and of Δ^7, $\Delta^{8(9)}$ and $\Delta^{8(14)}$ stenols, are less influenced by trimethylsilyl ether formation, but the derivatives still afford satisfactory mass spectra. The combination of gas chromatographic and mass spectrometric information allows positive identification of any of the sterols examined, whereas application of either technique alone may give inconclusive results.

INTRODUCTION

NATURALLY OCCURRING sterols generally occur in complex mixtures, and gas chromatography is of paramount importance in their analysis. Extensive use is made of trimethylsilyl ethers by virtue of their excellent chromatographic properties (1). Through the use of these and other derivatives, together with the application of selective stationary phases, not only separation, but also a high degree of characterization, can be accomplished (2). In the past few years, more definitive structural identification has been greatly aided by combined gas chromatography-mass spectrometry (3). Trimethylsilyl ethers have been found generally to yield informative mass spectra (4), and in certain instances they afford highly characteristic modes of fragmentation from which structural details may be inferred. For example, compounds containing a methylcarbinol group, such as 20-hydroxypregnanes, are recognizable by the abundant ion of m/e 117 (or m/e 116 in 17α-hydroxylated analogues), produced by α-cleavage of their trimethylsilyl ethers (4,5). In the sterol series, Δ^5-3β-hydroxysteroids, examined as their trimethylsilyl ethers, yield a major ion of m/e 129 which, although not uniquely characteristic, is frequently indicative of this structural type (3,6,7).

The present work was undertaken with the aim of exploring and extending the range of the correlations indicated by the studies of Eneroth et al. (3). Salient features of our data have already been reported (7,8). Since the completion of this survey, other publications have appeared which bear on the same topic (9-11). Our results provide evidence for two new structural correlations which are of diagnostic value. It is clear that the full utility of trimethylsilyl ethers in the identification of steroids has not yet been realized. The results also indicate that a combination of GLC and mass spectrometric data may be used for the conclusive identification of sterols.

MATERIALS AND METHODS

The sterols examined were purified commercial samples except for the following: pregn-5-en-3β-ol, desmosterol, cholest-5-en-3β, 24α-diol, cholest-5-en-3β, 25-diol, cholest-5-en-3β-ol-24-one, dihydroagnosterol, lanosterol and 5α-lanost-8-en-3β-ol, zymosterol, stigmastanol, 5α-cholest-8(14)-en-3β-ol, 5α-cholestan-2β-ol, 4β-methylcholesterol, 4, 4-dimethylcholesterol, 4α-methyl-5α-cholest-7-en-3β-ol, and cholest-4-en-3β-ol (prepared by borohydride reduction of cholest-4-en-3-one).

Trimethylsilyl ethers were prepared by treatment of the sterol (0.1-1 mg) in dry pyridine (10-20 μl) with hexamethyldisilazane (10-20 μl) and trimethylchlorosilane (2-5 μl) at room temperature. The reagents were removed in a stream of nitrogen and the residue was extracted with hexane or chloroform. In most instances the resulting solution was used directly for gas chromatography and mass

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TABLE I

Relative Retention Times (Cholestane = 1) and Methylene Unit Values of Sterol Trimethylsilyl Ethers on 1% SE-30, 225°

Parent sterol	Retention data for TMS Ethers	
	Relative retention	MU value
Pregn-5-en-3β-ol	0.43	25.10
Desmosterol	2.36	30.60
Zymosterol	2.92	31.30
7-Dehydrocholesterol	2.92	31.30
Cholest-4-en-3β-ol	2.44	30.70
Cholesterol	2.47	30.75
5α-Cholest-7-en-3β-ol	2.99	31.35
5α-Cholest-8(14)en-3β-ol	2.51	30.80
5α-Cholestan-2β-ol	2.14	30.25
5α-Cholestan-3β-ol	2.67	31.00
5α-Cholestan-3α-ol	2.03	30.10
5β-Cholestan-3β-ol	2.01	30.05
5β-Cholestan-3α-ol	2.11	30.25
Cholest-5-ene-3β, 24α-diol	5.73	33.45
Cholest-5-ene-3β, 25-diol	6.10	33.65
Cholest-5-en-3β-ol-7-one	5.22	33.20
Cholest-5-en-3β-ol-24-one	4.48	32.65
Ergosterol	3.20	31.50
4β-Methylcholesterol	2.80	31.15
4α-Methyl-5α-cholest-7-en-3β-ol	3.64	32.00
Campesterol	3.40	31.75
Stigmasterol	3.74	32.10
β-Sitosterol	4.39	32.60
4, 4-Dimethylcholesterol	3.78	32.10
Stigmastanol	4.48	32.65
Dihydroagnosterol	3.69	32.00
Lanosterol	4.23	32.40
Lanost-8-en-3β-ol	3.81	32.15

spectrometry. The purity of the derivatives was also checked by thin-layer chromatography (12).

Gas chromatographic separations were carried out with a Barber-Colman Model 5000 instrument and with a Pye Argon Chromatograph. The former instrument employed U-tube columns (6 ft or 12 ft), the latter straight 4-ft columns. Glass columns were used, packed with 1% SE-30 on silanized Gas Chrom P (100-120 mesh). Gas chromatographic procedures were carried out as previously described (13,14). Methylene unit (MU) values (15) were determined with reference to saturated unbranched long-chain hydrocarbons.

Combined gas chromatography-mass spectrometry was effected with an Atlas CH4 mass spectrometer as modified by R. Ryhage (16) to allow fast scanning of gas chromatographic effluents. In the later stages of the work, samples (ca. 1 μg) were introduced via a standard commercial gas chromatograph (Barber-Colman Co.). Full details of the general technique have been given by Leemans and McCloskey (17) for the instrument used in this investigation. The gas chromatographic columns were operated at 200-235 C (according to the molecular weight of the derivative concerned) with helium (30-40 ml/min) as carrier gas. The Ryhage-Becker molecular separator was kept at a temperature near that of the column. The ion source temperature was 250 C, the operating pressure ca. 2 x 10^6 mm Hg, the electron energy 20 ev, and the accelerating voltage 2.5-3.0 Kv. Mass spectral scanning times (m/e 28-500) were 4 to 9 sec. At least two scans were recorded during the emergence of each peak, to permit assessment of "bias" in the spectra (due to changing concentration) as well as to check the homogeneity of the peak. Perfluorokerosene and perfluorotributylamine (17) were used as mass markers.

Mass spectrometric data are not fully reported here but have been supplied to the Mass Spectrometry Data Centre, Atomic Weapons Research Establishment, Aldermaston, Berks, England. Representative mass spectra are included here as line diagrams, and the 10 most abundant ions in each spectrum are recorded (Table II). This is, of course, an arbitrary selection whereby it excludes ions of obvious significance; these are discussed separately.

RESULTS AND DISCUSSION

Gas Chromatographic Data

Relative retention times and methylene unit (MU) values for 28 sterol trimethylsilyl ethers are listed in Table I. As expected for the non-selective stationary phase SE-30, certain closely related sterol derivatives show almost identical retention behavior. Examples are (a) desmosterol, cholest-4-en-3β-ol, cholesterol and 5α-cholest-8(14)-en-3β-ol; (b) zymosterol, 7-dehydrocholesterol and cholest-7-en-3β-ol; (c) 4,4-dimethylcholesterol, dihydroagnosterol and lanost-8-en-3β-ol; and (d) cholest-5-en-3β-ol-24-one, β-sitosterol, stigmastanol and lanosterol, in which similarity of retention behavior may also occur fortuitously for markedly different structures.

Through the use of selective phases, with the free sterols or with various derivatives, separations of many of these steroids can be achieved and evidence of structure can be inferred. However, this indirect characterization procedure is applicable only with difficulty to the complex mixtures of sterols which are frequently encountered. The great value of mass spectrometric information secured in direct conjunction with retention data is thus apparent. For example, Fig. 1 depicts the mass spectra of the trimethylsilyl ethers of group (a) mentioned above; their distinctive characteristics are evident.

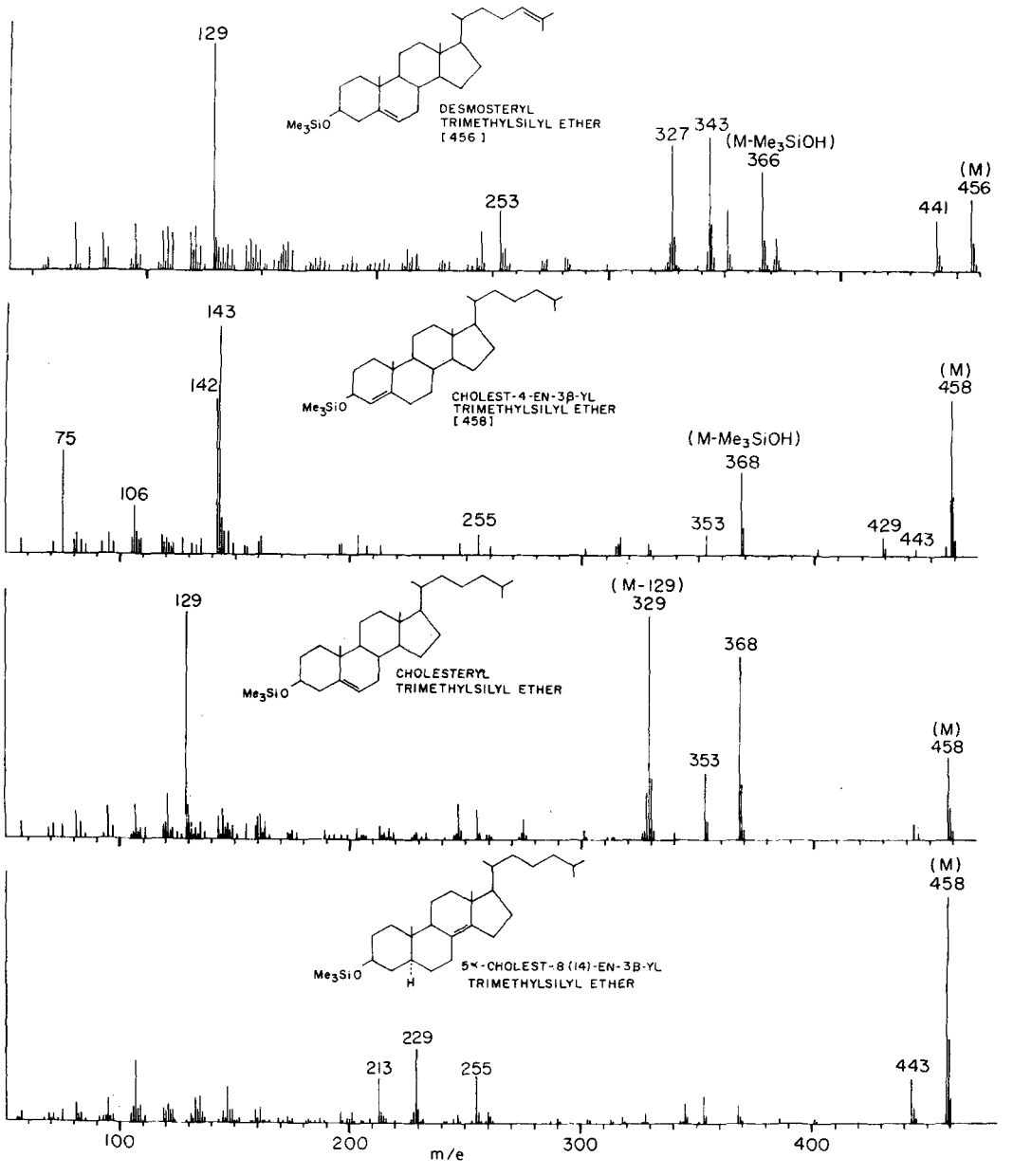


FIG. 1. Mass spectra of 4 closely-related steroid trimethylsilyl ethers which have similar retention behavior on 1% SE-30.

Mass Spectrometric Data

For convenience of presentation, the 10 most abundant ions of each mass spectrum have been selected for citation in Table II. In addition the relative abundance of the molecular ion is included for each example. The observed relative abundances were subject, of course, to some variation, but the values shown are representative of spectra recorded without serious "bias" due to concentration changes. Certain ions, notably that of m/e 75 (largely $(CH_3)_2SiO^+H$) from trimethylsilyl groups) and that of m/e 69 are of no diagnostic value but are recorded as observed. Intensities are cited as percentages of the base peak abundance.

The results provide confirmation of a number of correlations noted in the literature, and considerably extend their scope. Several new

TABLE II

Sterol Trimethylsilyl Ethers

	Base Peak	Molecular Ion % of Base Peak	Principal Ions in Mass Spectrum								
Pregn-5-en-3β-ol	129[j]	4	245[b]	163	121	107	95	123	284[c]	109	145
$C_{24}H_{42}OSi$ (374)			(40%)	(28%)	(24%)	(18%)	(16%)	(14%)	(13%)	(12%)	(9%)
Desmosterol	129[j]	32	343[i]	327[b]	366[e]	456	253[h]	351[d]	441[a]	69	95
$C_{30}H_{52}OSi$ (456)			(59%)	(55%)	(44%)	(32%)	(27%)	(27%)	(22%)	(21%)	(20%)
Zymosterol	456	100	441[a]	229	213	107	351[d]	147	109	135	69
$C_{30}H_{52}OSi$ (456)			(37%)	(25%)	(24%)	(24%)	(21%)	(16%)	(16%)	(14%)	(13%)
7-Dehydrocholesterol	351[d]	37	325[e]	456	364	131[k]	144	119	199	366[c]	253[h]
$C_{30}H_{52}OSi$ (456)			(80%)	(37%)	(29%)	(22%)	(20%)	(16%)	(13%)	(10%)	(10%)
Cholest-4-en-3β-ol	143	68	458	142	75	368[e]	106	145	353[d]	255[h]	161
$C_{30}H_{54}OSi$ (458)			(68%)	(68%)	(45%)	(36%)	(21%)	(9%)	(9%)	(9%)	(8%)
Cholesterol	129[j]	100	329[b]	368[e]	458	353[d]	121	247	107	95	255[h]
$C_{30}H_{54}OSi$ (458)			(95%)	(80%)	(34%)	(30%)	(19%)	(18%)	(15%)	(15%)	(14%)
Cholest-7-en-3β-ol	458	100	255[h]	443[a]	229	213	107	353[d]	147	161	135
$C_{30}H_{54}OSi$ (458)			(60%)	(18%)	(18%)	(14%)	(13%)	(10%)	(10%)	(8%)	(7%)
Cholest-8(14)-en-3β-ol	458	100	229	107	255[h]	213	443[a]	147	353[d]	135	133
$C_{30}H_{54}OSi$ (458)			(33%)	(27%)	(21%)	(20%)	(19%)	(16%)	(12%)	(12%)	(11%)
5α-Cholestan-2β-ol	216	95	460	215	370[e]	230	145	144	129	445[a]	355[d]
$C_{30}H_{56}OSi$ (460)			(95%)	(95%)	(85%)	(60%)	(60%)	(60%)	(56%)	(40%)	(40%)
5α-Cholestan-3β-ol	215	77	460	445[a]	216	106	217	75	230	355[d]	370[e]
$C_{30}H_{56}OSi$ (460)			(77%)	(68%)	(45%)	(41%)	(40%)	(37%)	(36%)	(34%)	(33%)
5α-Cholestan-3α-ol	215	25	370[e]	355[d]	230	460	148	306	106	369	108
$C_{30}H_{56}OSi$ (460)			(66%)	(46%)	(37%)	(25%)	(21%)	(20%)	(20%)	(18%)	(17%)
5β-Cholestan-3β-ol	370[c]	2	355[d]	215	108	257[h]	216	230	142	106	135
$C_{30}H_{56}OSi$ (460)			(25%)	(25%)	(22%)	(14%)	(10%)	(10%)	(8%)	(8%)	(7%)
5β-Cholestan-3α-ol	370[c]	1	215	355[d]	257[h]	216	230	274	75	162	161
$C_{30}H_{56}OSi$ (460)			(47%)	(21%)	(19%)	(17%)	(16%)	(13%)	(10%)	(8%)	(8%)
Cholest-5-ene-3β, 24-diol	145	7	413	159	129[j]	503	327[f]	323	69	546	243
$C_{33}H_{62}O_2Si_2$ (546)			(80%)	(48%)	(32%)	(24%)	(19%)	(16%)	(16%)	(7%)	(7%)

Cholest-5-ene-3β, 25-diol	131[i]	2	75	129[j]	271	327	456[e]	366[g]	255[h]	111	245
$C_{38}H_{62}O_2Si_2$ (546)			(17%)	(16%)	(11%)	(8%)	(7%)	(5%)	(3%)	(3%)	(2%)
Cholest-5-en-3β-ol-7-one	472	100	382[c]	174	75	129[j]	367[d]	142	161	187	457[a]
$C_{30}H_{52}O_2Si$ (472)			(38%)	(37%)	(32%)	(28%)	(20%)	(20%)	(18%)	(14%)	(9%)
Cholest-5-en-3β-ol-24-one	129[j]	35	382[c]	343[b]	472	127	367[d]	255[h]	342	416	121
$C_{30}H_{52}O_2Si$ (472)			(81%)	(81%)	(35%)	(28%)	(26%)	(21%)	(17%)	(16%)	(16%)
Ergosterol	363[d]	58	337[e]	468	378[c]	253[h]	131[k]	69	144	125	211
$C_{31}H_{52}OSi$ (468)			(60%)	(58%)	(30%)	(29%)	(29%)	(29%)	(28%)	(16%)	(12%)
4β-Methylcholesterol	343[b]	18	129	382[c]	472	135	367[d]	109	121	185	457[a]
$C_{31}H_{56}OSi$ (472)			(44%)	(25%)	(18%)	(18%)	(9%)	(9%)	(5%)	(5%)	(3%)
4α-Methyl-5α-cholest-7-en-3β-ol	472	100	269[h]	121	161	147	382[c]	367[d]	227	457[a]	243
$C_{31}H_{56}OSi$ (472)			(65%)	(20%)	(17%)	(17%)	(17%)	(16%)	(15%)	(15%)	(14%)
Campesterol	129[j]	32	343[b]	382[c]	472	367[d]	342	121	95	261	107
$C_{31}H_{56}OSi$ (472)			(94%)	(77%)	(32%)	(31%)	(21%)	(18%)	(15%)	(15%)	(14%)
Stigmasterol	83	42	129[j]	255[h]	394	139	484	69	57	351	355[b]
$C_{32}H_{56}OSi$ (484)			(67%)	(53%)	(52%)	(46%)	(42%)	(28%)	(28%)	(23%)	(23%)
β-Sitosterol	129[j]	37	357[b]	396[c]	486	381[d]	356	121	255[h]	107	95
$C_{32}H_{58}OSi$ (486)			(93%)	(80%)	(37%)	(30%)	(21%)	(20%)	(16%)	(16%)	(15%)
4, 4-Dimethylcholesterol	357[b]	2.5	129[j]	443	149	210	123	199	396	109	135
$C_{32}H_{58}OSi$ (486)			(25%)	(14%)	(9%)	(8%)	(8%)	(5%)	(5%)	(4%)	(4%)
Stigmastanol	215	37	75	216	473[a]	398[c]	217	106	488	383[d]	230
$C_{32}H_{60}OSi$ (488)			(88%)	(68%)	(46%)	(44%)	(43%)	(39%)	(37%)	(36%)	(34%)
Dihydroagnosterol	498	100	253[h]	240	393[d]	408[c]	369	295	145	157	161
$C_{33}H_{58}OSi$ (498)			(41%)	(24%)	(23%)	(15%)	(11%)	(10%)	(10%)	(9%)	(8%)
Lanosterol	393[d]	44	498	483[a]	109	135	95	241	187	227	83
$C_{33}H_{58}OSi$ (498)			(44%)	(39%)	(26%)	(18%)	(12%)	(9%)	(9%)	(8%)	(8%)
Lanost-8-en-3β-ol	395[d]	24	485[a]	500	135	227	95	83	187	175	161
$C_{33}H_{60}OSi$ (500)			(30%)	(24%)	(14%)	(7%)	(7%)	(7%)	(6%)	(6%)	(6%)

Molecular ion is underlined.

[a] M-15
[b] M-129
[c] M-90
[d] M-105 (90, 15)
[e] M-131
[f] M-129 (90, 129)
[g] M-180 (2 x 90)
[h] M-90-sidechain
[i] M-sidechain-2H
[j] $(CH_3)_3SiOCH\text{-}CH = CH_2$
[k] $(CH_3)_3SiOCH\text{-}CH_2CH_3$
[l] $(CH_3)_3SiOC(CH_3)_3$

observations of value in structural diagnosis have also been made. The data are discussed below according to structural features.

Certain regularities may be observed in the mass spectra of stanols. Both 5β-stanol derivatives studied gave a base peak of (M-90) and a molecular ion of low intensity; the principal feature distinguishing the 3β- and 3α-epimers was the strong peak at m/e 108 characteristic of the former (3,6). The four 5α-stanol trimethylsilyl ethers examined gave base peaks at m/e 215 (marginally more intense at m/e 216 for 5α-cholestan-2β-ol), characteristic of the tricyclic nucleus resulting from loss of the side chain, $C_{(15)}$-$C_{(17)}$ and the elements of trimethylsilanol.[2] These derivatives also yielded substantial peaks due to molecular ions. The spectra of the 5α-stanol derivatives are remarkable for their exhibition of many ions of comparable abundance. 5α-Cholestan-2β-ol trimethylsilyl ether is a striking example; the intensities of the 10 strongest peaks range from 40 to 100%. This compound is of interest in affording important peaks at m/e 145 and 144, attributable to fragments arising, with and without hydrogen transfer by cleavage of $C_{(1)}$-$C_{(10)}$ and $C_{(4)}$-$C_{(5)}$. A further strong peak at m/e 129 doubtless represents a 3 carbon fragment from ring A bearing the trimethylsilyloxy group (see below).

All five stanol trimethylsilyl ethers gave normal peaks at m/e 257 corresponding to loss of the side chain together with the elements of trimethylsilanol. A major peak was also observed in every case at m/e 230; this presumably represents the loss, in addition, of C_{16} and C_{17}.

Stenols

Δ^4-*Cholesten-3β-ol.* The trimethylsilyl ether of this sterol (Fig. 1 ii) showed a mass spectrum strikingly different from that of cholesterol (Fig. 1 iii). As noted elsewhere (8), this is fortunate in view of the difficulty of separating Δ^4- from Δ^5-stenols by gas-liquid chromatography. The base peak occurred at m/e 143 and was accompanied by a strong peak at m/e 142. These are ascribed to fragments comprising the trimethylsilyloxy group and a 4 carbon unit derived from ring A. Note that the isomeric 5α-cholestan-3-one enol trimethylsilyl ether affords a similar pair of peaks[3]: that at 142 in this case is the expected ion from retro-Diels-Alder fragmentation. Major peaks at m/e 143 and 142 were also noted in the spectrum of androst-4-en-3β, 17β-diol *bis*-trimethylsilyl ether, and subsequent observations have confirmed that these peaks are characteristic of Δ^4-3-trimethylsilyloxy-steroids.

Δ^5-*Stenols.* Twelve derivatives of this class were examined, six of which contained additional functional groups. (The $\Delta^{5,7}$ dienols are dealt with separately.) For six simple Δ^5-stenol trimethylsilyl ethers, the two strongest peaks were invariably at m/e 129 and M-129, and the occurrence of this pair of ions is characteristic of this structural type. We have pointed out (7) that the fact that the ion of m/e 129 is obtained from 4-alkylcholesterol trimethylsilyl ethers is in harmony with the expectation that it represents the trimethylsilyloxy group together with $C_{(1)}$-$C_{(3)}$, rather than $C_{(2)}$-$C_{(4)}$ as was tentatively proposed earlier (3). The relative stabilization of the (M-129) peak in the 4-alkylcholesterol derivatives was also noted (Fig. 2). Similar observations have been discussed in detail by Diekman and Djerassi (11), who have also confirmed the origin of the ion of m/e 129 by examining deuterium-labeled derivatives. It should be emphasized that fragments of m/e 129 may arise from other structural types, notably from 17-trimethylsilyloxysteroids; we have also drawn attention above to such an ion from a 2β-trimethylsilyloxysteroid. In our experience, however, the accompanying fragments of m/e M-129 are found as major peaks only for 3β-trimethylsilyloxy-Δ^5-steroids. Examples additional to the sterol derivatives cited here include the trimethylsilyl ethers of pregnenolone (m/e 259, 21%); dehydroepiandrosterone (m/e 231, 16%, androst-5-en-3β, 17β-diol (m/e 305, 66%) and pregna-5, 16-dien-3β-ol-20-one trimethylsilyloxime (m/e 244, 23%).

Within the sterol group, the following summaries of data show that an intense peak at m/e 129 is itself highly characteristic for the Δ^5-3β-trimethylsilyl ether group. (The 3α epimers may also be expected to yield this ion, but have not been examined). The case of 5α-cholestan-2β-yl trimethylsilyl ether is the only exception we have met so far. The five other saturated sterol derivatives examined gave peaks at m/e 129 ranging from 0-4% in relative abundance. (In contrast, Sjövall and Vihko (5) reported relative intensities of 20-30% for this ion, in the spectra of the trimethylsilyl ethers of androsterone, etiocholanolone and epiandrosterone.) The deriva-

[2]For free stigmastanol the ion of m/e 215 showed 95% the abundance of the base peak at 398 (M-18).

[3]C. J. Brooks and M. M. Campbell, unpublished observation.

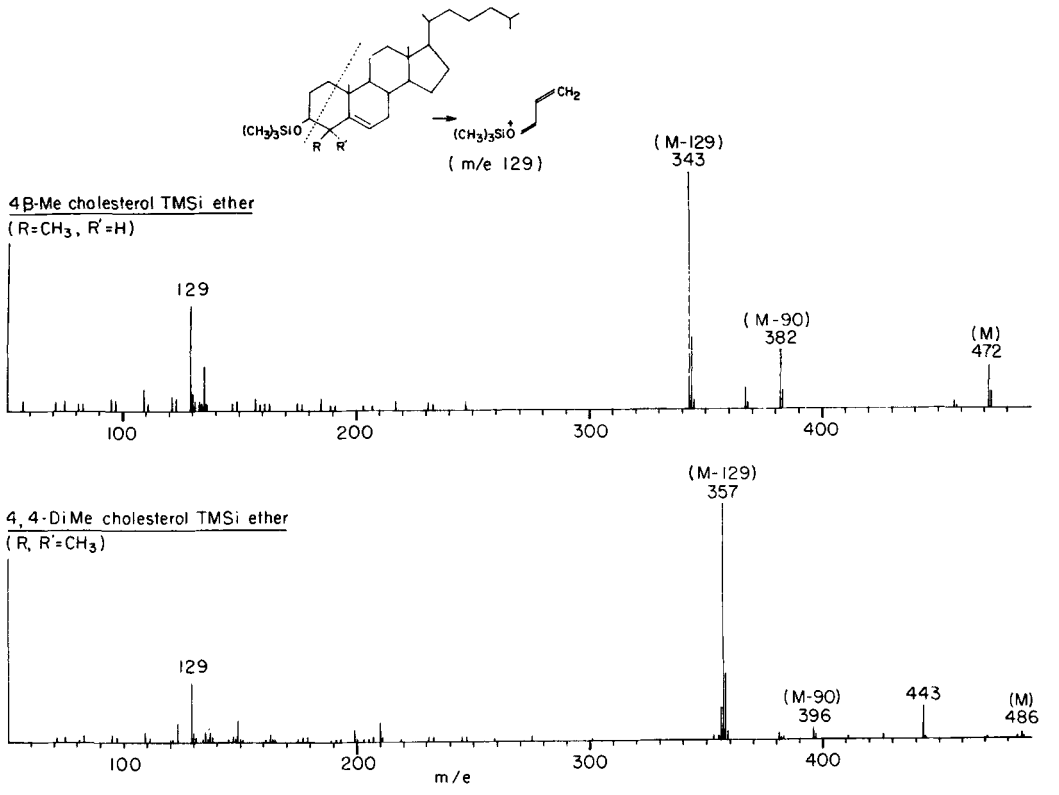


FIG. 2. Mass spectra of 4β-methyl- and 4, 4-dimethylcholesteryl trimethylsilyl ether, illustrating the characteristic major peaks at m/e 129 and(M-129), and the relative stabilization of the latter ion by 4-alkyl substituents.

tives of 10 unsaturated sterols (other than Δ^5) gave peaks at m/e 129 ranging from 0 to 7%, whereas the corresponding intensities for the 12 Δ^5-sterol trimethylsilyl ethers ranged from 16 to 100% (mean, 68%).

Desmosterol. The expected major peaks at m/e 129 and M-129 were observed, together with a peak at m/e 366 due to loss of trimethylsilanol. The most characteristic ions, however, were those of m/e 253 and 343, corresponding to loss of the side chain (with and without trimethylsilanol) together with two nuclear hydrogen atoms. The former ion is also present (31% of base peak) in the spectrum of desmosteryl acetate (Fig. 3 i), while the ion of m/e 343 is equivalent to that of m/e 271 which is the base peak in the spectrum of free desmosterol (Fig. 3 ii). Similar ions of m/e 343 have been reported (10) for the trimethylsilyl ethers of 24-methylene- and 24-ethylidenecholesterol, while in the analogous Δ^7-isomers they constitute the base peaks. Ions of m/e 253 were also found in all four examples. It would thus appear that derivatives of many Δ^{24}-stenols undergo loss of two nuclear hydrogen atoms together with the side chain. However, where a nuclear double bond is present in ring C, the process is less significant. Zymosterol trimethylsilyl ether yields ions at m/e 343 (8%), 345 (4%), 253 (4%) and 255 (7%), indicating no great predominance of hydrogen transfer from the nucleus. For the derivatives of lanosterol, lanost-8-en-3β-ol, and dihydroagnosterol, ions from loss of the side chain were of low abundance. The case of a Δ^{22}-sterol, stigmasterol, is discussed below.

Stigmasterol. The observed spectrum of the trimethylsilyl ether is in good agreement with that reported by Eneroth et al. (6), who drew attention to the preponderance of the peak at m/e 255 (due to loss of the side chain and of trimethylsilanol) over that at m/e 253 resulting from the additional loss of two nuclear hydrogen atoms. The presence of the 24α-ethyl substituent and of a Δ^{22} rather than a Δ^{24} bond thus reverses the situation described for desmosteryl trimethylsilyl ether, as far as the combined loss of the side chain and the

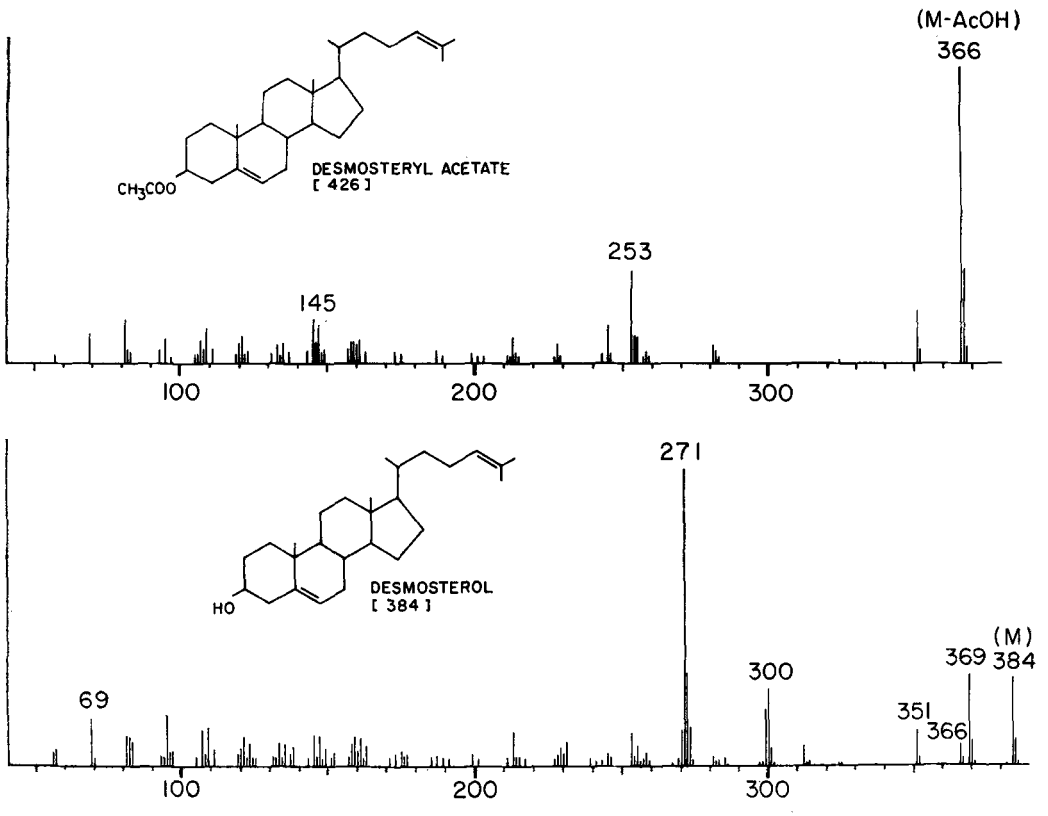


FIG. 3. Mass spectra of desmosteryl acetate and desmosterol, indicating the preponderance of peaks resulting from loss of the side chain together with two additional hydrogen atoms.

trimethylsilanol moiety is concerned.

Peaks at m/e 129 (67%) and (M-129) (23%) again characterize the Δ^5-3β-trimethylsilyloxysteroid structure. These are of course the strongest peaks in the spectrum of β-sitosteryl trimethylsilyl ether, but the introduction of the Δ^{22} bond leads to a new base peak at m/e 83. This presumably arises from C(24)-C(29), but the mechanism of formation is not known.

Cholest-5-en-3β, 24α-diol. The *bis*-trimethylsilyl ether gave a base peak at m/e 145, due to simple α-cleavage of the side-chain ether group to give the fragment $((CH_3)_2CH\text{-}CHOSi(CH_3)_3)^+$. The alternative α-cleavage at C(24)-C(25) elides the isopropyl group to give an ion of m/e 503, which suffers loss of trimethylsilanol affording the second most intense peak at m/e 413. These two losses are attested by metastable peaks at 463 (546→503) and 339 (503→413), respectively. The fragments serve to locate the side-chain ether group. The origin of the ion of m/e 159 is less clear. It may result from β-cleavage (C(22)-C(23)) or from the grouping (C(20)-C(24)-$OSi(CH_3)_3$) after elision of C(25)-C(27).

Cholest-5-en-3β, 25-diol. The base peak of the *bis*-trimethylsilyl ether, at m/e 131, is characteristic of the dimethylcarbinol ether group, and results from simple α-cleavage at C(24)-C(25). All other peaks were less than 20% as abundant. They included the expected fragment at m/e 129.

3β-Hydroxycholest-5-en-7-one. (Fig. 4 i). The presence of the conjugated ketone group confers stability on the molecular ion of the trimethylsilyl ether, which is the base peak. The spectrum is otherwise notable for the presence of two odd-electron fragments at m/e 174 and 142, the origin of which has not been determined. The usual peak is observed at m/e 129, but the complementary ion of m/e M-129 is not formed, doubtless because of the destabilizing effect of the 7-keto group on this allylic carbonium ion.

3β-Hydroxycholest-5-en-24-one (Fig. 4 ii). The 24 keto group does not affect the prevalence of fragments of m/e 129 and M-129 in

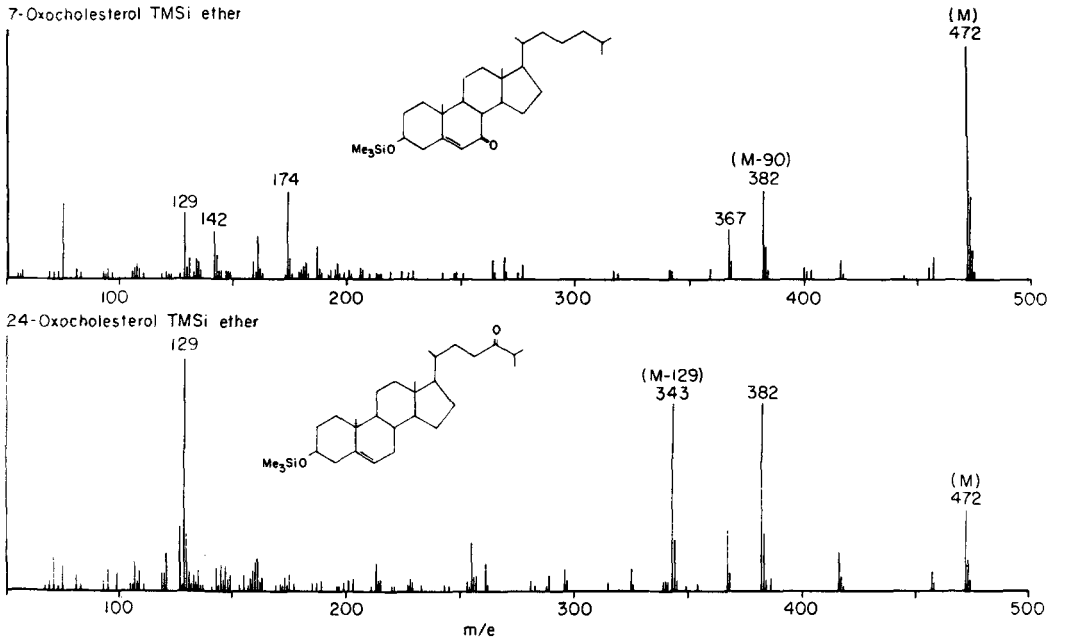


FIG. 4. Mass spectra of 7- and 24-oxocholesteryl trimethylsilyl ether, indicating the suppression of the ion at m/e (M-129) in the former.

the spectrum of this trimethylsilyl ether. A peak at m/e 127 is ascribed to the ion $(C_8H_{15}O)^+$ derived from the side chain.

Δ^7-Stenols

Trimethylsilyl ethers of Δ^7-cholestenol and its 4α-methyl analogue have been examined (Fig. 5). Closely parallel spectra are observed, each with the molecular ion as base peak. (The 70 ev spectrum reported by Knights (10) for Δ^7-cholestenyl trimethylsilyl ether shows the molecular ion as the second most abundant peak). Next in abundance are the ions at m/e 255 and 269 respectively, representing the nuclei. Fragments at m/e 229 and 213, representing further degradation of ring D, are found for Δ^7-cholestenyl trimethylsilyl ether, as outlined by Knights. Analogous peaks occur at m/e 243 and 227 in the 4α-methyl derivative.

$\Delta^{8(9)}$-Stenols

Derivatives of $\Delta^{8(9)}$-lanostenol, lanosterol and zymosterol have been examined. The spectra of the first two are closely parallel, showing base peaks at m/e M-105, with abundant molecular ions, also ions at m/e M-15. Other fragments are of minor incidence. The absence of fragments representing the nucleus (to be expected at m/e 297 and 295 if the methyl substituents were retained) is a notable feature of the spectra of these $\Delta^{8(9)}$-trimethylsterol derivatives (cf., however, dihydroagnosterol). For zymosteryl trimethylsilyl ether the molecular ion is also the base peak, and in this case the spectrum includes major ions at m/e 229 and 213, as in the Δ^7-cholestenol derivative; the peak at m/e 255, however, is still of low abundance (7%).

$\Delta^{8(14)}$-Stenols

The mass spectrum of cholest-8(14)-en-3β-yl trimethylsilyl ether (Fig. 1 iv) was generally similar to that of the Δ^7-isomer. It would appear that the trimethylsilyl ether group, apart from its stabilization of the molecular ion, confers no striking fragmentation behavior on sterols of this type.

$\Delta^{5,7}$-Dienols

The trimethylsilyl ethers of 7-dehydrocholesterol and ergosterol (Fig. 6) gave very characteristic spectra, with the base peak at m/e M-105, owing to elimination of trimethylsilanol probably with the C-19 methyl group. The second most abundant ion occurs at m/e M-131, and a complementary fragment at m/e 131 is observed among the most intense peaks. The formation of these ions, which together characterize the $\Delta^{5,7}$-dienol trimethylsilyl ether structure, may be rationalized as follows: Peaks at m/e 129 and M-129 were found to

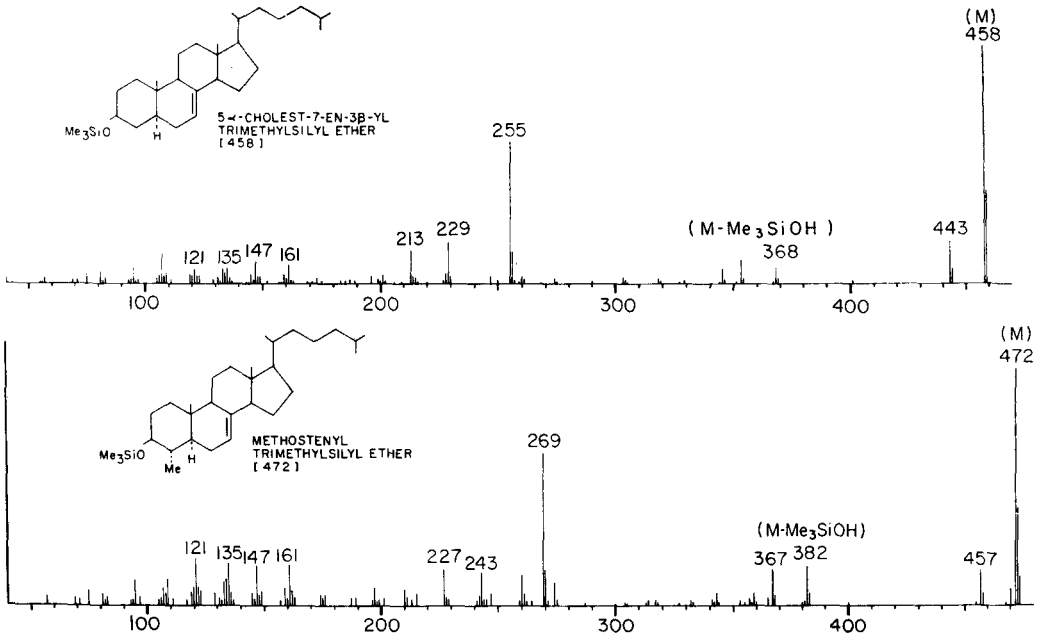
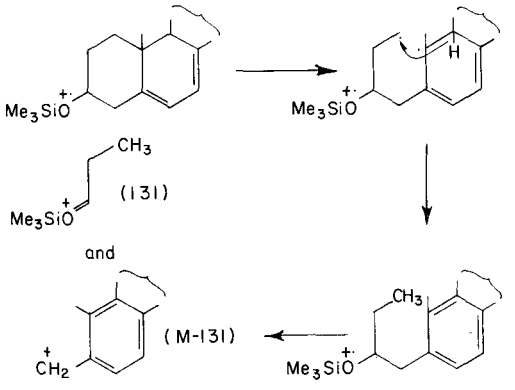


FIG. 5. Mass spectra of 5α-cholest-7-en-3β-yl and methostenyl trimethylsilyl ether illustrating the close parallelism of fragmentation, showing that the structural difference is at a nuclear site.



be of low abundance. Both compounds also afford a major ion of m/e 144 which appears to comprise the trimethylsilyl ether group and C(1)-C(4); its precise origin remains to be determined. A peak at m/e 199 (from 7-dehydrocholesteryl trimethylsilyl ether) and its analogue at m/e 211 (from ergosteryl trimethylsilyl ether) evidently represent fragments derived from rings C and D with the side chain attached. Both compounds show peaks at m/e M-90 due to elimination of trimethylsilanol; an "anomalous" peak at m/e M-90-2 in the dehydrocholestrol derivative evidently ensues from an accompanying dehydrogenation.

$\Delta^{7,9(11)}$-Dienols

Only one example, the trimethylsilyl ether of dihydroagnosterol, has been examined. The molecular ion constitutes the base peak. The next most intense peaks, at m/e 253 and m/e 240, seem likely to be due to fragments retaining rings A, B and C with respectively five and four methyl or methylene residues. The odd-electron ion of m/e 240 has not been observed as a major peak in any of the other sterol trimethylsilyl ether spectra in this series.

Reproducibility of the Mass Spectra of Trimethylsilyl Ethers

A brief comment on this topic is required in view of the generalization by Diekman and Djerassi (11) that "minor variations of conditions have an important effect on the mass spectra of trimethylsilyl ethers of steroids." Specifically, these authors cited data recorded for cholesteryl trimethylsilyl ether under various conditions, indicating dependence on 1) the mass spectrometer used, 2) the inlet system and ion source temperature, and 3) the method of synthesizing the silyl derivative.

In our experience, only factor 2 appears likely to lead to major variations, which are to

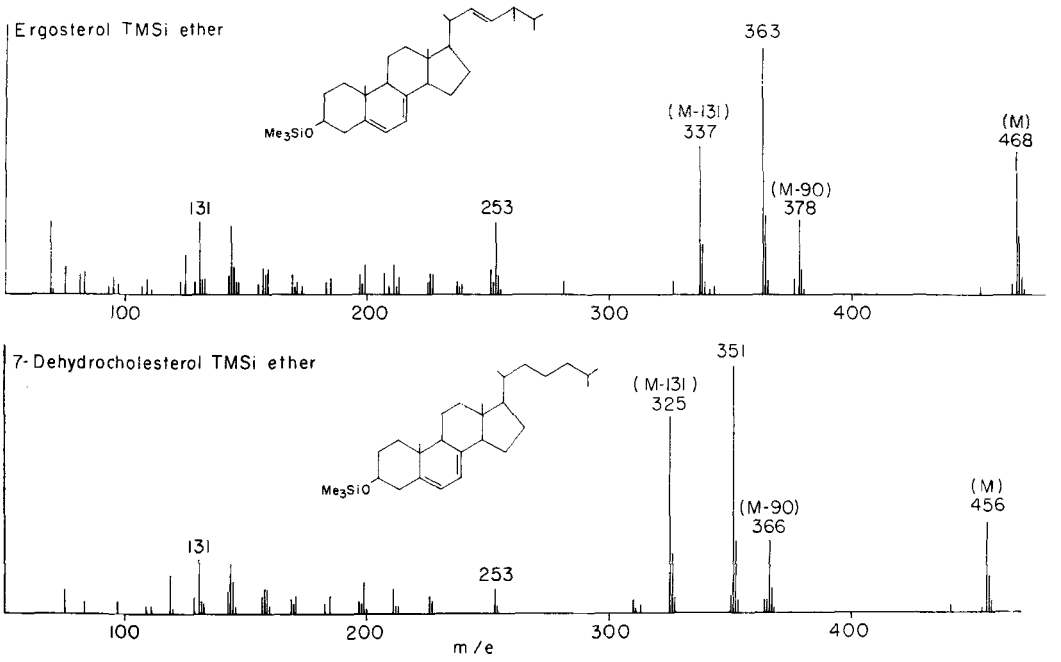


FIG. 6. Mass spectra of ergosteryl and 7-dehydrocholesteryl trimethylsilyl ether showing the characteristic peaks at m/e 131, M-131 and M-105, and disclosing that the structural difference is in the side-chain.

be expected, for example, when the sample is introduced by a conventional heated inlet system and may not reach the ion chamber with its original structure intact. In accordance with this view, the two such instances cited by Diekman and Djerassi led to lower-than-expected relative abundances for the molecular ions.

We have not observed significant differences between the mass spectra of an individual sterol trimethylsilyl ether whether prepared with hexamethyldisilazane or *bis*-trimethylsilylacetamide.

Our personal experience in this field is limited to an Atlas CH-4 spectrometer (modified according to Ryhage) and an LKB 9000 instrument, with either direct "probe" sampling or a gas chromatographic inlet system. Together with information in the literature, based on the use of other mass spectrometers, our observations suggest that there is in fact remarkable consistency in the mass spectra of sterol trimethylsilyl ethers recorded under a variety of conditions. Relevant data for cholesteryl trimethylsilyl ether, including examples taken at random from our files, are quoted in Table III. Close agreement is observed be-

TABLE III

Characteristic Ions in the Mass Spectrum of Cholesteryl Trimethylsilyl Ether: Relative Abundances Observed in Spectra Recorded Under Various Conditions

	Relative abundance (Percentage of base peak)					
Ion, m/e	(1)	(2)	(3)	(4)[a]	(5)	(6)
458	34	45	22	11	45	45
443	7	8	20	4	13	10
368	80	80	52	40	90	65
353	30	30	30	24	35	30
329	95	100	100	62	100	98
301	4	3	4	3	3	4
275	9	8	8	5	6	8
255	13	11	16	13	12	11
247	18	14	16	14	14	14
229	3	2	—	3	2	2
213	6	4	—	9	8	7
129	100	75	—	100	50	100

(1) Present work. Modified Atlas CH-4, GC inlet system (20 ev).

(2) LKB 9000, GC inlet system (20 ev). Recorded at Glasgow by C. J. W. Brooks.

(3) LKB 9000, as in (2) but 70 ev. Data from Knights (10).

(4) Modified Atlas CH-4, GC inlet system. Data estimated from Fig. 2, ref. 3.

(5) 180C Instrument, gas inlet system at 210C. Data estimated from Fig. 3, ref. 3.

(6) MS-9, direct inlet. Data from Diekman and Djerassi (11).

[a] These data were recorded during the analysis of a mixture of sterol trimethylsilyl ethers, and the peak scanned was not wholly homogeneous.

tween the spectrum recorded by Diekman and Djerassi with an MS-9 instrument at 70 ev (direct inlet) and those observed by gas chromatography-mass spectrometry at 70 or 20 ev.

From these and other observations, we conclude that the mass spectra of sterol trimethylsilyl ethers, recorded under different conditions, do not show variations to a degree likely to impair their use for qualitative characterization. Unduly large variations may frequently be traced to the use of an unsuitable sampling system or to extraneous causes. We recognize the need for stringent control of conditions wherever quantitative application is intended for mass spectrometric data.

Role of Trimethylsilyl Ethers in the Characterization of Sterols

From these results it is possible to discern certain salient features of the use of trimethylsilyl ethers for the characterization of sterols.

1) A peak due to the molecular ion is always observed. With a few exceptions, this is of high relative abundance even in those instances where well-defined fragmentations are favored. For derivatives of sterols possessing unsaturation in the vicinity of ring C, it is frequently the base peak. Accordingly, trimethylsilyl ethers appear to be of general value for defining the molecular weights of sterols.

2) Trimethylsilyl ethers of Δ^5-3β-ols yield strong peaks at m/e 129 and M-129. This general behavior is typical of all simple Δ^5-3β-yl trimethylsilyl ethers, and the former ion, in particular, is formed even in the presence of a 7 keto group. The formation of the ion of m/e M-129 serves to distinguish the Δ^5-3β-ol structure from other groups (17β-ol, 2β-ol) which, as their trimethylsilyl ethers, also afford peaks at m/e 129.

3) Trimethylsilyl ethers of the two $\Delta^{5,7}$-3β-ols examined yield no appreciable peaks at m/e 129 or M-129. Instead, characteristic ions at m/e 131 and M-131 are found. Although further examples have not been available, there seems to be no reason to doubt that these ions result from a specific mode of fragmentation which serves to identify the $\Delta^{5,7}$ system in these sterols.

4) The trimethylsilyl ether of Δ^4-cholesten-3β-ol differs from the Δ^5-isomer in yielding no significant ion at m/e 129. The abundant ions at m/e 143 and 142 appear to be diagnostic for the Δ^4-isomers. They are found also for androst-4-en-3β, 17β-diol *bis*-trimethylsilyl ether and related C_{19}-steroids.

5) The location of side-chain hydroxyl substituents is strongly indicated by the ions resulting from α-cleavages of their trimethylsilyl ethers.

The above regularities appear to us to substantiate the utility of trimethylsilyl ethers in the characterization and structural identification of hydroxyl-substituted steroids.

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Fatty Acids of Unusual Double-Bond Positions and Chain Lengths Found in Rat Skin Surface Lipids[1]

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ABSTRACT

The fatty acids of rat skin surface lipids comprise four main skeletal types of chains which occur both as saturates and monoenes and range from C_{12} to C_{38}: straight even, straight odd, iso and anteiso (the latter two identified by GC retention data only). Two unidentified series of branched monoenes also occur in trace amounts.

Reductive ozonolysis of monoenes reveals two characteristic double-bond position patterns, one for the straight even chain series and the other for the straight odd chain series. The straight even chain pattern comprises four series, of which $\omega7 >> \omega9 > \omega5 > \omega11$; the straight odd chain series in contrast shows a large number of ω series with irregular distribution. The biosynthesis of the even chain fatty acid monoenes can be thought of as occurring in two stages: synthesis of 14:Δ9, 16:Δ9, 18:Δ9 and 20:Δ9, with 16:Δ9 predominating; elongation of these chains mostly by 1, 2, or 3 C_2 units but up to the unusually long lengths by 11 C_2 units. For the formation of the former, two schemes by known pathways are proposed.

Iso and anteiso chains which are nearly all saturated comprised ⅓ the total fatty acids.

INTRODUCTION

In a classic study, the position of unsaturation of the free fatty acids of human hair fat was found to be mainly at Δ6 for most of the fatty chains of this lipid (1). With the advent of gas chromatography and the assistance of a simple micro technique for the determination of double-bond position of methyl esters (2), the earlier work was largely confirmed (3, 4). It was further shown that the position of the double-bonds for nearly all of the chains was either at Δ6 or could be derived from Δ6 by chain extension or degradation at the carboxyl group by an integral number of C_2 units. Recently, the positions of the fatty chains of the wax alcohol monoenes occurring in human scalp skin surface lipid were determined, and a scheme was proposed for the common build-up of the chains of both the fatty acids and the fatty alcohols (5). It was proposed that fatty chains of different carbon skeletal types were first built up mainly to C_{16}, desaturated at Δ6, then further extended by one of two patterns: a short pattern which formed the fatty acids and a long pattern which formed the fatty alcohols.

To be able to study the biochemistry of these processes conveniently, we investigated the double-bond pattern of the rat skin surface lipid monoene fatty acids. To our surprise an entirely different pattern was revealed. In this paper we describe and discuss our findings.

EXPERIMENTAL PROCEDURES

Figures 1A and 1B summarize diagramatically the preparation, work-up and analysis of rat skin surface lipid fatty acids. The skin surface lipids from three male Sprague Dawley rats (average wt 208 g), fed a Purina Chow diet ad libitum, were extracted on the first, seventh and eighteenth day of the experiment as follows. After anesthesia with ether, the feet, tail and area of the anus were wiped with fat-free cotton pledgets soaked in hexane, the entire body exclusive of neck and head was immersed momentarily three or four times in an 800-ml beaker containing 350 ml redistilled hexane. (All solvents used in this study were redistilled.) The pooled extracts were filtered through coarse sintered glass and the solvent removed on a rotary evaporator. Only the extracts from the eighteenth day were used, the yield for the three rats being 448.9 mg.

A 224.4-mg portion was saponified for 2 hr in 20 ml of 10% KOH (w/v) in ethanol-water (9:1, v/v) under reflux in a nitrogen atmos-

[1] 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, ω = terminal carbon atom, iso = a straight chain with a methyl group at the ω-1 position, anteiso = a straight chain with a methyl group at the ω-2 position, Δn = a double bond between the n^{th} and the $(n+1)^{th}$ carbon atom from the carbonyl group of the fatty acid or ester, ωn = a double bond between the ω-n^{th} and the ω-$(n-1)^{th}$ carbon atom where n is an integer, aldester = aldehyde methyl ester, Me = methyl, GLC = gas-liquid chromatography, TLC = thin-layer chromatography.

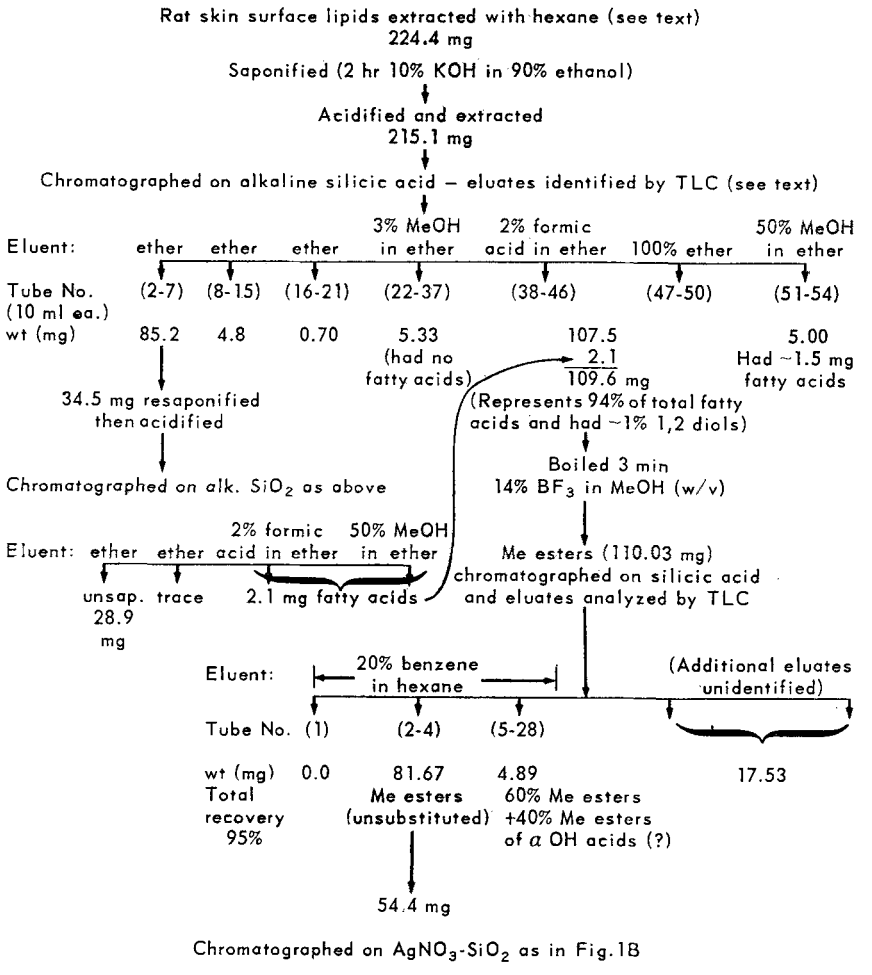

Fig. 1A. Preparation, work-up and analysis of rat skin surface lipid fatty acids.

phere. The reaction mixture was diluted with an equal volume of water, acidified with 6 N H_2SO_4, then extracted with four washes of freshly redistilled ether. These washes were pooled and counterwashed with water (yield 215.1 mg).

Separation of saponifiable from unsaponifiable matter was carried out by column chromatography on alkaline silicic acid (6). Thirteen grams activated silicic acid, 100-200 mesh (Unisil of Clarkson Chemical Co., Williamsport, Pa.) made alkaline was packed into a column (14.5 × 1.6 cm i.d.) and washed with ether until the eluate was free of residue. The lipid of the saponification reaction was layered on to the column and eluted as outlined in Fig. 1A. Thin-layer chromatography (TLC) of the eluates was performed on Rouser's adsorbent (7), and developed multilinearly, first with chloroform-acetone-acetic acid (80:20:1, v/v/v) to 60% of the total distance of the plate, followed by hexane-ether (95:5, v/v) to 80% of the distance of the plate, followed by hexane to the top of the plate, the plate being air-dried for 10 min after each development. Fatty acids were present only in tubes 38-54, and the bulk of the unsaponifiable matter appeared in tubes 2-7. To test the completeness of the saponification, 34.5 mg of the eluate of tubes 2-7 was resaponified for 2 hr with 15 ml ethanolic KOH as previously described, and the reaction mixture was worked up as before. TLC showed that an additional 7% fatty acids were released. These were included with the main bulk of fatty acids. The unsaponifiable fraction was saponified a third time and yielded no more fatty acids.

Methyl esters of the fatty acids were made with methanolic BF_3 (8) and were chromatographed on a column (13.2 × 1.0 cm i.d.) of

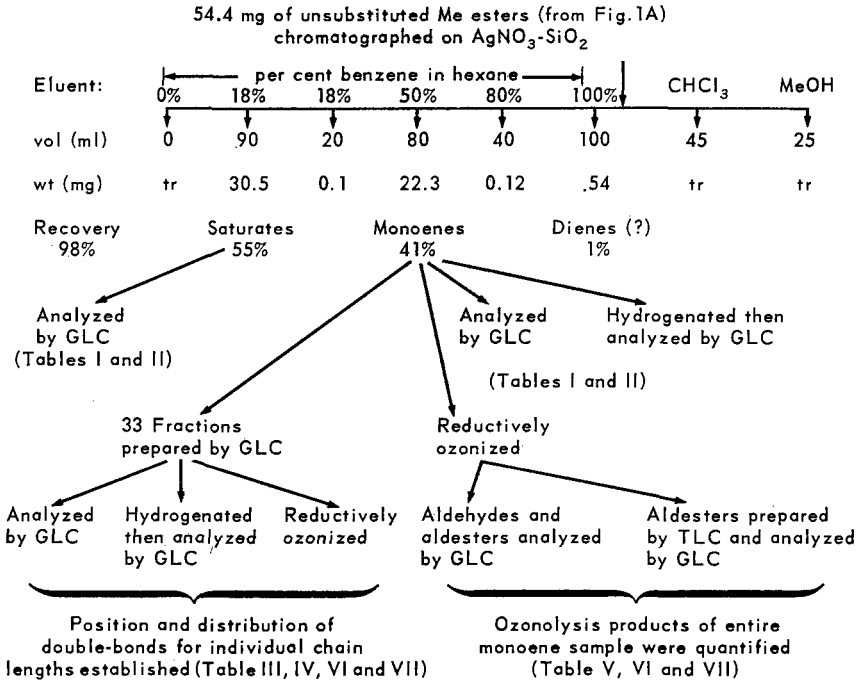

Fig. 1B. Preparation, work-up and analysis of rat skin surface lipid fatty acids.

silicic acid (Unisil, 100-200 mesh) in order to remove hydroxy fatty acid methyl ester, any nonesterified acids and traces of unsaponifiable matter which TLC showed to be present. An aliquot (54.4 mg) of the bulk of the unsubstituted methyl esters, tubes 2-4, was chromatographed on a column (13.2 × 1.0 cm i.d.) of silicic acid impregnated with 25% silver nitrate (Adsorbosil CABN, Applied Science Laboratories, Inc., State College, Pa.) to separate saturates from monoenes and polyenes (Fig. 1B).

The saturates were analyzed by gas-liquid chromatography (GLC). A portion of the monoenes was separated into 33 fractions by preparative GLC and these were analyzed separately by analytical GLC, hydrogenation followed by GLC, and reductive ozonolysis followed by GLC as previously described (2-5). A portion of the total monoene methyl esters was also subjected to the same chemical and GLC procedures.

RESULTS

Table I gives the weight percentages of each member of the eight series of fatty acids found: straight even, straight odd, iso[2] and anteiso,[2] with the saturated and the monoene members of each type of carbon skeleton. To determine the minor components and the maximum chain lengths for each series, a large sample of the saturates or the monoenes was injected into the gas chromatograph and run well beyond the retention time of the highest detected member at maximum instrument sensitivity. Note the very wide range in chain length for each series, e.g., C_{14} to C_{38} for the straight even monoenes. Note also that the amounts of the homologues of each series do not show a simple rise and fall of relative amount with increasing chain length but show one or more distinct maxima.

Table II summarizes the data of Table I. About 60% of the chains are straight even, of which nearly 2/3 are monoenes, whereas only 4% are odd, and half of these are monoenes. In contrast the branched chains make up about 1/3 of the total, nearly all are saturated and there is about twice as much iso as anteiso.

Table III gives the ozonolysis data for the straight even monoenes as determined on fractions of a given chain length obtained by preparative GLC. In every case the main aldehydes yielded were C_9, C_7, and C_5, with C_7 always in greatest amount. The aldesters are almost entirely of odd chain length, and suggest a possible mode of extension or degradation of

[2]Tentatively identified (see footnote b Table I).

TABLE I
Weight Per Cent of the Fatty Acids of Rat Skin Surface Lipids[a]

	Straight					Branched			
	Even		Odd		Carbon	Iso[b]		Anteiso[b]	
C atoms in chain	Sat. %	Monoene %	Sat. %	Monoene %	number on JXR[c]	Sat. %	Monoene %	Sat. %	Monoene %
12	.02	ND							
13			.04	ND	12.7			.04	ND
14	.76	.10			13.67	.88	ND		
15			.61	.08	14.72			1.21	ND
16	9.62	1.32			15.65	5.21	.10		
17			.54	.53	16.74			3.23	.08
18	2.62	7.84			17.66	3.04	.10		
19			.11	.26	18.71			1.25	.08
20	1.39	5.53			19.65	4.61	.15		
21			.07	.26	20.71			2.40	.07
22	1.54	4.76			21.64	2.40	.15		
23			.26	.20	22.72			1.51	.04
24	4.91	4.09			23.68	2.75	.10		
25			.41	.26	24.71			1.60	.03
26	1.39	2.53			25.68	1.36	.07		
27			.02	.18	26.71			.38	ND
28	.18	2.86			27.64	.18	ND		
29			.006	.15	28.71			.10	
30	.14	3.41			29.68	.07			
31			.006	.12	30.70			.06	
32	.07	4.46			31.68	.02			
33			.006	.09	32.71			.04	
34	.006	1.77			33.6	ND			
35			ND	ND	34.7			ND	
36	<.006	.20			35.6				
37					36.7				
38	ND	<.006			37.6				
39					38.7				
40		ND							

[a] Does not include hydroxy or other substituted fatty acids. Weight percentages are of the methyl esters determined from areas of peaks from GLC charts (see text). ND indicates the chain length at and above which nothing could be detected when GLC charts were examined at that expected position.

[b] The assignment of the the terms "iso" and "anteiso" are based solely on carbon number data when the sample was run on the nonpolar liquid phase JXR.

[c] Carbon numbers were determined by the method of Woodford & van Ghent (9).

Δ9 chains by C_2 units (last column) as will be discussed later. Table IV gives ozonolysis data for the odd chain length monoenes. This series constitutes about 3 mole per cent of the total monoenes. The straight odd monoenes show many more position isomers than adjacent straight even homologues.

Table V lists the ozonolysis products of a sample of the entire fatty acid monoenes of rat skin surface lipids. Again C_7 aldehyde predominates, with C_9 next, confirming the data of Table III. The even chain aldehydes reflect the contribution of the odd chain length monoenes (Table IV). Traces of what appear to be aldehydes with fractional carbon numbers are also seen. The aldester chains obtained from ozonolysis of the entire fatty acid methyl esters (Table V) were 96% odd, 3% even and about 1% branched. The latter seem to comprise two distinct series. These series of peaks persisted on the GLC charts, when an aldester fraction obtained by preparative TLC of the total ozonized sample was run.

TABLE II
Weight Per Cent of Fatty Acids of Rat Skin Surface Lipids[a]

	Saturated	Monoene	
Straight, even chains	22.6	38.9	
Straight, odd chains	2.1	2.1	
Totals	24.7	41.0	
Total straight chains			65.7
Iso	20.5	0.7	
Anteiso	11.8	.3	
Totals	32.3	1.0	
Total branched chains			33.3
Polyenes (not included above)			1.0
Grand Total			100.0

[a] Data is summarized from Table I.

DISCUSSION

Surface lipids of rat skin comprise a group of fatty acid esters chiefly of mono- and dihydric wax alcohols and sterols (10-13). Most of these are very likely of sebaceous gland origin although components derived from keratinizing epidermal cells and resident bacteria

TABLE III

Position Isomers at Each Even Chain Length of the Rat Skin Surface Lipid Fatty Acid Monoenes (as Methyl Esters) Determined by Reductive Ozonolysis

No. of C atoms in a straight chain	Mole per cent of monoenes[a]	Aldehyde (C atoms in chain)	Aldester (C atoms in chain)	Deduced structure	Per cent at each chain length[b]	Possible mode of extension or degradation of Δ9 chains by C_2 units
14	0.39	7	7	14:Δ7	81	16:Δ9 – C_2
		5	9	14:Δ9	18	14:Δ9 no change
16	4.37	9	7	16:Δ7	4	18:Δ9 – C_2
		7	9	16:Δ9	80	16:Δ9 no change
		5	11	16:Δ11	16	14:Δ9 + C_2
18	23.32	9	9	18:Δ9	36	18:Δ9 no change
		7	11	18:Δ11	54	16:Δ9 + C_2
		5	13	18:Δ13	10	14:Δ9 + 2 C_2
20	14.92	11	9	20:Δ9	2	20:Δ9 no change
		9	11	20:Δ11	10	18:Δ9 + C_2
		7	13	20:Δ13	86	16:Δ9 + 2 C_2
		5	15	20:Δ15	2	14:Δ9 + 3 C_2
22	11.74	11	11	22:Δ11	1	20:Δ9 + C_2
		9	13	22:Δ13	9	18:Δ9 + 2 C_2
		7	15	22:Δ15	88	16:Δ9 + 3 C_2
		5	17	22:Δ17	3	14:Δ9 + 4 C_2
24	9.29	11	13	24:Δ13	1	20:Δ9 + 2 C_2
		9	15	24:Δ15	17	18:Δ9 + 3 C_2
		7	17	24:Δ17	80	16:Δ9 + 4 C_2
		5	19	24:Δ19	3	14:Δ9 + 5 C_2
26	5.33	10	16	26:Δ16	tr	
		9	17	26:Δ17	14	18:Δ9 + 4 C_2
		8	18	26:Δ18	1	
		7	19	26:Δ19	82	16:Δ9 + 5 C_2
		5	21	26:Δ21	3	14:Δ9 + 6 C_2
28	5.62	9	19	28:Δ19	10	18:Δ9 + 5 C_2
		8	20	28:Δ20	tr	
		7	21	28:Δ21	87	16:Δ9 + 6 C_2
		5	23	28:Δ23	3	14:Δ9 + 7 C_2
30	6.28	9	21	30:Δ21	14	18:Δ9 + 6 C_2
		7	23	30:Δ23	82	16:Δ9 + 7 C_2
		5	25	30:Δ25	2	14:Δ9 + 8 C_2
32	7.72	9	23	30:Δ23	13	18:Δ9 + 7 C_2
		7	25	30:Δ25	83	16:Δ9 + 8 C_2
		5	27	30:Δ27	4	14:Δ9 + 9 C_2

[a] The total mole per cent of even chain lengths of this table (88.98%) does not include chain lengths at C_{20}, C_{26} and C_{28} which amount to 3.22% of the total monoenes since the unincluded even chain lengths were not ozonized. The remaining 7.8% of the monoenes are odd and branched.

[b] Determined by the relative amounts of aldehydes or aldester yielded for each chain length.

may also be present to a lesser extent. The monoene fatty acids analyzed in this study could be derived from any of these sources.

Interpretation of the double-bond pattern found for these acids is thus hazardous, not only because of the complex origin of the material, but also because the analysis itself represents a static picture of what must in reality be many dynamic biosynthetic processes. Despite these difficulties, however, certain regularities stand out in the pattern. This applies especially to the monoene acids of even chain length which constitute by far the major group (Table II). As was already pointed out, the even chain monoene fatty acid methyl esters yielded only odd chain aldehydes and odd chain aldesters (Tables III and V). Furthermore, the fact that ozonolysis yielded primarily C_7, C_9, C_{11} and C_{13} aldehydes for all the even chain fatty acids shows that all even chain fatty acids belong to four ω series (Table VI). Both the yield of aldehydes from ozonolysis of the total fatty acid methyl ester sample of monoenes, and the yield of aldehydes determined from a summation of the position isomer data of individually collected fractions present essentially the same quantitative picture as to the amounts of each series, i.e., $\omega 7 >> \omega 9 > \omega 5 > \omega 11$.

The aldester data corroborate this picture and give information about chain extension. If we assume that primary desaturation occurs on any chain at Δ9, we get the chain extension pattern shown in Table VII. Like the aldehydes of Table VI, this pattern was computed from aldester data of the ozonolysis of the total fatty acid methyl esters and from position

TABLE IV

Position Isomers at Each Odd Chain Length of the Rat Skin Surface Lipid Fatty Acid Monoenes (as Methyl Esters) Determined by Reductive Ozonolysis

No. of C atoms in chain	Mole per cent of monoenes[a]	Aldehyde (C atoms in chain)	Aldesters (C atoms in chain)	Deduced structure	Per cent at each chain length[b]
15	0.30	8	7	15:Δ7	2
		7	8	15:Δ8	49
		6	9	15:Δ9	37
		5	10	15:Δ10	12
17	1.66	11	6	17:Δ6	9
		10	7	17:Δ7	1
		9	8	17:Δ8	8
		8	9	17:Δ9	14
		7	10	17:Δ10	23
		6	11	17:Δ11	35
		5	12	17:Δ12	10
19	0.74	11	8	19:Δ8	1
		10	9	19:Δ9	10
		9	10	19:Δ10	6
		8	11	19:Δ11	17
		7	12	19:Δ12	37
		6	13	19:Δ13	28
21	0.67	13	8	21:Δ8	7
		12	9	21:Δ9	10
		11	10	21:Δ10	17
		10	11	21:Δ11	18
		9	12	21:Δ12	5
		8	13	21:Δ13	6
		7	14	21:Δ14	17
		6	15	21:Δ15	10
		5	16	21:Δ16	12

[a] The total mole per cent of odd chain lengths of this table (3.37%) does not include the odd chain lengths from C_{23} to C_{35} inclusive, which amount to 1.86% of the total monoenes since the unincluded odd chain lengths were not ozonized. The remaining 94.77% are even and branched.

[b] Determined by the relative amounts of aldehyde or aldester yielded for each chain length.

TABLE V

Mole Per Cent of Ozonolysis Products Yielded from a Sample of the Total Fatty Acid Monoenes (as Methyl Esters) of Rat Skin Surface Lipids

Aldehydes		Aldesters				
		Straight chain			Branched chain	
No. C atoms in chain	Mole %	No. C atoms in chain	Mole % Odd	Mole % Even	Carbon number on JXR	Mole %
5	2.7	6		.5		
6	3.2	7	.94			
6.6	tr	8		.32		
		9	14.6		9.6	.13
7	78.0	10		.70	10.7	.19
7.7	tr	11	20.2		11.6	.06
8	.91	12		.67	12.7	.16
		13	21.2		13.6	.10
8.75	tr	14		.25	14.7	.12
9	14.5	15	12.3		15.6	.05
		16		.13	16.7	.06
9.8	tr	17	8.7			
10	tr	18		.083		
10.7	tr	19	3.2			
11	.7	20		.01		
		21	3.3			
Total 100%		22		.07		
		23	4.7			
		24		.01		
		25	4.4			
		26		.01		
		27	2.8			
		Subtotal	96.34	2.79		.87
		Total				100%

TABLE VI

Mole Per Cent of Various Series of Even Chain Monoene Fatty Acids of Rat Skin Surface Lipids

Aldehyde	Deduced ω series	Mole per cent aldehydes from GLC total fatty acid sample[a]	Mole per cent aldehydes from position isomer data[b]
C_5	ω5	2.8	5.4
C_7	ω7	80.8	76.2
C_9	ω9	15.4	17.8
C_{11}	ω11	1.0	.6
	Total	100.0	100.0

[a] Data from Table V. Total odd aldehydes from C_5 to C_{11} (actual mole per cent = 96.5) assumed to be 100%.

[b] Computed from Table III. Total odd aldehydes from C_5 to C_{11} (actual mole per cent = 89.0) assumed to be 100%.

isomer data of individually collected fractions. Again, both sets of data are quantitatively in agreement. This shows the internal consistency of the data. It also shows that more chains are extended by 1,2 or 3 C_2 units than to any other length, but that chains can be extended to at least 9 and very likely 11 C_2 units. The possible mode of extension of Δ9 chains to form the various position isomers found, is shown in the last column of Table III. If primary desaturation of any chain is at Δ9, then the only way to explain a C_7 aldester is by degradation of the chain by one C_2 unit. Any degradation of a Δ11, Δ13, . . . Δ27 chain would not be detected from these data.

The presence of very long chain straight and branched saturated acids as well as odd and even monoene acids (Table I), suggests that all the fatty acids are elongated to very long lengths by a common mechanism. Our data on rat skin surface lipid fatty acid chain lengths confirm and extend those of Nikkari and Haahti (11) who report chain lengths up to C_{34} with 26:1 being the last unsaturate. They did not report data on double-bond positions.

Apart from the sphingo lipids, fatty acids of extremely long chain length do not appear to have a widespread occurrence in biological systems. However, chain lengths of C_{26} and C_{27} have been reported for the sphingo lipids and these appear to be derived by extension of C_{20} but not C_{18} or lower chain lengths (14). It is also possible that fatty acids of very long chain lengths have not been generally detected because many GLC studies have employed columns and temperatures where the emergence of such chains would be inordinately long and the peaks so flat that they could scarcely be differentiated from the base line. For the detection of the upper limits of the chain lengths of the rat skin surface lipid fatty acid we injected large samples of fatty acids on an 18 in. o.d. column of 3% JXR and carried out the analysis at maximum sensitivity well beyond where C_{40} would have emerged.

It should not be inferred that only one chain extension pattern is operating. The relative proportion of ω9, ω7 and ω5 for each chain above C_{20} is about 15:83:2 (Table III), whereas the proportion among C_{18} isomers is 36:54:10. This suggests that some additional oleic acid, perhaps in another pool, is formed and does not become further extended. The relative increase of saturated chains in the vicinity of C_{24} could be due to an additional chain extension process similar to the sphingo lipid fatty acids operating simultaneously.

The starting point for the chain extension pattern discussed above was for chain lengths C_{14}, C_{16}, C_{18} and C_{20}, all desaturated at Δ9. Biosynthesis of such chains can be explained by either of 2 schemes (Fig. 2) based on processes known to occur in other biological systems:

Scheme 1 consists of four separate processes: De novo synthesis of even saturated fatty acid chains mainly to C_{16} by the fatty acid synthetase system (15, 16); desaturation at Δ9, possibly by the NADPH+H^+, oxygenase system (17); further extension of the chains from 1 to 11 C_2 units; and degradation of the chains by at least 1 C_2 unit.

Scheme 2 consists essentially of anaerobic

TABLE VII

Extension Pattern of Monoenes of Rat Skin Surface Lipid Fatty Acid (as Methyl Esters)

Aldester	No. C_2 units extended above C_{16}	Mole per cent aldesters from GLC of total fatty acids[a]	Mole per cent aldester from position isomer data[b]
C_7	−1	.9	.6
C_9	0	15.2	13.9
C_{11}	1	21.0	16.8
C_{13}	2	22.0	18.4
C_{15}	3	12.8	13.7
C_{17}	4	9.0	9.5
C_{19}	5	3.3	5.5
C_{21}	6	3.4	6.9
C_{23}	7	4.9	7.2
C_{25}	8	4.6	7.2
C_{27}	9	2.9	.3
C_{29}[c]	10(?)		
C_{31}[c]	11(?)		
	Total	100.0	100.0

[a] Data from Table V. Total aldesters C_7 to C_{27} (actual mole per cent = 96.4) assumed to be 100%.

[b] Computed from Table III. Total aldesters C_7 to C_{27} (actual mole per cent = 89.1) assumed to be 100%.

[c] Assumed to be present since monoene methyl esters of C_{36} and C_{38} were detected.

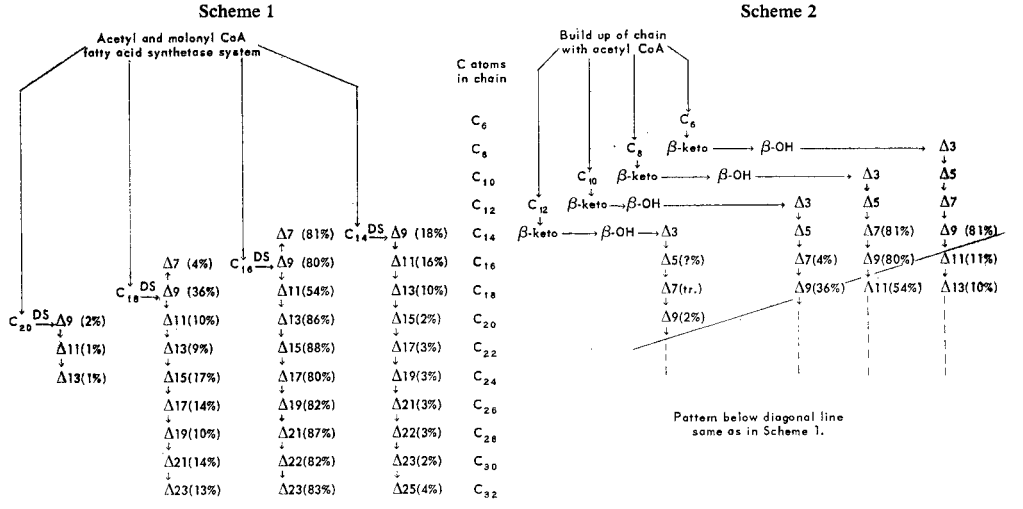

FIG. 2. Two schemes for the biosynthesis of the monoenoic fatty acids of even chain length of rat skin surface lipids. Arrows down (↓) indicate elongation; arrows up (↑) indicate chain degradation; arrows horizontal (→) indicate transformation at a given chain length; DS indicates desaturation (see text). Percentages represent relative amounts of position isomers at each chain length.

synthesis of monoene fatty acids (18) with further chain elongation: De novo synthesis of saturated chains to C_6, C_8, C_{10}, and C_{12}, with C_8, predominating; addition of a C_2 unit to form a β-keto derivative; reduction of the β-keto derivative to the β-hydroxy derivative; dehydration of the β-hydroxy derivative to a *cis* Δ3 unsaturated acid; elongation of the fatty chains by 2 to 3 C_2 units to yield C_{14}, C_{16}, C_{18}, or C_{20}; and further chain elongation from 1 to 11 C_2 units (same as Scheme 1, step 3).

It is noteworthy that 33% of all the fatty acids of rat skin surface lipid are branched chain, of which only 1% is monoene. This would imply that desaturation enzymes react preferentially with straight chain substrates or that branched substrates are not accessible to the enzymes. Unfortunately, branched chain monoeneoic fatty acids occurred in such minute amounts as to preclude structure analysis. Odd straight chain fatty acids on the other hand constitute only 4.2% of the total rat skin surface fatty acids, and half of these are monoene. The large number of position isomers of these acids suggests additional mechanisms are operative as well for the desaturation of these acids. In contrast, human skin surface lipid fatty acid and fatty alcohol monoenes showed a double-bond pattern for the branched and odd chains, very similar to that of the even chains (5). There the double-bond pattern for all types of carbon skeletons seem to fit Scheme 1 except that the desaturation occurs mainly at Δ6, although ~5% of the chains were at Δ9. Indeed, the fatty acids of the diol diesters of vemix caseosa (unpublished) showed patterns for both straight odd and even chains very similar to those of the rat.

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The Incorporation of ^{14}C-Glycerol into Different Species of Diglycerides and Triglycerides in Rat Liver Slices

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ABSTRACT

The relative rates of de novo synthesis of species of diglycerides and triglycerides from ^{14}C-glycerol were examined in rat liver slices. Diglycerides containing one or two double bonds per molecule and triglycerides containing four or more double bonds per molecule represented 70% and 60% respectively of the newly synthesized diglycerides and triglycerides. The newly synthesized triglycerides were more unsaturated than the endogenous triglycerides. Our results suggest that a nonrandom synthesis of species of diglycerides occurred followed by an almost random utilization of the various diglyceride species for the biosynthesis of triglycerides.

INTRODUCTION

In liver 1,2-diglycerides can be considered to serve as a common precursor for the biosynthesis of glyceryl esters triglycerides, lecithin and cephalin. Since the formation of all three types of lipid requires reaction at only the 3 position, the ordered structure of the diglycerides would be expected to remain as a common feature of the product glyceryl esters. Saturated and unsaturated fatty acids are predominantly present at the 1 and 2 positions respectively of triglycerides, lecithin and cephalin (1-3), but differences in fatty acid composition of these types of lipids have been observed. For example, stearic and arachidonic acids, which are major components of rat liver lecithin and cephalin, are minor components in rat liver triglycerides (1, 4). Such structural studies indicate that the distribution of fatty acids in glyceryl esters is not a random process, but rather that mechanisms exist in the cell which control both the positional occurrence and the extent of occurrence of a given fatty acid in different glyceryl esters.

Attempts to demonstrate the reactions which control the location of a given fatty acid have not been overly successful. Experiments testing the specificity for certain diglycerides in the enzymic acylation of diglycerides to produce triglycerides have been reported (5, 6); however, the significance of these studies was obscured by differences in solubility of different diglycerides. Likewise, no appreciable specificity has been observed in the formation of phosphatidic acid by acylation of glycerol-3-phosphate (7) or in the hydrolysis of phosphate from phosphatidic acid (8).

In the present study the de novo synthesis of diglycerides and triglycerides from ^{14}C-glycerol was examined to see if all types of triglyceride were formed at similar rates. Our results indicate that a degree of selectivity occurred in the synthesis of triglycerides by rat liver, and that the de novo synthesis of triglyceride did not lead to a duplication of the pre-existing distribution of molecular species of triglycerides.

EXPERIMENTAL

Incorporation of ^{14}C-Glycerol into Liver Lipids

Male Sprague-Dawley rats (175-240 g) were sacrificed by decapitation. The body cavity was immediately opened, and the liver was perfused with cold Locke's solution (9) devoid of calcium containing 0.027 M trisodium citrate until the liver attained a creamy color. Liver slices were prepared with a Briggs-Stadie microtome.

Liver slices (2 to 3) were incubated in Krebs-Ringer bicarbonate buffer (9) containing one-half the normal amount of calcium with 100 μmoles of ATP, 0.2 mg of reduced coenzyme A and 10 μc of 1-^{14}C-glycerol (10 μc/μm). The final volume of the incubation mixture was 7.5 ml. Incubations were performed at 37C with constant shaking in an atmosphere of 5% carbon dioxide and 95% oxygen. Liver slices were removed at indicated time intervals, rinsed, and immediately frozen between 2 blocks of dry ice.

Extraction of Lipids

After the weight (200-400 mg) of the frozen tissue had been recorded, the sample was homogenized in 5 ml of chloroform-methanol (1:1, v/v) containing 100 μl of a solution of 1% 2,6-di(t-butyl) 4-methylphenol in chlo-

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roform with a Virtis microhomogenizer for 1 min. An additional 2.5 ml of chloroform was added, and the homogenization was continued for approximately 30 seconds. Additional chloroform-methanol (2:1) was added to give a final volume of 20 ml, and the samples were allowed to remain at room temperature for approximately 30 min. The extracts were washed once with 0.2 volumes of 0.58% sodium chloride and once with "pure upper phase" as described by Folch et al. (10). The samples were evaporated to dryness using a rotary evaporator which was flushed with nitrogen, dissolved in chloroform and stored at –15C.

Isolation of Neutral Lipids

Neutral lipids were separated from polar lipids using DEAE-cellulose columns as described by Rouser et al. (11). Diglycerides and triglycerides were isolated by thin-layer chromatography (TLC) using Mallinckrodt silicic acid of less than 200 mesh. The plates were developed 4 cm in diethyl ether-petroleum ether (4:1). After drying for 15 min at room temperature, they were developed in diethyl ether-petroleum ether (12:88) to a distance of 18-20 cm. The lipids were visualized under ultraviolet light after spraying the plate with a solution of 0.2% 2′,7′-dichlorofluorescein in methanol. The bands of triglycerides (R_f = 0.70) and diglycerides (R_f = 0.30) were scraped from the plate and the lipids were eluted with methanol-ether (1:9).

An aliquot of the recovered triglyceride fraction revealed a radiochemical purity of less than 90% when chromatogrammed with carrier diglycerides and triglycerides. Accordingly, the whole triglyceride samples were routinely rechromatographed on thin layer plates as described above; to give a radiochemical purity of greater than 95%.

Similarly, the isolated diglycerides were found to be contaminated with small amounts of cholesterol and an unidentified impurity. Since these contaminants yielded anomalous bands in the subsequent separation of the diglyceride fraction into species, the diglycerides were further purified by TLC in a solvent of chloroform-acetone (96:4) with a layer of boric acid-Adsorbosil-1 (12). Approximately 90% of it was located in the 1,2-diglyceride fraction and more than 95% of the isotope was recovered. The remainder of the radioactivity was found in nearly equal amounts in monoglyceride, 1,3-diglyceride and triglyceride fractions.

Separation of Di- and Triglycerides into Molecular Species

The separation of diglycerides into individual species was achieved using thin layer plates containing 5% (w/w) silver nitrate Adsorbosil-1. Thin layers (0.3 mm) were activated at 125C for 75 min and stored over a saturated solution of calcium chloride in a metal cabinet.

Diglycerides prepared from pig liver lecithin by the action of phospholipase C (13) were added as a carrier to all diglyceride samples to facilitate location of the samples in subsequent steps. The plates were developed in a solvent system composed of diethyl ether-petroleum ether-benzene-methanol (45:35:30:2). After spraying the plates lightly with a solution of 0.1% of 2′,7′-dichlorofluorescein in methanol five discrete bands were visible under ultraviolet light. These bands were found to contain one to five or more double bonds per

TABLE I

Fatty Acid Composition of the Carrier Diglyceride Resolved by Silver Nitrate-TLC

Band[a]	Fatty acid (Mole %) 16:0	18:0	18:1	18:2	18:3	20:3	20:4	22:4	22:6	Band designation
4	20	29	45	5	2	—	—	—	—	
[b]	19	30	44	6	2	—	—	—	—	Monoene
5	13	35	4	49	—	—	—	—	—	
[b]	14	33	7	46	—	—	—	—	—	Diene
7	10	44	4	—	18	25	—	—	—	
[b]	10	42	3	10	17	28	—	—	—	Triene
8	12	43	4	—	1	1	35	5	—	
[b]	13	48	4	—	—	—	36	—	—	Tetraene
9	17	41	3	—	1	1	1	—	28	
[b]	17	41	3	—	1	4	—	—	34	Polyunsaturated
Total	16	35	20	13	3	4	4	1	4	

[a] The TLC-plate was divided into ten bands. Less than 3% of the total fatty acids was located in bands other than those indicated in this table.

[b] The second row represents a separate experiment.

molecule by means of GLC analysis of the methyl esters derived from the diglycerides. The fatty acid composition of these bands is indicated in Table I. Less than 3% of the total diglyceride sample applied to the TLC plate was recovered in areas other than the five bands described in Table I.

The method used to separate the triglyceride fraction of rat liver into its component species has been described in a previous communication from this laboratory (14).

Elution of Di- and Triglycerides from Silver Nitrate-Silicic Acid

The bands of silicic acid that contained the separated glycerides were scraped from the thin layer plates and transferred into small test tubes. A solution of 1% sodium chloride in 90% methanol was added in portions (approximately 0.5 ml) with vigorous mixing until the characteristic red color of the silver-dichlorofluorescein complex was destroyed. The glycerides were extracted with 5 ml of ether-methanol (9:1). After centrifugation, the supernatant solution was transferred to a scintillation vial. The residue was washed two additional times. The combined extracts were evaporated to dryness under a stream of nitrogen with gentle heating.

The triglycerides were counted in 7.0 ml of a scintillation fluid containing 4 g of 2,5-diphenyloxazole and 50 mg of p-bis[5-phenyloxazoyl]-benzene per liter of toluene. Diglycerides were counted in 10.0 ml of scintillation fluid prepared with dioxane (15). The recovery of radioactivity applied to the plate was greater than 90% in either case.

Gas-Liquid Chromatography

These methods have been described in an earlier paper (14).

MATERIALS

All solvents were analytical reagent grade and were used without further purification. The petroleum ether had a boiling point range of 30-60C. DEAE-cellulose (DE-23) was obtained from Reeve Angel Corp.

RESULTS

The time course of incorporation of ^{14}C-glycerol into diglycerides and triglycerides is shown in Fig. 1. The total counts incorporated into neutral lipids increased in a linear fashion for 2-3 hr. Preliminary results showed that optimal yields of lipid soluble ^{14}C-glycerol were obtained when ATP and CoA were added to the incubation medium. In addition, the lower Ca^{++} content led to more radioactivity in the

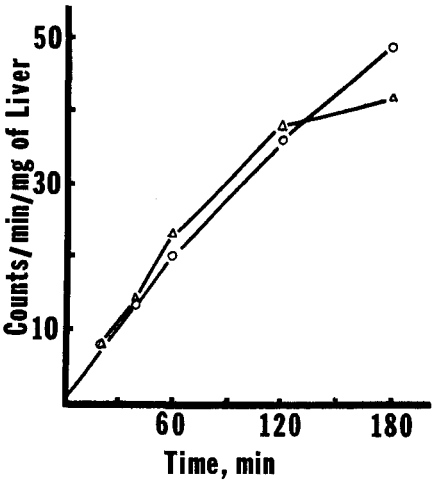

FIG. 1. The incorporation of ^{14}C-glycerol into diglycerides and triglycerides in rat liver slices. ○——○ Diglycerides, △——△ Triglycerides.

neutral lipids. The total counts present in the diglyceride and triglyceride fractions were nearly equal at each time interval measured. Since the quantity of triglyceride in rat liver is much greater than that of diglyceride, the specific activity of the diglyceride pool at a given time interval was much greater than that of the triglyceride pool.

The percentage of distribution of ^{14}C-glycerol among the species of triglycerides at various time intervals is shown in Table II. After 20 min of incubation approximately 70% of the incorporated label was located in three fractions, the SMD, SD_2 and the polyunsaturated species. The other six species indicated contained less than 30% of the incorporated label. The distribution of incorporated glycerol among the species had been established at the first time point measured. This pattern was not altered upon extended incubation even though the total incorporation of ^{14}C-glycerol increased several fold.

The mole percent distribution of triglyceride species of unincubated rat liver and two incubated samples is shown in Table III. The SM_2, SMD and polyunsaturated subfractions of triglycerides were the major components to accumulate ^{14}C-glycerol, and they comprised 16, 24 and 22% respectively of the total mass represented by the triglyceride fraction. The weight distribution of species of triglycerides did not demonstrably change during the course of the incubation.

Comparison of the distribution of ^{14}C-glycerol among species to the weight distribution of species shows that the de novo synthesis of

TABLE II

Incorporation of ^{14}C-glycerol into Species of Triglycerides

	Percentage Distribution of Counts					
	20 min	40 min	60 min	90 min	120 min	180 min
Subfractions[a]	%					
S_3	1	0	1	1	0	1
S_2M	3	2	1	2	3	3
SM_2	8	5	6	6	6	5
S_2D + M_3	8	7	10	8	9	8
SMD	16	15	16	15	16	14
M_2D	7	5	5	5	4	4
SD_2	11	13	15	14	14	12
MD_2	6	7	5	6	5	5
Polyunsaturated[b]	42	46	39	44	44	47

[a] S,M and D are used as abbreviations for saturated, monounsaturated, and diunsaturated fatty acids respectively. The subscript refers to the number of times a given acid appears in the same species of triglyceride.

[b] Polyunsaturated triglycerides represent several slow moving bands which were combined. These triglycerides contain at least one fatty acid containing five or more double bonds or they contain six or more double bonds by virtue of the presence of more than one unsaturated fatty acid.

TABLE III

Distribution of Individual Species of Triglycerides in Rat Liver

Species	Unincubated[a]	20 min[b]	120 min[b]
	Mole %		
S_3	1	1	2
S_2M	6	4	8
SM_2	14	16	20
S_2D + M_3	8	8	8
SMD	26	24	23
M_2D	8	7	4
SD_2	11	10	9
MD_2	5	6	5
Polyunsaturated	22	23	21

[a] The value represents the average of three different triglyceride samples isolated from whole liver. The values are corrected for cross contamination.

[b] A portion of the triglyceride sample from liver slices incubated with ^{14}C-glycerol was separated into species by silver nitrate-TLC. The individual species of triglycerides were converted to methyl esters for analysis by GLC.

triglycerides from glycerol did not produce new molecular species in proportion to their endogenous abundance. Certain species, SM_2 and SMD, incorporated approximately one-half the amount of isotope that would be predicted from their original abundance. Conversely, the polyunsaturated species incorporated approximately twice the amount of isotope that would be predicted from the weight of this subfraction.

In Table IV the distribution of ^{14}C-glycerol among the species of diglycerides is shown. Shorter periods of incubation were examined in this experiment since no alterations in labeling pattern were observed with species of triglycerides from incubations of 20 min or longer. As indicated earlier, the observable bands were due to the molecular species of added diglyceride that had been prepared from pig liver lecithin; however, no appreciable amount of radioactivity was detected in the other areas in which bands were not discernible. In the present experiments, the diglycerides were separated according to the total number of double bonds per molecule. Thus a diglyceride containing 2 monoenoic acids and a diglyceride containing a saturated acid and a dienoic acid were not separated from one another. In contrast to the results with triglycerides, the distribution of ^{14}C-glycerol among the species of diglycerides was predominantly in the more saturated species rather than in the unsaturated species. At all time points examined, approximately 70% of the isotope was found in the monoenoic and dienoic diglycerides. With increasing times of incubation, the isotopic content of the tetraene fraction gradually increased. This percentage increase was compensated by a decrease in the relative radioactivity of the diene fraction. Other species contained a more or less constant percentage of the incorporated ^{14}C-glycerol throughout the course of the incubation.

When the ^{14}C-diglycerides were subjected to methanolysis with sodium methoxide, 2-4% of the recovered counts appeared in the methyl esters regardless of the length of the incubation period. The recovery of counts in methyl esters plus glycerol ranged from 90-93%. Thus,

TABLE IV

The Incorporation of ^{14}C-glycerol into Species of Diglycerides

Number of double bonds	Percentage Distribution of Counts									
	5 min[a]	10 min[a]	15 min[a]	20 min[a]	30 min	40 min[a]	60 min[a]	90 min[a]	120 min	180 min
	%									
1	24	26	24	24	25	23	28	28	27	29
2	51	48	48	46	43	41	40	40	40	42
3	5	5	5	5	5	5	4	4	4	4
4	6	7	8	10	11	14	14	13	12	11
5	10	12	10	10	9	14	10	10	13	11
Origin	3	2	5	5	7	4	4	6	2	3

[a] These values are the average of two separate experiments.

the labeling patterns observed are representative of the incorporation of ^{14}C-glycerol into the glycerol portion rather than the fatty acid of the diglyceride.

DISCUSSION

The present experiments were designed to consider two questions. First, does the biosynthesis of triglycerides from diglycerides involve a random acylation of diglyceride units from a pool of diglycerides or does the synthesis preferentially select certain diglyceride units? Secondly, are fatty acids randomly incorporated into the newly-synthesized species of diglycerides? The pathway by which ^{14}C-glycerol can be incorporated into these pools of lipids is summarized below.

Glycerol
↓
Glycerol-3-phosphate
↓
1,2-Diacylglycerol-3-phosphate
↓
1,2-Diglyceride
↓
Triglyceride

The de novo synthesis of triglycerides from glycerol did not yield the same relative amounts of species which are endogenous to the entire liver. The differences that we observed in the distribution of mass and radioactivity can be attributed to the presence of a separate pool which is rapidly synthesized and does not represent the triglycerides of the whole liver. Stein and Shapiro (16) suggested the presence of two pools of triglyceride, one of which rapidly equilibrates with plasma triglyceride and a second pool which is relatively inert. Other workers (17, 18) have also found that those triglycerides synthesized for secretion into plasma represent a more active pool.

Approximately 70% of the ^{14}C-glycerol incorporated into diglycerides was located in the monoene and diene fractions. On the other hand, approximately 60% of the total radioactivity in triglycerides is present in those species of triglycerides containing four or more double bonds per molecule. Since acylation of the 3 position can introduce further unsaturation into the molecule, it is not surprising that the degree of unsaturation of the product triglyceride is greater than that of the precursor-diglycerides. The unsaturated character of the 3 position of rat liver triglycerides has been emphasized in a recent report from this laboratory (14).

To compare the degree of unsaturation of the newly-formed triglycerides to their precursor diglycerides, we asssumed that the fatty acid composition at the 3 position might be similar to that reported. If the diglyceride units were randomly esterified in a non-correlative manner, we could predict the expected quantities of isotope in the triglyceride species by multiplication of the mole fractional occurrence of a given fatty acid at the 3 position of triglyceride times the percentage distribution of isotope in the newly-synthesized diglycerides. For example, diglyceride species containing 2 double bonds per molecule constituted 46% of the total radioactive diglycerides. This species of diglyceride will lead to the formation of SDX or MMX triglycerides (X denotes the fatty acid esterified to the 3-position). If X is saturated, the triglyceride will contain only two double bonds whereas if X is a monene the triglyceride will contain three double bonds, etc. For simplicity, we considered only the total number of double bonds per molecule. The extent to which each type of diglyceride would be expected to produce the different triglyceride species is shown in Table V.

TABLE V
Predicted Distribution[a] of Species in Newly-Synthesized Triglycerides

Precursor[b] diglyceride	Expected number of double bonds in triglyceride						
	0	1	2	3	4	5	≥6
				%			
Monoene	—	3	9	5	1	1	6
Diene	—	—	6	18	9	1	12
Triene	—	—	—	1	2	1	1
Tetraene	—	—	—	—	1	4	5
Polyunsaturated	—	—	—	—	—	—	15
Predicted	0	3	15	24	13	7	39
Observed[c]	1	3	16	16	18	6	42

[a] The expected (predicted) percentage of isotope in species of triglycerides is estimated by multiplication of the percentage of isotope in a given species of diglyceride times the fractional occurrence of fatty acids at the 3 position of endogenous triglycerides. The composition of the 3 position is 12, 39, 20, 3, 3 and 23% respectively for saturated, monoenoic, dienoic, trienoic, tetraenoic and unidentified acids (14).

[b] These values are taken from Table IV, 20 min.

[c] These values are taken from Table II, 20 min.

For those triglycerides containing 1, 2, 5 and 6 or more double bonds per molecule, the predicted values for radioactivity agree very closely with the observed values. These calculations, however, predict somewhat higher and lower values respectively for those triglycerides containing four and five double bonds per molecule. Similar calculations for other periods of incubations yielded analogous results. The close correlation between the predicted and observed values leads us to believe that very little selectivity occurred in the

utilization of diglyceride species for triglyceride biosynthesis, and that acylation of the diglycerides proceeded with non-correlative specificity as had been previously suggested (14).

The observed distribution of ^{14}C-glycerol among diglyceride units indicates that the newly-synthesized diglycerides were composed of about 25, 45, 5 and 10% respectively of monoenoic, dienoic, trienoic and tetraenoic diglycerides. The total amount of ^{14}C-glycerol in all diglyceride fractions increased several-fold with time. The only significant variations observed in the percentage distribution of isotope among the diglyceride units were a two-fold increase in the tetraenoic fraction and a slight decrease in the relative percent of label found in the diene fraction. The increase in radioactivity of the polyunsaturated diglycerides could arise in part from the hydrolysis at the 1 position of a radioactive triglyceride containing a polyunsaturated fatty acid at the 3 position. This reaction would be expected to contribute greater amounts of isotope to diglycerides with longer times of incubation, and would eventually tend to produce triglycerides with similar acid composition at the 1 and 3 positions. The small percentage of saturated and trienoic diglycerides compared to the large percentage of monoenoic and dienoic diglycerides indicates that the acylation of glycero-3-phosphate was not a random process, but rather that certain diglyceride units were formed in preference to others.

The diglyceride pool in livers of young rats that had been fasted and refed carbohydrate was reported to contain approximately 40, 30 and 20% respectively of monoene, diene plus triene and tetraene diglycerides (19). The content of monoenoic acids was presumably higher than normal due to the increased stearoyl-CoA desaturase activity under these conditions (20, 21). In view of the known increase in monoene diglyceride units and decrease in polyene diglyceride units of glycerolipids evoked by fasting and refeeding (13), our results on the newly synthesized diglycerides would seem to reflect the distribution of mass among diglycerides.

Lecithins may also form diglycerides by the reverse action of CDP-choline:diglyceride choline phosphotransferase, although this reaction does not represent net synthesis of diglyceride. Bjørnstad and Bremer (22) demonstrated a rapid reversibility of the CDP-choline:diglyceride choline phosphotransferase reaction in vivo in rat liver; however, it is not known whether all species of lecithin take part equally in this reversible reaction. Elovson showed that ^{14}C-stearic acid appeared in rat liver phospholipids at a much faster rate than would be predicted from the amount of isotope appearing in the diglycerides, whereas oleic acid derived from stearic acid appeared more rapidly in diglycerides than in phospholipids (19). He suggested that the stearic acid was preferentially incorporated into liver phospholipids via acylation of monoacyl phospholipids rather than by the action of the aminophosphotransferase on the diglyceride unit.

Nevertheless, transfer of ^{14}C-glycerol from phospholipids to triglycerides via diglyceride was expected to be minimal at early time points in these experiments since the labeled phospholipid would be greatly diluted by the large size of the endogenous pool. Thus, the contribution of radioactivity to the diglyceride pool by units derived from choline and ethanolamine phospholipid should be very small compared to that of ^{14}C-diglyceride arising by de novo synthesis.

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Predicting the Positional Distribution of Docosahexaenoic and Docosapentaenoic Acids in Aquatic Animal Triglycerides

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ABSTRACT

The positional distribution of 22:6 and 22:5 in aquatic animal triglycerides can be predicted by simple proportionality equations of the type $y = kx$. The mole % 22:6 at the 1, 2, or 3 position (y) is obtained by multiplying the proportionality constant for that position (k_1, k_2, or k_3) times the mole % 22:6 in the total triglycerides (x). For fish and invertebrate triglycerides, $k_1 = 0.28$, $k_2 = 2.06$, and $k_3 = 0.66$. For marine mammal blubber triglycerides, $k_1 = 0.94$, $k_2 = 0.22$, and $k_3 = 1.84$. The same equations apply to both 22:6 and 22:5.

INTRODUCTION

As EXPERIMENTAL DATA on the triglyceride composition of natural fats has accumulated, numerous workers have sought to define the distribution rules by which Nature assembles fatty acids into triglycerides. Such hypotheses have proven useful in estimating the triglyceride composition of natural fats. Brockerhoff and co-workers (1-5) have pointed out the general tendency of long-chain polyunsaturated fatty acids (22:6, 22:5, and 20:5) to be preferentially esterified at the 2 position in fish and invertebrate triglycerides and at the 3 position in marine mammal triglycerides. Further study has now revealed that the positional distribution of 22:6 and 22:5 in aquatic animal triglycerides can be predicted from the fatty acid composition of the total triglycerides using simple proportionality equations. Suitable equations are reported here for two classes of animals: (a) fish and invertebrates, and (b) marine mammals.

DISCUSSION

Docosahexaenoic Acid

The positional distribution of 22:6 in the triglycerides of 29 fish, invertebrate, and turtle fats has been reported in the literature. These include 3 species of freshwater fish (5), 11 species of marine fish (1, 2, 5, 6), 5 marine invertebrates (1, 5, 7), and a marine turtle (5). When the mole % 22:6 in the 2 position (y_2) of these fats is plotted against the mole % 22:6 in the total triglycerides (x), a linear relationship is observed (Fig. 1). Similar linear plots are also obtained for the 1 and 3 positions. Mathematical equations to describe these relationships can be developed in the following manner. Application of the method of least squares yields the equations: $y_1 = 0.53 + 0.22\ x$, $y_2 = 0.49 + 1.97\ x$, and $y_3 = -0.32 + 0.66\ x$. Since the y-intercept of each of these equations is considerably less than the experimental error in the analytical techniques (5, 8), it is obvious that $y \approx 0$ where $x = 0$ as expected. This produces simple proportionality equations of the type $y = kx$. Proportionality constants can then be chosen so that the sum of the standard errors for y_1, y_2, and y_3 has a minimum value, and so that $y_1 + y_2 + y_3 = 3.00\ x$. The resultant formulas and the standard errors for y and k are shown in Table I. The predicted and found values for several typical species are reported in Table II.

Considering the wide range of animal species

TABLE I

Formulas for Predicting the Positional Distribution of 22:6 in Aquatic Animal Triglycerides

	Position	Equation	Sample size	Standard error of y	Standard error of proportionality constant
Fish & Invertebrates	1	$y_1 = 0.28x$	16	1.07	0.05
	2	$y_2 = 2.06x$	29	2.29	0.09
	3	$y_3 = 0.66x$	16	1.77	0.09
Blubber of Marine Mammals	1	$y_1 = 0.94x$	4	1.82	0.25
	2	$y_2 = 0.22x$	9	0.98	0.11
	3	$y_3 = 1.84x$	4	1.34	0.18

Nomenclature: y_1 = mole % 22:6 at 1 position of triglycerides; y_2 = same for 2 position; y_3 = same for 3 position; x = mole % 22:6 in total triglycerides.

TABLE II
Positional Distribution of 22:6 in Typical Aquatic Animal Triglycerides

Animal	Tissue	Mole % 22:6						
		Total found	1 position Found	1 position Calc.	2 position Found	2 position Calc.	3 position Found	3 position Calc.
Fish and Invertebrates								
Mackerel (5)	body	9.0	2	3	20	19	5	6
Barndoor skate (5)	liver	17.7	5	5	37	36	11	12
Speckled trout (5)	body	3.0	1	1	7	6	1	2
Cod (5)	liver	15.7	3	4	32	32	12	10
Squid (5)	liver	17.3	4	5	38	36	10	11
Lobster (5)	hepato-pancreas	7.7	3	2	15	16	5	5
Scallop (5)	hepato-pancreas	14.0	5	4	29	29	8	9
Marine Mammals								
Sei whale (5)	blubber	8.3	6	8	3	2	16	15
Polar bear (4)	blubber	7.3	7	7	2	2	13	13
Harp seal (5)	blubber	14.7	15	14	3	3	26	27
Harbor seal (5)	blubber	4.7	3	4	1	1	10	9

surveyed and the varied fatty acid composition of their diets, the positional distribution of 22:6 in fish, invertebrate, and turtle fats shows striking regularity. The calculated amount of 22:6 at each position is within 2% absolute of the experimental value in 55 of the 61 results examined. Only the periwinkle snail (5) and the liver oil from one barndoor skate (2) show a major deviation (> 3% absolute) from the predicted values. A second analysis of the same sample of skate liver oil (5, 9) does show good agreement with predicted values (see Table II), so the deviant lipolysis results (2) are probably erroneous. The deviation of periwinkle triglycerides from the predicted values remains unexplained, although the completely herbivorous diet of this marine snail might possibly be a contributing factor. If the deviant periwinkle and skate results are eliminated from the statistical calculations, then the standard error of y is reduced to 0.95, 1.57, and 1.33 for the 1, 2, and 3 positions respectively.

Marine mammal blubber triglycerides also show a linear relationship between the mole % 22:6 at each position and the amount of 22:6 in the total triglycerides. However, the proportionality constants for marine mammals are different from those found for fish and invertebrates. Data are available on the positional distribution of 22:6 in the blubber fats of 4 whales (2, 5, 6), 4 seals (2, 4, 5), and a polar bear (4). Mathematical analysis of these data in the manner described above yields the formulas and the standard errors for y and k listed in Table I. The standard errors for the proportionality constants in the marine mammal equations are larger than for the fish and invertebrate equations due to the smaller sample sizes. A comparison of typical predicted and found values is presented in Table II. The calculated amount of 22:6 at each position is within 2% absolute of the experimental value in all of the 17 results examined.

Decosapentaenoic Acid

The positional distribution of 22:5 in aquatic animal triglycerides parallels that of 22:6 (1-5), and one would expect the same proportionality

TABLE III
Positional Distribution of 22:5 in Aquatic Animal Triglycerides[a]

Animal	Tissue	Mole % 22:5						
		Total found	1 position Found	1 position Calc.	2 position Found	2 position Calc.	3 position Found	3 position Calc.
Fish								
Barndoor skate (5)	liver	3.3	1	1	7	7	2	2
Barndoor skate (2)	liver	3.0	-	-	7	6	-	-
American eel (2)	muscle	4.0	-	-	6	8	-	-
Marine Mammals								
Whale (6)	blubber	3.9	-	-	1	1	-	-
Whale (6)	blubber	5.2	-	-	< 1	1	-	-
Polar bear (4)	blubber	7.0	6	7	2	2	13	13
Harp seal (5)	blubber	5.3	4	5	1	1	11	10
Harp seal (2)	blubber	3.0	-	-	< 1	1	-	-
Harbor seal (4)	blubber	3.0	2	3	1	1	6	6
Harbor seal (2)	blubber	3.4	-	-	< 1	1	-	-

[a] Only samples containing 3.0% or more 22:5 are listed.

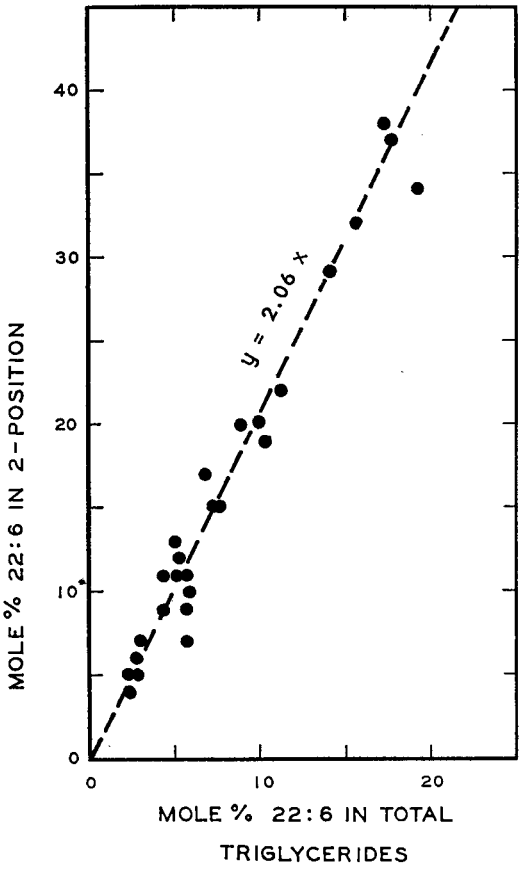

FIG. 1. Relationship between the % 22:6 esterified at the 2 position and the total % 22:6 present in fish and invertebrate triglycerides.

equations to apply to both acids. An empirical comparison shows this to be true (Table III), and the positional distribution of 22:5 can be predicted within 2% absolute in all cases. This must be regarded as a tentative conclusion, however, since the 22:5 content of aquatic animal fats is so low (< 3% in all but 10 samples) that a meaningful statistical evaluation cannot be performed.

The positional distribution of 20:5 in aquatic animal triglycerides resembles that of 22:6, but the similarity is not close enough to allow mathematical prediction. The positional distribution of long chain polyunsaturated acids in other types of animals may differ from the two groups discussed above, but insufficient data are available to draw any conclusions.

Metabolic Implications

If the positional distribution of 22:6 and 22:5 in aquatic animal triglycerides can be predicted by simple proportionality equations of the type $y = kx$, then it seems likely that only two factors are involved in determining this distribution. The proportionality constant k represents the affinity of the acids for esterification at a given position. This affinity might be determined by: (a) the specificity of the acyl transferase associated with each position, (b) the ratio in which 22:6 and 22:5 are compartmentalized between the acylation systems for the 1, 2, and 3 positions, or (c) a combination of (a) and (b). The factor x must represent the amount of 22:6 or 22:5 in the pool of fatty acids available for triglyceride synthesis. The existence of different proportionality equations for the three separate positions lends support to the conclusion of Slakey and Lands (10) that the acylations of the 1, 2, and 3 positions proceed independently of one another.

Attempts to find similar distribution patterns for other fatty acids in animal triglycerides have been unsuccessful. Available evidence indicates that 22:6 and 22:5 are not synthesized de novo in mammals, fish, and invertebrates but are obtained in the diet or are derived from exogenous precursors by chain elongation. Other acids such as palmitic and oleic may be of both exogenous and endogenous origin. Perhaps different rules govern the esterification of exogenous and endogenous acids; and the positional distributions observed for other acids are the combined result of several different pools supplying acyl groups to a single position of the triglyceride molecules.

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Human Serum α-1-Lipoprotein Patterns Revealed by Starch Gel Electrophoresis

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ABSTRACT

The recognition of different serum α-1-lipoproteins patterns was made possible by improved lipoprotein staining and separation methods. Three common and some less common patterns were found. The common α-1-lipoprotein patterns, here called S, M and F, were found to differ in their relative frequency in men and women, and to be associated with significantly different serum α-1-lipoprotein concentrations.

INTRODUCTION

A VARIETY OF ZONE electrophoretic methods have been used for separating human serum lipoproteins (1-6). Various supporting media have been used, including agar gel (1), starch granules (2), starch gel (3) or polyacrylamide gels (4,5). Paper has also been used, and a recent improvement in the paper method (6) has made possible a useful means of classifying some of the dyslipidemias involving primarily disorders of the low density lipoproteins (7).

These methods have been of great aid in uncovering and understanding the polymorphism of many human serum proteins, but none has yet revealed variation among the serum α-1-lipoproteins. This inability may reflect the rarity of molecular variation in the serum α-1-lipoproteins or may be merely technical.

Assuming the latter, attempts were made to alter the starch gel electrophoretic method for better resolution of the serum lipoproteins. This report will describe the progress so far made. It will include a description of a modified oil red O staining technique which produces intense staining of α-1-lipoproteins, and will detail a modification in the usual starch gel electrophoretic method which permits the recognition of the several α-l-lipoprotein patterns. The three most common patterns found will be demonstrated, and the results of some initial studies into the basis of these pattern differences will be mentioned.

METHODS

Subjects

A total of 231 unrelated fasting healthy Caucasian persons (120 males and 111 females) were studied. Their ages ranged from 16 to 80 years, with a mean age of 45 years. All were without known dyslipidemia. They were members of an executive health program, blood donors or persons examined and found in good health.

Serum Preparation

Venous blood was collected from all subjects before breakfast after an overnight fast and allowed to clot for at least 1 hr. Serum was separated from the clotted blood by centrifugation for 20 min at 2,500 rpm. For optimal results, serum samples were kept at refrigerator temperatures and studied within 8 hr of collection.

Electrophoresis

A 12% starch gel was made with a tris-borate-versene (TBV) buffer (8). The hydrolyzed starch used was obtained from the Connaught Medical Research Laboratories, Toronto, Canada. Only half the lots of this starch proved satisfactory for α-1-lipoprotein separations. The stock TBV buffer solution contained trishydroxyaminomethane (Tris) 0.9 M; boric acid, 0.5 M; and the versene, disodium ethylenediaminetetraacetate (Na_2EDTA), 0.02 M. The buffer solution used for preparing the starch gel contained 1 part of the stock TBV buffer solution and 19 parts of deionized water; it had a final boric acid concentration of 0.025 M and a pH of 8.5. The anodal bridge chamber solution contained Tris 0.54 M, boric acid 0.3 M and Na_2EDTA 0.012 M; and the anodal electrode chamber contained a 10% sodium chloride solution. Both the cathodal bridge and electrode chambers were filled with a borate buffer, containing 0.3 M boric acid and 0.06 M sodium hydroxide. Only these buffer solutions were used in the studies reported here.

With the TBV, or other buffers, lipoprotein "trailing" was always conspicious, suggesting either lipoprotein deterioration or interaction with the starch during the period of electrophoresis. Attempts to reduce lipoprotein de-

terioration by adding a variety of enzyme inhibitors to the gel, and to reduce lipoprotein-starch interaction by using starch extracted with fat solvents did not significantly reduce lipoprotein trailing.

To reduce possible starch leaching of lipoprotein lipid during electrophoresis, lipophilic sites in the starch gel were saturated with various fatty acids. The addition of 50 mg of lauric acid per 600 ml of the starch mixture (4.1×10^{-4} M) provided a striking improvement in lipoprotein resolution, particularly the α-1-lipoprotein. Remarkably, other lauric acid concentrations (from 0.5×10^{-4} to 1.2×10^{-3} M), as well as 0.5 to 1.0×10^{-4} M concentrations of even numbered chains of saturated fatty acids of the homologous series from C_8 to C_{18} provided conspicuously inferior lipoprotein resolution. The lauric acid was dissolved in hot TBV buffer, tranferred quantitatively to the molten starch mixture, which was then vigorously mixed before degassing, pouring and molding the gel. It was the lauric acid addition in appropriate concentration which provided the necessary technical improvement which made possible the subsequent study of whole serum lipoproteins.

Optimal separations occurred when each slot contained 50 μl of serum, and the number of samples studied in a single gel did not exceed eight. After gelation of the starch mixture at room temperature, the gel was allowed to cool in the refrigerator for about 2 hr before the samples were added to the sample slots and the gel covered with petrolatum. Electrophoresis was conducted at 4C, for 22 hr, at 130v, measured across the gel. Gel slicing was performed as previously described (9).

Protein Staining

The top slice of each starch gel was surface stained for 1 min with an aqueous solution of amido black (1 g/100 ml of 2% acetic acid) (9), then rinsed thoroughly with water, and destained for many hours with a mixture of ethanol-water-acetic acid (50:50:10, v/v/v). Occasionally, a bottom slice was stained by totally immersing it in the above dye for 15 min; after water rinsing, it was destained in 2% acetic acid.

Lipid Staining

The bottom slice of the starch gel was stained with oil red 0. To visualize the α-1-lipoprotein, it was necessary to improve the existing lipoprotein staining methods. The method developed provided immediate precipitation of all the proteins within the gel, selective adsorption of the dye by lipid without significant nonspecific protein or background staining, and intense staining. The dye was prepared as follows: A mixture of 2 g of oil red 0 in 1 l of CH_3OH (technical grade) was heated with constant stirring to a slow boil, filtered while hot, and allowed to cool. Equal volumes (150 ml) of this saturated oil red 0 solution and a 50% solution of trichloracetic acid (500 g trichloracetic acid made to 1 l with deionized water containing 1% $AlCl_3$) were mixed just before use, and then poured into a plastic staining chamber to completely cover the bottom slice of the gel. The closed chamber was incubated for 16 hr at 37C. Staining at room temperature was inadequate.

Rubber gloves were always used when handling the gel. The gel was removed from the staining solution, and rinsed briefly under a gentle stream of water. Additional destaining was unnecessary. The rinsed gel was then placed on a piece of glass and photographed as described below. Despite total immersion of the dye mixture only the upper surface of the gel stained well. This necessitated the use of reflected, rather than transmitted light, for photographic purposes.

The high trichloracetic acid concentration was necessary to maintain a relatively high oil red 0 concentration in solution when the methanolic solution of the dye was mixed with water. The precipitation of some dye on mixing the methanolic and aqueous solutions did not preclude intense staining. The addition of aluminum chloride to the stain seemed to reduce the minimal background staining of the starch gel and to act as a mordant.

Photography

Photographic reproductions were made with the Polaroid MP-3 industrial camera using type 55 P/N or type 52 polaroid film, and with 35 mm Kodak Plus X pan or high-contrast copy film.

A green filter (Kodak Wratten #40) was used when photographing the lipoproteins with these films. This filter was chosen as a result of a spectrophotometric scan of oil red 0 in heptane. Three absorption peaks were noted as 242, 355 and 512 mμ. Of the peaks in the visible range the 512 mμ peak was 1.6 times that of the 355 mμ peak. The use of a green filter with a 512 mμ band pass markedly improved the quality of the black and white photographic reproductions.

The gel slices stained for protein were photographed using a red filter (Tiffen, Photar Red I).

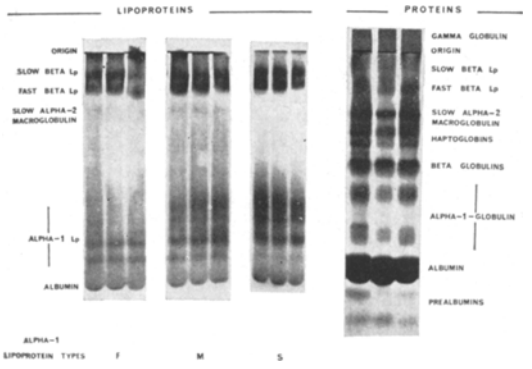

FIG. 1. Oil red 0 staining components in serum after electrophoresis in a lauric acid starch gel. At the right, three examples of the amido black staining of the serum proteins are displayed for comparison. The gamma globulin has been retouched. At the left, examples of the oil red 0 staining of the serum lipoproteins may be seen. All oil red 0 staining components migrate anodally. Three major regions of staining are identifiable: one near the origin, representing the two β lipoprotein components; a second in the post-albumin region, representing the multiple α-1-lipoproteins; and a third, representing the staining of albumin. A minor staining of the slow α_2 macroglobulin is also seen. Nine different serum lipoprotein samples are displayed. Three examples of each of the three major patterns of α-1-lipoproteins (F, M, and S) which have been discriminated by the methods described here are demonstrated.

Color photographs of each gel were made with 35 mm Kodachrome II film.

Lipoprotein Isolations

Lipoprotein fractions of densities < 1.006, 1.006-1.019 (VLDL), 1.019-1.063 (LDL), 1.063-1.21 (HDL) and > 1.21 were separated by flotational methods described elsewhere (10) using a 30.2 rotor in the Spinco Model L preparative ultracentrifuge. Each fraction was purified by reflotation, accomplished by carefully overlaying the fraction with 8 volumes of salt solution of the density used for the original isolation, and spinning at 30,000 rpm for an additional 24 hr. Each fraction was desalted by dialyzing the lipoprotein fractions versus the TBV buffer. To establish the relationships between the lipoproteins in whole serum and those separated by ultracentrifugal flotation, the electrophoretic migrations of the five dialyzed fractions and those of the lipoproteins in whole serum were simultaneously compared.

Finally, to determine the quantitative significance of the different α-1-lipoprotein patterns, the HDL fractions were isolated from 64 different serums, which had been previously characterized as to their α-1-lipoprotein pattern by electrophoresis, and each HDL fraction was analyzed for its content of protein (11), cholesterol (10), phospholipid (12) and triglyceride (13).

RESULTS

Oil Red 0 Staining Components in Serum

In Fig. 1, the oil red 0 staining components in 9 different sera are shown. Three regions are stained by the oil red 0 dye: the region between the origin and the slow α_2 macroglobulin; the α_1 globulin (post-albumin) region; and albumin. The first lipid-staining region contains two bands called the fast and slow β lipoproteins, and as will be shown, these correspond to the LDL, and VLDL, respectively. The post-albumin α_1 globulin area contains four or five well defined lipoprotein bands and these are called α-1-lipoprotein, and as also will be shown, these correspond to the HDL. Albumin also stains and, interestingly, its front stains intensely. Very faint staining of the α_2 macroglobulin is not uncommonly seen.

Reproducibility of the Electrophoresis and Staining

This was studied in several ways. Duplicate samples of sera were compared in the same gel. In 15 of 109 such comparisons the patterns were dissimilar; in all of these 15 comparisons, one of the pair had been placed in an outer lane of the gel. Thus, samples run in outer lanes are not included in the α-1-lipoprotein studies described below.

The effect of serum storage at 4C on the reproducibility of the electrophoretic pattern was also determined by repeating the serum electrophoresis daily for up to 4 days. Four effects of aging were noted: the preciseness in the band separations was lost; the migration rates of the bands changed; some bands were lost; and new bands appeared. Therefore, to avoid these effects, sera were studied *on the day of blood withdrawal.* These alterations presumably reflect changes in lipoprotein structure and it is interesting that they can be induced by aging serum and that these differences are revealed by this method.

The Electrophoresis of Serum Lipoproteins Separated by Ultracentrifugal Flotation

The electrophoretic appearance and migration of the serum lipoprotein fractions, isolated by ultracentrifugal flotation, were compared with those seen in fresh whole serum and in whole

TABLE I
Alpha-1-Lipoprotein Patterns in Healthy Persons

	Number	% Common			% Uncommon		
		F	M	S	F-M	M-S	U
Women	111	0	38	52	6	2	2
Men	120	18	42	16	8	8	8

F-M and M-S indicate that the patterns seen could not be classified as either F or M, or M or S, respectively. U refers to patterns not fitting any of the three common categories.

serum which had been exposed to salt and dialyzed. The correspondence of oil red 0 staining components in whole serum with isolated lipoprotein fractions is diagramed in Fig. 2. As can be seen in Fig. 2, chylomicra and other lipoproteins of density < 1.006 which float when centrifugated at 27,000 g for 1 hr do not enter the gel. Lipoproteins of density 1.006-1.019 (VLDL) enter the gel very slowly and are seen as an ill-defined band emanating from the origin; these probably correspond to the slow β lipoproteins seen in the whole serum separation, and are probably altered somewhat during the period required for their isolation. Lipoproteins of density 1.019-1.063 (LDL) enter the gel as a rather sharp band and migrate as does the fast β lipoprotein seen in whole serum. Lipoproteins of density 1.063-1.21 (HDL) migrate in the post-albumin region and correspond to the α-1-lipoproteins. After salt exposure their migration is slower and the pattern, though very similar, shows some of the changes attributable to aging alone. The fraction of density > 1.21 contains the remainer of the serum proteins, including albumin; only the albumin stained for lipid, presumably reflecting its fatty acid content (14). The rate of migration of a molecule within a starch gel is determined to a major degree by its molecular size (15); the rates of migration of the ultracentrifugal isolates in the starch gel are appropriate with what is known of the molecular size of each (16).

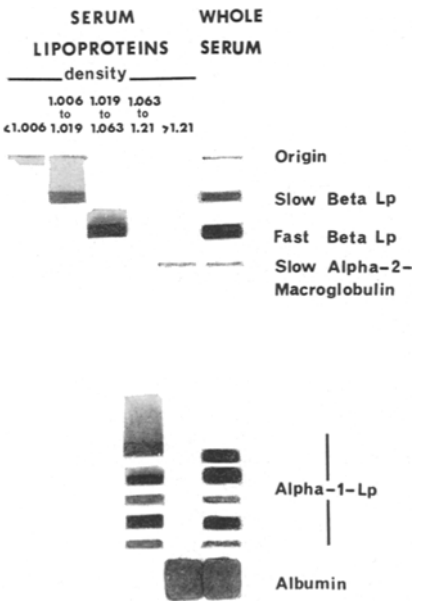

FIG. 2. The identification of the oil red 0 components in serum separated by starch gel electrophoresis. This drawing is a schematic representation of the migration of five lipoprotein fractions isolated by ultracentrifugal flotational methods. Each fraction, but one, corresponds with one of the oil red 0 staining components seen in whole serum. The lipoproteins of density < 1.006 do not enter the gel; the slow β lipoproteins correspond best with lipoproteins of density 1.006 to 1.019; the fast β component with those of density 1.019 to 1.063; and the α-1-lipoproteins with those of density 1.063 to 1.21. The fraction of density > 1.21 contains albumin and the slow α_2 macroglobulins. This drawing is based on the results of 30 separate serum fractionations and electrophoretic studies of these fractions.

α-1-Lipoprotein Patterns

Although several patterns of fast and slow β lipoproteins were discernible, they were more difficult to group than were the α-1-lipoproteins. Therefore, only the patterns displayed by the latter will be described in detail. Three common α-1-lipoprotein patterns were distinguished. These are shown in Fig. 1, and are called α-1-F. α-1-M and α-1-S. The F, M and S allude to intensity of oil red 0 staining, faint (F), moderate (M) and strong (S); in addition, F has 4 bands and others, 5, although the two slowest migrating fourth and fifth bands are not always well separated in the M and F patterns.

The reproducibility of the α-1-lipoprotein pattern found in a given person was determined. Ten persons were studied, on the average, once every 8 weeks for 24 weeks. Of the 10 studied eight had reproducible patterns in 22 consecutive tests; in two individuals one studied twice and the other, six times, two different patterns were seen: an S pattern changed to a pattern not yet named, and an F pattern changed to an M.

The three common patterns, F, M and S were seen in both sexes but a sex difference was readily apparent. In women the S pattern was most common, the M less so, and the F was rare. In men the M pattern was most com-

mon, but both the S and F patterns were frequently seen. The distribution of these three patterns in 120 men and 111 women are tabulated in Table 1.

HDL Protein and Lipid and Their Relation to the α-1-Lipoprotein Pattern

The protein and lipid contents of 64 different HDL fractions were determined. These fractions were obtained from 14 serums showing the S pattern, 36 showing the M pattern, and 14 the F pattern. Each group of α-1-lipoprotein patterns (S, M and F) was associated with significantly different mean HDL lipid and protein concentrations. Highest concentrations were found in HDL fractions isolated from serum showing the S pattern; the lowest mean concentrations were found in those with the F pattern; and the mean concentrations were intermediate for those with the M pattern. Though concentration differences were significant, the relative lipid and protein composition of the HDL for each pattern was comparable. Thus, the three common patterns (S, M and F) appear to reflect α-1-lipoprotein concentration differences, but qualitative differences in the protein component of the lipoproteins have not been excluded.

COMMENT

The method of separating the serum lipoproteins described here depended on the development of an improved oil red O stain, the addition of a specific fatty acid in an exact concentration to the starch, and on the performance of the serum lipoprotein electrophoretic study on the day of the serum collection. This method of lipoprotein staining provides intense lipoprotein staining and avoids nonspecific protein staining; also, by virtue of little background staining, it avoids the inconvenience of destaining. The improved α-1-lipoprotein resolution provided by the addition of lauric acid to the gel, suggests that similar approaches may improve the resolution of the β lipoproteins.

These developments, the reproducibility of the method, the reasonably clear correlations of the migration of isolated serum lipoprotein fractions and whole serum lipoproteins, may prove to have some general usefulness. An immediate contribution is the delineation of differences within the class of human serum α-1-lipoproteins. The disc electrophoretic system of Narajan and Narajan (4) has shown a similar multicomponent aspect of the α-1-lipoproteins but differences between persons were not seen. The present method has permitted the delineation of three common human serum α-1-lipoprotein patterns (F M and S) in 83% of 231 men and women. The basis of the differences in the common pattern appears at the moment to be merely concentration differences in the serum α-1-(high density) lipoproteins. As has been reported in preliminary form, however, the methods described here give promise of delineating genetically determined variants of α-1-lipoproteins (17).

This methodology has also provided insights into the alterations which occur in the high density lipoproteins, in addition to those already described by Levy et al. (18). The observed lability of this fraction suggests that presently available descriptions of the physical and chemical characteristics of the intact lipoproteins are probably only approximately correct.

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Structure and Intraglyceride Distribution of Coriolic Acid[1]

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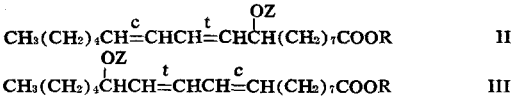
ABSTRACT

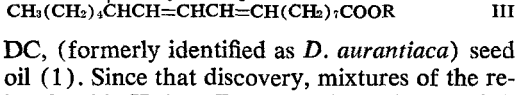
Coriolic [(*R*)-13-hydroxy-*cis*-9, *trans*-11-octadecadienoic] acid (III, R=Z=H) was isolated as the methyl ester from two *Coriaria* seed oils in 66 and 68% yields. The double bonds and hydroxyl group were located by periodate-permanganate oxidation before, and chromic acid oxidation after, hydrogenation of the double bonds. Alternatively the positions of the functional groups were indicated by a convenient micro-ozonolysis-gas-liquid chromatographic procedure. Determination of products from partial hydrolysis of the *Coriaria* oils with pancreatic lipase (EC 3.1.1.3) revealed a preference of the corioloyl group for the 1,3-positions in triglyceride molecules. The possible significance of coriolic acid as an intermediate in the biogenetic conversion of linoleic acid to conjugated trienoic acids is discussed.

INTRODUCTION

INTEREST IN HYDROXYDIENOID fatty acids from plant lipids was initiated by the discovery of dimorphecolic [(*S*)-9-hydroxy-*trans*-10,*trans*-12-octadecadienoic] acid (I, R = H), the major fatty acid in *Dimorphotheca sinuata*



I



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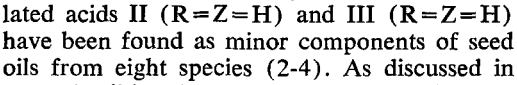

III

DC, (formerly identified as *D. aurantiaca*) seed oil (1). Since that discovery, mixtures of the related acids II (R=Z=H) and III (R=Z=H) have been found as minor components of seed oils from eight species (2-4). As discussed in more detail in a later section, both II and III are logical key intermediates in the biogenetic conversion of linoleic acid to conjugated trienoic acids. In view of the general specificity of enzymatic reactions for products as well as substrates however, such biogenetic involvement of the hydroxydienoid acids is best corroborated if they occur separately and in optically active form. Provided their assignment of double-bond geometry based on biogenetic considerations is correct, the acid isolated by Badami and Morris as the methyl ester in small yield from *Calendula officinalis* L. seed oil and characterized as (*S*)-9-hydroxy-10,12-octadecadienoic acid meets these conditions of separate occurrence and optical activity for II (5). Support for the biogenetic involvement of III is given in the present report.

Part of this work has been described briefly in a preliminary communication (6), in which we reported evidence establishing Structure III (R=Z=H) for the major fatty acid of *Coriaria nepalensis* Wall. (family Coriariaceae) seed oil and proposed the name coriolic acid. Subsequently, Serck-Hanssen (7) supported Structure III and showed a thin-layer chromatogram suggesting the presence of corioloyl groups in several other seed oils.

EXPERIMENTAL SECTION

Seed cleaning, grinding, and extraction were performed as described by Earle et al. (8). Hydrobromic acid titrations were conducted according to the procedure described by Harris et al. (9) except that our samples were only one-tenth as large. We have observed that oils containing I, II plus III, or III rapidly consume hydrobromic acid when titrated at 55C, but there is no significant reaction at 3C.

Infrared (IR) spectra were obtained for thin films of oils or methyl esters between sodium chloride plates with a Perkin-Elmer Infracord Model 137 spectrophotometer. For quantitative *cis,trans* conjugated diene determination (10), carbon disulfide served as the solvent in a 1-mm sodium chloride cell used with a Perkin-Elmer Infracord Model 337 spectrophotometer. Ultraviolet (UV) spectra were recorded for methanolic solutions with a Beckman DK-2A spectrophotometer. Thin-layer chromatography (TLC) was carried out on 20 × 20 cm glass plates, coated to a thickness of 250 μ with Silica Gel G. The chromatograms were developed by capillary ascent of *n*-hexane-diethyl ether-acetic acid (70:30:1), and the spots were made visible with iodine vapor. Unless otherwise specified, gas-liquid chromatography (GLC) was isothermal and was accomplished with a Resoflex 446 column according to con-

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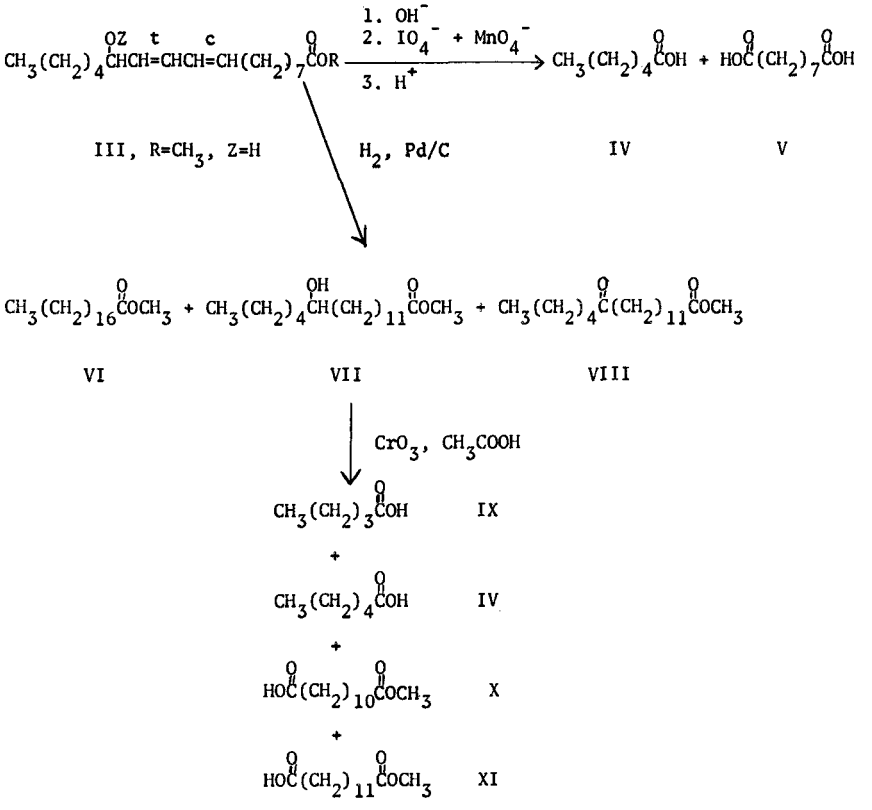

Fig. 1

ditions detailed in earlier publications (11). Melting points were determined on a Kofler micro hot stage.

Hydrogenations were conducted at atmospheric pressure and room temperature with 10% Pd/C catalyst, and the chromatographic procedure used to separate the three products from each pure hydroxydienoid methyl ester has been reported by Dolev et al. (12).

The *C. nepalensis* Wall. mixed methyl esters were prepared by treating the seed oil with sodium methoxide in methanol at room temperature for 4 hr (13). Methyl coriolate (III, R = CH_3, Z = H) was isolated from the mixed methyl esters by countercurrent distribution (CCD) in a 200-tube Craig-Post apparatus with preequilibrated *n*-hexane-acetonitrile, 40 ml each of upper and lower phases. Methyl coriolate was saponified under nitrogen in 1N potassium hydroxide in refluxing 95% ethanol for 30 min. The procedure of von Rudloff (14) was followed for periodate-permanganate oxidation. Oxidation with chromic acid was effected under conditions previously described (15).

The *Coriaria myrtifolia* L. seed oil was subjected to methanolysis under conditions described by Marinetti (16) for 30 min, and the resulting mixed esters were chromatographed on a silica gel column (12). The nonoxygenated esters were eluted rapidly with *n*-hexane-diethyl ether (9:1). The solvent ratio was then changed to 7:3 to elute quickly the easily isomerized methyl coriolate, which was off the column after less than 2 hr in contact with the adsorbent.

The microhydrogenation-GLC procedure of Beroza and Sarmiento (17) was followed. The micro-ozonolysis-GLC procedure was modified from that of Stein and Nicolaides (18). In our laboratory, samples are taken at several intervals during ozonization, reduced with triphenylphosphine, and analyzed by GLC to reveal early formation and subsequent disappearance of intermediates (19).

Partial hydrolysis of *C. nepalensis* seed oil, catalyzed with pancreatic lipase (EC 3.1.1.3), was accomplished by the procedure of Mattson and Volpenhein (20). The resulting lipolysate was treated with diazomethane (21) to convert

TABLE I

Products of Catalytic Hydrogenation of Methyl Dimorphecolate (I, R=CH_3)[a] and Methyl Coriolate (III, R=CH_3, Z=H)[b]

Methyl ester	Percentage of total product	ECL[c]	Melting point		
			Reported[d]	Found	Mixed
Stearate (VI) from I (R=CH_3)	16	18.3	.....	.....	.....
Stearate (VI) from III (R=CH_3, Z=H)	1	18.0	.....	.....	.....
9-Hydroxystearate	63	24.2	50.3–50.6	54–55	48–57
13-Hydroxystearate (VII)[e]	80	24.4	52.0–53.5	56–57	
9-Ketostearate	21	23.5	47.5–48.0	49–50	42–50
13-Ketostearate (VIII)[f]	19	23.6	47.3–47.7	49–50	

[a] In 95% ethanol.

[b] In *n*-hexane.

[c] Equivalent chain-length; see Reference 11.

[d] See Reference 24. Reported melting points for the hydroxy esters were determined with *dl* mixtures.

[e] Found: C, 73.0; H, 12.4. $C_{19}H_{38}O_3$ requires: C, 72.6; H, 12.2.

[f] Found: C, 72.6; H, 12.1. $C_{19}H_{36}O_3$ requires: C, 73.0; H, 11.6.

free acids to methyl esters and then hydrogenated in ethanol. Silylation and temperature-programmed GLC were carried out as previously described (22). More recently developed procedures were used for lipolysis of *C. myrtifolia* seed oil and analysis of the products by silylation and temperature-programmed GLC (23).

RESULTS AND DISCUSSION

Isolation and Characterization of Methyl Coriolate (III, R = CH_3 Z = H). Absorptivity of *C. nepalensis* seed oil at 233 mμ was 62.1. From this value and the molar absorptivity of subsequently isolated methyl coriolate (27,063), a corioloyl group content of 71% was calculated. Hydrobromic acid titration at 55C indicated 69% of corioloyl groups in the oil vs. less than 1% when the titration was conducted at 3C. After CCD of the mixed methyl esters from the oil for 600 transfers, the non-oxygenated esters had been removed in upper-phase fractions singly withdrawn from the last tube of the apparatus; and the methyl coriolate was found on a plot of weight vs. tube number as a symmetrical peak between tubes 90 and 140. The yield of the compound from these tubes was 66% of the sample subjected to CCD.

Important spectral properties of the methyl coriolate have already been reported (6). Its *cis,trans* conjugated diene content, determined by the method of Chipault and Hawkins (10), was 95%. Double bonds and the hydroxyl group were located by oxidative degradation before and after hydrogenation as shown in Figure 1. Compounds IV and IX were identified as such by GLC; V, X, and XI were identified by GLC after treatment with diazomethane.

The isomeric 9-hydroxy-10,12-diene (II, R=CH_3, Z=H) would have given the same products on periodate-permanganate oxidation but, as shown in Table I, the melting points of VII and VIII were depressed by admixture respectively with authentic methyl 9-hydroxystearate and methyl 9-ketostearate. Moreover chromic acid oxidation of the 9-hydroxystearate would have given as major products nonanoic and decanoic acids, no trace of which were detected after oxidative cleavage of VII.

Stereochemistry at C-13 and the geometry of double bonds in methyl coriolate were determined respectively by ORD and NMR data presented previously (6). Since then the geometry of the double bonds has been confirmed chemically (25).

Absorptivity of *C. myrtifolia* seed oil at 233 mμ was 64.5, indicating a corioloyl content of 74%; hydrobromic acid titration showed 72% at 55C and 2% at 3C. Isolation by column chromatography gave a 68% yield of methyl coriolate. Its UV and IR spectra were identical with those of methyl coriolate from *C. nepalensis*, but these spectra do not distinguish between II and III (R=CH_3, Z=H) (25). Better evidence in support of the identity of the hydroxy-dienoid esters from the two *Coriaria* oils was provided by TLC. Samples of methyl coriolate from both sources gave single spots with the same R_f. Under the conditions employed, TLC of the mixture of II and III (R=CH_3, Z=H) from *Xeranthemum annuum* seed oil (4,25) produced two spots with respective R_f values

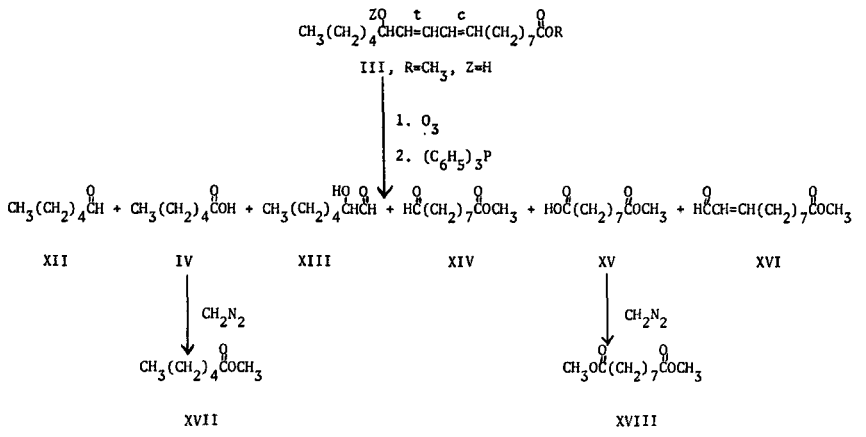

Fig. 2

slightly lower than and equal to that of methyl coriolate.

Microhydrogenation-GLC of methyl coriolate from *C. myrtifolia* gave peaks with the same ECL values as VI-VIII, which verify chain length and the presence of the hydroxyl group. The positions of the functional groups were confirmed by micro-ozonolysis-GLC. Substances identified are shown in Figure 2 and include XIII and the transient XVI as distinguishing products. Data in Table II are for the ester from *C. myrtifolia*, but the one from *C. nepalensis* gave essentially the same results. On the other hand, methyl dimorphecolate (I, R=CH_3), which served as a model of the alternative 9-hydroxy-10,12-diene structure, gave significantly different results both qualitatively and quantitatively.

Intraglyceride Distribution of Corioloyl Groups. The structures of many new fatty acids from unusual seed oils have been determined (26). We have carried one step farther the chemical characterization of selected examples of the parent oils by investigating distribution between the 2- and the 1,3-positions on glycerol of the new fatty acyl moieties in them. Such investigations have shown that nonconjugated unsaturated 18-carbon fatty acids with oxygen-containing substituents usually show preference for the secondary hydroxyl group of glycerol (22,23,27). The corioloyl group has now been found to be much more concen-

TABLE II

Micro-ozonolysis-GLC of Methyl Coriolate

Compound[a]	Found ECL[b]		Predicted ECL[c]		Percentage in products[d]		
	ApL	R-446	ApL	R-446	15 sec	100 sec	100 sec + CH_2N_2
XII	5.5	5.5	5.2	5.2	7	15	10
IV	6.7	11.8	6.8	11.9	0	3	....
XVII	6.0	6.0	6.0	6.0	....	....	2
XIII	7.5	10.6	7.4	10.4	20	10	7
XIV	10.8	15.1	10.8	15.0	30	48	43
XVIII	11.8	15.8	11.7	16.0	....	....	6
XVI	12.8	17.1	12.9	17.4	5	0	0

[a] Compound XV shown in Figure 2 was not eluted under the conditions employed. Presence of XVIII in the methyl esters (last column) shows that some XV was present in the 100-sec product.

[b] See References 11 and 19. Since temperature-programmed GLC was used, ECL values were determined from a linear relationship between retention time and chain length rather than the semilogarithmic relationship which holds under isothermal conditions. ApL = Apiezon L; R-446 = Resoflex 446.

[c] Empirically determined effects of α-hydroxyl groups and α, β-unsaturation were used to predict ECL values for XIII and XVI. Reference samples were available to determine ECL values for the other compounds listed.

[d] Quantitative data from R-446 chromatograms. Unidentified peaks amounted to 38% at 15 sec, 24% at 100 sec, and 32% after methylation of the 100 sec product. Presumably among the unidentified peaks at 15 sec was one representing 4-hydroxy-2-nonenal, for which there was insufficient information to predict an ECL.

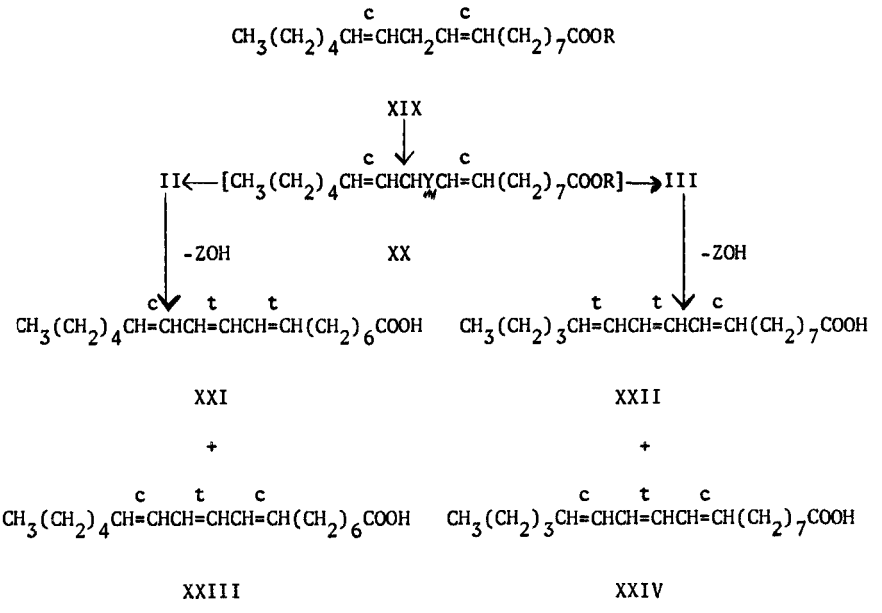

Fig. 3

trated in the 1- and 3-positions than in the 2-position in *C. nepalensis* and *C. myrtifolia* seed triglycerides.

When a triglyceride is subjected to pancreatic lipase (EC 3.1.1.3)-catalyzed partial hydrolysis, the acyl moieties attached to the primary hydroxyl groups are selectively removed (28). The monoglycerides left in the resulting mixture are, therefore, glyceryl esters of acids esterified to the 2-positions in the original substrate. Our approach to determination of intraglyceride distribution involves conversion of all lipolysate components into derivatives that are sufficiently volatile to permit analysis by GLC. In one procedure free fatty acids are converted to methyl esters by treatment of the total lipolysate with diazomethane. All free hydroxyl groups are then silylated with a mixture of hexamethyldisilazane and trimethylchlorosilane in pyridine (22). Since amine hydrochlorides, formed as by-products from the silylating reagents, catalyze the facile dehydration of corioloyl moieties to conjugated trienes, we first hydrogenated the methylated lipolysate (hydrolysis 56% complete) from *C. nepalensis* seed oil. After silylation and temperature-programmed GLC, examination of the appropriate region of the chromatogram revealed that the monoglycerides contained only 32% 13-hydroxymonostearin and 5% 13-ketomonostearin. This total of 37% monocoriolin-derived products is much less than the corioloyl content of the original oil (66-71%, depending on the method of analysis). Therefore, in the original oil, far fewer corioloyl groups were attached to the 2- than to the 1- and 3-positions.

Recently we have further simplified our analysis of lipolysates by omitting the preliminary treatment with diazomethane, since silyl esters of fatty acids proved as suitable as methyl esters for GLC (23). Bis(trimethylsilyl)acetamide was adopted as a preferable silylating agent; and with it two *C. myrtifolia* seed oil lipolysates, respectively 30% and 48% hydrolyzed, were derivatized without prior hydrogenation. Subsequent gas chromatograms of both showed 42% monocoriolin in the monoglycerides, again as opposed to a much higher corioloyl content (68-74%) in the original oil. Agreement of results for 30% and 48% lipolysates indicates that the apparent intraglyceride distribution is not distorted owing to a combination of 2- to 1- and 3-monoglyceride isomerization and preferential removal of the corioloyl group by the enzyme as the lipolysis proceeds to different extents. The slightly lower result for the lipolysate from *C. nepalensis* oil is possibly attributable to some hydrogenolysis of 13-hydroxymonostearin to monostearin.

Possible Biochemical Significance of Coriolic Acid. Figure 3 combines selected key features from three postulated biogenetic schemes for conversion of linoleic acid to the conjugated trienoic acids (notably XXI-XXIV) found in seed oils (5,29). Initial attack may

be envisioned as taking place at the methylene group activated by two adjacent double bonds in linoleic acid or a derivative (XIX, R=H, coenzyme A, carnitine, acyl carrier protein, a diacylglyceryl moiety, or the remainder of a phospholipid). In the hypothetical intermediate XX, Y might represent an unpaired electron or attachment to an enzyme. Whatever its exact nature, XX probably has a transient existence. Efforts have consistently failed to demonstrate formation of 11-hydroperoxides either by enzymatic oxidation of linoleic acid (30,31) or by autoxidation of methyl linoleate (32). Elimination of HOZ from II and III, in which OZ may be OOH, OH, or a better leaving group, would give trienes XXI-XXIV.

Two lines of evidence support involvement of coriolic acid or a derivative of it in some such scheme as Figure 3. First, oxidation of linoleic acid in the presence of soybean lipoxidase preparations gives predominantly (33) or exclusively (34) the corresponding hydroperoxide (III, R=H, Z=OH). Second, the postulated schemes on which Figure 3 is based all predict isomerization of the double bond nearer the hydroxyl group in II and III to the thermodynamically more favorable *trans* form during formation of these derivatives, e.g., by allylic rearrangement or S_N2' displacement. Coriolic acid represents the first characterized, probable intermediate in Figure 3, in which the double-bond stereochemistry expected from the biogenetic considerations is unequivocally proved rather than merely assumed.

The observed conversion of linoleic acid into a mixture of 80-90% II (R=Z=H) and 10-20% III (R=Z=H) by a prostaglandin-synthesizing enzyme of animal origin (35) suggests that these acids may be important in animal, as well as plant, biochemistry. Also, this observation may presage the eventual isolation of plant lipoxidases with the expected product specificity to lead from linoleic acid into the left side of Figure 3 just as the above mentioned soybean lipoxidases lead to the right.

ACKNOWLEDGMENTS

Mrs. Mary A. Spencer performed the hydrobromic acid titrations; Mrs. Clara E. McGrew and her associates, elemental analyses. C. K. Lyon of the Western Utilization Research and Development Division, ARS, USDA, Albany, Calif., provided a sample of methyl dimorphecolate; and Quentin Jones, Crops Research Division, ARS. USDA, Beltsville, Md., made arrangements for collection and botanical identification of seeds.

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Hydroxy Fatty Acids From Cerebrosides of the Central Nervous System: GLC Determination and Mass Spectrometric Identification

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ABSTRACT

A method has been developed for GLC determination of hydroxy fatty acids (HFA) from cerebrosides of the central nervous system.

Identification of HFA present in complex biological mixtures was achieved by the use of the combination gas chromatography—mass spectrometry. GLC separations and mass spectrometric determinations were carried out on the trimethylsilyl derivatives of HFA, which show excellent GLC characteristics and favorable cleavages to determine the position of OH groups on the aliphatic chain. The mass spectra of these derivatives present some unusual rearrangement ions, whose composition has been studied with the aid of deuterium labeled analogs.

INTRODUCTION

THE OCCURRENCE OF HYDROXY fatty acids (HFA) in nature has been established by several investigators and has been recently reviewed by Markley (1) and Downing (2). HFA are mainly present in sphingolipids such as cerebroside, cerebroside sulfate and ceramide, of several animal tissues (3-6), and their concentration is particularly high in nervous tissues (5). Whereas most naturally occurring HFA can have the hydroxyl group in various positions on the aliphatic chain, HFA present in sphingolipids have the alcohol functional group only in the α position (5,6).

The biological importance of cerebrosides has prompted us to isolate and identify, the HFA and unsubstituted fatty acids (FA) of this lipid fraction.

Analytical methods for the isolation of cerebrosides, and for the isolation and structural determination of HFA from cerebrosides have been developed (see review by Radin 7). The availability of precise techniques to handle small quantities of biological material is a condition necessary to this problem. Our procedure is based on the purification of cerebrosides by column chromatography and the subsequent separation of HFA-containing cerebrosides (HFA-psy)[1] from cerebrosides containing unsubstituted fatty acids (FA-psy).[1] The composition of HFA and FA fractions is finally determined by GLC and the identity of each component established by using a combination of gas chromatography and mass spectrometry. GLC separation and mass spectrometric identification of HFA is carried out on the methyl esters of their trimethylsilyl (TMS) derivatives. Some unusual features of the mass spectra of these compounds led us to investigate in some detail their fragmentation under electron impact and the results are reported in this paper.

EXPERIMENTAL

Lipid Extraction

Central nervous tissues have been used. in all experiments. Lipids were extracted using three portions of chloroform-methanol (2:1) mixtures in a nitrogen atmosphere. After evaporation to dryness, lipids were reextracted with Chloroform-methanol (2:1) in order to remove additional nonlipid contaminants (8).

Preparation of Cerebrosides

Cerebrosides were separated from the other lipid classes using a combination of column and thin-layer chromatography (TLC).

Chromatographic columns (10 cm × 1 cm I.D.) were prepared using 1 g of DEAE cellulose as adsorbent. Washing of the adsorbent and packing of the column with the DEAE in the acetate form were carried out according to the procedure described by Rouser et al. (8).

Approximately 20 mg of the total lipid extract were applied to the column and, after the elution of neutral lipids (free sterols) with chloroform, cerebrosides, lecithin and sphingomyelin were obtained using chloroform-methanol (9:1) as eluting solvent. The other lipid classes were removed from the column with methanol and, finally, with chloroform-methanol-ammonia-ammonium acetate (4:1:20 ml per liter:0.1 M) (8). After a final wash with methanol, followed by acetic acid, the column could be reused (8).

The separation of cerebrosides from phosphatidyl choline and sphingomyelin and the

[1]Shorthand notation, see Burton, R. M. in G. Schettler (ed.), "Lipids and Lipidoses," Springer-Verlag, New York-Heidelberg, 1967, p. 123.

subfractionation into cerebrosides containing unsubstituted fatty acids and cerebrosides containing α-hydroxy fatty acids were obtained using TLC techniques.

Silica Gel G (Merck; (25 g of the powder)) was used as adsorbent slurried in 50 ml of water and spread over 20 × 20 cm glass plates, to give a 0.3-mm thick layer. Plates were heat activated at 120C for 1 hr, cooled for 10 min in the air and stored in a large desiccator until used. The samples were applied to the chromatograms in a continuous band and the separation of the compound was obtained using chloroform-methanol-acetic acid (80:15:5) as developing solvent system. Spots were visualized under UV light after spraying the plates with 0.001% aqueous Rhodamine 6G. Two well-separated bands corresponding to cerebrosides with unsubstituted and hydroxy fatty acids were obtained. The purity of the separated compounds was checked by subsequent gas chromatographic analysis of their fatty acids.

The same type of separation of cerebrosides can be obtained from the total lipid extracts without prior column chromatographic fractionation. However preparations of FA-psy and HFA-psy from direct TLC of total lipid extracts contained some impurities detectable by GLC. For this reason the combination column chromatography—TLC is preferred.

Preparation of Fatty Acid Methyl Esters

The spots corresponding to FA-psy and HFA-psy were visualized with Rhodamine 6G as previously described, then scraped directly into test tubes by means of a small metal spatula attached to the lip of each tube. Methyl esters were prepared by adding 2 ml of 6% sulfuric acid in methanol to the silica gel containing the cerebroside fraction. The tubes were sealed under nitrogen and heated at 110C overnight (9). The extent of the methylation was determined by measuring the amount of methyl esters formed. The determination was based on photodensitometric measurements after TLC of the methyl esters and methyl ester standards. The methylations were in the order of 92-94% complete.

Preparation of Trimethylsilyl Derivatives of Hydroxy-Fatty Acids

About 2 mg of HFA methyl esters was dissolved in 100 μl of a mixture of hexamethyldisilazane-trimethylchlorosilane-pyridine (10:1:1) and allowed to stand at room temperature for 30 min. The samples were evaporated to dryness under vacuum and the residue dissolved in CS_2 for GLC analysis.

Methyl nonadeuterio-trimethylsilyloxy palmitate was prepared by treating 100 μg of methyl α-hydroxypalmitate with 0.2 ml of a 5% solution of bis-perdeuteriotrimethylsilylacetamide containing small amounts of monosilylacetamide in pyridine and heating for 5 min at 80C. The preparation of deuterium labeled trimethylsilyl derivatives and their use in mass spectrometry has been recently described by McCloskey et al. (10).

GLC Separation of Hydroxy-Fatty Acids

GLC analyses were carried out using a Carlo Erba gas chromatograph Fractovap Model GB, equipped with a flame ionization detector. A 2 meter, 3 mm I.D., glass U column, packed with 1% SE-30 on 100-120 mesh Gas-Chrom P (previously acid washed and silanized) was used. Separations of HFA-TMS were carried out by programming the temperature from 175C to 275C at 2C per minute. The flash heater temperature was 280C. Carrier gas: nitrogen 30 ml/min.

Mass Spectrometric Analysis

Mass spectra were obtained on LKB model 9000 combination gas chromatograph—mass spectrometer. The gas chromatograph was equipped with a coiled glass column, packed with 1% SE-30 on acid washed silanized Gas-Chrom P. The conditions used for the separation of HFA derivatives were the same as those used with the conventional gas chromatograph. The helium separator was maintained at 250C and the ion source at 290C. Ionizing potential was 70 ev and ionizing current was 20μA. Spectra were recorded in 5 sec (m/e 1-400) on the apex of the gas-chromatographic peak.

RESULTS AND DISCUSSION

It has been reported that methyl esters of both short (11,12) and long chain HFA (13) can undergo GLC analysis without protecting the hydroxyl group through derivative formation. However we found it necessary to prepare derivatives in order to obtain satisfactory quantitative results for GLC determination. Several derivatives were taken into consideration. Acetoxy derivatives are stable compounds and are easily prepared (7,11,14) but show relatively long retention times, when compared with other derivatives. Methyl ethers have also been used (15-17) and show good GLC properties; some difficulties arise, however, in their quantitative preparation. Trifluoroacetates (18) have short retention times but they are relatively unstable at the temperature used and it is rather difficult to obtain pure trifluoro-

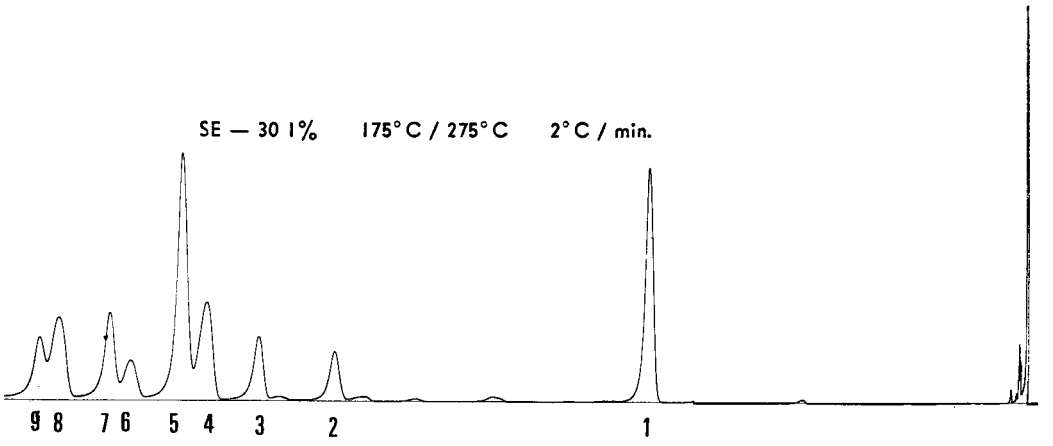


FIG. 1. Analysis of alpha-hydroxy fatty acid methyl esters (as TMS derivatives) of beef brain cerebrosides. Conditions used are described in Methods.

Peak n°	1	C 18 : 0	Peak n°	6	C 25 : 1
	2	C 22 : 0		7	C 25 : 0
	3	C 23 : 0		8	C 26 : 1
	4	C 24 : 1		9	C 26 : 0
	5	C 24 : 0			

acetic anhydride for their preparation. Trimethylsilyl derivatives, previously used in GLC analysis of HFA (7,19), have therefore been used because they are highly thermostable and volatile, have very low polarity, and their quantitative preparation is easily achieved.

A gas chromatogram of HFA of beef brain cerebrosides is reported as an example in Fig. 1. The HFA of biological mixtures were characterized in terms of Relative Elution Temperature according to Schmidt and Wynne (20), using the methyl ester of N-eicosanoic acid as an internal standard. Final identification was achieved by means of combination gas chromatography—mass spectrometry.

Quantitative response of HFA trimethylsilyl ethers was checked using the TMS derivatives of methyl α-hydroxystearate and α-hydroxyhexacosanoate. Results are reported in Table I.

The use of the combination gas chromatography-mass spectrometry allows a rapid identification of the HFA present in complex mixtures of biological origin when reference spectra are available. A distinct advantage of this method is that a preliminary isolation and purification step is not necessary, making it possible to obtain complete structural information with a very small amount of material.

TMS derivatives are very well suited for this purpose because the trimethylsilyl group "labels" the position of the hydroxyl group giving well-stabilized α-cleavage ions and have been widely used in the study of hydroxy compounds (21-26). Their basic fragmentation mode has been recently reviewed by Budzikiewiez et al. (27).

Several TMS derivatives of authentic methyl esters of α-hydroxy fatty acids were prepared and their mass spectra recorded. In Fig. 2 is reproduced the mass spectrum of methyl α-trimethysilyloxy-palmitate, which is a representative of the compounds studied. In order to gain a deeper insight into the fragmentation mechanisms, ethyl α-trimethylsilyloxy-palmitate (Et α-(TMS)pal), trideuteriomethyl α-trimethylsilyloxy-palmitate (^{2}H-Me α-(TMS) pal), and methyl α-nonadeuteriotrimethylsilyloxy — palmitate (Me α-(^{2}H-TMS) pal) were prepared. Their mass spectra are reproduced in Figs. 3, 4, and 5, respectively. The mass spectra of the methyl esters of α-trimethylsilyloxy fatty acids show the same principal mode of fragmentation.

The molecular weight is indicated by the

TABLE I

Quantitative GLC Response to Saturated α-Hydroxy Fatty Acids

α-hydroxyhexacosanote: α-hydroxystearate		
Weight ratio	Area ratio	Relative error
		%
0.333	0.356	6.9
0.665	0.694	4.4
1.330	1.321	0.7
2.660	2.810	5.6

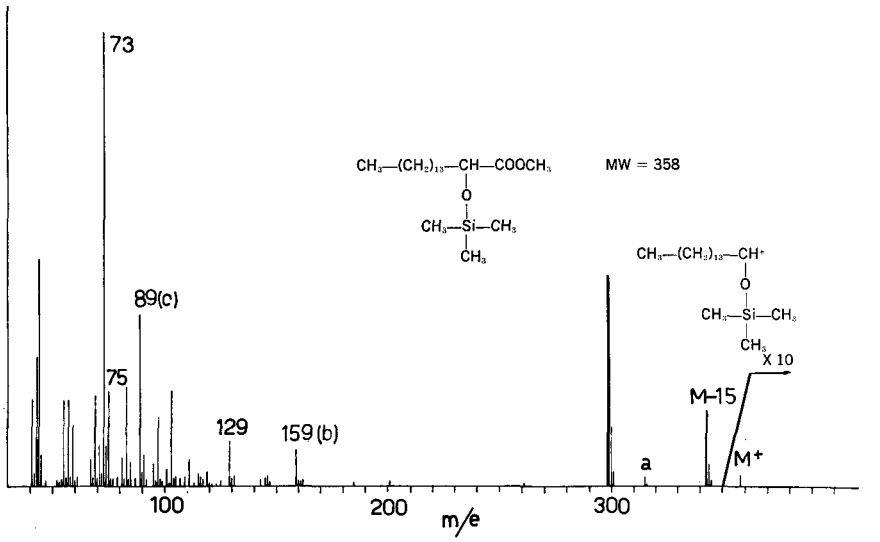


FIG. 2. Mass spectrum of methyl α-trimethylsilyloxy palmitate.

presence of a low intensity molecular ion and by an intense M-15 peak, due to loss of a methyl from the trimethylsilyl group, as shown by shift to M-18 in the Me α-(²H-TMS)pal derivative (Fig. 5). Below the M-15 peak a significant ion is found at m/e 315 (a). This ion shifts to m/e 329 in the ethyl ester analog.

In this case the presence of a metastable (calculated 303.1 for the transition m/e 357 → 329; found 303.1) shows that ion "a" arises from elimination of 28 from the M-15 ion. Ion "a" is shifted to m/e 318 in the mass spectrum of the ²H-Me α-(TMS)pal derivative (Fig. 4), and to m/e 321 in the mass spectrum of Meα(²H-TMS)pal (Fig. 5). Ion "a" must therefore contain the methyl (or ethyl) of the ester group and only two methyl groups of the trimethylsilyl ether. These facts and the presence of a M-15-28 ion also in the mass spectrum of the TMS derivative of methyl lactate suggest that M-15-28 peak (ion "a")

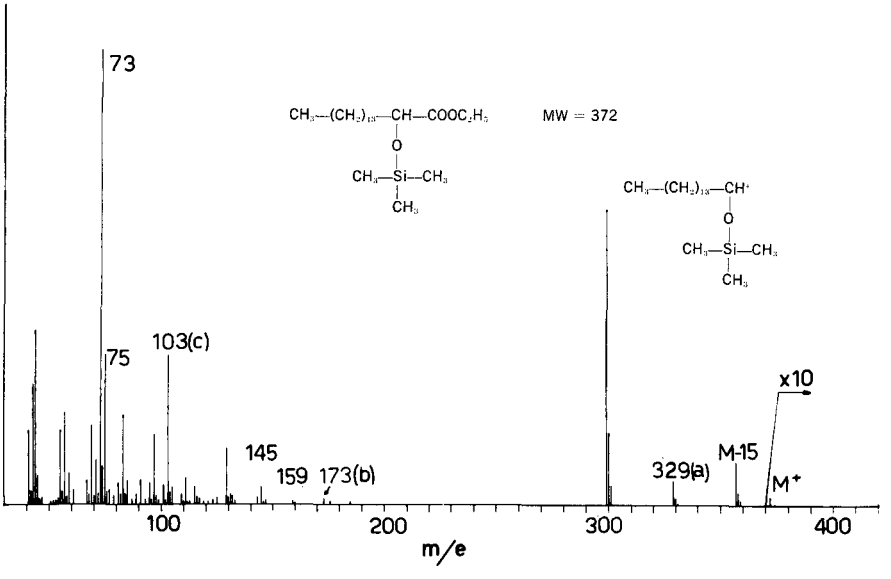


FIG. 3. Mass spectrum of ethyl α-trimethylsilyloxy palmitate.

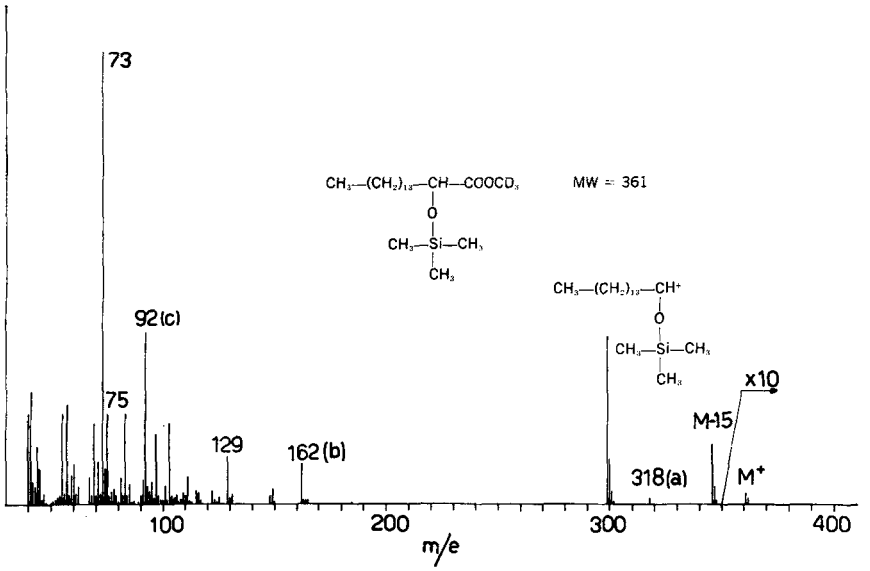
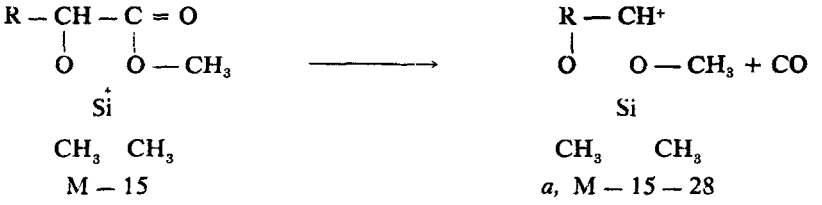


FIG. 4. Mass spectrum of trideuteriomethyl α-trimethylsilyloxy palmitate.

corresponds to elimination of CO from the M-15 ion. A reasonable mechanism would involve the transfer, via a five member intermediate, of the methoxyl group to the charge bearing silicon atom of the M-15 ion and followed by elimination of CO:

Indirect evidence for this mechanism is supplied by the absence of ion "a" in the spectra of TMS derivatives of hydroxy fatty acids in which the hydroxyl group is not on carbon 2, but is much further removed on the aliphatic chain such as the case of 9-hydroxy palmitic



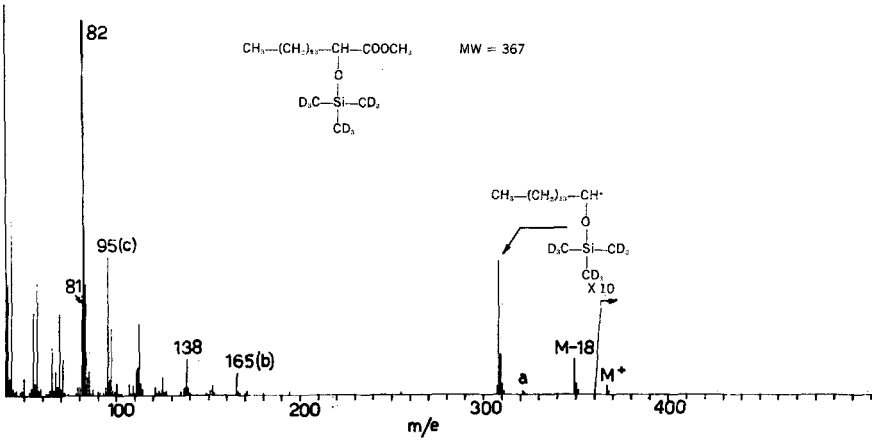


FIG. 5. Mass spectrum of methyl α-nonadeuteriotrimethylsilyloxy palmitate.

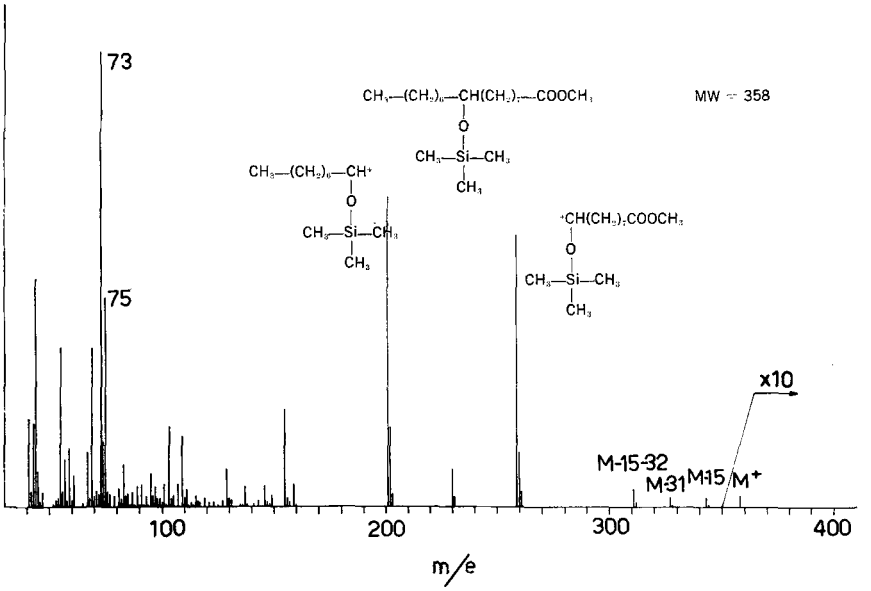
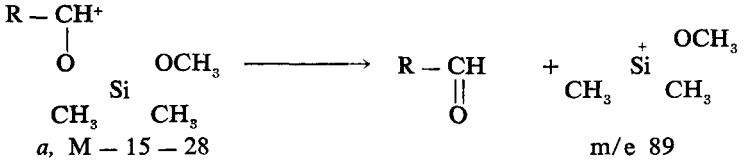


FIG. 6. Mass spectrum of methyl 9-trimethylsilyloxy palmitate.

acid (Fig. 6) and ricinoleic acid (Fig. 7).

Further decomposition of ion "a" results in ion "c" (m/e 89), as shown by presence of a metastable peak (calculated 25.1 for the transition m/e 315 → 89; found 25.2). Ion "c" shifts to m/e 103 in the mass spectrum of the ethyl ester (Fig. 3), to m/e 92 in the mass spectrum of the ^{2}H-Me α-(TMS)pal analog (Fig. 4), and to m/e 95 in the mass spectrum of Me α-(^{2}H-TMS)pal (Fig. 5). This shows that ion "c" still contains two methyls of the trimethylsilyl group and the methoxyl (or ethoxyl) of the ester group. A mechanism can be visualized in which ion "c" is derived from ion "a" by elimination of the aliphatic chain as an aldehyde:



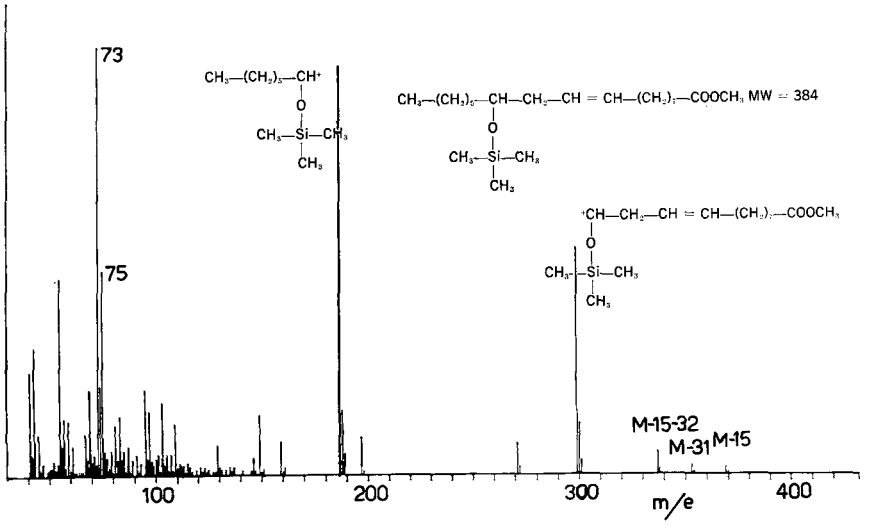


FIG. 7. Mass spectrum of 12-trimethylsilyloxy oleate.

The composition of the ion at m/e 89 is, therefore, the same as for the analogous fragment which is found in the mass spectra of the TMS derivatives of primary alcohols (27); however, its formation follows a different path.

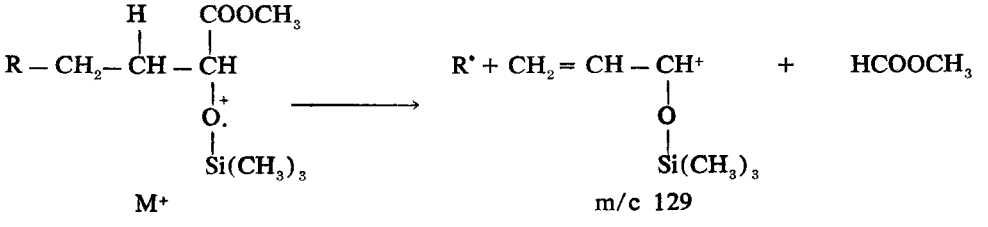
In the high mass region the most intense peak (m/e 299, Fig. 2) is due to cleavage α to the trimethylsilyloxy group and elimination of the carbomethoxyl group. The diagnostic importance of this fragment is easily understood since it confirms the molecular weight, and because the loss of the carbomethoxyl indicates that the hydroxyl group is on the α carbon. It is of interest to note that cleavage between the α and the β carbon atoms give rise, on the contrary, to a very low intensity peak (m/e 161, Fig. 2), because of the destabilizing effect of the adjacent carbonyl.

All the mass spectra of TMS derivatives of α-hydroxy fatty acids, both saturated and unsaturated, examined so far in the lower mass range exhibit the same series of major fragment ions at m/e 159,129,103,75 and 73, besides the previously discussed fragment at m/e 89.

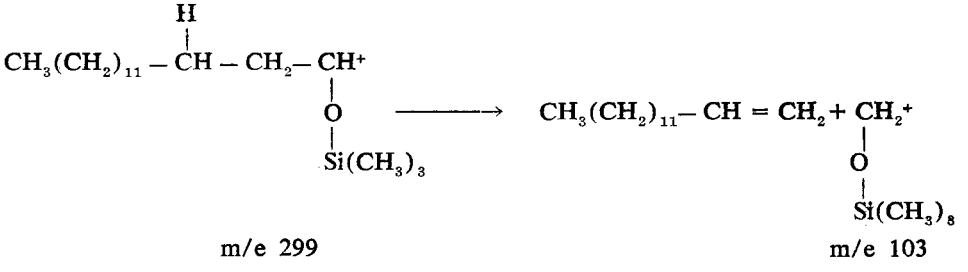
The peak at m/e 159 "b" is not present in the mass spectrum of the ethyl ester analog, and is replaced by two low intensity peaks at m/e 173 and 145 (Fig. 3). Ion "b" shifts to m/e 162 in the mass spectrum of the ^{2}H-Me α-(TMS)pal (Fig. 4), and to m/e 165 in the mass spectrum of Me α-(^{2}H-TMS)pal (Fig. 5). This shows that ion "b" contains the methyl (or ethyl) of the ester group and only two methyls of the trimethylsilyl group. The evidence presently available does not permit us to suggest a tentative structure for this fragment.

The peak at m/e 129, which shifts to m/e 138 in the mass spectrum of Me α-(^{2}H-TMS) pal, contains the trimethylsilyl ether group and, probably, three carbon atoms of the aliphatic chain and four hydrogens. A peak at m/e 129 is frequently found in the mass spectra of trimethylsilyl ethers (22,28) and it is found to be the base peak of TMS derivatives of 3,17-hydroxy-Δ^5-steroids (28). The peak at m/e 129 is not found however in the mass spectrum of coprostanyl trimethylsilyl ether (22), indicating that steric factors play an important role in the formation of this ion.

A probable mechanism could involve a hydrogen of the carbon atom α to the trimethylsilyl group:



More than one mechanism is probably operating in the formation of the fragment at m/e 103, which shifts to m/e 112 in the mass spectrum of Me α-(^{2}H-TMS)pal. A metastable peak at 35.5 indicates that, at least in part, this ion is derived from the α cleavage ion at m/e 299 (calculated 35.4 for the transition m/e 299 → 103)



The peak at m/e 73 is found to be the base peak in all the mass spectra of the compounds studied and corresponds to the trimethylsilyl ion ($(CH_3)_3Si^+$), as shown by the shift to m/e 82 in the mass spectrum of Me α-(^{2}H-TMS)pal. The peak at m/e 75 (m/e 81 in the mass spectrum of Me α-(^{2}H-TMS) pal) corresponds to what could be expected for loss of a methyl group from trimethylsilanol $(CH_3)_3SiOH$ (90-15).

It is interesting to note that the formation of the McLafferty rearrangement product is strongly suppressed in these compounds by the presence of the α-trimethylsilyloxy group, and

gives rise to a very low intensity peak at m/e 162.

In Fig. 6 and Fig. 7 are reproduced the mass spectra of the TMS derivatives of methyl 9-hydroxypalmitate and of methyl ricinoleate, respectively.

It is evident that the fragmentation follows a somewhat different pattern when the TMS group is not α to the carboxyl group.

Peaks at M-15-28 and m/e 89 are not present. It is of interest to note, however, that a prominent ion of m/e 89 was found in the mass spectra of 4- and 5-hydroxyacids by Wyatt et al. (29). These spectra are characterized, on the contrary, by the presence of two very intense peaks, which correspond to cleavage α to the trimethylsilyloxy group (m/e 201 and 259 for the TMS derivative of 9-hydroxy palmitate, and m/e 187 and 299 for the methyl ricinoleate derivative).

The molecular ion is very small and it is sometimes not visible at the sample pressures used. The molecular weight is, however, clearly indicated by a series of peaks corresponding to M-15, M-31 and M-15-32. This latter peak corresponds very likely to elimination of methanol from the M-15 ion.

The results of this work show that the TMS derivatives of α-hydroxy fatty acids are very well suited for a GLC determination. Furthermore, their mass spectra allow an unambiguous identification of α-hydroxy fatty acids present in complex biological mixtures when the combination gas chromatography — mass spectrometry is used.

The results of an extensive investigation on the composition of the α-hydroxy acids of cerebrosides of a number of animal species of different phylogenetic position determined by this method will be reported elsewhere.

ACKNOWLEDGMENTS

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Uptake of Cerebroside, Cholesterol and Lecithin by Brain Myelin and Mitochondria

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ABSTRACT

The uptake of emulsified labeled lipids by rat brain myelin and mitochondria was studied. Cerebroside and lecithin uptakes were greatly stimulated by addition of salts, particularly those containing divalent cations. Cholesterol uptake was not influenced by salts. Increasing concentrations of detergent (non-ionic) were inhibitory. Delipidated membranes took up much less lipid, but pretreatment with lecithin partially restored the ability to take up cerebroside and cholesterol. The lipid uptake appears to be nonenzymatic and appears to depend on the size of the emulsified particles. The possible role of such a phenomenon in membrane formation and maintenance is discussed.

INTRODUCTION

SUBCELLULAR PARTICLES have been shown to take up lipids and other substances by processes which seem to be nonenzymatic and which probably involve only the surface membrane. Such studies have been made with liver and adipose tissue mitochondria and microsomes, using labeled palmitate (1); brain particles, with gangliosides (2); liver mitochondria and plasma membranes, with steroids (3); and liver reticulosomes, using phospholipids (4). While not every one of these studies investigated the point, the uptakes appear to be adsorptive in nature. Whether the lipid molecules or micelles become attached at specific membrane sites is not known, nor is it clear whether the uptake is related to subsequent metabolic conversions or incorporation into specific substructures. In the case of uptake studies with partially delipidated mitochondria (5), the lipids apparently enter the original sites in the membranes.

In some cases, the lipid composition of membranes has been shown to change with time or under different environmental conditions. Brain myelin composition is different in young and older animals (6, 7), consistent with the observations that the fatty acid composition of brain lipids changes with age (8, 9). It is not evident from these studies whether only the recently formed membranes have differing composition, or whether all the membranes change with age.

The finding that subcellular particles can take up lipids raises the question as to whether this is not the normal process by which membranes are formed or maintained in the process of turnover. The possibility that membranes are self-assembling is supported by recent work from the Institute for Enzyme Research (10) and other laboratories.

The study described here has been made with the above questions in mind, using brain myelin. This particle was examined partly because of its metabolic inertness and partly because of its chemical simplicity. Brain mitochondria were also examined by way of comparison.

MATERIALS AND METHODS

Myelin and Mitochondria

A modification of the method of Autilio et al. (11) was used for the preparation of myelin. Rat brains weighing a total of about 7.5 g were homogenized in 30 ml of 0.32 M sucrose and the homogenate was filtered through cheesecloth. The filtrate was brought up to 52 ml with the same sucrose solution, then layered over 0.656 M sucrose in three 32 ml centrifuge tubes. Centrifugation in a swinging bucket rotor (SW 25.1) for 40 min at 40,000×g yielded a dense interfacial layer which was diluted with an equal volume of water. The crude myelin suspension was purified by repetition of the layering step and final centrifugation in 0.25 M sucrose (11). The myelin was then resuspended in 5 ml of water.

The mitochondria were prepared from the particles sedimenting between 800×g (10 min) and 10,000×g (15 min), starting with a 10% homogenate in 0.25 M sucrose. The crude mitochondria were suspended in 0.25 M sucrose and layered over 0.9 M sucrose. Centrifugation for 60 min at 100,000×g yielded the mito-

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chondria in the pellet; these were suspended in 0.25 M sucrose.

The myelin suspensions were stored no longer than 4 days, as cerebroside uptake was found to diminish after 6 days. The mitochondria, based on tests with cerebroside and cholesterol, were used within 24 hr.

The purity of the myelin and mitochondrial preparations was checked by analysis of their lipids (by TLC) and by enzymatic assays. The TLC plates showed the usual assortment of brain lipids, the cerebroside spots being particularly heavy in the myelin extract and the hydroxy cerebroside spot being barely visible in the mitochondrial extract. Also in semiquantitative agreement with published data for the lipid composition of these particles was the finding of relatively more cholesterol and less lecithin and ethanolamine lipids in myelin.

Assay of the myelin for acetylcholine esterase gave a specific activity of 0.6 units/mg protein, to be compared with 4.8 units/mg in the M_1 (0.9) fraction of synaptic membranes (12). Mitochondrial contamination in the myelin was shown to be very low by assay of activity toward succinate with INT (iodonitrotetrazolium violet). The myelin contained 1.0 units/mg protein, while purified mitochondria contained 13.5 units/mg (13). Microsomal contamination was estimated from the content of glutamine transferase: myelin activity was 2.8 units/mg while the activity in purified microsomes was 31.4 units/mg (14). It is evident that the myelin, as with most subcellular preparations, was not completely pure; it should be noted that the experimental conditions of incubation resulted in additional purification. Moreover, it was found (see Results) that myelin and mitochondria were qualitatively the same with respect to lipid uptake.

Incubation Conditions

The lipid emulsions were prepared by adding solutions of lipid and detergent to chloroform, evaporating to dryness, then sonicating with water by means of a dipping probe for 2 min. A Branson Sonifier was used; the entire lower part and vial were covered with a polyethylene bag to retain radioactive spray. The detergent used was G-2159, a polyoxyethylene stearate made by Atlas Chemical Industries, stored in chloroform. This detergent was found to be a superior emulsifier for lipids. Usually 20 μg of cerebroside + 60 μg of detergent, or 10 μg of cholesterol + 1 mg of detergent were used in each incubation tube. The emulsions were stored no longer than 4 days. They exhibited a faint bluish tint in transmitted light. Control tests under various incubation conditions, with and without the addition of absorbent cotton as trapping agent, showed no sedimentation of the emulsions under the conditions of assay.

Aliquots of salts, lipid emulsion (0.4 ml) and particle suspension were added in total volume of 1 ml to 15-ml Corex centrifuge tubes. The suspensions were shaken gently 10 or 15 min at 37C, then 4 ml of cold 0.25 M sucrose were added, the mixture was agitated, and the whole was centrifuged at 18,000×g for 15 min. The pelleted particles were suspended in 4 ml of sucrose and centrifuged again. The pellet was suspended in 1 ml of water and aliquots were taken for radioactivity and protein determination. Usually each incubation tube contained myelin or mitochondria equivalent to 600 μg of protein.

The uptake of lipid was measured by adding 4 volumes of chloroform-methanol (2:1), centrifuging, and drying a part of the lower layer in a counting vial. The lipid was taken up in 10 ml of a scintillation mixture based on toluene-ethanol (95:5). In the case of ^{45}Ca, the pellet was suspended in 0.3 ml of water and 5 ml of XDC scintillation solvent (15) were added. The mixture was transferred to a counting vial with a 5-ml rinse of XDC.

Reagents

Stearoyl cerebroside labeled with tritium in the 6-position of the galactose was described previously (16). ^3H-Cholesterol was obtained from Schwarz BioResearch, Inc., and was found to give a single radioactive spot on TLC with hexane-ether. ^{32}P-Lecithin was prepared by incubation of labeled phosphate with liver slices (17). Radioautography of the product, after TLC with chloroform-methanol-water (24:7:1), showed a single radioactive spot. Carrier was added to produce compounds of the following specific activities: cerebroside and cholesterol — 2,000 cpm/μg; $CaCl_2$ — 5,000 cpm/μmole.

Egg yolk phospholipids (mainly lecithin and ethanolamine phosphoglycerides) were prepared by cellulose chromatography according to the method of Lea et al. (18). Lecithin was isolated from this preparation by silica gel chromatography. This was used to dilute the ^{32}P-lecithin so as to give 4,000-6,000 cpm per incubation.

Other Methods

Protein was determined by the method of Lowry et al. (19). Thin-layer chromatography was on Silica Gel G, using as visualization re-

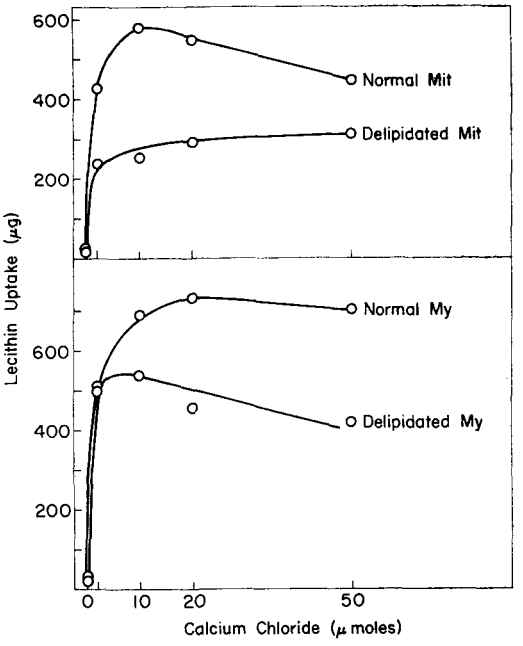

FIG. 1. Uptake of lecithin as a function of calcium ion concentration and delipidation. Incubations contain 1 mg of labeled lecithin and particles equivalent to 0.6 mg of protein, in a total volume of 1.2 ml. Upper curves: mitochondria. Lower curves: myelin.

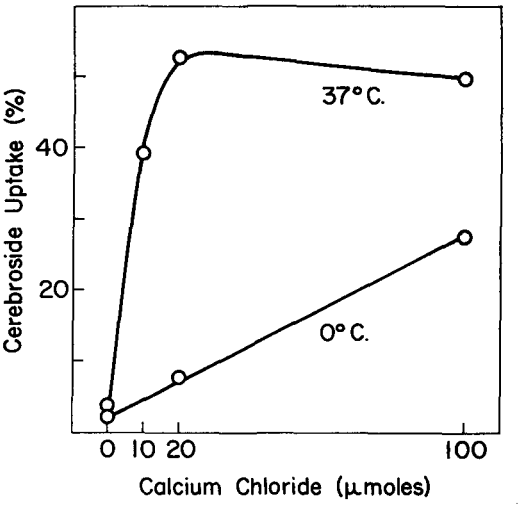

FIG. 2. Effect of Ca^{++} concentration on cerebroside uptake by mitochondria. Tubes contained 1.2 mg protein, 20 μg cerebroside, and 40 μg detergent.

agents, alkaline bromthymol blue, phosphomolybdic acid, or ninhydrin.

The delipidation procedure for the cell particles was based on the method of Lester and Fleischer (20). A suspension of particles containing 20-25 mg protein in 2 ml of water was added to 10 ml of acetone and the mixture was stirred 5 min. The solution was removed after centrifugation at 15,000×g for 10 min and the particles were washed twice with 6 ml of 0.25 M sucrose, centrifuging as above. About 30% of the protein was lost from both particles by this procedure, and incubations were corrected for this so that equal weights of protein were in each incubation tube.

All materials were kept at 0-4C except where noted otherwise.

RESULTS AND DISCUSSION

Effect of Salts

The uptake of cerebroside by myelin was found to be greatly augmented by the presence of salts (Table I). Divalent ions were much more effective than monovalent ions, and little difference was seen between the members within each class. A linear relationship between uptake and monovalent ion concentration was seen up to 0.2 M; the divalent ions showed a rapidly increasing effect up to about 0.005 M and a leveling off at about 0.02 M. Cholesterol uptake by myelin, on the other hand, was only slightly affected by salts. Without added salt, 42% of the cholesterol was taken up, while 46% was taken up in the presence of 5 μmoles of Ca^{++}.

Lecithin, like cerebroside, showed a marked dependence on salt concentration but differed in that there was some inhibition at higher Ca^{++} concentrations (Fig. 1, upper curves). The inhibitory effect with myelin was some-

TABLE I

Effect of Salt Concentration on Cerebroside Uptake by Myelin

Amount of cation (μatoms)	Ca^{++}	Mg^{++}	Mn^{++}	Na^{+}	K^{+}	Li^{+}
0.5	4	4	6	2	3	3
1	6	7	17			
2	12					
3	19					
4	35					
5	55	59	59	3	3	3
10	64	63				
20	76	81	84	6	7	6
50	80	80	71	11	11	10
100	80			18	17	17
200				20	23	21

Incubation conditions: 20 μg cerebroside and 40 μg detergent; myelin equivalent to 700 μg protein; cations present as chlorides. Some of the columns are averages of several experiments. Data are percent of cerebroside taken up by the particles.

TABLE II

Uptake of Cerebroside and Cholesterol by Normal and Lipid-Depleted Myelin and Mitochondria as a Function of Calcium Concentration (Percent of Incubated Sample)

Substrate	Amount of $CaCl_2$ (μmoles)	Myelin		Mitochondria	
		Normal	Delipidated	Normal	Delipidated
Cerebroside, 20μg	0	7	5	2	3
	2	15	10	.....	.....
	5	44	17	4	.....
	10	63	22	8	.....
	20	61	.....	10	4
	50	.....	.....	17	.....
	100	.....	.....	23	6
Cholesterol, 10 μg	0	.....	.....	15	9
	10	39	22	.....	.....
	20	.....	.....	17	9
	100	39	22	.....	.....

Incubation conditions: With cerebroside, 40 μg of detergent and membranes equivalent to 700 μg of protein. With cholesterol, 1 mg of detergent, myelin equivalent to 600 μg protein, mitochondria equivalent to 1 mg protein. Total volume, 1 ml.

what weaker than with mitochondria, but was reproducible.

Mitochondria resembled myelin in the need for ions in cerebroside uptake (Fig. 2). The Ca^{++} dependency for cerebroside was seen at low temperature also, but the amount of uptake was less in the time interval studied.

As with myelin, mitochondrial uptake of cholesterol was little affected by Ca^{++}. Mitochondria equivalent to 240 μg of protein, under the usual conditions, took up 15% of the cholesterol without Ca^{++} and 17% with Ca^{++}.

A comparison of $MgSO_4$ with $MgCl_2$ (5 μmoles) for the effect on myelin uptake of cerebroside showed the anion to be unimportant here.

It is interesting that the uptake of both a non-ionic lipid (cerebroside) and a phospholipid was greatly influenced by salt concentration. In the case of the phospholipid, the Ca^{++} is apparently adsorbed to the micelles (21,22), and one could postulate that the Ca^{++} then acts to bind the micelles to negatively charged groups on the membranes. This explanation seems somewhat less probable in the case of cerebroside binding, and it also does not explain the effect of the monovalent ions. Perhaps the ions, of all types, act by changing the charge distribution on the membrane surfaces, causing a configurational change which favors lipid binding (as by bringing lipophilic groups out to the surface). It is well known that divalent ions affect the swelling of mitochondria, with a consequent change in membrane structure, and it is possible that myelin reacts similarly.

This interpretation is supported by an experiment with labeled Ca^{++}, in which we incubated myelin (0.6 mg protein) with 4 μmoles of $CaCl_2$ in the usual way. The uptake of Ca^{++} was 0.057 μatoms, a value in good agreement with the concentration of total divalent ions found in myelin preparations by Gerstl et al. (23). Apparently most or all of the divalent metal ions in our preparation exchanged with the aqueous Ca^{++}. (This bound Ca^{++} could be removed by incubation with unlabeled Ca^{++}.) When lecithin emulsions were added to the ^{45}Ca and myelin, the amount of Ca^{++} uptake was not noticeably affected. It may be concluded that the configurational change in the membrane induced by the surrounding ions is not the result of a strong linkage, and that the membrane-lipid complex, once formed, loses its surrounding ions during the washing step without uncoupling of the membrane-lipid linkage. However, the sensitivity of this experiment depends on how much Ca^{++} is required for the binding. In the lecithin experiment, about 0.06 μmoles of lecithin was taken up. If the lecithin particles were bound mole-for-mole with labeled $CaCl_2$, the observed uptake of Ca^{++} would be 0.06 μatoms above the control value. The experimental procedure would detect an uptake of 1/10 this amount, so the molar ratio of Ca^{++} to lecithin in the micelles could be 1:10 or less.

Effect of Membrane Delipidation

Samples of myelin and mitochondria were partially delipidated with acetone (final concentration about 85%) and compared with unprocessed particles. The relationship between uptake of lecithin and Ca^{++} concentration is shown in Fig. 1. It can be seen that normal myelin is more effective than mitochondria in taking up lecithin (per milligram of protein), perhaps due to its higher lipid content. The same relationship is seen with the lipid-depleted membranes, which take up somewhat less lecithin. This reduced uptake is consistent with the above idea, that uptake is due primarily to the lipid portion of the membrane surfaces.

In all four systems, Ca^{++} greatly increases the amount of uptake. At higher levels of Ca^{++} there is reduced effectiveness; perhaps this means that Ca^{++} becomes attached to the lecithin micelles at the higher concentrations and such micelles are not as readily taken up by the membranes.

A similar comparison with cerebroside and cholesterol is shown in Table II. It is evident

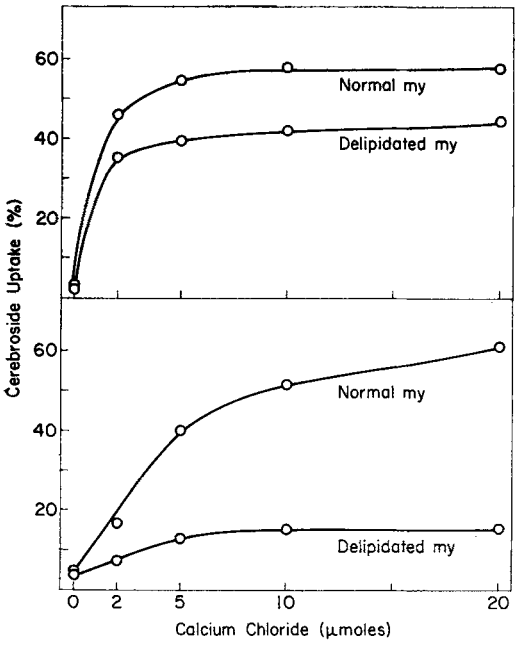

FIG. 3. Uptake of cerebroside emulsions by myelin and lipid-depleted myelin. Upper curves: 20 μg cerebroside + 60 μg detergent + 100 μg lecithin. Lower curves: same, but without lecithin.

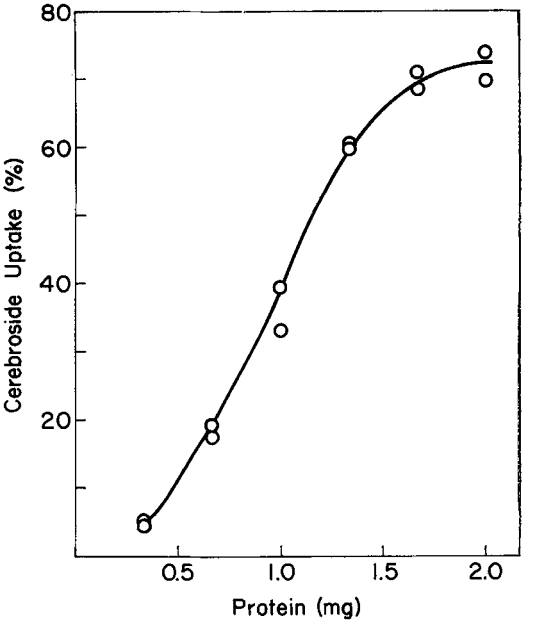

FIG. 4. Dependence of cerebroside uptake on quantity of mitochondria. Tubes contained 20 μg cerebroside + 40 μg detergent + 20 μmoles calcium chloride.

that both normal mitochondria and myelin take up cerebroside, but that myelin is much more effective (see also Figs. 4 and 5). In this sense, cerebroside could be considered as being more specifically bound to myelin than to mitochondria, which contain little cerebroside (24,25). Cholesterol also shows preferential uptake by myelin, but not as specifically. The effect of lipid depletion, as with lecithin, was a marked decrease in lipid uptake by both types of cell particles. The delipidation effect was markedly greater with increased Ca^{++} levels and was somewhat greater for cerebroside than for lecithin. As noted before, cholesterol uptake was not affected by calcium.

The uptake of cerebroside by delipidated myelin could be appreciably enhanced by incorporating lecithin into the cerebroside-detergent micelle (Fig. 3). (The mixed micelle was made by evaporating all three compounds from organic solvent and emulsifying them simultaneously.) With normal myelin, lecithin acted to enhance cerebroside uptake at low levels of Ca^{++}, but there seemed to be little effect at high levels. With depleted myelin, there was a marked enhancement at all levels. Since we have shown that lecithin is more

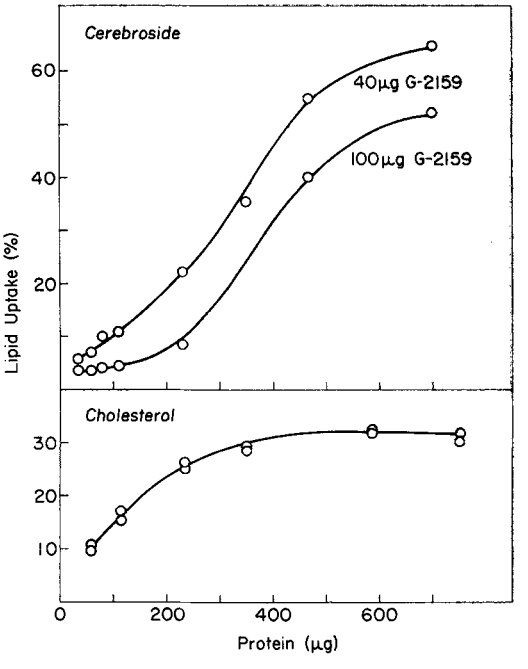

FIG. 5. Uptake of lipids by increasing amounts of myelin, and effect of additional detergent. Upper curves: 20 μg cerebroside + 40 or 100 μg detergent. Lower curve: 10 μg cholesterol + 1 mg detergent. Tubes contained 5 μmoles $CaCl_2$.

TABLE III

Composition of Lipids Taken up by Myelin from Lecithin-Cerebroside Mixtures

Experiment number	Amount of Ca^{++} (μatoms)	Weight of lipids (in μg)					
		Initial emulsion			Taken up by myelin		
		Lecithin	Cerebroside	L/C	Lecithin	Cerebroside	L/C
1	0	1840	20	92	7	0.15	48
2	2	1840	20	92	187	2.10	89
3	10	1840	20	92	222	2.52	88
4	0	500	20	25	6	0.22	30
5	2	500	20	25	76	3.39	23
6	10	500	20	25	105	4.20	25
7	0	256	20	12.8	4	0.81	5.3
8	2	256	20	12.8	47	9.49	5.0
9	10	256	20	12.8	46	9.22	5.1

L/C = ratio of weights, lecithin:cerebroside.

Incubation conditions: myelin equivalent to 600 μg protein; 60 μg of detergent in each; 1 ml total volume; incubated 10 min at 37C.

strongly attracted to delipidated myelin than is cerebroside, apparently the lecithin in the mixed micelle is simply carrying the cerebroside along with it.

To test this explanation, we prepared mixed micelles containing different ratios of lecithin: cerebroside and determined the uptake of both labeled lipids with normal myelin (Table III). Looking at the data obtained from incubations which contained Ca^{++} and higher amounts of lecithin (Experiments 2,3,5, and 6), we see that the ratio of lipids complexing with the myelin was the same as the ratio in the original emulsion. Thus, under these conditions the micelles were taken up by the membrane *in toto*, as predicted by the above suggestion that the cerebroside simply accompanied the lecithin. In the case of the emulsions containing a low amount of lecithin (Experiments 7-9), the ratio of incorporated lipids is unexpectedly low and independent of Ca^{++} concentration.

It is interesting that the total weight of cerebroside taken up decreased with increasing lecithin content in the emulsion. This is probably not an inhibitory or competitive effect, but simply a reflection of the finite capacity of the myelin portions to take up lipid.

Lecithin could be shown to enhance cerebroside uptake into delipidated myelin by a different mechanism. Depleted myelin was incubated in the usual way with 2 mg of lecithin and 20 μmoles of $CaCl_2$ for 10 min, then washed twice. On subsequent incubation with cerebroside-detergent emulsion and 20 μmoles Ca^{++}, the myelin took up 25% of the cerebroside. If the Ca^{++} or the lecithin was omitted from the preincubation step, only 10% of the cerebroside was taken up in the second incubation. This increase in cerebroside uptake is apparently due to the partial relipidation of the myelin by preincubation with the complete system (lecithin + calcium).

The uptake enhancement by phospholipid could be demonstrated also for cholesterol and mitochondria (Table IV). The data on the right

TABLE IV

Uptake of Cholesterol from Phospholipid and Detergent Emulsions by Normal and Delipidated Mitochondria

Amount[a] of mitochondria	Cholesterol + Phospholipid				Cholesterol + Detergent	
	Normal mitochondria		Delipidated mitochondria		Delipidated mitochondria	
(mg)	(–Ca^{++})	(+Ca^{++})	(–Ca^{++})	(+Ca^{++})	(–Ca^{++})	(+Ca^{++})
0.2	3.0	28	2.2	19	3.7	3.6
0.4	4.5	36	.....	.....	.....	.....
0.6	4.8	40	3.5	39	8.3	8.3
0.8	5.1	47	4.0	36	10.9	10.2
1.0	4.7	49	4.7	40	12.5	12.0

[a] Weight of mitochondrial protein.

All tubes contained 10 μg cholesterol, emulsified with 100 μg phospholipid or 1 mg detergent. Calcium chloride concentration, where added, was 20 mM. Data are percentage of cholesterol taken up by the particles.

side of the table illustrate for lipid-depleted mitochondria the same lack of dependency on the presence of Ca^{++} noted before. The data for emulsions made with egg phospholipids and cholesterol show less uptake of cholesterol in the absence of Ca^{++} but considerably increased uptake in the presence of 20 μatoms of Ca^{++}. Both the enhancement and Ca^{++} effects were presumably mediated through the phospholipid portion of the micelles, and the cholesterol was simply carried along like the cerebroside.

Capacity of Membranes

The amount of lipid taken up was compared with different amounts of membranous particles. Fig. 4 shows the relationship between cerebroside and mitochondria. The uptake was rather linear over most of the range studied, but leveled off at about 70% uptake. This failure to take up all of the emulsified lipid may mean that about 30% of the micelles were of a size which could not be taken up by the mitochondria. The plausibility of this explanation is supported by the finding that groups of particles of two distinct sizes can readily be produced in simple lipid emulsions (26-28).

A similar situation was seen with myelin and cerebroside and cholesterol (Fig. 5). Here too the maximal cerebroside uptake was in the neighborhood of 70%, so the two membranes did not seem to differ with respect to the types of micelles that could be taken up. Cholesterol leveled off at 30% of the incubated lipid. In the case of mitochondria, a mixed phospholipid-cholesterol emulsion was seen to level off at a higher percentage, about 50% (Table IV, left side). Delipidated mitochondria apparently accepted a smaller range of micelles (about 39%).

The temperature-dependency experiment shown in Fig. 2 seemed to show that myelin might have a lower capacity for cerebroside at a low temperature. However, further work showed that the uptake is simply slower; after 3 hr at 0C the uptake had nearly leveled off at about 60%. Time studies at 37C showed uptake leveled off around 15 min but became erratic with longer periods, evidently owing to progressive breakup of the myelin. Cholesterol uptake by myelin at 37C leveled off at 10 min.

The amount of cerebroside taken up by mitochondria equivalent to 1 mg of protein was 8 μg. Assuming the lipid:protein ratio for brain mitochondria is 0.6 (29), the ratio of cerebroside to mitochondrial lipid is 13 μg/mg. The corresponding calculation for myelin yields an uptake of 7 μg/mg myelin lipid (assuming myelin is 25% protein and 75% lipid).

Detergent Effects

In Fig. 5 may be seen the effect of increasing the amount of detergent in the cerebroside emulsion. At all levels of myelin there was decreased uptake. With 1 mg of detergent there was no uptake at all. The same interference was seen when NaCl was used instead of $CaCl_2$, suggesting the detergent did not act by binding Ca^{++}. It should be noted that the cholesterol incubations contained 1 mg of detergent, yet appreciable lipid was taken up. This difference is undoubtedly related to the fact that much more detergent was found necessary to emulsify the cholesterol. Most of the 1 mg of detergent in the cholesterol emulsion must be in the mixed micelle, but most of the 1 mg in the cerebroside emulsion may be in separate micelles of pure detergent. The pure detergent micelles may compete effectively for the lipid binding sites.

The idea that two kinds of detergent molecules can exist in lipid emulsions—free and lipid-bound—and that the free detergent can compete with the lipid micelles for attachment to membranes or enzyme-lipid substrate complexes, might be of general use in lipid biochemistry. It may explain the frequently observed stimulatory action of detergents or bile salts on lipid-acting enzymes, but inhibitory effects when high concentrations of detergents are used. It also may explain, conversely, why cholesterol protects red blood cells against the lytic action of lysolecithin, which is an excellent detergent (30).

A small part of the inhibitory effect of free detergent could be shown to arise from its solubilizing effect on myelin itself. Incubation for 10 min with 1 mg of detergent, followed by the usual centrifugal washing, was followed by extraction of the lipids in the pellet and supernatant solutions with chloroform-methanol (2:1). The lipids were quantified by photometric scanning of TLC plates, and it was found that roughly 35% of the myelin lipids was lost in the washes when no detergent was present, and about 50% was lost in the detergent incubation. The detergent caused loss of all classes of lipids that were seen on the chromatogram, but ethanolamine phosphoglycerides were lost preferentially. The myelin pellets were not noticeably different in volume.

Nature of the Lipid-Membrane Linkage

The reversibility of the uptake phenomenon was studied by incubating the washed cerebro-

TABLE V
Recovery of Myelin Protein After Incubation and Washing

Incubation medium	Protein in pellet (μg)	(% lost)
Water alone	570,513	23
5 μatoms Ca^{++}	540,530	24
40 μg detergent	350,366	49
Detergent + 20 μg cerebroside	493,450	33
Detergent + cerebroside + Ca^{++}	563,543	21

Protein weights are from duplicate experiments; percentages are averaged.

Incubation conditions: myelin equivalent to 700 μg protein, 10 min at 37C, two washes with 4 ml sucrose solution.

side-myelin complex with a fresh portion of nonradioactive cerebroside-detergent emulsion in Ca^{++}. A decrease in radioactivity of 18% was observed, but this was equal to the decrease observed in the control incubation (water and $CaCl_2$ alone). Evidently the bound cerebroside cannot readily dissociate or exchange with cerebroside micelles in the medium. An additional incubation with unlabeled cerebroside emulsion resulted in similar additional loss, but a third incubation did not result in further loss. It is likely that part of the myelin (and its attached cerebroside) is more labile to exposure to the incubation medium and breaks off in the form of very small particles, as noted in the experiments on detergent effects.

The loss of myelin during incubation and washing was followed also by measuring the protein portion (Table V). It is evident that a small part of the myelin was lost in the procedure and that the detergent alone increased the loss appreciably. However, the detergent that is complexed with cerebroside, especially in the presence of Ca^{++}, had no effect on the loss. This is to be expected from the previous discussion. Centrifugation of the first wash, following incubation, yielded an additional 4% of the protein in the case of the complete system; with detergent alone, the additional recovery was 12%. This difference presumably represents myelin which had been dissociated by the free detergent, then reaggregated on dilution with the sucrose. Similar reaggregation of membranes solubilized by bile salts has been reported (10).

The lipid uptake is apparently a physical phenomenon, rather than enzymatic. This was indicated by the lack of interference on incubating cerebroside with myelin and puromycin (5.4 μg) and iodoacetamide (200 μg). Myelin heated 15 min at 90C showed considerably better uptake, Ca^{++} still being required; mitochondria behaved similarly. Uptake was about 92% with both cell components; evidently the new surface exposed by the heat denaturation was able to adsorb a wider range of emulsion particle sizes.

A similar augmentation was produced in mitochondria by $HgCl_2$ and 2,4-dinitrophenol (1 μmole each), but Ca^{++} reduced the extent of the effect. Mitochondria equivalent to 650 μg protein were incubated with cerebroside and 20 μmoles $CaCl_2$; the uptake was 17%. In the presence of Hg^{++} and DNP, the uptakes were 34% and 55% respectively; if Ca^{++} was omitted, the uptakes were 92% and 85% respectively.

GENERAL DISCUSSION

The Significance of Particle Size in Emulsions

We have shown that our lipid emulsions cannot be taken up completely by brain membranes and have suggested that only emulsified particles within a certain range of sizes can be effective. We also noted that excessive amounts of detergent, which may be expected to produce smaller lipid particles, interfere with the uptake process. It is therefore likely that the inactive lipid particles are the smaller ones. The smaller particles presumably contain a relatively high ratio of detergent to lipid; if detergent molecules were taken up preferentially one would predict that these smaller particles would be taken up in preference to the larger ones. This inconsistency suggests that the interfering effect of high detergent concentrations is not due to competitive uptake of detergent, as suggested in the section on detergent effects, but to production of particles that are too small.

It seems clear that work with lipid emulsions will have to take into consideration the factor of particle size, and the effect of additives of various sorts (lipoidal and nonlipoidal) on the particle size of the emulsion. This problem is relevant not only to in vitro enzyme work but to physiological matters, such as the tendency of circulating lipids to be taken up by organs and arterial walls. The phenomenon of the stabilizing or labilizing action of lipids on lysosomes and circulating blood cells may involve uptake of emulsion particles of specific sizes.

The Self-Assembly of Membranes

Our findings are consistent with the idea that membranes are formed, in part at least, by a self-assembling process. Some evidence for

specificity of assembly was obtained; we found lecithin to be taken up more efficiently than cerebroside or cholesterol, especially by lipid-depleted membranes. The uptake of cerebroside by mitochondria might seem a sign of *non*-specificity, as the cerebroside content of some mitochondrial preparations has been found to be low (25) and might actually represent some contamination by myelin.

It is now well known that cerebroside deposition in brain does not become appreciable until myelin deposition begins on a vigorous scale. In rats this is about 10 days of age (9). Lecithin deposition is already quite active before this time and it is tempting to think, drawing upon our findings on relipidation with lecithin, that cerebroside deposition cannot begin until lecithin first condenses with myelin protein. In agreement with this concept are the reports (6,7) indicating that myelin from young mice and rats is relatively rich in lecithin, but low in cerebroside and cholesterol.

Since myelin lipids undergo attrition by some process of turnover (25), there must be some mechanism for replacing the lipid molecules. Perhaps the mechanism involves an uptake process similar to those observed in this study. It is unlikely that myelin is destroyed and replaced in toto as the component lipids are destroyed at different rates. If myelin (and other membranous particles) lose their component lipids from assorted regions, the membranes isolated from animals at any moment of life must have regions that are deficient in lipids. Presumably these are the sites which took up lipids in our incubation experiments.

In agreement with this viewpoint is the finding by Omura et al. (31) that the lipids of liver microsomes exhibit a turnover rate that is higher than that of the microsomal proteins. Thus, there must always be some empty sites available for uptake of new lipid molecules.

Some recent studies with whole membranes, their disaggregation, and their reaggregation indicate that membrane components can be reassembled into a complex much like the original one. The cell membranes of a microorganism have been disaggregated with anionic detergents, then dialyzed until the membrane reformed (32). It was necessary to dialyze against 0.01 M Ca^{++} or Mg^{++}, a concentration we found quite effective for lipid uptake. The reformed membrane was a little thinner than the original membrane but showed the same triple-layer pattern. The reformed membrane had a lower protein content than the original, possibly because the composition of the final product depends on the proportions of the components in the "soluble" form.

Of particular interest is the study by Pollack et al. (4) in which it was shown that liver reticulosomes (minute particles occurring in the microsomal fraction) could take up lipids from a crude phospholipid emulsion. Electron microscopy revealed conversion of the particles to membranous particles and the authors hypothesized that the reticulosomes are the precursors of membranes. They are visible best during the period of most active growth, presumably because lipid synthesis does not quite keep pace with the reticulosomal protein synthesis. Thus the reticulosomes, once they have combined with lipid, could be considered as "protomembranes" which are transported and assembled into the larger membranes of specialized structures.

Some evidence for such a process in brain comes from a study we made of the fatty acid distribution in lipids from different brain particles (33). The acids of sphingomyelin and cephalins (ethanolamine and serine lipids) were examined. It was found that the distribution of acids was very similar in synaptosomes (nerve terminals) and microsomes of gray matter. Myelin had a considerably different pattern, and the microsomes from white matter had a pattern intermediate between those of myelin and gray microsomes. These results indicate that microsomes have a characteristic composition, whether from gray or white matter, but they also include "protomembrane" particles characteristic of adjoining membranes. Thus, white microsomes include "protomyelin" fragments and gray microsomes include "protosynaptosomes"; these particles are in the process of being transported to the zones where they will be assembled into place.

Comparison with Other Lipid Uptake Studies

Cholesterol uptake by L5178Y tissue-culture cells showed several points of similarity (34). The cholesterol, once taken up, was not removable by exchange. Delipidation impaired sterol uptake, but the effect of relipidation was not studied. Heated cells (60C for 30 min) were also as effective as normal cells. It is interesting that uptake was observed when horse serum was used to emulsify the cholesterol, as well as when a non-ionic detergent was used.

Another study demonstrated palmitic acid uptake by mitochondria and microsomes (1). Here the lipid was kept in solution by complexing with serum albumin, so it would appear

that the particle size was much lower than in our emulsions. The uptake was not much less at 0C than at 37C and it leveled off after about 10-30 min. In this and the above study the particles were suspended in salt-containing medium, so the influence of cation concentration was not investigated.

It is obvious that the conditions used in these and our uptake studies were far from physiological and the various proposals must be considered highly tentative.

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Dietary Induced Alterations in Swelling Characteristics and Endogenous Phospholipase A_2 Activity of Rat Liver Mitochondria

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ABSTRACT

The present study describes the rapid alterations in the fatty acid patterns of phosphatidyl choline and phosphatidyl ethanolamine from rat liver mitochondria induced by corn oil feeding to EFA-deficient rats. Simultaneous changes occurring with comparable rates were observed in the swelling properties and phospholipase A_2 activity of the mitochondria. Mitochondria isolated from the liver of EFA-deficient rats exhibited a high tendency to swell and a high phospholipase A_2 activity in comparison with those prepared from normal rat liver. Feeding of corn oil for 48 hr to the EFA-deficient rats completely reduced this high rate of swelling and phospholipase A_2 activity to the normal level. In the same time eicosatrienoic acid, a characteristic fatty acid constituent of phospholipids from EFA-deficient animals, was replaced by the more common fatty acids, linoleic and arachidonic. The possible relationship between fatty acid constituents of phospholipids, swelling properties and phospholipase A_2 activity in rat liver mitochondria are discussed.

INTRODUCTION

RECENT STUDIES in this laboratory have shown that the molecular composition of rat liver lecithin can be influenced to a great extent by dietary changes (1,2). Feeding of corn oil to EFA-deficient animals which are characterized by high amounts of 18:0/20:3-,[2] 16:0/20:3-, and 18:1/18:1-lecithins in the liver, causes a rapid disappearance of these molecular species and a concomitant increase in 16:0/18:2,- 18:0/18:2,- 16:0/20:4- and 18:0/20:4-lecithin (2). In agreement with their molecular makeup, the lecithins from the EFA-deficient and the corn-oil-fed rats gave on the Langmuir-Adam trough liquid expanded monolayer films exhibiting force-area curves with only limited differences (1). It was concluded tentatively that these shifts in molecular species induced by dietary changes are attempts of the organism to maintain constant the physico-chemical properties of the membrane phospholipids at least to some extent. This does not imply, however, that the phospholipid composition which completely meets the characteristics of the normal membrane is always attained by the organism. In 1963 Johnson (3) reported that mitochondria isolated from livers of EFA-deficient rats undergo more rapid swelling, either spontaneously or in the presence of swelling agents than do those from normal rats. The author suggested that the swelling tendency in liver mitochondria from EFA-deficient animals is associated with altered respiratory and/or phosphorylating mechanisms rather than with a membrane alteration. On the other hand, de Pury and Collins (4) reported that the fragility of mitochondrial membranes from EFA-deficient animals could be explained by assuming that the structural proteins have some special sites for hydrophobic binding of arachidonic acid in which phospholipids containing this fatty acid are bound more firmly than other phospholipids. A parallelism has been demonstrated recently between certain types of swelling and the phospholipase A_2 activity of the mitochondria (5). Because the phospholipase A_2 may play an important role in the deacylation-reacylation mechanism by which the dietary-induced changes in the fatty acid composition of mitochondrial phospholipids may be brought about, it was of interest to examine the rate of change of fatty acid composition, swelling and mitochondrial phospholipase A_2 upon addition of corn oil to the diet of EFA-deficient rats.

METHODS AND MATERIALS

A corn oil diet containing 50 cal/100 carbohydrates, 16.8 cal/100 proteins, 33.3 cal/100 corn oil and the required amounts of salts and vitamins was supplied to rats which had been

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[2] 18:0/20:3-lecithin is used to designate (1-stearoyl-2-eicosatrienoyl)-lecithin.

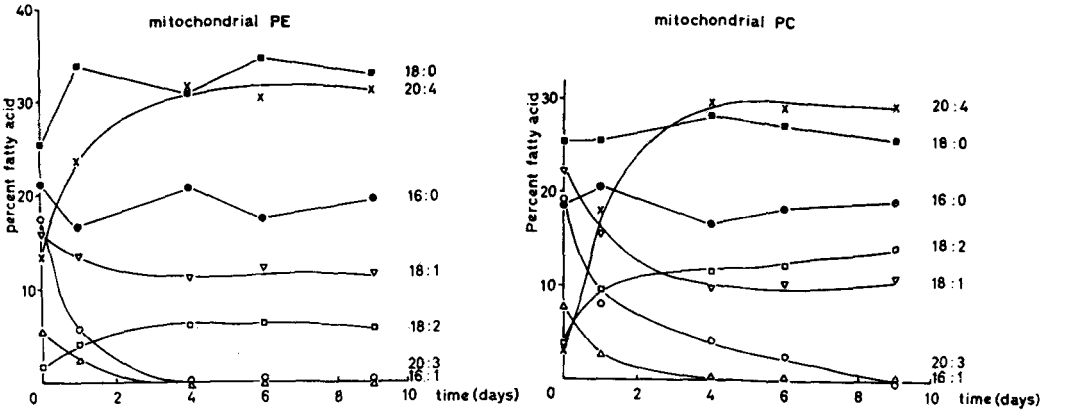

FIG. 1 Alterations induced in the fatty acid composition of phosphatidyl choline and phosphatidyl ethanolamine from liver mitochondria by feeding of corn oil to EFA-deficient rats as a function of time. Time zero represents the fatty acid composition of EFA-deficient rats. Each point resulted from duplicated analyses, the experimental error being smaller than the normal relative deviation of 5% in gas chromatography.

raised on an EFA-deficient diet for a period of 3 months. The control rats were fed ad libitum, a regular chow-pellet diet. At each indicated time 2 rats were sacrificed. The livers were quickly isolated and, after pooling, homogenized in ice-cold 0.28 M sucrose. The mitochondria were prepared as described before (6). An aliquot of the mitochondria was extracted according to the procedure of Bligh and Dyer (7) followed by 2 additional washings of the aqueous layer with chloroform. Phosphatidyl choline and phosphatidyl ethanolamine were isolated by means of thin-layer chromatography on silica using chloroform-methanol-water (65:35:4, v/v/v) as a developer. Synthetic samples of phosphatidyl choline and phosphatidyl ethanolamine were used as reference substances. After spraying with an 0.01% solution of Rhodamine 6G in water, the spots were outlined under ultraviolet light. The phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) fraction were scraped from the plates and eluted exhaustively with methanol-chloroform (80:20, v/v). The fatty acid composition of the obtained PC and PE was determined by the techniques reported in earlier studies (1,2). The methylesters derived from the fatty acid constituents were analyzed using an F&M gas chomatograph, equipped with a flame-ionisation detector and an EGSS-X (Applied Science, U.S.A.) column. The temperature of the column was maintained at 185 C. The simultaneous spectrophotometric assay of mitochondrial swelling and estimation of hydrolysis of exogenous ^{14}C-labeled phosphatidyl ethanolamine were carried out as follows: Mitochondria prepared from the EFA-deficient rats or EFA-deficient rats which had been transferred to the corn oil diet were incubated for the indicated lengths of time. These reaction mixtures contained 10 mg of mitochondrial protein and 15 mμmoles 2-[1-^{14}C]-linoleoyl phosphatidyl ethanolamine (1.1×10^5 cpm)/ml isotonic Tris-KCl, pH 7.4. At the indicated times a 1.0 ml aliquot was pipetted into 2.0 ml methanol at 0 C for the determination of phospholipase A_2 activity by the hydrolysis of exogenous phosphatidyl ethanolamine as described earlier (6), and an 0.04 ml aliquot was pipetted into 3 ml cold isotonic Tris-KCl for determination of the optical density at 520 mμ. In these experiments the initial optical density was about 0.450 units. In Fig. 2 swelling is expressed as negative change in absorbancy at 520 mμ.

RESULTS AND DISCUSSION

Fig. 1 represents the results obtained from fatty acid analysis of the mitochondrial PC and PE from EFA-deficient rats and those which had been on the corn oil diet for the indicated period of time. A rapid increase was noted in the amount of arachidonic acid, and to a lower extent, of linoleic acid when corn oil was fed to EFA-deficient rats. A concomitant decrease in eicosatrienoic and oleic acid is apparent, these changes being nearly complete within 48 hr. Similar but somewhat more rapid changes are found in the fatty acid composition of PE, especially in the disappearance of eicosatrienoic acid. In this connection it is of interest to note that PE is better hydrolyzed

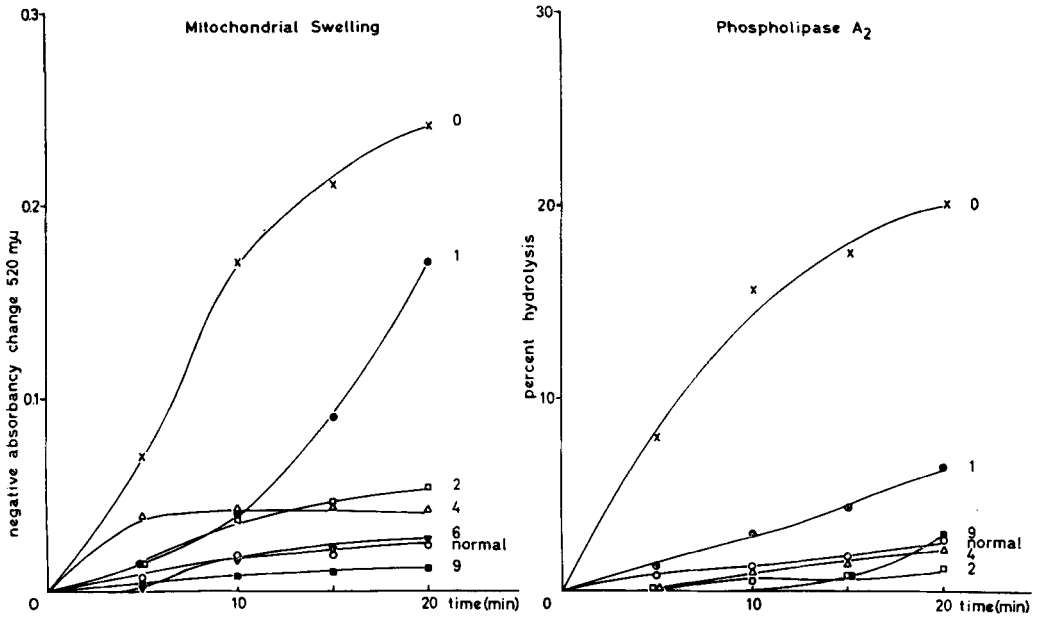

FIG. 2. Changes induced in swelling and phospholipase A_2 activity towards exogenous substrates of rat liver mitochondria by feeding of corn oil to EFA-deficient rats as a function of time. Details are given in the Method section.

than PC by the mitochondrial phospholipase A_2 (6). The saturated fatty acids did not reveal any notable variation during these experiments except in PE, where an initial increase in the content of stearic acid is to be noted. These results compare favorably with earlier studies in which the changes of the individual molecular species of total liver lecithin were determined after corn oil feeding to EFA-deficient rats as a function of time (2).

Marked alterations in the swelling characteristics and phospholipase A_2 activity of the mitochondria were observed (Fig. 2). Mitochondria from the EFA-deficient animals (indicated in Fig. 2 as 0 days on corn oil diet) were about 10 times as active in hydrolyzing exogenous PE than those from normal rats. An analogous situation can be seen in the swelling properties; whereas the mitochondria from normal rats underwent no swelling during 20 min incubation, those from EFA-deficient animals exhibited a high degree of swelling during the same incubation time. Feeding of corn oil during 24 hr caused a marked decrease in both the swelling and phospholipase A_2 activity. After 48 hr there was no appreciable change. The values obtained after 48 hr of corn oil feeding are essentially the same as those from the normal rat liver mitochondria. At present it cannot be stated whether the increased phospholipase A_2 activity is due to alterations in the membrane which favor an increase in the enzymatic activity or whether there is an elevated level of enzyme under these conditions. Also, it is not known if the swelling of the mitochondria from EFA-deficient rats is due directly to an increased level of phospholipase A_2 activity or due to changes in physico-chemical properties brought about by the changes in the fatty acid composition, either in the membrane as proposed by Collins (4) or in an alteration in the electron transport system and/or an oxidative phosphorylation as suggested by Johnson (3). If the latter were to be the primary cause it would be possible for the swelling effect to be manifested by an increased phospholipase A_2 activity since it has been shown that the level of compounds such as ATP could effect phospholipase A_2 activity (8).

This study shows that changes in the fatty acid composition of mitochondrial PE and PC, swelling and in phospholipase A_2 activity occur at comparable rates upon feeding of corn oil to EFA-deficient rats. Although it is impossible to draw definite conclusions at present, these observations are consistent with the concept that the replacement of certain major molec-

ular species (1-stearoyl-2-eicosatrienoyl)- and (1-palmitoyl-2-eicosatrienoyl) by other species (1-stearoyl-2-arachidonoyl) and (1-palmitoyl-2-arachidonoyl)-lecithin when corn oil is fed to EFA-deficient rats can be brought about, at least in part, by a selective hydrolysis of the 2-acyl group of the phospholipids followed by reacylation of the lysoderivative giving rise to the new species.

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SHORT COMMUNICATIONS

The Behavior of Proteolipids on Dextran Gel Columns Eluted With Organic Solvents

THE SEPARATION OF WATER-SOLUBLE, non-lipid material and gangliosides from other lipids in chloroform-methanol extracts of tissue has usually been accomplished by partition (1,2). However, the variations of this method thus far described have the disadvantage of either not completely segregating the gangliosides into the upper (water-methanol) phase or, if such a transfer is complete, of losing a small fraction of other lipids into that phase. Furthermore, after partition, extensive dialysis is required to separate the gangliosides from water-soluble non-lipids.

In 1965, Siakotos and Rouser (3) introduced the use of a dextran gel (G-25 beaded Sephadex) column for total separation of gangliosides from the other lipids and from water-soluble, non-lipid material. Since this is an extremely useful procedure in the initial separation of components of a lipid extract, it is of some interest to examine the behavior of proteolipid protein on this type of column, particularly in view of the prominent part proteolipids play in the quantitive analyses of the elements that comprise the myelin sheath (4).

Adult cerebral white matter was extracted and partitioned by the method of Folch et al. (1). It is necessary to exercise great care when evaporating the lower phase of a Folch extract. If two phases form while the volume is being reduced, almost all of the protein is denatured and cannot be eluted from a dextran gel column. The results are improved if the evaporation is not carried to complete dryness but a loss still occurs (5). We have avoided this loss by evaporating the lower phase in vacuo after adding sufficient chloroform to maintain the sample in a single phase until dry. This prevents the denaturation of the proteolipid protein which is apt to occur at a chloroform-methanol/water-methanol interface. In most instances, five volumes of chloroform were required for each volume of lower phase to be evaporated. If this ratio was adhered to, it was seldom necessary to interrupt the evaporation because of cloudiness. The residue was immediately redissolved in chloroform-methanol (19:1, v/v) saturated with water, and aliquots were taken for the estimation of proteolipid protein by a modification of the method of Lowry et al. (6) designed to measure protein in lipid extracts (7). The remainder of the redissolved extract was applied to a G-25 beaded Sephadex column and eluted with the solvent mixtures outlined by Siakotos and Rouser (3):

1) Chloroform-methanol (19:1, v/v) saturated with water.
2) 850 ml of chloroform-methanol (19:1, v/v) + 170 ml of glacial acetic acid; saturated with water.
3) 850 ml of chloroform-methanol (9:1, v/v) + 170 ml of glacial acetic acid, saturated with water.
4) Methanol-water (1:1, v/v).

Approximately 100 ml of each solvent mixture was used for each 10 ml of column volume. The proteolipid content of each of the eluates was then measured.

As can be seen in Table I most of the proteolipid applied to a Sephadex column is eluted. However, the amount recovered is not constant or reproducible. The variation in recovery does not appear to be related to column size, the amount of material placed on the column or the nature of the sample. Aliquots of the same white matter extract placed on different columns gave different results, and the amount of proteolipid recovered from the same column varied from time to time.

Most of the proteolipid was found in fraction 1. Fractions 2 and 3 accounted for 0.582-1.52% of the proteolipid recovered, while fraction 4 accounted for 0.135-0.78%. The amount of material in fractions 2-4 was not sufficient to determine the amino acid composition; thus we cannot be certain that it did not differ qualitatively from fraction 1. However, when fraction 1 was reapplied to Sephadex columns, a

TABLE I
Recovery of Proteolipid from G-25 Sephadex Columns

Sample No.	Volume of column (cc)	Amount of proteolipid applied (mgm)	% Recovery of proteolipid
1	9	7.22	96.5
2	9	6.38	90.1
3	26	38.89	93.5
4	26	34.28	96.9
5	26	29.02	91.0
6	26	22.74	96.1
7	26	18.39	74.2
8	26	18.21	90.2
9	26	17.89	93.3
10	26	17.42	81.6
11	26	15.23	88.9

similar distribution occurred in these eluates, suggesting that the original separation was not necessarily due to a difference in structure.

The proteolipid that remains on the column apparently cannot be eluted by the repeated application of solvent mixtures. Columns that had been used several times for the separation of these samples were left standing in methanol-water (1:1, v/v) and then eluted once again with chloroform-methanol (19:1, v/v) saturated with H_2O; the amount of proteolipid recovered varied from 14.4 to 32.0 γ, although in any single run on columns of this size several milligrams of proteolipid often cannot be accounted for.

We feel that a plausible explanation for the loss of proteolipid on these columns and the inability to elute it with further solvent is that it has been denatured. This possibly is the result of using solvent systems saturated with water. The amount of proteolipid protein in solutions of C/M (19:1, v/v) saturated with H_2O left standing at room temperature for periods of up to 16 days diminishes steadily with time; at 8 days 58.4 ± 8.0% of the original is present, and at 16 days 43.9 ± 2.2%. A translucent ring of precipitate often coats the walls of the cylinders at the level of the meniscus. This occurs despite the fact that two phases are not formed. On the other hand, the amount of proteolipid in the lower phase of a C-M (2:1) extract (1) remains constant at room temperature for long periods of time.

We suggest that if it is desired to estimate the proteolipid protein in a lipid extract that an aliquot of the original extract be partitioned and the protein in the lower phase measured. The remainder of the original extract may then be chromatographed on a G-25 beaded Sephadex column.

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Effect of Bile Salts on Hydrolysis of Cholesteryl Oleate by Rabbit Aorta

THE RATIO OF GLYCOCHOLANIC to taurocholanic acids (G/T) present in bile may be correlated with species susceptibility to experimental atherosclerosis (1). Thus, G/T is low in the bile of the resistant rat and dog and high in the susceptible rabbit. Attempts to reduce hypercholesteremia by oral administration of taurine have been ineffective in man (2) and rabbit (3) but have had some effect in the rat (3).

An increase in aortic ester cholesterol has been observed in human and in experimental atherosclerosis. Zemplenyi (4) has demonstrated that the lipolytic capacity of aortas of different animal species is related to their susceptibility to experimentally induced atherosclerosis. In view of the observation of Vahouny et al. (5) of an absolute bile salt requirement for cholesterol esterase activity, we investigated the possibility that taurocholic acid might enhance the hydrolysis of cholesteryl oleate by aorta preparations.

Two types of experiment were conducted. In the first, rabbit aortas were excised and freed of adhering tissue; small segments (100–150 mg) were then placed in 4 ml of Krebs-Ringer phosphate buffer, pH 7.4, according to the method of Zemplenyi (4). A solution of 4-^{14}C-cholesteryl oleate was prepared by adding a solution of 900 μg of carrier cholesteryl oleate and 2 μc 4-^{14}C-cholesteryl oleate in 1.13 ml of acetone to 225 mg albumin in 45 ml of buffer. The acetone was removed by dialysis. One milliliter of the cholesteryl oleate solution was added to the suspension of aorta slice in buffer, and the mixture was incubated at 37 C for 3 hr with shaking. The tissue and medium were exhaustively extracted with chloroform-

similar distribution occurred in these eluates, suggesting that the original separation was not necessarily due to a difference in structure.

The proteolipid that remains on the column apparently cannot be eluted by the repeated application of solvent mixtures. Columns that had been used several times for the separation of these samples were left standing in methanol-water (1:1, v/v) and then eluted once again with chloroform-methanol (19:1, v/v) saturated with H_2O; the amount of proteolipid recovered varied from 14.4 to 32.0 γ, although in any single run on columns of this size several milligrams of proteolipid often cannot be accounted for.

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We suggest that if it is desired to estimate the proteolipid protein in a lipid extract that an aliquot of the original extract be partitioned and the protein in the lower phase measured. The remainder of the original extract may then be chromatographed on a G-25 beaded Sephadex column.

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Effect of Bile Salts on Hydrolysis of Cholesteryl Oleate by Rabbit Aorta

THE RATIO OF GLYCOCHOLANIC to taurocholanic acids (G/T) present in bile may be correlated with species susceptibility to experimental atherosclerosis (1). Thus, G/T is low in the bile of the resistant rat and dog and high in the susceptible rabbit. Attempts to reduce hypercholesteremia by oral administration of taurine have been ineffective in man (2) and rabbit (3) but have had some effect in the rat (3).

An increase in aortic ester cholesterol has been observed in human and in experimental atherosclerosis. Zemplenyi (4) has demonstrated that the lipolytic capacity of aortas of different animal species is related to their susceptibility to experimentally induced atherosclerosis. In view of the observation of Vahouny et al. (5) of an absolute bile salt requirement for cholesterol esterase activity, we investigated the possibility that taurocholic acid might enhance the hydrolysis of cholesteryl oleate by aorta preparations.

Two types of experiment were conducted. In the first, rabbit aortas were excised and freed of adhering tissue; small segments (100–150 mg) were then placed in 4 ml of Krebs-Ringer phosphate buffer, pH 7.4, according to the method of Zemplenyi (4). A solution of 4-^{14}C-cholesteryl oleate was prepared by adding a solution of 900 μg of carrier cholesteryl oleate and 2 μc 4-^{14}C-cholesteryl oleate in 1.13 ml of acetone to 225 mg albumin in 45 ml of buffer. The acetone was removed by dialysis. One milliliter of the cholesteryl oleate solution was added to the suspension of aorta slice in buffer, and the mixture was incubated at 37 C for 3 hr with shaking. The tissue and medium were exhaustively extracted with chloroform-

TABLE I

Influence of Sodium Glycocholate and Taurocholate Upon Hydrolysis of 4-^{14}C-Cholesteryl Oleate by Rabbit Aorta

Moles of bile salt	Taurocholate	Glycocholate
Exp't. 1, Aorta Slices[a]	dpm/mg aorta slice	
10^{-6}	6.18 (4.23-7.40)[b]	3.17 (2.20-3.80)
10^{-5}	9.15 (6.95-12.20)	4.50 (2.40-5.70)
Exp't. 2, Aorta Homogenate[c]	dpm/mg N	
8×10^{-7}	125	90
4×10^{-6}	178	128
10^{-5}	200	113

[a] Average of 3 experiments, individual aortas.

[b] Range.

[c] Average of duplicate incubations, single homogenate of 3 pooled aortas.

methanol (2:1) and the chloroform phase dried over anhydrous Na_2SO_4 and evaporated under N_2. The residue was dissolved in ethanol from which the free cholesterol was precipitated with digitonin. The cholesterol digitonides were assayed for radioactivity by liquid scintillation spectrometry.

In a second series of experiments the solutions of cholesteryl oleate and bile salt were added to 1 ml of aorta homogenate prepared according to the method of Howard and Portman (6). After incubation at 37 C for 2 hr the free cholesterol was extracted and assayed as described above. In both types of experiment control vessels containing no bile salts were used and the results corrected for the extent of hydrolysis observed in their absence.

The results (Table I) indicate that, compared to sodium glycocholate, sodium taurocholate enhances the hydrolysis of cholesteryl oleate by preparations of rabbit aorta. Cholesteryl oleate was used because it is the predominant cholesteryl ester of the atheromatous plaque. In view of the reports of preferential hydrolysis of various cholesteryl esters by rabbit aorta (7), and since the ratios of biliary bile acids may vary under certain conditions such as the hyperthyroid state (8), these experiments are being extended to include several common cholesteryl esters and bile acid conjugates. Ultimately we will also study the aortas of other animal species.

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[Received June 5, 1968]

Fatty Acid Methyl Esters in Grasshopper Eggs

DURING AN INVESTIGATION of the lipids of eggs from the grasshopper, *Melanoplus bivittatus*, a lipid component was noticed that had the same R_f value as the fatty acid methyl esters of long-chain fatty acids. Further studies reported here indicated that the fatty acid methyl esters are not formed as artifacts of extraction. To our knowledge, fatty acid methyl esters of long-chain fatty acids have not been previously isolated and characterized in insect eggs, although they have been reported to be present in other animal tissues (1,2).

It is essential to remove all low molecular weight alcohols from the solvents, since we were able to detect the corresponding esters after extraction with chloroform-ethanol and chloroform-isopropanol (2:1, v/v). The organic solvents used for both extraction and chromatography were treated to remove any contaminating low molecular weight alcohols (3) and were tested by gas chromatography for contaminating methanol.

Egg lipids were extracted by homogenizing the eggs in diethyl ether and then filtering the homogenate through a medium porosity fritted glass funnel. This lipid extract contained most-

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ly free fatty acids, with lesser amounts of sterol, triglycerides, sterol esters and hydrocarbons. Fatty acid methyl esters amounted to 1% to 2% of the total lipid (4).

The component corresponding to the fatty acid methyl esters was isolated by preparative thin-layer chromatography on silica gel using a solvent system of hexane-diethyl ether (90:10, v/v). The adsorbent containing the methyl esters was scraped from the plate and eluted with benzene purified by fractional distillation. Thin-layer chromatography of the purified preparation gave only one spot corresponding to fatty acid methyl esters in hexane-diethyl ether (85:15, v/v or 90:10, v/v). When high activity thin-layer chromatograms were run in hexane-diethyl ether (90:10, v/v) the spot began to separate into three spots, all above triglycerides and below wax esters. We observed this phenomenon with standard fatty acid methyl ester mixtures containing saturated, monoene, diene and triene fatty acid methyl esters.

The identification of the isolated material as methyl esters of fatty acids was confirmed by means of infrared and nuclear magnetic resonance spectroscopy. The infrared spectra of the natural product and a known methyl ester mixture were virtually identical. A 60 Mc NMR spectrum of the same material verified the methyl ester assignment via a typical methoxy peak at 3.6 ppm(δ).

The purified fatty acid methyl esters were analyzed by gas-liquid chromatography on a 4 ft × ¼ in O.D. glass column packed with 15% ethylene glycol succinate on Gas Chrom Z (Applied Science Labs, State College, Pa.). The column oven temperature was 170 C. Quantitation was accomplished using a standard mixture of fatty acid methyl esters; no corrections were necessary. The fatty acid methyl ester composition is 9% 16:0, 3% 16:1, 2% 18:0, 35% 18:1, 26% 18:2 and 24% 18:3.

The role that fatty acid methyl esters might play in insect eggs is unknown. However, McFarlane and Henneberry (6) have postulated that fatty acids and fatty acid methyl esters play a role in growth and development in insects. They showed that certain of the C-12 to C-18 fatty acids and their methyl esters inhibited the growth of a cricket, *Gryllodes sigillatus* (Walk.) when these substances were presented in such a way that they enter the body through the cuticle, but did not inhibit when presented in fairly large amounts (1%) in the diet. Methyl linolenate and methyl laurate stimulated growth in the house cricket, *A. domesticus* (7,8). MacFarlane also demonstrated that methyl palmitate inhibited the growth of larvae of *Gryllodes sigillatus* (Walk.) (9). The effect of the chemical took place during the first 11 days of exposure; after that, additional exposure had a negligible effect. These observations plus the presence of methyl esters in the egg suggest that specific levels of natural fatty acid methyl esters may be necessary for normal physiological development.

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[Received May 20, 1968]

Irreversible Enzyme Inhibition by a Phosphatidic Acid-Like Lipid

DURING AN INVESTIGATION of phospholipid metabolism in mice a potent irreversible enzyme inhibitor was found in crude preparations of phosphatidic acid. Alpha-glycerolphosphate dehydrogenase from mouse liver and glucose-6-phosphate dehydrogenase were particularly sensitive. The inhibitor appears to be an oxidized form of phosphatidic acid and is probably not naturally occurring but is generated from phosphatidic acid during its preparation by exposure to oxidizing conditions.

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Phosphatidic acid was prepared enzymatically from various lecithins by the procedure of Kates (1). The inhibitor was separated from

TABLE I

The Inhibition of α-Glycerol Phosphate Dehydrogenase by Phosphatidic Acid and Other Preparations

Preparation	mμmoles[1]	% enzyme activity remaining[2]
Phosphatidic acid, commercial	218	107
Phosphatidic acid, crude[3]	103	52
Inhibitor, purified	16	26
Lecithin, egg, commercial	410	94
Lysolecithin	910	98

[1]Determined as phosphorus.

[2]Activity of α-glycerol phosphate dehydrogenase in the presence of the indicated amount of chemical, expressed as percentage of control: Assay system consisted of 0.10 ml mouse liver enzyme (prepared by homogenizing fresh mouse liver in 4 parts water containing 1.0 mM EDTA pH 7.0 and centrifuging at 100,000 × *g* for 1 hr. The supernatant was used without further purification), 0.2 μmoles NADH, 1.0 μmole dihydroxyacetone phosphate, 60 μmoles tris buffer pH 7.5, 1.6 μmoles EDTA, and water and additions. Final volume: 1.3 ml.

[3]Prepared from commercial egg lecithin.

phosphatidic acid by chromatography of an aqueous emulsion of the crude phosphatidic acid on Sephadex G-25 followed by thin layer chromatography on silica gel G in diisobutylketone-acetic acid-water (80:50:7). The inhibitor migrated with a slightly lower Rf than phosphatidic acid and hence is slightly more polar. The inhibitor was assayed routinely with α-glycerolphosphate dehydrogenase from mouse liver (Table I). The inhibitor was found to contain phosphorus which was entirely released by treatment with wheat germ acid phosphatase but not by 1 *N* sulfuric acid at 100C for 10 min. Assuming one atom of phosphorus per molecule of inhibitor, a molecular weight of 737 is obtained. The average molecular weight of the disodium salt of phosphatidic acid from rat liver, for example, is 745 (2). The acyl/phosphorus ratio was found to be 1.90. The inhibitory activity was destroyed by wheat germ acid phosphatase and by mild alkaline hydrolysis under conditions which are known to release α-glycerolphosphate from phosphatidic acid. Alpha-glycerolphosphate was detected by paper chromatography in such hydrolysates. Methanol, which also precipitates the inhibitor, caused its destruction following incubation in 0.1 M phosphate buffer pH 7.5 for three hr, whereas incubation in buffer alone caused only a slight loss.

Commercial phosphatidic acid and phosphatidic acid purified in our laboratory by column chromatography on silicic acid (2) were inactive. Commercial lecithins from egg yolk and soy bean as well as lecithins prepared from egg yolk and beef liver by the method of Folch et al. (3) are themselves inactive but all yield inhibitor-containing phosphatidic acid preparations. A typical preparation from commercial egg lecithin contained approximately 10% inhibitor as determined by weight and phosphorus assay following isolation by chromatography on Sephadex G-25. The inhibitor can be produced from inhibitor-free phosphatidic acid by bubbling oxygen into chloroform solution or by repeated evaporation of an inhibitor-free phosphatidic acid solution in peroxide-containing ether. The inclusion of butylated hydroxytoluene in the phosphatidic acid preparations suppressed inhibitor formation during exposure to oxidizing conditions. When butylated hydroxytoluene is included in all phases of phosphatidic acid preparation the inhibitor content is markedly diminished. However, the exposure of the various lecithins, used for the preparation of the phosphatidic acids, to these oxidizing conditions does not result in an increased yield of inhibitor in the phosphatidic acid made from the treated lecithins. Finally, purified inhibitor preparations exhibit an absorption maximum at 275 mμ, which is characteristic of oxidized lipids.

Several dehydrogenases were inhibited by the presence of the inhibitor although to markedly different degrees. Alpha-glycerolphosphate dehydrogenase and glucose-6-phosphate dehydrogenase were the most sensitive. In the dilute conditions of the normal assay system competitive inhibition kinetics were found with apparent Ki values shown in Table II. However, when α-glycerolphosphate dehydrogenase and glucose-6-phosphate dehydrogenase were preincubated with the inhibitor for 15 min the enzymes were irreversibly and totally inhibited. When substrate was included in the preincubation mixture the enzymes were afforded only temporary protection since continued preincubation resulted in total loss of activity which could not be restored by adding excess substrate.

Recently, we learned that Wills (4) reported the inhibitory effect of 0.4 mM linoleic acid on several enzymes not including dehydrogenases. However, the fact that our inhibitor is destroyed by mild alkaline hydrolysis and by treatment with acid phosphatase and appears to

TABLE II

Apparent Ki's for Competitive Inhibition of Various Dehydrogenases by the Inhibitor

Dehydrogenase	Apparent Ki mM
α-Glycerolphosphate	0.026
Glucose-6-phosphate	0.043
Alcohol	0.30
Lactic	1.9
Sorbitol	4.0

be many times more potent on a molar basis than free linoleic acid suggests that its effectiveness as an enzyme inhibitor is not due merely to its content of unsaturated fatty acids. We suggest that the inhibitor reported here is an oxidized form of phosphatidic acid.

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The Occurrence of 5α-Cholestan-3β-ol (Dihydrocholesterol) in Human Skin Surface Lipid

ALTHOUGH MUCH HAS BEEN WRITTEN about the sterols of human skin surface lipids, no sterol other than cholesterol has hitherto been unambiguously identified. The data in this paper show that 5α-cholestan-3β-ol occurs in this lipid.

A fraction of saturated sterols was prepared from 0.752 g of human scalp skin surface lipid as previously described (1). Steps summarizing the preparation are: chromatography of the surface lipid on silicic acid to obtain a mixture of wax esters plus sterol esters; saponification of this mixture; acetylation of the nonsaponifiable fraction; chromatography of the nonsaponifiable acetates on SiO_2-$AgNO_3$ to obtain a mixture of acetates of saturated wax alcohols and sterols; and separation of the saturated acetates into a urea adduct forming fraction (which comprised the bulk of the material) and a non urea adduct forming fraction (~1.5 mg).

Preliminary gas chromatography of the non-urea adduct forming fraction on 3% JXR showed a major peak and two minor ones in the cholesteryl acetate region and minor amounts of low molecular weight material. The rest of this paper deals with the identification of this major sterol acetate peak, which will be designated as human skin surface lipid stanol or HSSL-stanol.

Stereochemistry about positions 3 and 5 of the stanols predicts four isomers: 5α-cholestan-3β-ol (dihydrocholesterol); 5α-cholestan-3α-ol (epidihydrocholesterol); 5β-coprostan-3β-ol (coprosterol); and 5β-coprostan-3α-ol (epicoprosterol).

TABLE I

GLC Retention Data for Cholesterol, a Saturated Sterol from Human Skin Surface Lipid (HSSL-Stanol) and the Epimers of Cholestanol and Coprostanol

	Relative Retention Data[a] on SE 30/NGS[b]			Relative Retention Data[c] on SE 52/XE 60[d]			
	Parent compound	Acetate	TFA[e]	Parent compound	Acetate	TFA[e]	TMSi[f]
Cholesterol	2.5	3.1	1.6	2.01	3.06	1.53	2.38
HSSL-Stanol	2.5	3.1	1.6	2.07	2.93	1.62	2.50
5α-Cholestan-3β-ol	2.5	3.1	1.6	2.04	2.93	1.62	2.50
5α-Cholestan-3α-ol	2.4	—	1.3	2.00	—	1.33	1.91
5β-Coprostan-3β-ol	2.1	2.6	1.3	1.81	2.40	1.30	1.85
5β-Coprostan-3α-ol	2.2	—	1.3	1.80	—	1.31	1.97

[a] Cholestane = 1.00 (6.9 min).
[b] Column temperature = 225C; Outlet flow rate = 100 ml/min. From Nair et al. (2).
[c] Cholestane = 1.00 (7.1 min).
[d] Column temperature = 230C; Outlet flow rate = 100 ml/min. From Nair et al. (3,4).
[e] TFA = Trifluoroacetates.
[f] TMSi = Trimethylsilyl ethers.

be many times more potent on a molar basis than free linoleic acid suggests that its effectiveness as an enzyme inhibitor is not due merely to its content of unsaturated fatty acids. We suggest that the inhibitor reported here is an oxidized form of phosphatidic acid.

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The Occurrence of 5α-Cholestan-3β-ol (Dihydrocholesterol) in Human Skin Surface Lipid

ALTHOUGH MUCH HAS BEEN WRITTEN about the sterols of human skin surface lipids, no sterol other than cholesterol has hitherto been unambiguously identified. The data in this paper show that 5α-cholestan-3β-ol occurs in this lipid.

A fraction of saturated sterols was prepared from 0.752 g of human scalp skin surface lipid as previously described (1). Steps summarizing the preparation are: chromatography of the surface lipid on silicic acid to obtain a mixture of wax esters plus sterol esters; saponification of this mixture; acetylation of the nonsaponifiable fraction; chromatography of the nonsaponifiable acetates on SiO_2-$AgNO_3$ to obtain a mixture of acetates of saturated wax alcohols and sterols; and separation of the saturated acetates into a urea adduct forming fraction (which comprised the bulk of the material) and a non urea adduct forming fraction (~1.5 mg).

Preliminary gas chromatography of the non-urea adduct forming fraction on 3% JXR showed a major peak and two minor ones in the cholesteryl acetate region and minor amounts of low molecular weight material. The rest of this paper deals with the identification of this major sterol acetate peak, which will be designated as human skin surface lipid stanol or HSSL-stanol.

Stereochemistry about positions 3 and 5 of the stanols predicts four isomers: 5α-cholestan-3β-ol (dihydrocholesterol); 5α-cholestan-3α-ol (epidihydrocholesterol); 5β-coprostan-3β-ol (coprosterol); and 5β-coprostan-3α-ol (epicoprosterol).

TABLE I

GLC Retention Data for Cholesterol, a Saturated Sterol from Human Skin Surface Lipid (HSSL-Stanol) and the Epimers of Cholestanol and Coprostanol

	Relative Retention Data[a] on SE 30/NGS[b]			Relative Retention Data[c] on SE 52/XE 60[d]			
	Parent compound	Acetate	TFA[e]	Parent compound	Acetate	TFA[e]	TMSi[f]
Cholesterol	2.5	3.1	1.6	2.01	3.06	1.53	2.38
HSSL-Stanol	2.5	3.1	1.6	2.07	2.93	1.62	2.50
5α-Cholestan-3β-ol	2.5	3.1	1.6	2.04	2.93	1.62	2.50
5α-Cholestan-3α-ol	2.4	—	1.3	2.00	—	1.33	1.91
5β-Coprostan-3β-ol	2.1	2.6	1.3	1.81	2.40	1.30	1.85
5β-Coprostan-3α-ol	2.2	—	1.3	1.80	—	1.31	1.97

[a] Cholestane = 1.00 (6.9 min).
[b] Column temperature = 225C; Outlet flow rate = 100 ml/min. From Nair et al. (2).
[c] Cholestane = 1.00 (7.1 min).
[d] Column temperature = 230C; Outlet flow rate = 100 ml/min. From Nair et al. (3,4).
[e] TFA = Trifluoroacetates.
[f] TMSi = Trimethylsilyl ethers.

Table I lists GLC retention data of the free stanols, and several derivatives on two liquid phases along with similar data for cholesterol and HSSL-stanol. Unaltered HSSL-stanol and its acetate, trifluoroacetate and trimethylsilyl ethers yielded relative retention data identical to 5α-cholestan-3β-ol and its derivatives on two different columns. These data and the fact that the HSSL-stanol acetate emerged from the SiO_2-$AgNO_3$ column with the saturated alcohol acetates would indicate that the HSSL-stanol is dihydrocholesterol.

It is not surprising that dihydrocholesterol is present in the sterol ester fraction of human skin surface lipid since this sterol is known to accompany cholesterol in most tissues. Its metabolic significance has not yet been elucidated.

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We thank Oscar Reiss for gifts of the two epimers of cholestanol and of coprostanol.

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[Received Sept. 19, 1968]

Fungal Spore Phospholipids and the Accumulation of Selected Chemicals

PHOSPHOLIPIDS CONSTITUTE one of the building blocks of cellular membranes. Therefore the phospholipid make-up of closely related organisms which have differing abilities to carry out a given membrane-located process is of interest. This communication reports on the phospholipids of spores from the fungi *Rhizopus nigricans, Alternaria oleracea* and *Neurospora sitophila.* These spores were shown by Miller et al. (1) to accumulate selected compounds and metal ions at characteristically different rates. Thus, 10 mg of *R. nigricans* spores accumulated 350 ppm of 2-heptadecyl-2-imidazoline in 1 min on the basis of spore weight, while 10 mg of *A. oleracea* and 10 mg of *N. sitophila* spores accumulated, in 1 min, 600 ppm and 1512 ppm, respectively, of the same compound. In addition, 10 mg of *N. sitophila* spores accumulated 583 ppm silver and 827 ppm cerium in 0.5 min, while *A. oleracea* accumulated 744 ppm silver and 2,255 ppm cerium in 0.5 min. Such cations and organic compounds actually entered the spores and were bound in characteristic proportions by the particulate (membranous) subcellular components, these components being, presumably, the mitochondria and fragments of the endoplasmic reticulum (2,3).

The same strains of *R. nigricans, A. oleracea* and *N. sitophila* that were used by Miller et al. (1) were used in the present study. The fungi also were cultured and harvested by methods employed by Miller and co-workers. Total lipids were extracted from the spores with chloroform-methanol (2:1, v/v) and the extracts were purified on Sephadex by the method of Siakotos and Rouser (4). Phospholipids were obtained from the purified total lipids by silicic acid column chromatography (5). The phospholipids were then subjected to thin-layer chromatography, deacylation, paper chromatography, hydrolysis and gas-liquid chromatography as previously described (5,6).

It was found that the three species of fungal spores contained similar phospholipid patterns; phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine and an unidentified phospholipid were the major components present. Therefore it seems unlikely that qualitative differences in phospholipid patterns can be invoked to explain the selective accumulation observed by Miller et al. (1). Moreover, the results of thin-layer chromatography suggested that the relative amounts of the phospholipids

Table I lists GLC retention data of the free stanols, and several derivatives on two liquid phases along with similar data for cholesterol and HSSL-stanol. Unaltered HSSL-stanol and its acetate, trifluoroacetate and trimethylsilyl ethers yielded relative retention data identical to 5α-cholestan-3β-ol and its derivatives on two different columns. These data and the fact that the HSSL-stanol acetate emerged from the SiO_2-$AgNO_3$ column with the saturated alcohol acetates would indicate that the HSSL-stanol is dihydrocholesterol.

It is not surprising that dihydrocholesterol is present in the sterol ester fraction of human skin surface lipid since this sterol is known to accompany cholesterol in most tissues. Its metabolic significance has not yet been elucidated.

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TABLE I
Fatty Acids of the Spore Phospholipids (Area %)

Fatty acid	*R. nigricans*	*A. oleracea*	*N. sitophila*
12:0	—	trace	2.4
14:0	13.2	4.9	2.4
16:0	16.4	21.7	29.6
16:1	6.7	trace	1.8
18:0	2.0	5.9	4.9
18:1	21.8	9.5	23.6
18:2	12.4	30.7	29.3
18:3	—	11.9	6.0
γ18:3	27.5	—	—
20:1	—	13.0	—
?	—	2.4	trace

did not vary greatly from organism to organism. On the other hand as shown in Table I, there were some notable differences in the quantities of 16-, 18- and 20-carbon unsaturated fatty acids derived from the spore phospholipids. Thus, on the basis of area per cent of GLC peaks, (Table I), *R. nigricans* contained about 3.7 times as much 16:1 fatty acid as *N. sitophila, N. sitophila* contained 2.5 times as much 18:1 fatty acid as *A. oleracea,* and *A. oleracea* contained 2.5 times as much 18:2 fatty acid as *R. nigricans* and 2.0 times as much 18:3 fatty acid as *N. sitophila.* Other quantitative differences are evident from an inspection of Table I.

It seems possible, therefore, that quantitative differences in fatty acids of the spore phospholipids could be among the factors that contributed to selective accumulation by the membranous subcellular particles (2,3). On the basis of lipoprotein subunit models of membrane structure (7,8,9), such a contribution of fatty acids could be made via van der Waals association of membrane fatty acid chains with specific membrane proteins.

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The Phosphatidyl Ethanolamine of *Pseudomonas aeruginosa* Grown in Hexadecane

THE LIPID COMPOSITION of bacteria changes with cultural conditions: composition of the medium, temperature and age of the culture (1). The amount of certain phospholipids correlates with Gram staining. Kates (2) has shown that in gram-positive bacteria virtually all the lipids associate with the cytoplasmic membrane, whereas in the gram-negative bacteria they could be found both in the cell membrane and in the cell wall. In previous work in which *Pseudomonas aeruginosa* was grown in media containing hexadecane as the sole carbon source, the presence of phosphatidyl ethanolamine was suggested (3). In the present work the presence and composition of phosphatidyl ethanolamine of *Pseudomonas aeruginosa* grown in hexadecane was carefully studied. The *Pseudomonas aeruginosa* belonged to the same strain studied previously (3). It was isolated by A. A. Solari from a petroleum contaminated soil and cultured in a mineral medium containing 0.2% n-hexadecane (99% pure, provided by Y. P. F. Florencio, Varela, Argentina).

The composition of the medium was: K_2HPO_4 (1 g); $MgSO_4 \cdot 7H_2O$ (0.2 g); $NH_4H_2PO_4$ (2 g); NaCl (5 g); and distilled water. *Pseudomonas aeruginosa* (1000 ml) was grown on agar slants for 24 hr at 37C. A suspension was made in 10 ml water and transferred to 250 ml of sterile mineral medium (pH 7.0) containing 0.2% n-hexadecane. The bacteria

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were incubated at 32C in a rotary shaker, 250 rpm, for 48 hr. The growth was followed by $NH_4H_2PO_4$ consumption, pH estimation and microscopic observation.

After repeated transfers, every 48 hr at first and then every 24 hr, the growth was inoculated into 2,700 ml of the same mineral medium with 0.2% n-hexadecane. The culture was incubated at pH 7.1 and 32C in a 5 liter New Brunswick (Model F 05) fermentor under a stream of air (2 liter/min). When 85% $NH_4H_2PO_4$ was consumed, the cells were harvested by centrifugation (34,000 × g, 0C). The cells were freeze-dried and extracted with chloroform-methanol (2:1) by the method of Folch et al. (4).

The lipids were separated by thin-layer chromatography (TLC) using silica gel G (Merck) and developed in chloroform-methanol-water (65:25:4). Eight polar fractions with R_f 0.15, 0.20, 0.35, 0.40, 0.50, 0.73, 0.80, and 0.95 were stained with iodine (Fig. 1). Phosphorus determinations every 3 mm showed that 55% was found in the spot F_v (R_f 0.50), located very near the standard of phosphatidyl ethanolamine. This spot was stained both with ninhydrin and, very weakly, with Dragendorff's reagent. It was eluted by the procedure of Davison et al. (5) and aliquots were used for ester function estimation by the procedure of Snyder et al. (6) modified by Renkonen (7) and enzymatic hydrolysis by snake venom phospholipase A.

The products of hydrolysis were separated by TLC on silica gel G with chloroform-methanol-water (65:25:4). Two spots were found, one having the same R_f as lysophosphatidyl ethanolamine and reacting positively with ninhydrin, the other corresponding to free fatty acids. The fatty acid composition of the lyso-derivative was analyzed by GLC and compared to the nonhydrolized fraction F_v (Table I) analyzed by the same procedure.

The structure of the molecule was also investigated by alkaline hydrolysis by the procedure of Dawson (9). Paper chromatography in phenol with water-acetic acid-ethanol (100:10:12) showed the presence of glycerol phosphoryl ethanolamine. The acidic hydrolysis of the water soluble fraction obtained after the transesterification of fraction F_v with 3N HCl in methanol, showed the existence of ethanolamine. The hydrolysis was performed by the procedure of Koning et al. (10). The base was recognized by paper chromatography in n-propanol-water (80:20, v/v). Glycerolphosphate was identified by paper chromatography

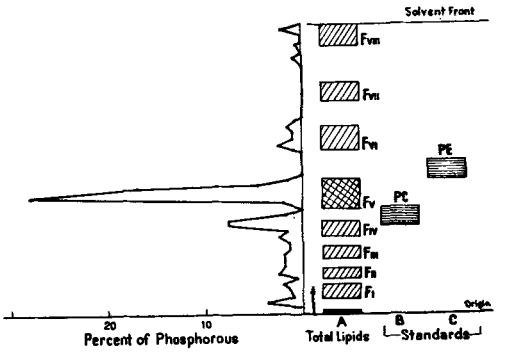

FIG. 1. Chromatogram prepared using Silica Gel G and chloroform/methanol/water (65:25:4). Spots were visualized with bromothymol blue. A) Total lipids; B) PC: Lecithin (from rat liver); C) PE: Phosphatidyl-ethanolamine (from rat liver).

in n-propanol-ammonia-water (60:30:10) after staining the chromatogram with Wade and Morgan's reagent (11). In other aliquots, phosphorus (12), glycerol (13), ethanolamine, serine (14), and choline (15) were quantitatively determined. Total glycerol was estimated after hydrolysis in 2N HCl at 125C for 48 hr.

The ratio for phosphorus:glycerol:ethanolamine (1:1:1.2), for ester function: phosphorus (2:1), and the identification of glycerolphosphate and phosphatidyl ethanolamine would identify phosphatidyl ethanolamine as the principal component of fraction F_v. This

TABLE I

Fatty Acid Composition of Fraction Fv

Fatty acid[a]	Equivalent chain length	Fraction Fv percent	Lysophosphatidyl ethanolamine[b]
14:0	14	1.6 ± 0.4[c]	1.5 ± 0.6
16:0	16	35.4 ± 2.0	22.8 ± 1.8
16:1	16.20	18.5 ± 1.0	8.4 ± 1.2
17.0	17	1.8 ± 0.5	0.8 ± 0.5
18:0	18	1.3 ± 0.6	2.9 ± 0.7
18:1	18.20	31.5 ± 0.7	30.0 ± 0.8
19:0	19	1.9 ± 0.2	5.4 ± 0.5
19:1	19.20	1.2 ± 0.1	2.8 ± 0.5
20:0	20	0.5 ± 0.03	4.6 ± 0.6
20:1	20.20	0.5 ± 0.2	1.0 ± 0.4
21:0	21	1.0 ± 0.6	1.3 ± 0.5
22:0	22	0.4 ± 0.03	2.8 ± 0.8
22:1	22.20	1.8 ± 0.2	1.6 ± 0.2

[a] Minor components make for 100%. First figure denotes number of carbon atoms, second figure, number of double bonds.

[b] Lysophosphatidyl ethanolamine was obtained by hydrolysis of Fraction Fv with phospholipase A followed by TLC.

[c] Standard error of the mean.

The column was 10% diethylene glycol succinate on Chromosorb W (80 to 100 mesh), operated at 150C.

was confirmed by investigating the reactivity with Phospholipase A; lysophosphatidyl ethanolamine and free fatty acids were obtained. Trace amounts of choline (P: Choline = 1: 0.006) and serine (P:serine = 1:0.004) indicated contamination of F_v with phosphatidyl choline and serine.

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Preparation and Properties of an Apoprotein Derivative of Human Serum β-Lipoprotein[1]

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ABSTRACT

The aim of this study was to develop a convenient method for the preparation and study of a soluble delipidated form of human serum β-lipoprotein. This was achieved by succinylation and delipidation with ether-ethanol (3:1). The succinylated apoprotein was soluble in either 0.13 M Tris-HCl buffer, pH 8.2 (for β-lipoprotein prepared by ultracentrifugation) or in the same Tris buffer to which 5 mM sodium decyl sulfate was added (for heparin-Mn precipitated β-lipoprotein). The immunological activity of β-lipoprotein or its apoprotein were markedly altered by succinylation. Whereas the succinylated β-lipoprotein appeared as one peak in the analytical ultracentrifuge, the succinylated apoprotein appeared as two. Under the electron microscope β-lipoprotein and succinylated β-lipoprotein were indistinguishable, appearing as uniform preparations of spherical particles 215 to 220 A in diameter.

INTRODUCTION

ALTHOUGH THE LIPID-FREE apoprotein of human serum α-lipoprotein has been extensively studied, the protein moiety of β-lipoprotein (β-LP) has not. Attempts to remove completely the neutral lipids or to strip away phospholipids from β-LP have usually produced gel-like products which were irreversibly aggregated (1-4). This difficulty in obtaining lipid-free β-apoprotein has greatly hampered its characterization.

Recently, delipidation in the presence of the detergent sodium dodecyl sulfate (5), reduction and alkylation after delipidation (6) or succinylation (7) prior to delipidation have been reported to yield a more soluble β-apoprotein. However, the insolubility of sodium dodecyl sulfate interferes with further study of the apoprotein preparation and this detergent cannot be removed completely, even by dialysis for several days (5).

It is the purpose of this communication to report methods which can conveniently be used for the preparation in high yield of a soluble succinylated delipidated derivative (s-β-apoprotein) of human serum β-LP. Two different methods for preparing large amounts of pure β-LP are described and compared as to convenience and properties of final product. The s-β-apoprotein derived from these two procedures is studied and contrasted with respect to aqueous solubility, immunological activity and sedimentation behavior. A portion of this work has been described in preliminary form (8, 9). A report on the properties of s-β-apoprotein has recently appeared from another laboratory (10) during the preparation of this paper.

MATERIALS AND METHODS

Succinic anhydride was obtained from Eastman Kodak Distillation Products Industries, Rochester, N. Y. All materials used were of the highest purity commercially available, except for sodium decyl sulfate and sodium dodecyl sulfate, which were recrystallized.

All of the lipoprotein and apoprotein fractions were extracted in chloroform-methanol (2:1, v/v), for estimation of triglyceride (11), cholesterol (12) and phospholipid (13). The lipids in the C/M extracts also were examined by thin-layer chromatography using both polar and nonpolar solvents (14). Protein was determined by the method of Lowry et al. (15). Paper electrophoretic (16) and immunological techniques used for the analysis of lipoproteins have been described previously (17, 18). For electrophoresis of s-β-apoprotein, 1% albumin was omitted from the buffer and 0.01% bromphenol blue was used as the protein stain. Antibodies to s-β-LP, or s-β-apoprotein were produced by three serial injections into the foot pads of white New Zealand rabbits of 1.25 ml of the antigen (5 to 8 mg protein) mixed with 0.75 ml of Freund's adjuvant (17). The antisera to s-β-LP, designated SB_1 and SB_2, and to s-β-apoprotein (SDB_1 and SDB_2) were harvested as previously described (17, 18). They reacted weakly and inconstantly with native β-LP. All four antisera strongly reacted with s-β-LP and SB_1, SDB_1, and SDB_2 reacted with s-β-apoprotein. The antibodies to β-LP which were used in this study have been characterized

[1]Presented at the AOCS Meeting, Washington, D. C., April, 1968.

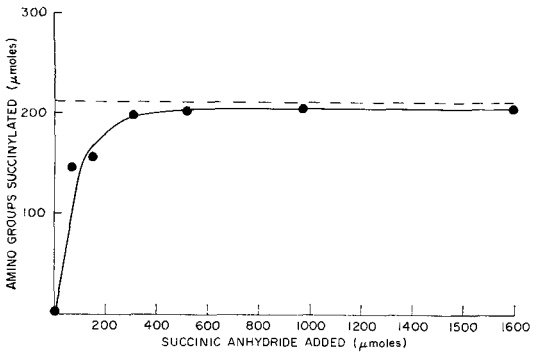

FIG. 1. Succinylation of β-lipoprotein. The dotted line represents the total number of amino groups present before addition of succinic anhydride. The differences between this value and the number of amino groups determined in subsequent samples were used to measure the quantity of amino groups succinylated at a particular time. After the addition of a measured quantity of succinic anhydride, 2 N NaOH was added with an automatic titrator until the pH was constant. An aliquot was then obtained for assay of free amino groups.

and described previously (17). All antisera were tested against a blank containing sodium

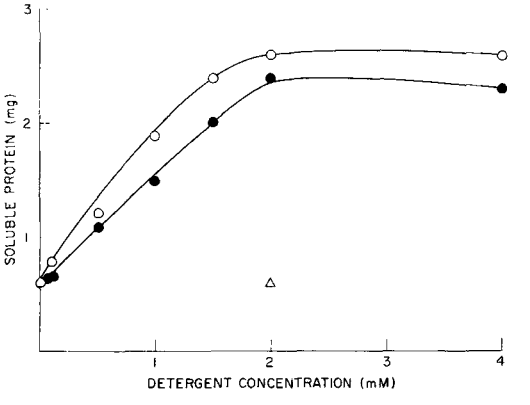

FIG. 2. Effects of sodium decyl sulfate and sodium dodecyl sulfate on the aqueous solubility of s-β-apoprotein prepared from heparin and manganese precipitated β-LP. Three mg of s-β-LP were delipidated (8). To the dry residue was added 1 ml of 0.13 M Tris buffer, pH 8.2, which contained varied quantities of sodium decyl sulfate (○) or sodium dodecyl sulfate (●). Non-succinylated β-apoprotein was incubated with Tris buffer containing 2 mM sodium decyl sulfate (△). The samples were incubated for 4 hr at 37C and aliquots of the aqueous phase were removed for protein assay. All of the visible residue from the succinylated preparations was soluble at concentrations of detergent of 2 mM or greater.

TABLE I

Solubility of Succinylated Apoprotein from β-Lipoprotein Prepared by Ultracentrifugation: Effect of Succinylation[a]

Incubation time (hr) of s-apoprotein	Fraction	Soluble protein[b] (mg)
4	Succinylated β-lipoprotein	3.9 ± 0.1
16	Non-succinylated β-lipoprotein	0.8 ± 0.03

[a] Succinylation, delipidation and solubilization of the delipidated products were performed as previously described (8).

[b] These values represent the total protein solubilized after delipidation of 24 individual samples ± standard error. The quantity of succinylated or native β-lipoprotein delipidated in each flask was 4.3 mg. Incubation was at 37C.

decyl sulfate when this substance was present, since it was noted that several antisera formed hazy precipitin bands with this detergent.

Negatively stained preparations of β-LP or s-β-LP were obtained on a Formvar-carbon coated grid using 2% potassium phosphotungstate, pH 7.0. The lipoprotein solutions contained 0.1 mg of protein per ml.

For the determination of sedimentation coefficients, distances from the maximum ordinate to the reference hole (x) were measured with a Nikon microcomparator. The sedimentation coefficient (s) was calculated from the slope of the line when log x was plotted against time. Values of s were corrected to standard conditions (19), assuming a v of 0.971 for s-β-LP (20) and 0.727 for s-β-apoprotein. The former value was obtained from the reciprocal of the average hydrated density while the latter value was calculated from the amino acid composition (5) by the method of Schachman (21) and neglected bound sodium decyl sulfate. Al-

TABLE II

Solubility of Succinylated Apoprotein from Heparin and Manganese Precipitated β-Lipoprotein: Effect of Sodium Decyl Sulfate[a]

Total incubation time (hr) of s-apoprotein	Soluble protein
4.5	0.4 ± 0.02
16	Gel[b] Sodium decyl sulfate added (5 mM)[c]
20	3.8 ± 0.1

[a] For details, please see explanatory notes for Table I. The quantity of succinylated β-LP delipidated in each flask was 4.4 mg. The total number of individual samples extracted was 24.

[b] Accurate pipetting from the gel was not possible.

[c] After 16 hr, sodium decyl sulfate was added to a final concentration of 5 mM. Incubation at 37C was continued for another 4 hr.

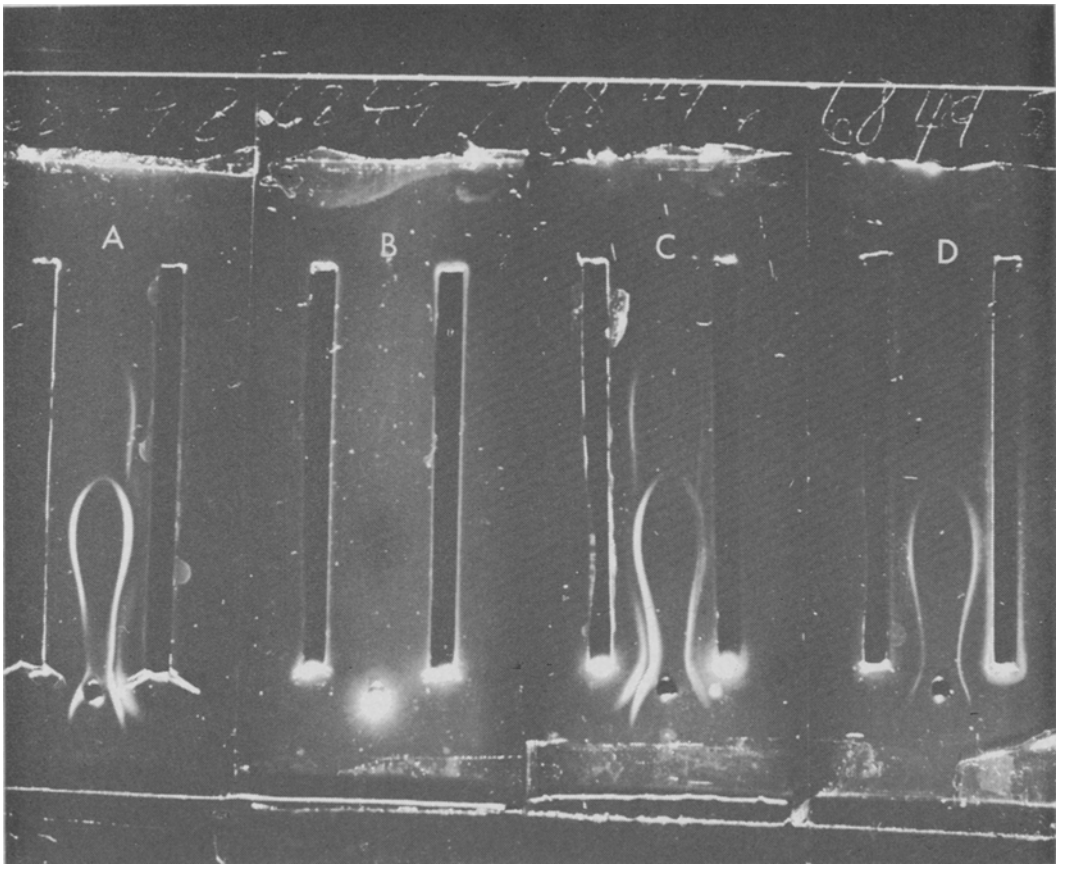

FIG. 3. Heterogeneity of s-β-LP on immunoelectrophoresis in 0.5% agarose. Samples of s-β-LP were placed in the 4 center wells and subjected to electrophoresis for 40 min. The following antisera were used: left trough A, R_1; right trough A, SDB_1; left trough B, R_2; right trough B, R_3; left trough C, SB_1; right trough C, SB_2; left trough D, R_7; right trough D, S_1A (17).

though these values of $\bar{v}$ are those of the non-succinylated lipoprotein and apoprotein, the recent data of Scanu et al. (10) would seem to justify their use in calculations involving the succinylated derivatives. A plot of $1/S_{20,w}$ vs. concentration was used for extrapolation to zero concentration, employing seven concentrations of protein (as s-β-LP or s-β-apoprotein) over a range from 1 to 5 mg. Approximate values were obtained for K from the expression, $S_{20,w} = S^{o}_{20,w}(1\text{-}Kc)$. These values were 48 and 111 ml/gm for ultracentrifugally prepared s-β-apoprotein in the presence and absence of 20 mM sodium decyl sulfate, respectively, and 9.2 ml/gm for s-β-LP.

The solutions of NaCl, NaBr and Tris-HCl buffer which were used in the preparation of β-LP, s-β-LP and s-β-apoprotein contained 0.01% EDTA, adjusted to pH 7.0. The final pH values of the 0.15 M NaCl-EDTA and of the 0.13 M Tris-EDTA solutions were adjusted to 7.0 and 8.2, respectively.

Plasma was collected from fasting, normal subjects by plasmaphoresis and stored at 4C.

TABLE III

Composition of β-lipoprotein and Succinylated β-apoprotein[a]

Preparation	Protein	% Composition Phospho-lipid	Triglyc-eride	Choles-terol
β-LP by ultra-centrifugation	24.2	25.1	4.7	46.4
β-LP by heparin -Mn precipitation	23.7	22.2	5.0	49.1
s-β-apoprotein no. 1	98.4	1.5	0.0	<0.1
s-β-apoprotein no. 2	98.7	1.2	0.0	<0.1
s-β-apoprotein no. 3	98.8	1.1	0.0	<0.1

[a] All analyses were done in duplicate.

Fractionation was usually begun within two weeks of collection. Procedures for the preparation of β-LP by precipitation with heparin and manganese in conjunction with ultracentrifugal separation between densities 1.019 and 1.063 (22), for succinylation and for delipidation have been described (8). β-LP was extensively succinylated with relatively small quantities of succinic anhydride (Fig. 1). Similar methods were employed when β-LP was prepared by ultracentrifugation alone, except that treatment with thrombin and precipitation with heparin and manganese were omitted. All β-LP used for this study was immunochemically pure.

The dry residue of s-β-apoprotein, prepared as previously described (8), was incubated at 37C with 0.13 M Tris-HCl buffer, pH 8.2 or with the same Tris buffer containing 5 mM sodium decyl sulfate. If higher concentrations of β-LP were delipidated, or rarely with low concentrations prepared with heparin and manganese, it was necessary to increase the concentration of sodium decyl sulfate to 20 mM to obtain complete solubilization.

RESULTS

Solubility of S-β-Apoprotein

Succinylation of β-lipoprotein led to a marked increase in solubility after delipidation with ether-ethanol (Table I). The method of preparation significantly affected the solubility of the s-β-apoprotein. When β-LP was prepared solely by ultracentrifugation, rapid and complete solubilization of the s-β-apoprotein was achieved. All of the protein recovered after delipidation was solubilized by this procedure. On the other hand, when precipitation with manganese and heparin was employed, only partial solubilization was achieved and gel formation occurred (Table II).

Complete solubilization of the s-β-apoprotein obtained by heparin and manganese precipitation was made possible by the further addition of 5 mM sodium decyl sulfate (Table II). Sodium decyl sulfate and sodium dodecyl sul-

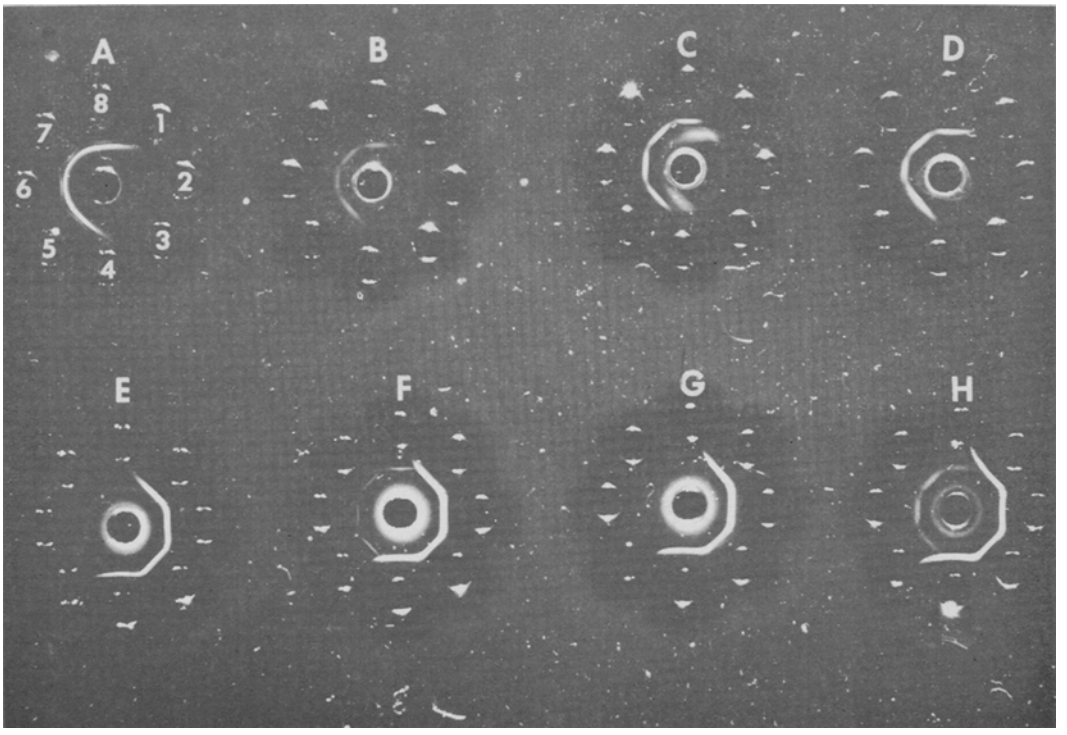

FIG. 4. Immunodiffusion patterns of β-LP and s-β-LP. The following antisera were placed in center wells: SB_1 in A, SB_2 in B, SDB_1 in C, SDB_2 in D, R_s in E, Hyland anti-β in F, S_1A in G and α-LpT in H (17). The following antigen preparations were placed in the outer wells: heparin and manganese precipitated β-LP in 1 and 4, ultracentrifugally prepared β-LP in 2 and 3, s-β-LP from precipitated lipoprotein in 5 and 8, and s-β-LP from ultracentrifugally prepared lipoprotein in 6 and 7.

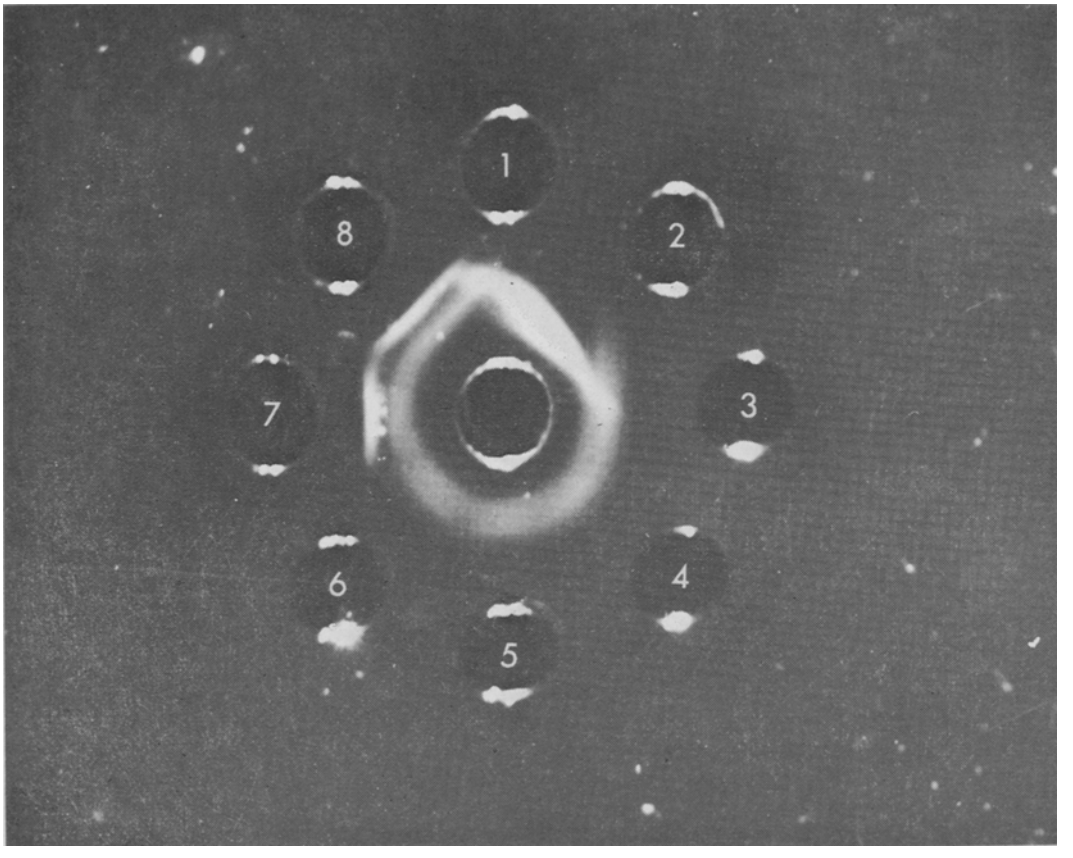

FIG. 5. Immunodiffusion patterns of β-LP. Effects of succinylation and delipidation. Antiserum SB_1 is in the central well. In outer wells clockwise from 1 are β-LP (1, 2), s-β-apoprotein (3-6) and s-β-LP (7, 8).

fate were equally effective in promoting solubilization (Fig. 2). Under these experimental conditions, there was little solubility of the delipidated material in the presence of 2×10^{-3} M sodium decyl sulfate unless the lipoprotein was succinylated. It was not necessary for the detergent to be present, however, during the delipidation procedure in order to achieve solubilization.

Composition of the Beta-LP and s-β-Apoprotein

No significant differences were found between the preparations of β-LP which were precipitated with heparin and those which were not. The extraction procedure removed more than 98% of the total lipid content of β-LP (Table III). No cholesterol (free or esterified) or triglyceride was detected by thin-layer chromatography. A small quantity of phospholipid, usually from 1.1% to 1.5%, could be detected.

Electrophoretic and immunological Properties of Beta-LP, S-β-LP and S-β-Apoprotein

Succinylation of β-LP from either method of preparation resulted in a more rapid electrophoretic migration on both albumin impregnated paper or on 0.5% agarose. On paper electrophoresis s-β-apoprotein migrated more slowly than s-β-LP and had a mobility similar to albumin.

Some, but not all, of the rabbit and sheep antisera to native β-LP retained activity with s-β-LP when examined by immunoelectrophoresis on 0.5% agarose. Heterogeneity of s-β-LP was indicated by the presence of at least two precipitin arcs of different mobility with antisera SB_1, SB_2 and SDB_1 (Fig. 3). Some antisera to β-LP reacted with s-β-LP, e.g., R_4, R_7 and S_1A, while others did not, e.g., R_2 and R_8 (Fig. 3). Those antisera to β-LP which did react, however, formed only the more slowly migrating precipitin arc.

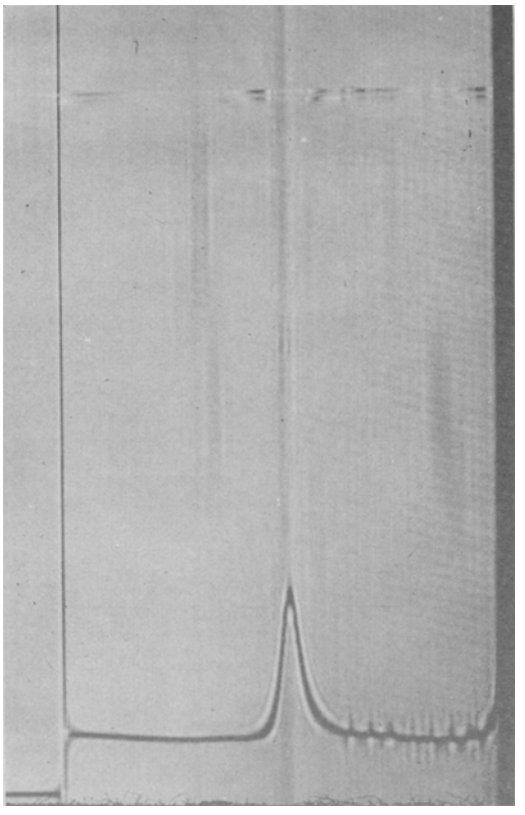

FIG. 6. Sedimentation velocity pattern of s-β-LP. The lipoprotein was prepared with heparin and manganese precipitation. The protein concentration was 0.9 mg/ml, the solvent 0.13 M Tris buffer, pH 8.2, and the bar angle 75°. The photograph was taken at 90 min after reaching speed. Temperature was 20C.

Beta-LP prepared by either heparin and manganese precipitation and ultracentrifugation or by ultracentrifugation alone was tested against a large number of antisera by the double-diffusion technique of Ouchterlony. Complete immunological identity of the lipoprotein preparations derived by these two methods was indicated (Fig. 4). Likewise, the succinylated derivatives of β-LP from these two types of preparations exhibited immunological identity (Fig. 4). The succinylated derivative, however, showed only partial immunological identity with the parent β-LP. As with immunoelectrophoresis, s-β-apoprotein reacted with certain antisera prepared against itself or against s-β-LP, and reacted inconstantly with a few, but not most antisera to β-LP. The immunological heterogeneity of s-β-LP again was demonstrated by the appearance of two precipitin lines with the antiserum SB_1, one of which was immunologically identical with the precipitin line of s-β-apoprotein (Fig 5).

Sedimentation Studies

Both β-LP and s-β-LP (Fig. 6) sedimented as single components in the analytical ultracentrifuge in 0.13 M Tris-HCl buffer (0.01% EDTA), pH 8.2. The $S^o_{20,w}$ value of the latter was 5 to 6. In sedimentation velocity experiments s-β-apoprotein exhibited heterogeneity independently of the method of preparation of β-LP and the presence or absence of sodium decyl sulfate (Fig. 7). The $S^o_{20,w}$ values of s-β-apoprotein from heparin-Mn precipitated β-LP were 4.2 and 5.6 for the slow and fast peaks of the preparation shown in Fig. 7 (in 20 mM sodium decyl sulfate). Although there were variations in the sedimentation coefficients with different preparations, two or more components were present in all instances. The $S^o_{20,w}$ values of ultracentrifugally prepared s-β-apoprotein were 3.3 and 6.0 for the slow components in the presence and absence of 20 mM sodium decyl sulfate, respectively. Although a fast component was definitely present, it was not possible to measure accurately its $S^o_{20,w}$ value. Sedimentation in 0.13 M Tris-HCl buffer (pH 8.2), which contained 0.15 M NaCl gave qualitatively similar results to those described above.

Electron Microscope Studies

Electron micrographs, prepared with the negative staining technique, indicated no differences between β-LP prepared by ultracentrifugation alone or prepared by an initial precipitation step. A typical preparation uniformly contained spherical particles of 215 to 220 A in diameter (Fig. 8). Succinylation did not alter the appearance of the lipoprotein particles.

DISCUSSION

The observation of Margolis and Langdon that diazotization of β-LP increases the solubility of the delipidated product (3) suggested to us that the addition of negative charge might increase the solubility of β-apoprotein. The results with succinylation provide strong support for this concept. When succinylation itself is insufficient to achieve solubilization of the apoprotein, viz. for β-LP obtained by heparin and manganese precipitation, the further addition of 5 mM sodium decyl sulfate permits complete solubilization. In comparison with sodium dodecyl sulfate, the decyl sulfate has the advantages of greater aqueous solubility and can be completely removed by dialysis. The mechanism of the decrease in aqueous solubility of the succinylated apoprotein from

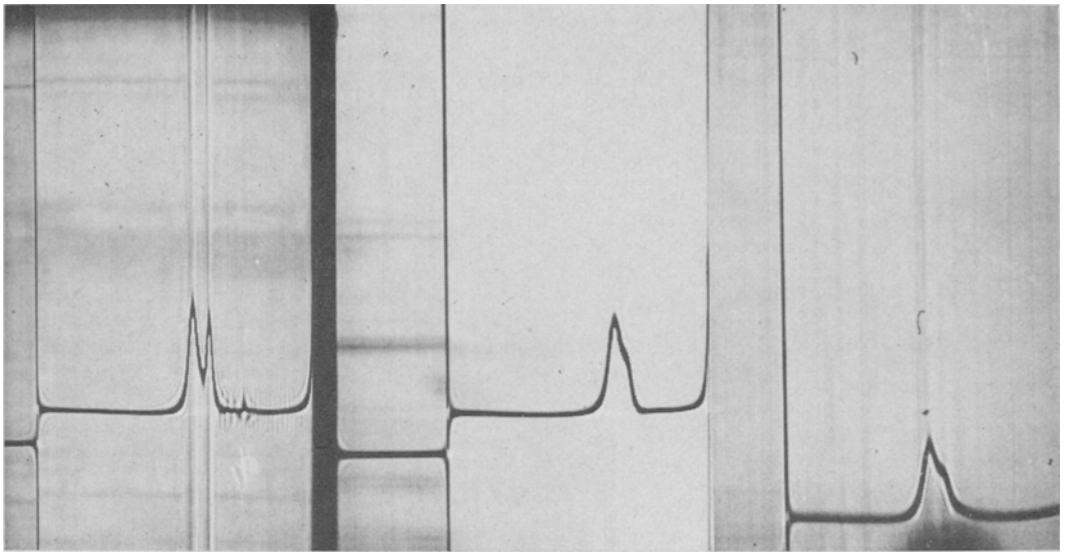

FIG. 7. Sedimentation velocity patterns of s-β-apoprotein. Experimental conditions were as follows: left frame, s-β-apoprotein from heparin-Mn treated β-LP, 4.4 mg protein per ml in 0.13 M Tris HCl buffer, 20 mM sodium decyl sulfate (pH 8.2), time after reaching speed was 130 min; middle frame, s-β-apoprotein from ultracentrifugally prepared β-LP, 4.0 mg protein per ml same solvent as above, time after reaching speed was 161 min; right frame, s-β-apoprotein from ultracentrifugally prepared β-LP, 4.2 mg/ml in 0.13 M Tris HCl buffer (pH 8.2), time after reaching speed was 129 min. For all three experiments, the bar angle was 75° and the temperature 20C.

heparin and manganese precipitation is not understood (Table I).

Although precipitation with heparin and manganese alters the solubility of delipidated β-LP, no alterations are detected in the immunological properties of the β-LP, and its succinylated and delipidated derivatives. Succinylation, which may introduce a strong haptenic group, alters the antigenic character of β-LP such that it reacts weakly with antisera to β-LP and shows only partial immunological identity with the parent molecule (Fig. 4). These results are in contrast to a recent report by Scanu et al. (10), in which s-β-LP failed to react with antisera to β-LP. It is not surprising, therefore, that delipidation of s-β-LP almost completely abolishes activity with most antisera to β-LP, although s-β-apoprotein reacts strongly with certain antisera to itself or to s-β-LP.

Heterogeneity of s-β-apoprotein is clearly demonstrated by sedimentation studies with s-β-LP. The finding of heterogeneity was a consistent one with all preparations, whether or not heparin and manganese precipitation was employed in the preparative procedure (Fig. 7). Previous failure to note heterogeneity of s-β-apoprotein in sedimentation experiments (5) may have been caused by the apparent homogeneity during the relatively early and middle stages of observation in the ultracentrifuge. This is particularly important at high concentrations of protein. Although a signifi-

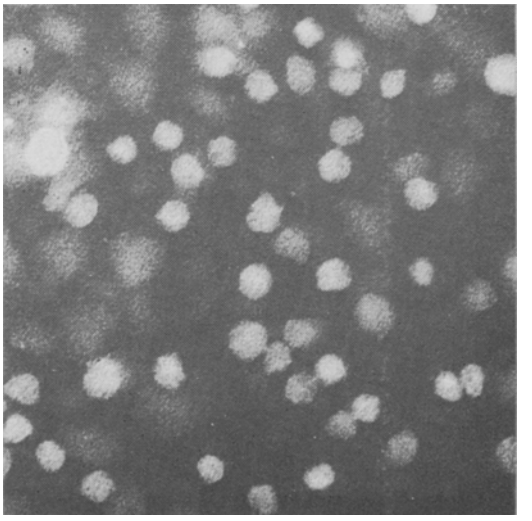

FIG. 8. Electron micrograph of β-LP with negative staining technique. A solution of heparin and manganese precipitated β-LP (0.1 mg/ml) was used. The magnification was 300,000.

cant decrease in the value of $S^{o}_{20,w}$ occurs when sodium decyl sulfate is added, ultracentrifugal heterogeneity is not dependent upon the presence of this detergent. Heterogeneity of s-β-apoprotein, recently described in sedimentation equilibrium and gel filtration experiments, has been attributed to aggregation (10).

Application of the methods described in this communication should facilitate study of a derivative of β-apoprotein by further application of other techniques of protein chemistry.

ACKNOWLEDGMENT

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Human Milk: Quantitative Gas-Liquid Chromatographic Analysis of Triglyceride and Cholesterol Content During Lactation

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ABSTRACT

Gas-liquid chromatography has been used to follow changes in the triglyceride composition of human colostrum and milk from one donor during the first 10 days postpartum and to compare the compositions obtained with those at later stages of lactation.

New triglycerides of low molecular weight appeared during the first 5 days postpartum. Lower molecular weight triglycerides ($< C_{48}$) continued to increase until the eighth day. Triglyceride C_{48} and total cholesterol content remained almost constant during the period of the study.

Comparison of triglyceride fatty acid composition on the third, sixth, and ninth days postpartum showed considerable specificity in all cases, but a tendency for randomization to occur as lactation proceeded.

The advantage of triglyceride over fatty acid analysis for this type of investigation is that in the former, new molecules appear. In the latter, only changes in the proportions of fatty acids occur.

INTRODUCTION

THE TECHNIQUE of GLC has enabled detailed analyses to be made of the fatty acid composition of human milk (1-8) and hence how this composition is affected by such factors as caloric balance (2) and type of fat in the diet (2,3,5,6). Related studies have been concerned with the absorption of human milk fat by newborn infants (7) and serum lipids in infants fed from the breast or on evaporated cow's milk (8).

Changes in human milk composition during the first 10 days postpartum have been studied with respect to gross changes in fat, lactose, and protein content (9), or with average values of fatty acid composition compiled from many reports (10). The change in fatty acid composition of human colostrum has only been followed during the first 3 days postpartum (4).

Temperature-programed GLC has been used for the analysis of intact triglycerides of mature human milk (11-13). It appeared timely, therefore, to apply this technique to follow changes in triglyceride composition of human colostrum and milk during the first 10 days postpartum and to compare the compositions obtained with those at later stages of lactation.

MATERIALS AND METHODS

Colostrum and Milk Samples

The donor was 19 years old, primipara, caucasian and discharged (14 days postpartum) from hospital in good health. The offspring was male, born 1 month prematurely, but with normal delivery.

Using manual expression, samples of colostrum or milk were collected on the second to tenth, twenty-first and thirty-fifth days postpartum. These samples were obtained from both left and right breasts at mid-feed (total time of feed 1 hr) on all days quoted except the seventh, when samples were taken at the beginning, middle, and end of the feed, using the right breast only (total time of feed ½ hr). The times of the beginning of the feeds were 3 PM on the second and third days, and 12 noon on all remaining days.

The donor's times of meals were: breakfast at 8 AM, lunch at 1 PM, tea at 4:30 PM, and supper at 6 PM. Throughout the first 10 days postpartum, the donor kept a record of the amount and composition of meals taken. This showed no striking variation in cow's milk consumed or the type of food eaten. An approximate evaluation of caloric intake showed a decline from about 3,000 to 2,000 calories during this period; the percentage contribution from protein, fat and carbohydrate remained relatively constant at 12%, 38%, and 50% respectively. Figures quoted for the U.K. are an intake of 2,600 calories of prepared food daily with a composition of 11.6%, 40.3%, and 48.0% of protein, fat, and carbohydrate respectively (14).

Dietary Fatty Acid Composition

Approximately one-third of the food provided on the tenth day postpartum was not consumed but was extracted and hydrolyzed by the method of Lough, Navia and Harris (15). The free fatty acids obtained were con-

TABLE I

Variation in Triglyceride and Cholesterol Composition of Human Colostrum and Milk According to the Day Postpartum. Values in Mole %

Triglyceride (carbon number)	Days postpartum										
	2(4)[a]	3(4)	4(4)	5(4)	6(5)	7(4)[b]	8(5)	9(4)	10(3)	21(2)	35(2)
32	0	0	0	0	0	0	0.1±0.0	0	0	0.2±0.0	0
34	0	0	0	0.2±0.05	0.2±0.0	0.2±0.0	0.4±0.05	0.2±0.05	0.2±0.0	0.4±0.0	0.2±0.0
36	0	0	0.2±0.0	0.5±0.05	0.5±0.05	0.6±0.05	0.9±0.1	0.5±0.05	0.4±0.1	0.6±0.1	0.5±0.0
38	0	0.3±0.05	0.6±0.05	0.9±0.0	1.0±0.1	1.4±0.0	2.0±0.2	1.3±0.1	0.9±0.1	1.2±0.1	1.1±0.05
40	0.2±0.0	0.9±0.1	1.2±0.05	1.9±0.1	2.3±0.1	2.6±0.2	3.2±0.3	3.1±0.2	2.2±0.1	2.3±0.1	2.2±0.1
42	0.9±0.05	2.0±0.2	2.4±0.1	3.7±0.2	4.1±0.2	5.0±0.1	5.6±0.4	5.3±0.3	4.2±0.05	4.3±0.1	4.4±0.05
44	2.7±0.3	4.4±0.2	5.3±0.1	7.0±0.3	7.4±0.3	8.3±0.3	8.9±0.4	9.2±0.5	7.7±0.1	7.0±0.2	8.0±0.1
46	6.0±0.6	9.8±0.8	9.9±0.3	11.4±0.2	12.1±0.3	11.8±0.0	13.1±0.4	13.5±0.3	12.0±0.2	11.3±0.3	12.5±0.1
48	11.4±0.1	15.0±0.8	14.0±0.3	14.3±0.2	15.0±0.3	14.6±0.0	13.9±0.2	15.4±0.1	14.7±0.2	14.3±0.2	14.6±0.2
50	22.8±0.1	22.8±1.0	21.0±0.1	19.8±0.2	20.1±0.3	19.7±0.2	17.5±0.3	19.2±0.3	19.4±0.3	19.3±0.1	18.7±0.1
52	38.0±0.8	31.0±2.0	31.5±0.8	28.1±0.6	27.1±0.7	25.7±0.3	24.5±1.1	23.9±1.2	28.6±0.6	29.4±0.3	26.8±0.0
54	14.9±1.1	11.4±1.0	11.6±0.7	10.0±0.3	8.9±0.3	8.3±0.3	7.9±0.3	7.4±0.4	8.4±0.2	8.6±0.2	9.5±0.05
56	3.1±0.5	2.5±0.3	2.5±0.1	2.4±0.1	1.4±0.2	1.7±0.05	1.9±0.2	1.2±0.1	1.2±0.05	0.9±0.1	1.6±0.05
Average carbon number	16.94	16.68	16.65	16.45	16.34	16.23	16.10	16.18	16.31	16.28	16.33
Free cholesterol	2.4±0.05	0.9±0.1	1.2±0.0	1.6±0.1	1.2±0.1	1.2±0.1	1.5±0.1	1.8±0.1	0.6±0.1	0.6±0.1	1.9±0.05

[a] Figures in parentheses denote number of determinations.

± Represents standard deviation, except for days 21 and 35 postpartum, where deviations between duplicates are given. Triglyceride compositions of milk obtained from the left or right breasts (or at the beginning, middle and end of feed at 7 days postpartum[b]), agreed within the standard deviations quoted for that day.

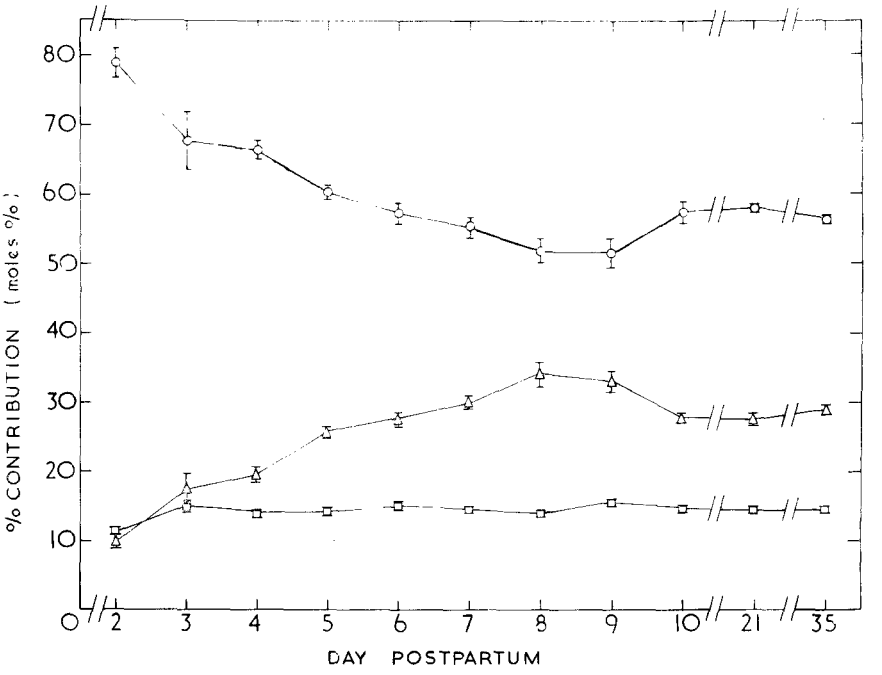

FIG. 1. Contribution of triglycerides on different days postpartum. Triglycerides $>$ C_{18} (o — o) triglycerides $<$ C_{18} (Δ — Δ) and triglycerides C_{18} ($\square$ — $\square$). Contributions are in mole %. The data are calculated from Table I.

verted to their methyl esters (16) and analyzed by GLC as described previously (12).

Colostrum and Milk Fat Composition

All samples were immediately shaken with 20 volumes of chloroform–methanol (2:1, v/v) and the lipids extracted as described previously (12). As soon as possible, 1–2 μl of the lipids (in chloroform) were analyzed by GLC for triglyceride composition. The remaining solution was taken to dryness in a stream of nitrogen, redissolved in n-hexane and stored over anhydrous sodium sulphate under nitrogen at –20C until further analyses could be carried out. During storage no change occurred in triglyceride or fatty acid composition that could be detected by GLC.

Analyses of fatty acid and triglyceride compositions were performed using the methods and apparatus already described (12, 13). In each case, molar correction factors were used for the range of fatty acid methyl esters C_4–C_{18}; fatty acid methyl esters $>$ C_{18} were assumed to have the same correction factors as C_{18}. Molar correction factors, obtained from standard mixtures of triglycerides varying from C_6 to C_{54} (i.e., representative of those found in milks), were also employed (13). Separation of triglycerides was according to total carbon number (i.e., the total number of carbon atoms in the fatty acid moieties). Free cholesterol was well separated from the triglyceride region and had a molar correction factor of 1.43 ± 0.03.

To determine the proportion of triglyceride in the colostrum and milk lipids, analytical thin-layer chromatography (12) was applied to each milk sample. This showed that the cholesterol ester and glyceryl ether diester concentrations were each less than 1% (w/w). The latter was tentatively identified by thin-layer chromatography of the lipid extracts of samples obtained on the third, sixth, and ninth days postpartum. These samples showed a minor component having the same R_f as chimyl dipalmitate. The solvent used was 10% diethyl ether in light petroleum (40–60C), based on that of Bollinger (17). Though phospholipid, free fatty acids, and mono– and diglycerides were detected, their concentrations were low and they did not interfere with the GLC of the triglycerides. This was confirmed as follows: A representative milk lipid extract —from which triglycerides, cholesterol ester, and glyceryl ether diester had been removed by thick-layer chromatography (12) — was analyzed by GLC using conditions for tri-

TABLE II

Fatty Acid Composition of Human Colostrum and Milk on Various Days Postpartum, in Mole %

Chain length of fatty acid	Days postpartum		
	3	6	9
C_{10}	0.4 ± 0.0	1.7 ± 0.0	2.8 ± 0.1
C_{12}	3.7 ± 0.0	9.7 ± 0.1	10.6 ± 0.1
C_{14}	8.1 ± 0.3	10.6 ± 0.1	11.4 ± 0.2
C_{16}	29.6 ± 0.1	28.8 ± 0.3	23.2 ± 0.3
C_{18}	57.2 ± 0.3	47.4 ± 0.4	50.1 ± 0.2
$>C_{18}$	0.9 ± 0.2	1.8 ± 0.3	1.9 ± 0.3
Average fatty acid chain length (Ca)	16.75 ± 0.07	16.32 ± 0.04	16.26 ± 0.01

± represents deviation between duplicate determinations on the same sample.

glyceride elution. No peaks were observed other than that of free cholesterol. Sufficient material was applied to the column to give a free cholesterol peak about 10 times the normal size obtained. Hence, human milk lipids can be analyzed for triglyceride composition by GLC without prior separation from other lipids. The small contamination with cholesterol ester and glyceryl ether diester has been ignored in this study.

Nevertheless, to obtain the fatty acid composition of the triglycerides present in colostrum and milk samples on the third, sixth, and ninth days postpartum, the lipid extracts were first purified by thick-layer chromatography (12). Methanolysis of the triglycerides was as described by Luddy et al. (18). The product was analyzed directly for both fatty acid and triglyceride composition. The latter analysis was used to determine that methanolysis had gone to completion, i.e., no intact triglyceride was detectable.

TABLE III

Fatty Acid Composition of Meals Taken on the Tenth Day Postpartum, in Mole %

Chain length of fatty acid	Mole %
C_4	1.3 ± 0.0
C_6	1.3 ± 0.0
C_8	1.1 ± 0.0
C_{10}	1.7 ± 0.2
C_{12}	3.5 ± 0.2
C_{14}	7.1 ± 0.6
C_{16}	30.3 ± 1.6
C_{18}	53.4 ± 2.5
$>C_{18}$	0.6 ± 0.05

± represents deviation between duplicate determinations on the same sample.

RESULTS AND DISCUSSION

The results in Table I show that there was a continuous appearance of new triglycerides of low carbon number during the first 5 days postpartum. The proportion of lower molecular weight ($< C_{48}$) triglycerides continued to increase until the eighth day postpartum and then tended to plateau with increasing time of lactation. The contribution of triglycerides C_{48} remained almost constant, though the reason for this is not clear. These results are expressed graphically in Fig. 1.

The fatty acid composition of the colostrum and milk triglycerides on the third, sixth, and ninth days postpartum (Table II) reflected this appearance of short-chain triglycerides. Read and Sarrif (4) had previously reported that the lauric and myristic acid content of human colostrum increases over the first 3 days of postpartum. The agreement we obtained between values for the average fatty acid chain-length (C_a) calculated (12) from triglyceride and fatty acid analyses (cf. Tables I and II) was good. Since triglycerides are separated according to total carbon number, fatty acids need only be distinguished on the basis of total carbon number (rather than on degree of unsaturation) to calculate C_a. The fatty acid composition shown in Table II for milk at 9 days of lactation is comparable to those found by a number of other workers (1,5,6,8) for this and later stages of lactation. Though small amounts of short-chain ($< C_{10}$) fatty acids were present in a typical day's diet (Table III), they were absent in the triglycerides of the colostrum and milk up to 9 days postpartum (Table II). That this was not due to loss in handling was seen from the correction factors used (13). Two reports of the presence of these fatty acids in human milk have shown that they represent at most 0.46% (19) and 2.2% (20) of the total fatty acids.

The free cholesterol content (Table I) showed no consistent change during the period under study and our values are similar to those obtained by other methods (10).

The fatty acid composition of the diet on the tenth day postpartum is shown in Table III. Further information on dietary and other extramammary contributions to milk fatty acids could be obtained by comparing blood plasma triglyceride fatty acid composition with that of colostrum and milk. Read et al. (4-6) have suggested that milk palmitic acid is derived mainly from extramammary sources, since its concentration in milk triglycerides remains fairly constant. The absolute amount of fat in

TABLE IV

Composition of Triglycerides of Human Colostrum and Milk on Various Days Postpartum. Values Found Experimentally and as Calculated Assuming Random Distribution

Triglyceride (carbon number)	Days postpartum								
	3			6			9		
	F[a]	R[b]	F:R	F	R	F:R	F	R	F:R
34	0	0		0.2	0.06	3.33	0.2	0.10	2.00
36	0	0		0.5	0.21	2.38	0.5	0.37	1.35
38	0.3	0.07	4.28	1.0	0.68	1.47	1.3	1.02	1.27
40	0.9	0.31	2.90	2.3	1.92	1.20	3.1	2.52	1.23
42	2.0	1.03	1.94	4.1	4.19	0.98	5.3	4.96	1.07
44	4.4	2.99	1.47	7.4	7.76	0.95	9.2	8.29	1.11
46	9.8	7.43	1.32	12.1	13.49	0.90	13.5	13.50	1.00
48	15.0	14.34	1.03	15.0	18.06	0.83	15.4	17.69	0.87
50	22.8	23.26	0.98	20.1	19.78	1.02	19.2	17.58	1.09
52	31.0	29.54	1.04	27.1	20.41	1.33	23.9	18.43	1.30
54	11.4	19.62	0.58	8.9	12.13	0.73	7.4	13.92	0.53
56	2.5	0.88	2.84	1.4	1.24	1.13	1.2	1.46	0.82
Average Ratio F:R			1.84			1.35			1.16

[a] F is the mole % of the triglyceride found experimentally.

[b] R is the mole % of the triglyceride assuming random distribution of the fatty acids.

human milk increases with day postpartum (4,9,10). However, it is not known whether the increase in amount of palmitic acid formed is due to preferential uptake or to increased mammary synthesis.

Read and Sarrif (4) have found that the fatty acid composition of antepartum colostrum was the same as that for colostrum at 1 day postpartum. It is of interest that though the donor in this study gave birth 1 month prematurely, the fatty acid composition of the colostrum on the third day postpartum (Table II) was very similar to that reported by Read and Sarrif.

The degree of specificity of triglyceride fatty acid composition was investigated as follows. The triglyceride compositions found on the third, sixth, and ninth days postpartum (Table I) were recalculated (21) assuming complete random distribution of fatty acids. To find how these two sets of data concurred, the ratio of found composition to random composition (F:R) has been determined for each triglyceride and an average ratio quoted for each of these days (Table IV).

On no day did F coincide with R. However, F approached R as the time of lactation increased. F:R on the ninth day (1.16) indicated a slightly more random distribution than that observed by Breckenridge and Kuksis (11) for a single sample of mature milk (F:R = 1.23, our calculation of their results).

Specificity of triglyceride fatty acid composition was seen (Table IV) with both low ($C_{34}-C_{40}$) and high ($C_{52}-C_{54}$) molecular weight triglycerides. More information could be obtained if the individual triglycerides were trapped as they emerged from the column and then specifically hydrolyzed.

In conclusion, the advantage of triglyceride over simple fatty acid analysis for this type of investigation is that in the former, new molecules appear, whereas in the latter, only changes in the proportions of existing fatty acids occur.

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The Lipid Components of White Potato Tubers *(Solanum tuberosum)*[1]

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ABSTRACT

Four Canadian varieties of potatoes were examined for their lipid composition. Lipids, extracted with chloroform-methanol, were shown by TLC and column chromatography to consist of 16.5% neutral lipids, 45.5% phospholipids and 38.1% glycolipids. Among the phospholipids and glycolipids, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, the galactolipids and the sterol glucosides were the major lipids. The predominant fatty acids were palmitic (19.5%), linoleic (44.8%) and linolenic (30.4%, in Kennebec). Analyses of the fatty acids of stored potatoes showed a marked decrease in linoleic acid and an increase in linolenic acid, in the Irish Cobbler and Sebago potatoes. β-sitosterol comprised 85.0% of total sterols. Nearly half of the carotenoids was lutein (xanthophyll), the others being α-carotene, β-carotene, an unidentified pigment and lutein epoxide.

INTRODUCTION

THE LIPIDS of white potatoes are a very complex mixture, including phospholipids, glycolipids, sterols and sterol glucosides, glycerides and fatty acids. Although they constitute only 0.5% of the dry weight, they are highly unsaturated and may therefore undergo significant chemical changes. For example, "off" flavors due to oxidative changes in dehydrated potatoes were demonstrated by Burton (1) and by Highlands et al. (2) and changes in the fatty acid composition of stored potatoes were reported by Mondy et al. (3) and Cotrufo and Lunsetter (4).

Except for descriptions of the fatty acids, relatively little has been published on the lipid composition of potatoes. As these lipids are rich in polar lipids (83.5%), further knowledge of their composition is necessary for a better understanding of their role in food quality and preservation.

The purpose of this investigation was to make a more detailed study of potato lipids by column, thin-layer and gas-liquid chromatographic methods.

EXPERIMENTAL PROCEDURE

Materials

Four Canadian varieties of potatoes, Netted Gem, Sebago, Irish Cobbler and Kennebec, used in this study, were grown at the Ottawa Research Station in 1964. Lipids were extracted from fresh potatoes, and also from potatoes stored at 21 C for 2.5 months in high relative humidity. The Netted Gem and Kennebec potatoes were chosen because of their excellent processing and storage qualities, and the Irish Cobbler and Sebago because of their fair-to-good storage quality.

Phospholipids used for TLC standardization were purchased from General Biochemicals, Chagrin Falls, Ohio; the fatty acid methyl esters from the Hormel Institute and Applied Science Laboratories; the sterols and tocopherols from Distillation Products Inc.

All solvents used in this study were reagent grade and were redistilled before use.

Extraction of Lipid

Samples (100 g potatoes) were homogenized for 2 min in 3.2 vol (w/v) of boiling ethanol. After standing for about 1 hr, with intermittent shaking, the homogenate was filtered, and the residue re-extracted twice with 3 vol of chloroform-methanol (2:1). The extracts were then pooled, the aqueous alcohol layer, which was tested and shown to contain negligible amounts of lipid was discarded, and the chloroform layer concentrated to dryness under nitrogen below 30 C. Crude extracts were freed from non-lipid materials by passage through a Sephadex G25 column (5). Lipids eluted with water-saturated chloroform-methanol (19:1) were used for column and thin-layer chromatography and were stored in benzene in a freezer without any sign of deterioration after several months.

Using the same procedure, lipids were also extracted from different parts of the potato

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tuber, corresponding to periderm, cortex, outer storage vascular parenchyma, inner storage vascular parenchyma and pith.

Column Chromatography

The Sephadex-purified lipid was then subjected to column chromatography, using a 0.9 × 13 cm silicic acid (Bio-Rad HA-325 mesh, Bio-Rad, Richmond, Calif.) column previously washed with methanol, chloroform and hexane. Samples (100 mg) were transferred onto the column in pure hexane, the elution was started with hexane and continued with 25 ml each of 2%, 5% and 50% ether in hexane, and then with 5%, 10%, 30% and 50% methanol in chloroform. After removal of the solvent in vacuum, the lipid residue was dissolved in chloroform and aliquots were tested by TLC.

Thin-Layer Chromatography

Thin-layer chromatography was performed on standard 20 × 20 cm chromatoplates coated with a 250 μ layer of Silica Gel G as described earlier (6). The solvent system hexane-diethyl ether-acetic acid (90:10:1) was used for the separation of neutral lipids and the system diisobutyl ketone-acetic acid-water (80:50:10) for the separation of polar lipids. Spots were located by exposure to iodine vapors or by spraying with 20% aqueous perchloric acid or 50% (v/v) sulphuric acid.

Individual lipids were isolated on a preparation scale by TLC. After development of the chromatoplates in the solvent systems mentioned above, the spots were visualized by spraying with rhodamin 6G in 0.25 M dipotassium hydrogen phosphate (7). The zones of interest were scraped off the plate, and the lipid was eluted from the silica gel with 5 ml of a mixture of chloroform-methanol (1:1) and used for the GLC analysis of their fatty acids.

Gas-Liquid Chromatography

The methyl esters of fatty acids were prepared in methanolic boron trifluoride. Samples (1-10 mg), obtained by TLC, were reacted in screw-cap tubes with 1 ml of boron trifluoride in methanol (Applied Science Laboratories), 1 ml of benzene, and 1 ml of methanol at 90 C for 45 min. Methyl esters were extracted with hexane and purified by TLC prior to their GLC. Their separation by GLC was performed on a 6 ft × 3/16-in. column of 5% DEGS on 60/80 mesh Gas Chrom P as already described (8). Standard mixtures of known fatty acid methyl esters were used for comparison of retention times. A column of 10% (W/W) polyvinyl acetate (AYAL 8285, Union Carbide) on 60/80 mesh Gas Chrom P was used as a check for possible peak overlap. Calibration of each methyl ester was done by using 17:0 as a marker and plotting the relative peak heights vs. the amounts injected.

Sterols and tocopherols were prepared by saponification of the crude lipid extracts. Samples (100 mg lipid) were heated in screw-cap tubes with 3 ml of 15% ethanolic potassium hydroxide, at 90 C for 60 min. Water was added to the reaction mixture and sterols were extracted twice with 6 ml of pure ether. The pooled ether extracts were evaporated and allowed to react with 0.1 ml of a mixture of hexamethyl disilazane and trimethylchlorosilane (molar ratio 1:1) in pyridine for about 30 min. Their separation and identification by GLC were done on a 3% XE-60 (supplied by D. Turner, Toronto) on Gas Chrom Q, 80/100 mesh column at a column temperature of 210 C.

Other Methods

Deacylation of fractions obtained by TLC or column chromatography was accomplished in 0.2 N methanolic potassium hydroxide at 40 C for 20 min. The hydrophylic portions of the polar lipids were identified by paper chromatography.

Carotenoids were fractionated by column chromatography as described previously (8) and their spectral absorptions were measured with a Bausch and Lomb spectronic 502 spectrophotometer. Quantitative measurements were made with a Bausch and Lomb 340 Spectrophotometer, at the maximum wave lengths, in hexane and chloroform. Some fractions were partitioned between hexane and 90% methanol (10). Others were tested for epoxides by the hydrochloric acid-ether color test (11).

RESULTS AND DISCUSSION

The lipid content of potatoes was relatively low for all varieties (about 0.5% of the dry weight), but their components were the most varied. As demonstrated with Netted Gem, neutral lipids amounted to 16.5%, whereas the phospholipids and glycolipids were respectively 45.5% and 38.0%. These latter, structural, lipids were present throughout the potato tissues. Their concentration was highest (3.1%) in the skin region, 0.6% in the cortex, and 0.4% in the outer storage vascular parenchyma, inner storage vascular parenchyma and pith.

TABLE I
Elution Pattern of Potato Lipids on Column Chromatography

Fractions	Composition of eluate	Volume (ml)	Lipid components[a]
1	2% ether in hexane	25	Pigment, sterol esters and triglycerides
2	5% ether in hexane	25	Triglycerides and fatty acids
3	50% ether in hexane	25	Free sterols and partial glycerides
4	5% methanol in chloroform	25	Esterified sterol glucoside,monogalactosyl diglyceride and sterol glucoside
5	10% methanol in chloroform	25	Digalactosyl diglyceride and cerebroside
6	30% methanol in chloroform	25	Phosphatidyl ethanolamine, phosphatidyl inositol and phophatidyl choline
7	50% methanol in chloroform	25	Phosphatidyl choline and phosphatidyl inositol

[a] As identified by TLC (Fig. 1) and paper chromatography (6, 8, 12).

Polar Lipids

Preliminary fractionation of total lipids for identification purposes was accomplished by silicic acid column chromatography, as shown in Table I. This provided enough material for deacylation and identification of the hydrophylic portion of the polar components by paper chromatography as described earlier (6). Individual components were measured quantitatively by gravimetric methods after their complete separation by TLC. Their relative percentages are presented in Table II.

One dimensional thin-layer chromatography allowed good separation of the major phospholipids and glycolipids (Fig. 1), but failed to show all the minor components, such as phosphatidyl glycerol or the sulfolipids. With the two dimensional system described earlier (6) using appropriate spray reagents, we had found 17 components. Some of them were detected in trace amounts and are not yet identified.

The esterified sterol glucoside, which is present in appreciable amounts in potatoes as well as in other tissues such as peas, soybean and wheat, has been fully described (6, 12). The structural composition of this polar lipid has been since confirmed by Kiribuchi et al (13).

Analyses carried out on the four varieties of potatoes studies showed few varietal differences in the lipid content or in the total fatty acid composition of fresh potatoes. Percentages of polar lipids were also similar: 83.6%, 84.6%, 83.6% and 82.0% respectively for the Netted Gem, Kennebec, Sebago and Irish Cobbler. A thin-layer chromatogram showing the polar lipid composition of these potatoes is shown in Fig. 1.

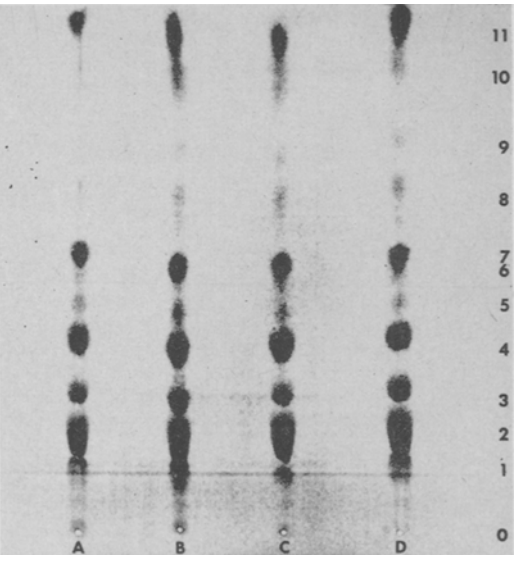

FIG. 1. Thin-layer chromatogram of potato lipids. Solvent system—diisobutyl ketone—acetic acid —water (8:5:1). Spots were revealed with iodine vapors. A, Netted Gem; B, Sebago; C, Irish Cobbler; and D, Kennebec. Spots were identified as: 1, phosphatidyl inositol; 2, phosphatidyl choline; 3, digalactosyl diglyceride; 4, phosphatidyl ethanolamine; 5, cerebroside; 6, sterol glucoside; 7, monogalactosyl diglyceride; 8, esterified sterol glucoside; 9, phosphatidic acid; 10, free sterols; and 11, triglycerides plus sterol esters.

Fatty Acid Composition

No important differences were shown between the overall fatty acid compositions of the four varieties. Within the lipid classes, however, marked differences were observed

TABLE II
Lipids of Potato Tubers (%)
(Netted Gem, 1967)

Components	mg/100 fresh tissue	Relative percentages
Neutral lipids	16.8	16.5
Phospholipids	46.4	45.5
Phosphatidyl choline	19.3	18.9
Phosphatidyl inositol	12.1	12.0
Phosphatidyl ethanolamine	14.9	14.6
Glycolipids	38.7	38.0
Monogalactosyl diglyceride	7.4	7.2
Digalactosyl diglyceride	7.3	7.1
Sterol glucoside	6.1	6.0
Esterified sterol glucoside	11.1	10.9
Cerebroside	7.0	6.9

TABLE III
Fatty Acid Composition of Fresh Potato Tubers
(Relative weight percent)

	16:0	16:1	18:0	18:1	18:2	18:3
Total Lipids						
NG[a]	17.5	0.5	4.9	0.7	52.0	24.2
S	17.6	0.4	4.7	0.8	45.1	31.2
IC	18.6	0.4	3.5	0.5	48.8	28.2
K	19.5	0.3	4.1	0.8	44.8	30.4
Phospholipids and Glycolipids						
PE						
NG	20.1	1.5	6.4	1.4	54.2	15.1
S	20.1	—	4.0	0.8	54.6	20.5
IC	21.6	0.5	2.7	0.4	56.8	18.1
K	21.1	0.4	3.5	0.6	56.0	18.1
PC						
NG	13.8	0.3	3.6	1.0	55.6	25.6
S	14.6	0.6	5.1	1.4	51.7	26.3
IC	19.1	0.7	3.5	0.8	54.4	21.6
K	20.1	0.5	4.9	1.1	49.4	23.5
PI						
NG	26.1	0.5	5.8	1.0	46.4	20.0
S	25.2	0.6	5.8	1.2	48.5	18.6
IC	30.0	0.3	4.0	0.6	50.8	14.4
K	26.9	0.6	4.5	0.6	45.1	22.1
MGal						
NG	4.9	0.2	1.5	0.7	60.1	32.6
S	6.9	0.6	1.9	0.5	37.8	53.4
IC	4.4	0.3	0.9	0.4	36.8	57.3
K	3.7	0.2	0.8	0.5	38.5	56.4
DGal						
NG	15.7	0.4	1.4	1.6	55.9	24.9
S	9.2	0.5	6.8	1.0	47.1	35.6
IC	12.4	0.4	4.4	0.7	48.6	33.6
K	9.6	0.3	5.0	1.1	46.2	37.7
ESG						
NG	54.3	1.1	11.0	1.0	24.3	7.2
S	61.3	1.3	13.0	1.6	17.3	4.0
IC	66.7	1.4	10.0	1.3	16.0	4.6
K	64.5	2.2	12.0	5.4	9.2	4.2

[a]NG, Netted Gem; S, Sebago; IC, Irish Cobbler; K, Kennebec. PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PC, phosphatidyl choline; MGal, monogalactosyl diglyceride; DGal, digalactosyl diglyceride; ESG, esterfied sterol glucoside.

(Table III). For example, palmitic acid (18.3% in total lipid), was found to predominate in the esterified sterol glucoside and also to occur in higher amounts in phosphatidyl inositol than in the other polar lipids. It was, in contrast, low in the mono and digalactosyl diglycerides. The other major fatty acids were linoleic (47.7%) and linolenic acids (28.5%). Fatty acids, such as myristic acid, with less than 0.3% are not shown in Tables III and IV.

The present results are in substantial agreement with the early data of Talbert and Smith (14) who reported 25% saturated acids (palmitic and stearic acids) and 30% linolenic acid. More recently, however, Cotrufo and Lunsetter (4) reported 31% saturated acids (myristic, palmitic and stearic acids) and 19.4% linolenic acid in the total fatty acid composition of potato. It is worth noting that Cotrufo and Lunsetter (4) used petroleum ether, a non-polar solvent, to extract the lipids whereas chloroform-methanol was used in this study. Extraction with chloroform-methanol yielded 0.5% lipid in comparison with 0.2-0.26% obtained by these authors.

Analysis of the fatty acids of the potatoes stored at 21 C for 2.5 months, showed changes (Table IV) particularly in the Sebago and Irish Cobbler varieties. Whereas the total fatty acids of the Kennebec and Netted Gem potatoes remained practically unchanged after storage, those of the Sebago and Irish Cobbler varieties showed a decrease in linoleic and an increase in linolenic acids. These changes were more marked in the varieties rated only fair-to-good for their storage quality. A similar increase in linolenic acid was also reported by Mondy et al. (3) in the Pontiac variety. We observed the same trend in the individual phospholipid components such as phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol. Galactolipids, however, gave more erratic results. These findings suggest

TABLE IV
Fatty Acid Composition of Stored Potato Tubers
(Relative weight percent)

	16:0	16:1	18:0	18:1	18:2	18:3
Total Lipids						
NG[a]	17.9	0.4	4.6	0.3	50.7	26.1
S	18.9	0.4	3.5	0.6	40.1	36.4
IC	17.6	0.5	3.1	0.8	39.4	38.7
K	19.6	0.5	3.7	0.5	44.7	30.9
Phospholipids and Glycolipids						
PE						
NG	17.6	0.5	3.6	....	57.3	20.7
S	20.0	0.5	2.6	0.5	47.8	28.3
IC	19.3	0.3	2.6	0.4	46.6	30.5
K	15.4	0.4	3.0	0.4	55.5	25.0
PC						
NG	16.7	0.5	4.8	0.5	50.8	26.5
S	18.8	0.5	3.5	0.9	39.6	36.3
IC	18.8	0.4	2.9	0.5	40.9	36.0
K	17.8	0.4	3.3	0.8	49.3	28.1
PI						
NG	26.9	0.6	5.0	0.5	44.0	29.5
S	24.8	0.5	3.1	0.5	37.1	33.7
IC	26.8	0.6	3.2	0.4	35.7	32.7
K	29.4	0.5	3.8	0.6	38.3	26.8
MGal						
NG	10.4	0.3	8.2	1.0	57.1	23.1
S	20.2	0.5	6.0	1.1	51.2	20.9
IC	15.1	0.5	6.8	1.0	39.4	36.8
K	10.7	0.3	6.6	1.0	49.8	31.5
ESG						
NG	46.9	1.2	10.3	1.2	31.2	8.2
S	61.5	2.0	8.9	1.1	16.3	8.2
IC	51.9	1.7	7.5	2.1	21.2	14.0
K	55.0	1.8	11.0	1.5	22.6	6.1

[a]Same as under Table III.

TABLE V
Column Chromatographic Separation and Percent Composition of Potato Tuber Carotenoids

FR	Composition of eluate[a]	Measured spectra, max mμ[a]				Relative percent[b]	Carotenoids
A	Hexane	426	449	475	(H)	16.3	β-carotene
B	5%E in hexane	422	442	468	(H)	6.4	α-carotene
C	50%E in hexane	434	454	476	(C)	14.2	unidentified
D	2%M in chloroform	424	455	483	(C)	48.5	lutein
E	5%M in chloroform	408	431	456	(C)	14.8	lutein epoxide

[a] Solvents: E, ether; M, methanol; H, hexane; C, chloroform

[b] Absorptivities values (15): β-carotene, 2650; α-carotene, 2710; lutein, 2380; lutein epoxide, 2400; unidentified pigment, as β-carotene.

that metabolic changes rather than oxidative deterioration took place.

Neutral Lipids and Unsaponifiable Materials

Neutral lipids (16.5%) were shown by TLC to be triglycerides together with free sterols and sterol esters, traces of partial glycerides and fatty acids. No difference was noted between varieties.

The sterols, analysed as their TMS derivatives, consisted of campesterol, stigmasterol and β-sitosterol in the ratio of 5.4:9.6:85.0. A search by GLC for tocopherols in potato lipid unsaponifiable matter showed that neither α-, β- nor γ-tocopherol was present in appreciable amounts.

Carotenoid Pigments

The carotenoids of potato were fractionated by column chromatography. Five components, whose absorption characteristics are presented in Table V, were resolved. The first fraction, which emerged with pure hexane, had the same absorptions as β-carotene. The second corresponded to α-carotene. The third one, which was not fully identified, was found to be hypophasic after partition between hexane and 90% methanol, which indicates that it is not a free xanthophyll, although it is related to lutein by its absorption characteristics. The fourth component, the most abundant of these pigments, co-chromatographed with authentic lutein and had, in addition, identical absorption characteristics. The last component to emerge on the column gave a positive test for epoxide and chromatographed with the same retention volume as lutein epoxide.

These five components, as measured at their maximum spectral absorptions in hexane and chloroform, and calculated according to the $E_1^{1\%}{}_{cm}$ values reported by Goodwin (15), amounted to 16.3%, 6.4%, 14.2%, 48.5% and 14.8%, respectively, for the β-carotene, α-carotene, the unidentified, lutein and lutein epoxide.

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Intracellular Distribution of Lipolytic Activity in the Female Gametophyte of Germinating Douglas Fir Seeds

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ABSTRACT

Acid (pH 5.2) and neutral (pH 7.1) lipase activity was studied in order to localize the sites of lipolysis in cellular fractions of catabolic organ of Douglas fir seed. Cellular particles were separated by differential centrifugation of the tissue homogenate and identified by electron microscopy. Emulsified native neutral lipids were provided as a substrate to protein body, mitochondrial, microsomal and soluble fractions, and endogenous lipids were used as a substrate for light and heavy fat body fractions. Little difference was observed in average specific activity of the two enzyme systems in dry seeds, but acid lipase activity increased sevenfold and neutral lipase activity fourfold during germination. Highest specific activity of both enzyme systems was found to be associated with the heavy fat bodies and the soluble fraction. Heavy fat bodies contained an ample quantity of endogenous substrate while the soluble fraction consisted of little substrate. Experimental data indicated that the soluble fraction was the source of lipases, and the heavy fat bodies were the site of in situ lipolysis.

INTRODUCTION

THE SEED OF DOUGLAS FIR (*Pseudotsuga menziesii* Franco) consists of 35% lipids, 32% protein, 29% fibers (seed coat), 1.8% minerals, 1.7% starch and sugars, 0.2% ribonucleic acid, 0.04% nucleotides and 0.03% deoxyribonucleic acids (1). During germination, the reserve lipids stored chiefly in the gametophytic tissue are utilized for the energy supply of growth and for the carbon skeleton of soluble metabolites and new cellular structures in the seedling (2). The reserve proteins are converted to soluble enzymes, amino acids, amides, small peptides, nucleotides, nucleic acids and structural proteins in the seedling (1).

Morphologically, the seed is composed of an outer thin seed coat, middle thick female gametophyte, and inner small embryo. Their weight distribution is respectively, 28%, 65% and 7% of the total dry weight. As revealed by electron micrographs, the female gametophyte is the storage site and consists mainly of fat bodies, protein bodies and nucleus in large parenchymatous cells. Mitochondria, endoplasmic reticula, ribosomes and plastids are present but very sparse. During germination, fat bodies decrease in size and number and acquire a vesicular, proteinaceous outer membrane indicating in situ lipolysis. Meanwhile, protein bodies become granulated, fragmented and finally solubilized relating in situ hydrolysis. Other organelles become conspicuous as the storage particles diminish. Some increase of ribosomes, mitochondria and amyloplasts is observed during the stage of rapid lipolysis (3).

Seed lipases are usually assayed with fat-free residues of dry or germinated seeds (4, 5). Whole homogenate (6) and fat particles (7, 8) of germinating seeds have also been studied for this type of enzymes. However, lipase activity was found to be localized around the cell wall and between aleurone grains (protein bodies) by in situ histochemical investigation in Scots pine seed (9). The compartmentalization of metabolic functions in cellular organelles is a well known fact but whether lipolysis is localized or not requires further study. This paper was thus initiated to discern the lipolytic activity in cellular organelles of storage organ of the Douglas fir seed during catabolism.

EXPERIMENTAL PROCEDURES

Material

Female gametophytes were dissected from seeds of following groups: air dried, stratified for four weeks at 2 C under moist conditions, and after 5, 10 and 14 days of germination.

Fractionation

All the procedures were conducted at about 1 C. Four grams of female gametophytic tissue was disinfected in 1% arasan, rinsed with sterile water and ground in a mortar with pestle in 10 ml 0.1 M Tris-HCl buffer at pH 7.5, containing 0.5 M sucrose and 0.001 M $CaCl_2$. The homogenate was filtered through four layers of cheese cloth, and the residue was ground again in the medium and filtered. The combined filtrate was centrifuged at 100 × g for 1 min to precipitate the cell debris, and tissue pieces. The supernatant was decant-

ed and centrifuged at 2,000 × g for 20 min to collect mainly protein bodies and a small number of nuclei and plastids. Mitochondria were spun down from the post-protein-body supernatant at 20,000 × g for 20 min, while light fat bodies were concentrated on the top of the medium by this procedure. The post-mitochondria supernatant was centrifuged at 105,000 × g for 2 hr to precipitate microsomes in the pellet, to float heavy fat bodies on the top, and to gather soluble fraction in the middle.

Chemical Analysis of the Fractions of Light and Heavy Fat Body and Soluble Fraction

The fat layers were lifted with a spatula, drain-dried on a piece of tissue paper, weighed and dried in a vacuum oven at 50 C for 12 hr. An aliquot of the soluble fraction was dried as above. The total lipids of the dried materials were extracted with chloroform-methanol (2:1, v/v), vacuum dried and weighed. The lipids were further analysed by quantitative thin-layer chromatography (10). The protein-nitrogen content of the different fractions was estimated by the micro-Kjeldahl method in the 10% ice-cold trichloroacetic acid precipitated pellets.

Assay Conditions and Analysis of Reaction Products

One half of each fraction, except the solubles, was suspended in 4 ml 0.1 M acetate buffer (pH 5.0) and the other half in 4 ml 0.1 M Tris-HCl buffer (pH 7.0). The pH of the resulting suspensions varied with the quantity of the pellet and ranged from 5.0 to 5.5 or from 7.1 to 7.3. A portion of the soluble fraction was adjusted to pH 5.0 with 0.1 M acetic acid. Lipase activity was assayed by incubating a reaction mixture of 3.2 ml containing 1 ml of either the suspension or the soluble fraction with 2 ml of substrate, 30 μ mole $CaCl_2$ as a cofactor, and 25 ppm streptomycin as a bacteriostat at 25 C for 12 hr with gentle shaking. The substrate for the light and heavy fat body fractions was endogenous glycerides and consequently 2 ml of the respective buffers were added in place of the substrate. The substrate for the other fractions was freshly prepared by homogenizing 1 g neutral oil which was extracted from Douglas fir seed with petroleum ether and vacuum dried in 60 ml of the acetate or the Tris-HCl buffer containing 5% purified gum arabic. Two milliliters of the prepared substrate contained approximately 100 μ eq of esterified long-chain fatty acids. At the end of incubation, the reaction was stopped by adding 1 mmole HCl, and the mixture extracted with 10 ml chloroform. The free long-chain fatty acids (FFA) in the extract were determined quantitatively (11). The qualitative change of neutral lipids before and after incubation was analysed by thin-layer chromatography (10). A tube of substrate without enzyme preparation was incubated simultaneously as the blank. Another zero hour control containing the suspension, substrate, $CaCl_2$ and streptomycin was also prepared.

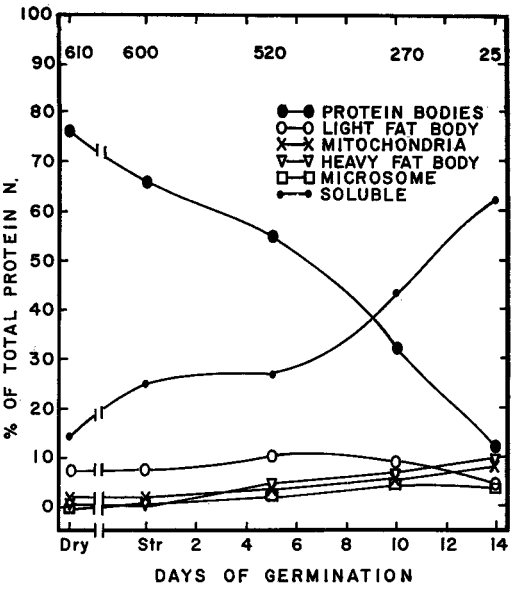

FIG. 1. Percentage distribution of total protein N in isolated cellular fractions of female gametophyte of Douglas fir seeds during germination. The total μg of protein N per gametophyte at different germination stages is listed at the top of the graph.

The lipase activity was estimated by the net increase of free fatty acids or by the difference between the total free fatty acids accumulated and the quantity of free fatty acids found in the blank and the zero hour control.

The specific activity of acid (pH 5.2) and neutral (pH 7.1) lipases in various fractions was calculated as the μ eq FFA produced per milligram protein N per hour. The protein N in each reaction tube was determined by the micro-Kjeldahl method in ice-cold 10% trichloroacetic acid precipitate of the chloroform extracted residue. The protein N in each fraction was obtained by multiplying the average N in each tube of the fraction by the total volume of the fraction.

Electron Microscopy

The identity and composition of the separated fractions were checked by electron

micrographs of comparable sets of material fractionated by the procedure described above. Each pellet was fixed in 1% osmium tetroxide or 2% potassium permanganate for 24 hr at 0C, dehydrated through ethanol series, embedded in Epon 812, sectioned with a diamond knife, stained with uranyl acetate and lead citrate, and viewed with a RCA EMU-2D electron microscope.

RESULTS AND DISCUSSION

The distribution of protein N in various fractions is shown in Fig. 1. The decrease in protein body N with germination provided the increase of soluble N and the N in other organelles. All the increases were slight since a major portion of protein body N is transported to the seedling for growth (1). Ultrastructural changes observed in this material during germination also indicated such a transfer (3). These observations and those presented in Figs. 2 and 3 supported the efficiency of the fractionation procedure. However, at the last two stages of germination, progressively more contamination in each fraction was observed; nuclei and amyloplasts were the dominant particles in the protein body fraction,

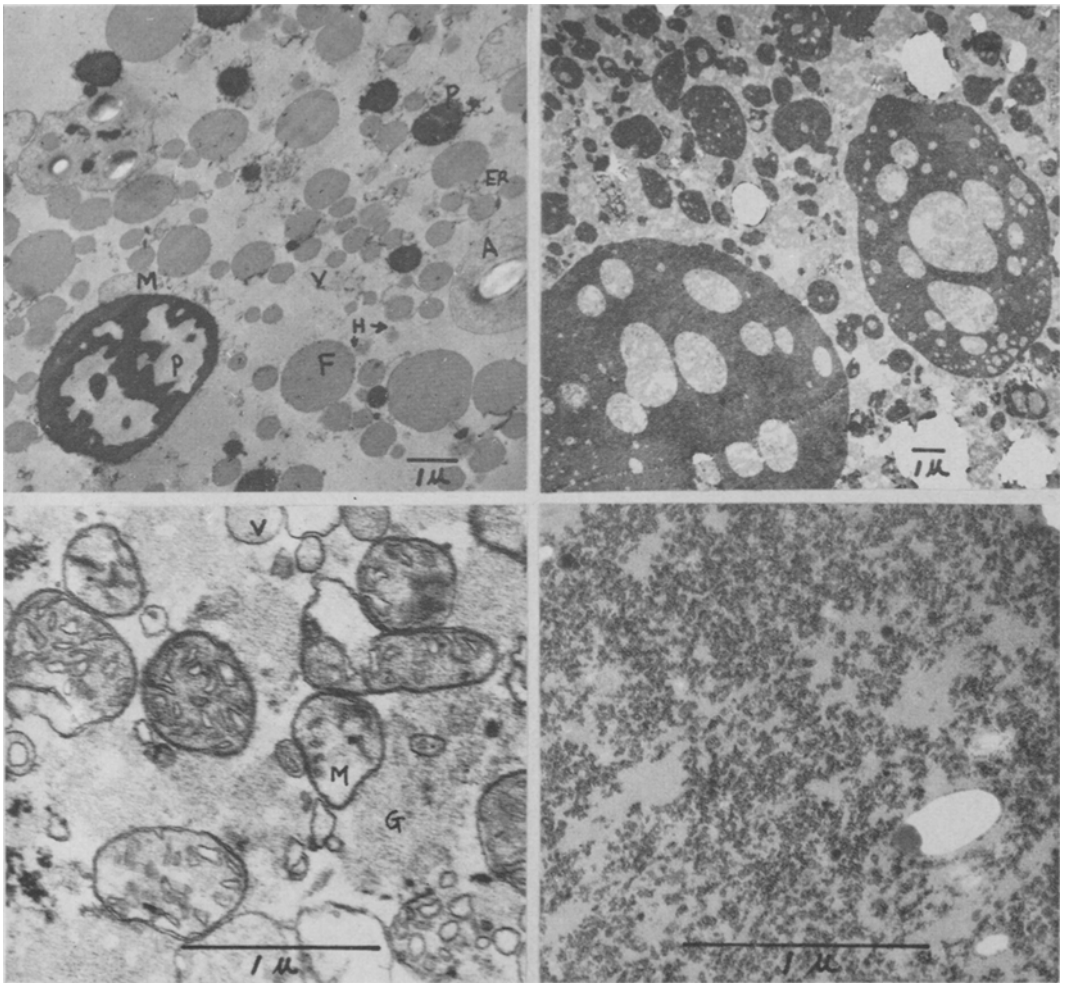

FIG. 2. Electronmicrographs of cellular fractions isolated from gametophyte on the fifth day of germination. Upper left—whole homogenate containing protein bodies (P), light fat bodies (F), amyloplasts (A), mitochondria (M), endoplasmic reticulum (ER), vesicles (V), heavy fat bodies (H) and scattered ribosomes. Upper right—protein body fraction showing different sized particles with no defined membrane. Lower left—mitochondrial fraction showing vesicles, granular substances (G) and mitochondria. Lower right—microsomal fraction containing mainly ribosomes. All osmium fixed.

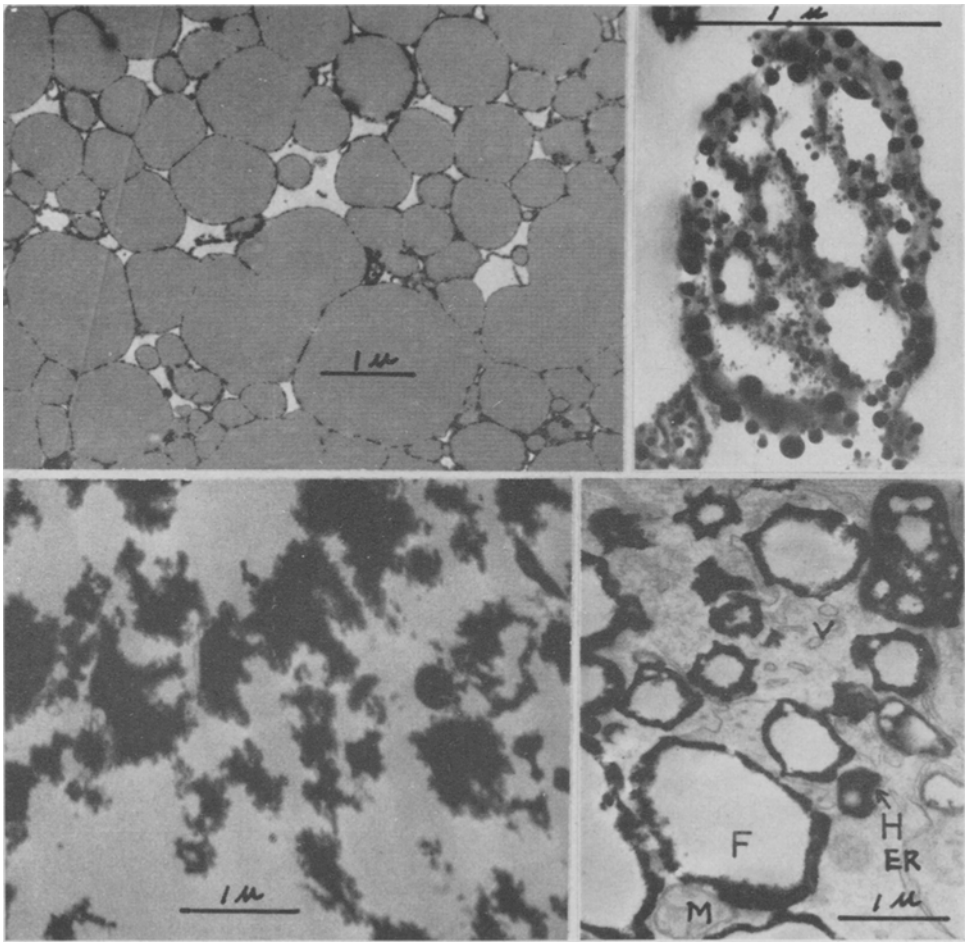

FIG. 3. Electronmicrograph of the gametophyte on the fifth day of germination. Upper left—light fat body fraction fixed with osmium. Upper right—a heavy fat body fixed with permanganate showing vesicular proteinaceous boundary. Lower left—isolated heavy fat bodies fixed with osmium. Lower right—a portion of a gametophytic cell fixed with permanganate showing the proteinaceous boundary around the fat particles and other organelles.

plastids and vesicles were common in the mitochondrial pellet, and the microsomal fraction consisted of numerous membrane-like materials with little ribosomes. The light and heavy fat body fractions were not contaminated at any of the stages of germination. The light fat body fraction contained coalesced and individual oil droplets with a thin proteinaceous outer boundary while the heavy fat bodies were irregular in shape, smaller and were surrounded with a thick proteinaceous zone. Both types of fat bodies were found in situ and appeared to be different only in density due to different compositions of lipids and proteins as shown below.

Further analysis of the fractions isolated from the material of five days of germination indicated that the light fat body fraction was composed of 10% water, 85% lipids, 2% protein and 3% others, and that the heavy fat bodies consisted of 32% water, 38% lipids, 12% proteins and 18% others. The lipids from the light fat body fraction contained 2% sterol esters, 84% triglycerides, 4% free fatty acids, 2% sterols, 4% diglycerides, 1% monoglycerides, and 3% phospholipids and other polar lipids. The lipids from the heavy fat body fraction consisted of 6% sterol esters, 70% triglycerides, 5% free fatty acids, 4% sterols, 9% diglycerides, 1% monoglycerides, and 5% phospholipids and others. The compositional differences of the two are obvious. The fact that more intermediates of lipolysis, proteins and emulsifiers, and less triglycerides were found in the heavy fraction than the light

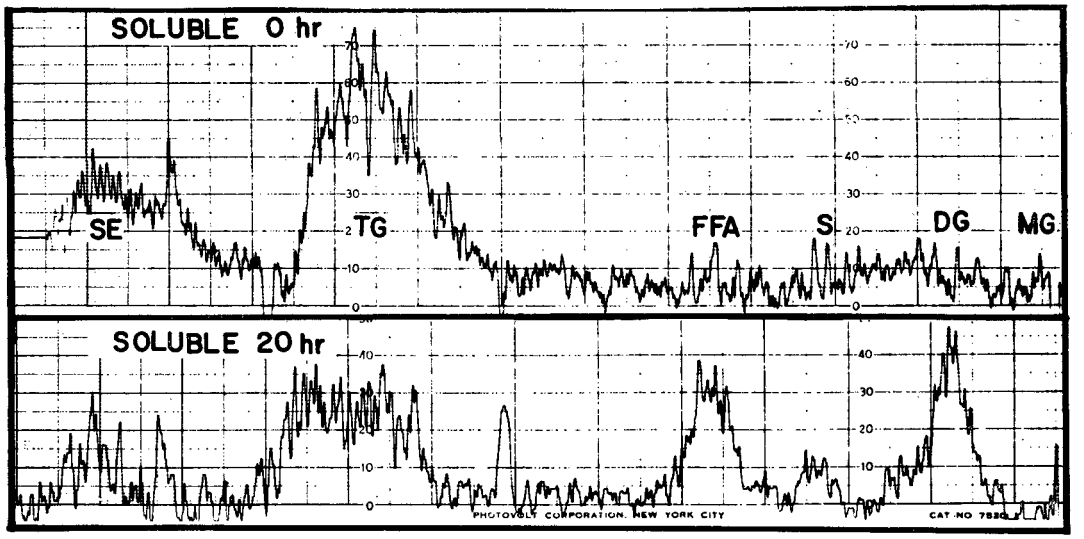

FIG. 4. Quantitative chromatograms of neutral lipids extracted from the incubation mixture of the soluble fraction with substrate. SE—sterol esters, TG—triglyceride, FFA—free fatty acids, S—sterols, DG—diglycerides and MG—monoglycerides. The chromatoplate was developed in hexane-diethyl ether-acetic acid (85:15:1 v/v).

fraction suggests a continuity between the two species of particles; the heavy fat bodies probably are more advanced lipolytic complexes than the light fat bodies in situ.

After correction for dilution by the homogenizing medium, the soluble fraction consisted of 97% water, 1.8% protein, 0.6% lipids and 0.8% others. The lipids were 25% triglycerides, 5% free fatty acids, 4% sterols, and 66% phospholipids and other polar lipids. These data indicate that the soluble fraction is poor in endogenous substrate of lipolysis.

The quantitative and qualitative changes of the substrate after incubation with the soluble fraction are illustrated in the quantitative chromatogram (Fig. 4). The reaction products of lipases, free fatty acids (FFA), diglycerides (DG) and monoglycerides (MG) increased while triglycerides (TG) and sterol esters decreased quantitatively during incubation. Ap-

TABLE I

Specific Activity[a] of Acid and Neutral Lipases in Cellular Fractions of the Female Gametophyte During Germination of Douglas Fir Seeds

Fraction	Dry Seed	Stratified Seed	Germinated Seed		
			5 days	10 days	14 days
Acid lipase					
Protein body	0.32 ± 0.02	0.46 ± 0.01	0.99 ± 0.21	1.44 ± 0.18	2.35 ± 0.40
Light fat body	1.70 ± 0.14	2.56 ± 0.31	5.30 ± 0.28	3.26 ± 0.51	1.74 ± 0.30
Mitochondrion	3.15 ± 0.35	3.40 ± 0.60	2.10 ± 0.33	1.30 ± 0.21	1.65 ± 0.33
Heavy fat body	17.00 ± 2.11	17.30 ± 3.05	16.70 ± 1.86	14.70 ± 2.80	12.15 ± 3.10
Microsome	1.50 ± 0.31	1.90 ± 0.11	1.50 ± 0.28	0.96 ± 0.45	4.20 ± 0.65
Soluble	14.70 ± 0.89	18.30 ± 3.50	17.10 ± 2.15	11.45 ± 1.86	24.10 ± 2.51
Average[b]	2.60	4.90	6.40	6.60	16.90
Neutral lipase					
Protein body	0.33 ± 0.09	0.59 ± 0.10	0.72 ± 0.18	1.50 ± 0.20	3.90 ± 0.42
Light fat body	1.38 ± 0.21	3.03 ± 0.18	2.56 ± 0.41	2.69 ± 0.30	2.43 ± 0.28
Mitochondrion	3.10 ± 0.24	5.30 ± 0.67	2.76 ± 0.32	1.76 ± 0.33	0.10 ± 0.03
Heavy fat body	11.20 ± 0.25	20.90 ± 1.98	11.35 ± 1.45	7.85 ± 0.24	7.73 ± 1.48
Microsome	1.90 ± 0.31	1.80 ± 0.20	1.60 ± 0.38	2.04 ± 0.28	7.90 ± 2.00
Soluble	13.20 ± 1.86	9.30 ± 2.30	6.60 ± 1.76	5.26 ± 1.33	10.90 ± 1.87
Average[b]	2.30	3.10	3.00	3.60	8.40

[a] Specific activity—μ eq FFA/mg protein N/hr. ± SD from four replications.

[b] Average specific activity— $\dfrac{\text{total } \mu \text{ eq FFA from all fractions}}{\text{total mg protein N}}$

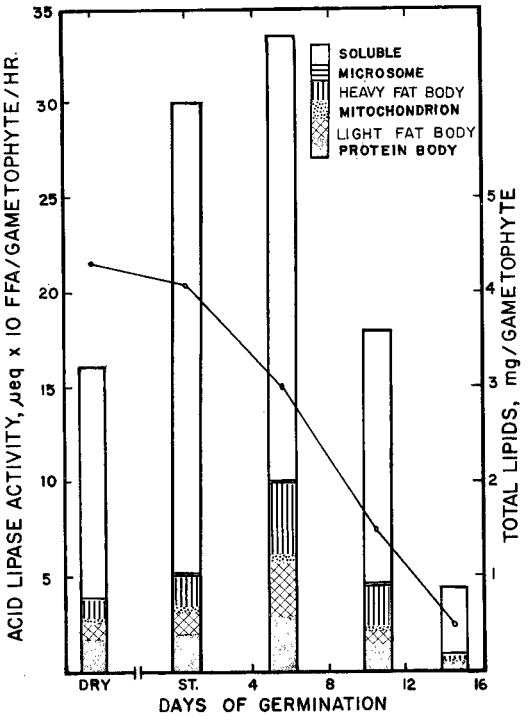

FIG. 5. Distribution of acid lipase activity in isolated cellular fractions of the female gametophyte of Douglas fir seeds during germination.

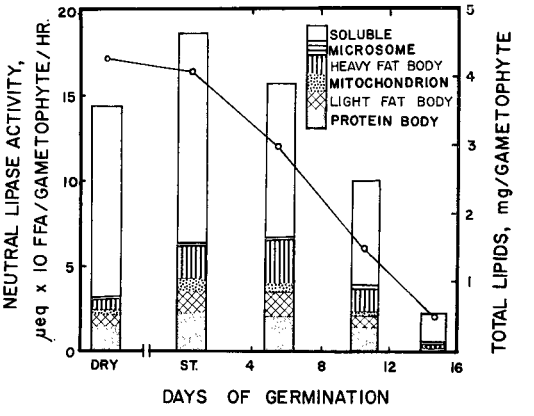

FIG. 6. Distribution of neutral lipase activity in isolated cellular fractions of the female gametophyte of Douglas fir seeds during germination.

parently esterases were present in the soluble fraction in addition to lipases. Other fractions also showed esterase activity under the incubation conditions employed particularly at the lower pH.

The specific activity of acid and neutral lipases in various fractions during germination is summarized in Table I. Highest specific activity was associated with the heavy fat bodies and the soluble fraction. This finding probably indicates that the source of lipases is in the soluble fraction while the in situ site of lipolysis is in the heavy fat bodies.

A definite but small increase of both acid and neutral lipase activity with germination was found in the protein body fraction. This trend might indicate the activity of plastids rather than protein bodies, since the frequency of plastids increased with germination in this fraction. The change of specific activity in the light fat body fraction was somewhat parallel with the fat utilization of the gametophyte (1). The mitochondrial fraction showed highest specific activity in the stratified seeds, while the specific activity of the microsomal fraction was highest at the last stage of germination. The significance of these observations is not clear at present.

The total activity of acid and neutral lipases in isolated cellular fractions of the female gametophyte is shown in Figs. 5 and 6, re-

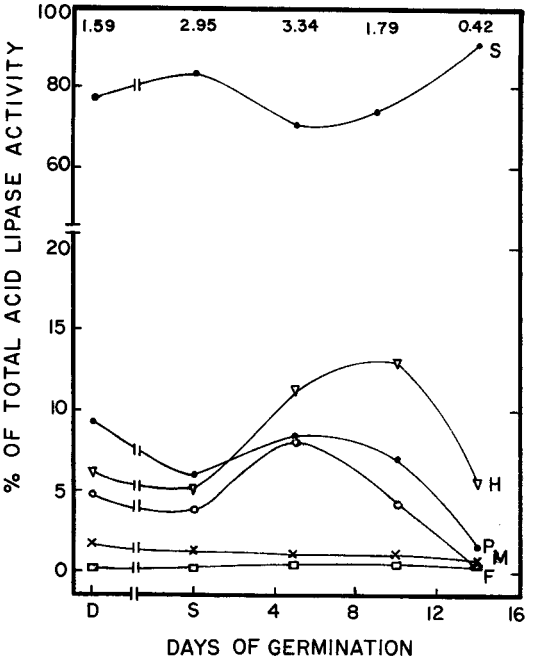

FIG. 7. Percentage distribution of acid lipase in isolated cellular fractions of the female gametophyte of Douglas fir seeds during germination. The total activity per gametophyte is listed on the top of the graph. S—soluble, H—heavy fat body, MC—microsomes, F—light fat body, M—mitochondrion and P—protein body.

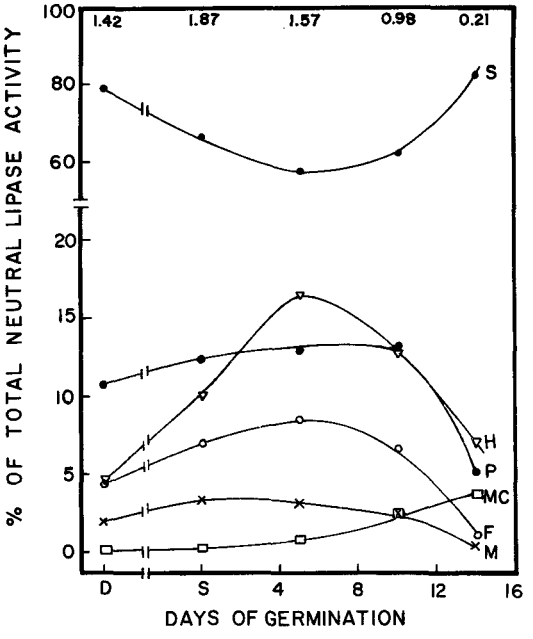

FIG. 8. Percentage distribution of neutral lipase in isolated cellular fractions of the female gametophyte of Douglas fir seeds during germination. The total activity per gametophyte is listed on the top of the graph. S—soluble, H—heavy fat body, MC—microsomes, F—light fat body, M—mitochondrion and P—protein body.

spectively. The total lipid content of the gametophyte is also indicated in the graphs for reference. The lipolytic potential appeared to be high in the dry seed, but little lipolysis occurred at this stage. Perhaps the hydration during grinding with an aqueous medium activated the pre-existing enzymes. Stratification increased the total acid lipases activity twofold, while neutral lipase activity only increased by 20%. The acid lipase activity increased further at the early germination stage, then decreased rapidly. The neutral lipase activity decreased with the advancement of germination. Even though the soluble fraction contained the highest percentage of lipase activity at any stage, a relative reduction was found on the fifth and tenth day of germination (Figs. 7 & 8). Concomitantly, a relative increase of both light and heavy fat body bound lipases was observed. This coincidence and the trend of fat utilization are probably related to the lipolytic role of the fat bodies.

The lipolytic activity in fractions of adipose tissue was found mainly in fat layers and soluble fractions (12-14). This paper observed comparable results, even though the lipolytic process is much more complicated in animal materials.

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Oxygenated Fatty Acids of Oil From Sunflower Seeds After Prolonged Storage[1]

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ABSTRACTS

Chemical analysis of a number of sunflower *(Helianthus annuus)* seed oil samples revealed a low and variable percentage of hydrogen bromide-reactive material. To characterize the compounds responsible for this reactivity, oil was extracted from selected introductions from Uruguay, Turkey, and Yugoslavia that had been subjected to prolonged storage. Two epoxy fatty acids and two conjugated dienolic acids were isolated from the methyl esters derived from these sunflower seed oils by using a combination of column chromatography and countercurrent distribution. The epoxy acids are *cis*-9,10-epoxystearic acid (0.5%) and *cis*-9,10-epoxy-*cis*-12-octadecenoic (coronaric) acid (2.2%). Characterization of the dienols revealed that they are 9-hydroxy-*trans*-10,*cis*-12-octadecadienoic acid (1.2%) and 13-hydroxy-*cis*-9,*trans*-11-octadecadienoic acid (1.3%). Fresher seed of some of these introductions contained less of the oxygenated components.

Oils from recently produced seed of selected high-oil Russian sunflower varieties, including some currently grown in the United States, contained no more than trace amounts of oxygenated acids. Though the relative contributions of genetic and environmental factors toward genesis of oxygenated acids are not established, increase of those acids in some sunflower lines as a result of storage has been demonstrated.

INTRODUCTION

SUNFLOWER *(Helianthus annuus)* has not achieved major crop status in the United States because of its susceptibility to attack by insects and diseases. Availability of other good oilseeds such as soybeans, cottonseed, flaxseed, peanuts and safflower has lessened the incentive to develop improved sunflower varieties. In the Soviet Union, however, sunflower is the major oilseed crop, and this status is probably the direct result of a successful program of plant selection and breeding to develop varieties that have desirable agronomic characteristics and that produce seeds of high oil content. The success of these high-oil Russian varieties has spurred interest in sunflower as a potential United States crop.

Presumably, a large proportion of the sunflower seed oil produced would be utilized by the edible oil industry. Previous reports (1-3) have indicated that sunflower seed oil contains small amounts of oxygenated acids, which might adversely affect its stability and nutritional properties. Morris and co-workers (1,2) obtained evidence for both hydroxy and epoxy acids in sunflower oil but did not isolate or characterize them structurally. We wish to report the isolation and identification of four oxygenated fatty acids from sunflower seed oil. The oil samples used in this study and the sample Morris used for his research were derived from the same group of stored seeds. As far as we can determine, these samples of sunflower seeds are the only ones known to yield oils containing oxygenated fatty acids. Samples of four Russian high-oil varieties harvested in 1966 were screened for oxygenated fatty acid content and were found to contain little, if any, of these acids.

EXPERIMENTAL PROCEDURES

Materials and Methods

Infrared (IR) spectra were determined with 1% solutions in carbon disulfide on a Perkin-Elmer Infracord, Model 137, and ultraviolet (UV) measurements were made on cyclohexane solutions with a Beckman DK-2A spectrophotometer. 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).

Thin-layer chromatographic (TLC) analyses of the oxygenated fatty acid esters were done on Silica Gel G developed with ether-hexane-acetic acid (30:70:1 or 20:80:1). Dihydroxy acid methyl esters were analyzed on Silica Gel

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TABLE I
Analysis of Sunflower (*H. annuus*) Seed Oils

Identity (crop year)	HBr equiv.[a] 0 C	25 C	55 C	UV[b]
Russian variety				
Armavirec (1966)	0.2	....	0.3	0.0
Peredovik (1966)	0.1	....	0.3	0.0
Smena (1966)	0.2	....	0.4	0.0
VNIIMK (1966)	0.2	....	0.3	0.0
Introductions from[c]				
Uruguay (1956) PI 162454	....	7.1 (2.9)[d]	....	2.3 (1.2)
Turkey (1956) PI 173704	....	7.5 (3.3)	....	2.7 (....)
Turkey (1956) PI 182777	....	9.6 (4.2)	....	2.9 (....)
Seed increase crop samples[c]				
Turkey (Ames, Iowa; 1966) PI 173704	0.5	....	1.2	0.0
Turkey (Ames, Iowa; 1966) PI 181994	0.5	....	1.0	0.0
Canada (Ames, Iowa; 1966) PI 201812-10	0.6	....	1.0	0.0

[a] Expressed as per cent epoxyoleate.

[b] Expressed as per cent *cis-trans*-conjugated diene, $A_{max}^{cyclohexane}$ 234 mμ.

[c] A limited number of representative samples from each group have been listed. Only PI 162454 was included in the group of seeds used for our characterization work.

[d] Parentheses contain values found when seed was received in 1956; other values represent oil extracted recently from the same seed accession.

G impregnated with boric acid (4) and developed with ether-hexane-acetic acid (40:60:1). TLC of the esterified diimide reduction products was done on Silica Gel G impregnated with $AgNO_3$ (5) and developed with ether-hexane (40:60). The spots on all analytical TLC plates were visualized by charring the plates at 120 C after they had been sprayed with a saturated solution of chromium trioxide in 50% aqueous H_2SO_4. Preparative plates were sprayed with 2',7'-dichlorofluorescein and viewed under UV light; then the separated components were recovered by the usual procedure.

Analysis of methyl ester samples by gas-liquid chromatography (GLC) was done as described previously (6) and free acid samples were analyzed on a polyester column.

Description of Seeds and Oils

Seeds of four high-oil Russian sunflower varieties (Armavirec, Peredovik, Smena, and VNIIMK) that were grown in northern United States or southern Canada in 1966 were obtained from Cargill, Inc. Dehulled seeds of these varieties were ground and analyzed for oil content by overnight extraction with petroleum ether (bp 30-60 C) in Soxhlet extractors. Solvent was removed in vacuo at 40 C, and the oils were titrated with HBr according to the procedure of Harris et al. (7) which utilizes the Durbetaki reagent (8) at two different temperatures. The results of these analyses, as well as those for other oil samples, are given in Table I. Titration values of the magnitude shown for the Russian varieties mean little because, as stated previously (7), a few tenths of a per cent of interfering autoxidation products are usually found in most oils. UV spectra of these four oils showed no maximum at 234 mμ; therefore, no attempt was made to isolate HBr-reactive materials from these samples. The petroleum ether extract of the hulls from one of the four seed samples had no unusual components as determined by IR and UV analysis.

A large group of sunflower seed samples that originated in a number of different countries were furnished by the North Central Regional Plant Introduction Station, Ames, Iowa, in 1956. Our screening results (3) indicated that oil from these seeds (dehulled) contained 2-4% of HBr-reactive materials. For our current work we selected four of these seed samples from Uruguay, Turkey, and Yugoslavia [plant introduction (PI) numbers 162454, 170390, 175725 and 184049]. The oils were extracted from these four seed samples (including hulls) and combined. This combined oil sample gave a UV spectrum with a maximum at 234 mμ equivalent to 2.3% of conjugated *cis,trans* diene (9).

Oxygenated Methyl Ester Concentrate

Sunflower seed oil was dissolved in a 3:1 mixture of methanol and diethyl ether, and this solution was stirred 4 hr at room temperature with sodium methoxide catalyst. The methyl esters were recovered by the usual ether extraction method. Separation of these mixed methyl esters into an oxygenated ester fraction and an ordinary ester fraction was accomplished on a 2.2 × 30-cm column of Adsorbosil CAB (100-140 mesh, Applied Science Laboratories, Inc.). Esters were applied to the column in 1.5-2.0 g batches dissolved in benzene; the column was then eluted with benzene to remove methyl esters of ordinary fatty acids and finally with benzene-methanol (9:1) to remove oxygenated methyl esters.

The oxygenated methyl ester concentrate (2.70 g from 37.0 g of mixed methyl esters) had infrared bands indicating hydroxyl (2.75 μ), conjugated *cis,trans* (10.18 and 10.54 μ), and epoxide (11.8 and 12.1 μ) absorption. Analysis of this concentrate by TLC revealed

three major spots, R_f 0.33, 0.36 and 0.60. Another relatively strong spot at R_f 0.45 turned pink within 5 min after spraying with acid and was, presumably, due to triterpene alcohols. Under the same conditions, methyl dimorphecolate (methyl 9-hydroxy-*trans,trans*-10,12-octadecadienoate) (10) had R_f = 0.31; methyl vernolate (methyl *cis*-12,13-epoxy-*cis*-9-octadecenoate (11), R_f = 0.67; methyl *cis*-9,10-epoxystearate, R_f = 0.62; and methyl coriolate (methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate) (12), R_f = 0.35. GLC analysis of the oxygenated methyl ester concentrate revealed approximately 10% of epoxystearate, 48% of epoxyoctadecenoate and 40% of conjugated octadecatrienoates. The conjugated trienes are presumably formed during GLC by dehydration of the original hydroxydienes (13). Titration of the concentrate with HBr at room temperature indicated 81% of reactive materials calculated as methyl epoxyoleate.

Countercurrent Distribution

The oxygenated methyl ester concentrate (2.70 g) was dissolved in 120 ml of acetonitrile (lower phase) and 120 ml of hexane (upper phase), and the solution was placed in the first three tubes of a 200-tube countercurrent distribution (CCD) apparatus. Lower phase (40 ml) was added to each of the remaining tubes, and the instrument was set to deliver 40 ml of upper phase at each transfer. After the 200 fundamental transfers had been completed, fraction collection was begun and was continued until 800 transfers had been made (600 fractions collected).

A fast-moving fraction (0.360 g) in transfers 214-263 apparently contained the sterol and triterpene components as indicated by the pink coloration it gave after TLC when sprayed with H_2SO_4/CrO_3 reagent. An epoxide, shown to be saturated by TLC on $AgNO_3$-impregnated Silica Gel G, was recovered from transfers 322-400 (0.250 g), and the corresponding monoenoic epoxide was recovered from transfers 412-520 (0.812 g). At the beginning of the run, a 1-in. piece of plastic tubing was used for an emergency repair to the CCD apparatus and this repair resulted in contamination of the solvent system with a plasticizer. The saturated epoxide fraction was the only one seriously affected; the contaminant could not be removed by column chromatography or crystallization.

The solvent in the CCD instrument was removed from all tubes except those containing dienol material and was replaced with fresh solvent. This technique was used to remove minor constituents which could contaminate the dienols during subsequent operations. The instrument was then operated on automatic recycle until 2,800 transfers had been made. In this technique, upper phase is not collected but passes directly from the last tube back to the first tube of the instrument. At this stage, the two dienols were partially resolved, 0.092 g of dienol I (higher R_f on TLC) was recovered from the leading edge of the peak (tubes 75-110), and 0.156 g of dienol II was recovered from the trailing edge (tubes 180-199 and 0-10). The vacated tubes were refilled with solvent, and the recycling operation was continued until a total of 5,500 transfers were completed. Additional quantities of dienol I (0.201 g) and dienol II (0.170 g) were recovered from tubes 70-125 and from tubes 160-199 and 0-30, respectively. Tubes 31-69 contained about 0.200 g of unseparated dienol mixture. Overall recovery from the CCD operation was 2.61 g, including a small, but undetermined, amount of contaminant.

Characterization of Saturated Epoxide

Analysis of the saturated epoxide fraction by GLC and by HBr titration demonstrated that it was about 60% pure. The ORD spectrum (c = 3.9, hexane) showed this fraction was dextrorotatory, but this result has little significance since the optical properties of the contaminant are not known. Saponification of a portion of this mixture, followed by crystallization of the products from ethyl acetate, yielded 40% of an epoxy acid, mp 54.0-55.0 C. No depression of the melting point was observed on admixture with authentic *cis*-9,10-epoxystearic acid, mp 53.0-54.0 C. The epoxy acid was converted to a dihydroxy acid, mp 92.5-93.5 C, by acetolysis followed by saponification (11). An admixture with authentic DL-*threo*-9,10-dihydroxystearic acid had an mp of 92.0-93.0 C. Comparison of the methyl esters of the unknown diol with those of the standard diol by TLC on boric acid-impregnated Silica Gel G (4) also demonstrated that both had the *threo* configuration.

The crude dihydroxy acid preparation (before crystallization) was cleaved by the periodate-permanganate method of von Rudloff (14) to determine the location of the original epoxy group and to detect other positional isomers, if present. Analysis of the monobasic cleavage products as free acids by GLC showed 92% nonanoic acid and small amounts of unidentified components. The dibasic fragments, analyzed by GLC of their methyl esters, contained 96% of nonanedioate but no dodecanedioate.

Characterization of Unsaturated Epoxide

The unsaturated epoxide fraction had a purity of 96% by HBr titration; GLC confirmed this result. IR showed no *trans* absorption. Analysis by TLC revealed that this epoxide was probably methyl coronarate since it migrated slower than methyl vernolate on Silica Gel G (2). This epoxy fraction gave a plain positive ORD spectrum, $[\alpha]_D^{24.5°}$ + 3.9° (c = 11.7, hexane), in contrast with the spectrum of methyl vernolate (15). A portion of the unsaturated epoxide was first subjected to acetolysis and then saponified (11), yielding a dihydroxy acid, mp 60.5-61.0 C (from ethyl acetate at −18 C). Oxidative cleavage (14) of this dihydroxy acid before crystallization yielded only hexanoic and nonanedioic acids as shown by GLC. The unsaturated diol consumed 0.97 mole equivalents of hydrogen (Pt catalyst) to yield a saturated diol, mp 92.5-93.0 C. No depression of the melting point was observed on admixture with authentic DL-*threo*-9-10-dihydroxystearic acid, mp 92.0-93.0 C. The saturated diol methyl ester, mp 67.0-68.0 C, had the same R_f value by TLC on boric acid-impregnated Silica Gel G as the methyl ester of the known *threo*-dihydroxy acid. Periodate-permanganate cleavage of the saturated diol gave nonanoic and nonanedioic acids as the major products (94%) and no other products in amounts larger than 1.5%. If vernolic acid had occurred in the unsaturated epoxy fraction, hexanoic and dodecanedioic acids would have been found in the cleavage products; these fragments were not observed.

Characterization of Dienol I

Dienol I gave an IR spectrum that showed hydroxyl (2.73 μ) and conjugated *cis,trans* (10.16 and 10.53 μ) absorption. The UV spectrum revealed $\lambda_{max}^{cyclohexane}$ 234 mμ (ε 29, 100), which also is indicative of a *cis,trans*-conjugated diene, although the absorption is slightly stronger than reported previously (16). Dienol I gave a plain negative ORD curve, $[\alpha]_{400}^{25°}$ −0.55° ± 0.10° (c = 2.9, hexane). Powell et al. (16) reported essentially the same spectrum for the 13-hydroxyoctadecadienoate from *Xeranthemum annuum* seed oil.

Periodate-permanganate (14) cleavage of dienol I (0.016 g) yielded hexanoic and nonanedioic acids as indicated by GLC analysis of the cleavage products as free acids and as methyl esters.

When hydrogenated in absolute ethanol over Adams catalyst, dienol I (0.0182 g) consumed 1.9 mole equivalents of H_2 to yield a saturated methyl ester (0.0151 g), mp 51.0-52.0C (from petroleum ether). The saturated hydroxy ester (0.012 g) was treated with 0.245 g of chromium trioxide (17) in 2.0 ml of acetic acid and 0.2 ml of water for 1 hr at room temperature. Pentanoic, hexanoic, dodecanedioic and tridecanedioic acids comprised the major portion (80%) of the cleavage products according to GLC analysis. Small quantities of numerous degradation products were also evident.

Partial diimide reduction of dienol I (0.120 g) with potassium azodicarboxylate (18) as the diimide source (19) was accomplished by utilizing reactants in the exact proportions described by Powell et al. (16). Analysis of the partial reduction mixture (0.109 g) by TLC on $AgNO_3$-impregnated Silica Gel G revealed four spots with R_f values of 0.31, 0.38, 0.52 and 0.68. This mixture was separated by preparative TLC on $AgNO_3$-impregnated Silica Gel G (1 mm thick), but because of overloading, only three discrete fractions could be isolated. The first fraction (highest R_f) was a saturated ester (0.052 g) having an IR band at 2.76 μ (OH), mp 51.0-51.5 C [lit. mp of methyl hydroxyoctadecanoates; racemic 13-hydroxy, 53.3-53.5 C; 13D-hydroxy, 56-57 C; racemic 9-hydroxy, 50.3-50.6 C; 9D-hydroxy, 53.0-53.5 C (12,20,21)]. No depression in mp was observed on admixture with authentic (±) methyl 13-hydroxyoctadecanoate. The IR spectrum of the next fraction (0.010 g), R_f 0.52, showed hydroxyl (2.74 μ) and isolated *trans* (10.34 μ) absorption. Hexanoic and undecanedioic acids were the only products obtained by oxidative cleavage of this fraction as demonstrated by GLC analysis of the fragments as free acids and also as methyl esters. The last fraction (0.032 g) contained both a *cis*-monoene and starting material as shown by IR (*cis,trans*-conjugation) and by two spots, R_f 0.37 and 0.32, on TLC. Oxidative cleavage of this fraction and analysis of the products as described above, indicated that the fragments were hexanoic and nonanedioic acids and a γ-lactone (5.64 μ IR band). The equivalent chain lengths (22) of the lactone were 10.7 in an Apiezon L column (nonpolar) and 15.9 in an LAC-2 R-446 (polyester) column (16).

Characterization of Dienol II

The IR spectrum of II showed hydroxyl (2.74 μ) and conjugated *cis,trans* (10.16 and 10.54 μ) absorption, and the UV spectrum indicated *cis,trans*-conjugated diene absorption, $\lambda_{max}^{cyclohexane}$ 234 mμ (ε 27,400). Dienol II

gave a plain positive ORD spectrum, $[\alpha]_{400}^{25°}$ + 2.3° ± 0.6° (c = 3.0, hexane). Oxidative cleavage of II yielded only hexanoic and nonanedioic acids. The product resulting from hydrogenation of dienol II (2.1 mole equivalents of H_2 consumed) had mp 48.0-49.0 C and was cleaved to nonanoic, decanoic, octanedioic, and nonanedioic acids by chromium trioxide.

Analysis of the partial diimide reduction products (0.145 g) from dienol II by TLC on $AgNO_3$-impregnated Silica Gel G indicated three spots, R_f 0.30, 0.48, and 0.64. The partially reduced mixture was separated as before by preparative TLC. The first fraction, R_f 0.64, contained a saturated hydroxy ester (0.055 g) which melted at 47.5-48.5 C. This mp was undepressed by admixture with authentic (±) methyl 9-hydroxyoctadecanoate. Residual starting material from the reduction was found in the second fraction (0.047 g), R_f 0.48, along with a *trans*-hydroxymonoene as demonstrated by IR bands at 2.76 μ (OH), 10.34 μ (isolated *trans*), and 10.17 and 10.54 μ (conjugated *cis,trans*). The permanganate-periodate cleavage products from the second fraction were hexanoic and nonanedioic acids, derived from the starting dienol, and octanoic and nonanedioic acids, derived from the *trans*-monoene. Hexanoic acid was the only monobasic acid fragment from cleavage of the last fraction (0.027 g) R_f 0.30, which was the *cis*-hydroxymonoene. A dibasic acid fragment (after esterification) was not observed, but a fragment giving strong γ-lactone (5.64 μ) and ester carbonyl (5.77 μ) absorption in the IR was one of the products and was shown by GLC to have equivalent chain lengths of 17.0 on a nonpolar column and 26.0 on a polyester column (16).

DISCUSSION

The dihydroxy acids derived from the epoxy acids of sunflower oil were identified as *threo*-9,10-dihydroxystearic and *threo*-9,10-dihydroxy-*cis*-12-octadecenoic acids. These results demonstrate conclusively that the original epoxy acids are *cis*-9,10-epoxystearic and *cis*-9,10-epoxy-*cis*-12-octadecenoic acids. Previous work in our Laboratory (15) revealed that the 9,10-epoxy acids from *X. annuum* seed oil both have the (9*R*,10*S*) configuration (23). Since the corresponding epoxides isolated here are also both dextrorotatory, they too must have the (9*R*,10*S*) configuration. None of the *cis*-12,13-epoxy-*cis*-9-octadecenoic (vernolic) acid was detected, either by TLC or by cleavage fragments, although Morris et al. (2) suggested on the basis of TLC results that a small amount of this acid occurs in sunflower seed oil. Powell et al. (15) also found a mixture of vernolic and coronaric acids in *Xeranthemum* seed oil.

Characterization of the diimide partial reduction products from the two dienols definitely establishes the location and configuration of the double bonds. Dienol I is 13-hydroxy-*cis*-9,*trans*-11-octadecadienoic acid and dienol II is 9-hydroxy-*trans*-10,*cis*-12-octadecadienoic acid. Both of these isomeric fatty acids were previously isolated from *Xeranthemum* oil (16), and the 13-hydroxy isomer is a major constituent of *Coriaria nepalensis* seed oil (12). The optical rotations of these dienols, while measurable, are lower than those of optically pure compounds (12) and this characteristic suggests that they are extensively racemized. However, since both the dienols and the epoxy acids are optically active, we conclude that they are the results of biological processes and not autoxidation.

The γ-lactones resulting from cleavage of the *cis*-hydroxymonoenes give retention characteristics (on GLC) that differ slightly from those previously reported (16), but this difference is probably caused by variations in column parameters.

As shown in Table I, earlier work (1-3) indicated that oils of sunflower seeds from Uruguay and Turkey contained HBr-reactive materials and also gave UV maxima at 234 mμ. The same was true of oils from seeds introduced from Yugoslavia and Canada. Analysis of recently extracted oil from the same seed accessories revealed that both the HBr equivalent and the UV absorption had more than doubled since the first analysis. Since seed kernels only were used in the original work, whereas whole seeds were used in the current work, one might suspect that the hull extract was responsible for the variation in amounts of HBr-reactive materials. However, our most recent information indicates that the hull extract contains little, if any, HBr-reactive material. The apparent increase in HBr-reactive materials during storage of the seeds introduced in 1956 is most interesting and we are investigating this aspect of the problem in more detail.

Seed oils from recently grown Russian varieties contain very low percentages of HBr-reactive materials by titration at either 0 C or 55 C (Table I). To establish whether the much larger quantities of such components found in other sunflower lines were due to age of seed or to genetic makeup, several selected introductions from Turkey and Canada that pro-

vided seed oils of substantial HBr reactivity were regrown in 1966 at Ames, Iowa, to produce fresh seed. Results of HBr titration of the oils from the Ames seed increases provided equivocal results. Their HBr uptake of 0.5-1.2% was several times that of the Russian varieties grown in the same crop year, yet below that found initially in the older seed from Turkey and Uruguay. Thus, further work is required to assess the relative importance of storage time and genetic composition in governing the amount of oxygenated acids in sunflower seed oil.

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Constituents of the Cotton Bud: XII. The Carotenoids in Buds, Seeds and Other Tissue[1]

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ABSTRACT

Eleven carotenoid pigments were found in the bud, leaf, flower petal, seedling and seed of the cotton plant; nine were identified and quantitated. The most abundant carotenoids in the green tissue of the cotton plant were β-carotene and lutein. Carotene hydrocarbons comprised 12% of the total carotenoids in the seed, 15% in the 1-day-old flower petal, 51% in the bud and 57% in green leaves. Only 5, 8-epoxy carotenoids were found in the flower petals and only 5,6-epoxides in the other tissue but both were present in the seed. The colorless phytoene precursors to the carotenoids comprised from 20% to 38% of the total carotenoid pigment in the growing tissue of the plant.

INTRODUCTION

THE CHEMICAL NATURE of the cotton plant (*Gossypium hirsutum* L.) with respect to its plant-host specificity to the boll weevil (*Anthonomus grandis*, Boheman) is presently being investigated by studying the saponified carotenoid pigments of the cotton bud (square), cottonseed and other plant tissue. Only α- and β-carotenes had been observed previously in cotton plant tissue (1).

The isolation and identification of the carotenoids described here were accomplished by following standard procedures (2,3). However, the molecular sensitivity of the carotenoid pigments to mild acidity and basicity, and to air and enzymic oxidation and reduction, often precluded any conclusions concerning their chemical nature in the plant.

Also since a boll weevil feeding stimulant (4) has been found in the lipid extracts of cotton buds, flowers and cottonseed, and to a lesser extent in other plant parts, the individual purified carotenoids were bioassayed for feeding stimulant activity (4). The results were negative, but efforts are continuing to isolate and bioassay the pigments as they exist in the plant before any chemical treatment in isolation.

EXPERIMENTAL PROCEDURES

Plant Material

Kilogram quantities of cotton buds containing the sepal, calyx, petal and bracts were harvested from mature Deltapine Smoothleaf cotton grown in the greenhouse. Cotton seedlings were gathered from greenhouse sand beds 1 week after planting, and the 1-day-old flower petals were obtained from small field plots. The cottonseed was of the same variety and was produced commercially during 1966.

Pigment Extraction

Plant material was macerated in an electric blender with acetone, the acetone was removed by filtration and the residue was extracted until all the pigment was removed. Four extractions resulted in the removal of 99.9% of the carotenoids. Successive acetone extracts were combined, and an equal amount of water was added. The aqueous acetone was then treated with ether (peroxide free) until two layers formed; the ether layer was dried over anhydrous sodium sulfate and the solvent was evaporated in vacuo at 40 C. Saponification of the residue was accomplished by the method described by Pattee and Purcell (5). The time interval between extraction of the pigments from plant tissue and their separation into individual fractions by column chromatography was 12-15 hr.

Chromatography

Column chromatographic separation of the total pigment into hydrocarbon and xanthophyll fractions was carried out on 5% aqueous deactivated alumina (Baker) with increasing amounts of acetone in petroleum ether (b.p. 38-60 C). The carotene hydrocarbons were separated by using column chromatography on magnesium oxide:Celite® (1:1 w/w). The column was developed with increasing amounts of acetone in hexane (5).

The hydroxy and epoxy carotenoids were separated by thin-layer chromatography (TLC) employed with: vegetable oil coated kieselguhr (6) in methanol-acetone-water (80:16:12); calcium hydroxide-silica gel (6:1 w/w) (3)

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TABLE I
Concentration of Carotenoids in Cotton Plant Parts[a]

Plant Tissue	Carotenoids (mg/kg fresh wt)	Ratio of Hydrocarbons to Oxygenates
Buds	44.3	37:63
Leaves	338.4	30:70
Flowers	7.0	16:84
Seedlings	24.0	37:63
Seed	0.6	12:88
Air dried buds	20.2	56:43

[a] Quantitative estimation of total carotenoids calculated using $E^{1\%}_{1cm}$ of 2505 for β-cartone at λ max.

developed in petroleum ether-benzene (98:2); and silica gel G developed in dichloromethane-ethyl acetate (80:20) (3). TLC plates were prepared with Brinkmann apparatus on 20 × 20-cm glass, 250μ bed depth, and developed in ascending fashion in solvent chambers saturated with vapor of the solvent to a height of 10 cm from the start.

Identification

The individual pigments were identified on the basis of comparison of chromatographic movements and visible spectra with those of authentic samples. Spectral data were obtained with a Beckman DK-2A ratio recording spectrophotometer in 1.0-cm matched cells. Partition coefficients were obtained by the method of Subbarayan et al. (7). Also, the effects of hydrochloric acid in aqueous methanol on the absorption spectra were studied by the method of Curl and Bailey (8).

The following authentic samples were available for direct comparison: violaxanthin, flavoxanthin, lutein and auroxanthin from the flower of yellow pansy *(Viola tricolor* L.) (9); α-carotene (10); phytoene (11); phytofluene (12); lutein 5,6-epoxide (isolutein) (13); and synthetic β-carotene was obtained from Hoffmann-La Roche. The lack of authentic samples is noted in the discussion of results.

RESULTS AND DISCUSSION

The quantitative estimates of the total carotenoids in the various cotton plant parts after saponification is given in Table I. The total carotenoid concentration in milligram per kilogram of fresh weight for each tissue was calculated by using $E^{1\%}_{1cm}$ of 2505 for β-carotene. Also, the total carotenoids from air dried cotton buds was estimated, but separation and identification of the component carotenoids was not accomplished because extensive chemical change had taken place. About 50% of the oxygenated carotenoids were lost by air drying.

Carotenoids of Fresh Cotton Plant Tissue and Cottonseed

The hydrocarbons of fractions I and II obtained from the alumina column (Table II) were separated by rechromatography on MgO-Celite (1:1 w/w) with acetone in hexane (1%, 2%, 4%, and 5%). β-carotene and phytofluene were the principal constituents of the hydrocarbon fraction of green tissue; much smaller amounts of β-carotene were found in the flower. The amount of phytoene and phy-

TABLE II
Carotenoids Obtained From Fresh Green Tissue of the Cotton Plant and From Cottonseed

Fraction No.[a]	Absorption maxima, nm in hexane	Identity	$E^{1\%}_{1cm}$	λ Max.	Solvent	Partition coefficient in 75% MeOH-hexane	Total carotenoids (%) Buds	Leaf	Flower	Seedling	Seed
I	285,(299)	Phytoene	1250	285	Pet. Ether	—	18.5	19.4	22.7	33.9	0.9
	364,349,330	Phytofluene	1350	349	Pet. Ether	—	14.6	10.8	11.5	10.8	0.3
	472,444,418	α-Carotene	2850	444	Pet. Ether	—	2.7	2.2	—	0.4	3.2
	468,446,(424)	β-Carotene	2505	446	Pet. Ether	—	25.1	21.2	4.3	20.5	7.6
II	441,(421)	Unknown	2505[c]	441	Pet. Ether	—	—	—	—	—	1.1
	(466),(437),(410),325	Vitamin A₁[b]	2505[c]	325	Pet. Ether	62.38[d]	—	—	—	—	4.0
III	470,443,418	Lutein	2160	470	CS_2	10:90	25.5	25.2	11.4	20.4	40.7
	471,442	Isolutein	2148	471	Benzene	0:100[d]	1.8	—	—	2.4	10.7
IV	440,422,400	Flavoxanthin	2505	422	Ethanol	—	—	—	5.0	—	—
	464,444,422	Violaxanthin	2216	444	Benzene	68:32	7.0	13.1	—	5.3	6.0
V	425,402,380	Auroxanthin	1850	402	Ethanol	75:25	—	—	19.1	—	4.1
	471,444,420,335	Neoxanthin[b]	2270	444	Ethanol	92:8	4.8	8.1	—	6.3	8.3
VI	453,425,402,318	Neochrome[b]	2505[c]	425	Ethanol	90:10	—	—	26.0	—	13.1

[a] Total pigment chromatographed on 5% aqueous deactivated alumina with increasing amounts of acetone in petroleum ether (b.p. 30-60 C).

[b] Tentative identification made by comparing the physical and chemical properties with those in the literature (15).

[c] Quantitative estimation calculated on basis of $E^{1\%}_{1cm}$ of 2505 for β-carotene.

[d] Partition coefficient in 95% methanol-hexane.

tofluene remained almost constant in all fresh tissue; this large percentage corresponds more closely to the percentage observed in ripening fruit (14) than to that in green tissue (15), Perhaps (14) the accumulation of the colorless polyene, phytoene, results from selective inhibition of carotenoid synthesis, and this may explain the relatively high concentration of phytoene in flower petals where carotene biosynthesis is low. However, it does not account for the high concentration in the tissue of buds, leaves and seedlings where carotenoid biosynthesis is high. Thus, in the highly photosynthetic cotton plant tissue, the biosynthesis of the phytoene precursors and their conversion to carotenoids appears to have reached a steady state.

Fraction II was eluted from the MgO-Celite column with 5% acetone in hexane. TLC of this fraction on calcium hydroxide-silica gel (6:1 w/w) in light petroleum-benzene (98:2) gave bands at R_f 0.10 and 0.45. The faster moving fraction (R_f 0.45) had two weak absorption maxima at 441 nm and 421 nm, but insufficient quantities of the material were present for identification. The slower moving band (R_f 0.10) was tentatively identified as vitamin A_1 since it had the column, TLC, spectral and partitioning properties of authentic vitamin A_1.

Fractions III to VI were separated into their components by reverse phase TLC on vegetable oil coated kieselguhr in methanol-acetone-water (80:16:12) (6). The 5,6-epoxides and diepoxides were eluted from the alumina column ahead of their corresponding 5,8-isomers. Isolutein was converted into flavoxanthin, violaxanthin into auroxanthin, and neoxanthin into neochrome by adding a trace of alcoholic hydrochloric acid. The pigment then tentatively identified as neoxanthin had spectral and partition coefficient properties corresponding to neochrome (15). Fraction VI contained a single pigment that was tentatively identified as neochrome since it had spectral properties and a partition coefficient consistent with the values cited in the literature (7, 15).

Valadon and Mummery (16) investigated the carotenoids of flowers from 11 varieties and species of plants and found that they qualitatively corresponded to those found in the rest of the plant. In the present study, the cotton flower petal contained only 5,8-epoxides; the other plant parts contained the less stable 5,6-isomers, and the seed contained both 5,6- and 5,8-epoxides (Table II). The absence of 5,6-epoxides in the flowers may be caused by metabolic conversion of this isomer to the 5,8-ipoxide; however, such a conversion could also be attributed to the decreased pH during senescence. This latter seems particularly likely since only a small concentration of 5,8-epoxides are present in the green tissue of most plants (15).

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The Purification and Specificity of a Lipase From *Vernonia anthelmintica* Seed[1,2]

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ABSTRACT

Acetone powders prepared from *Vernonia anthelmintica* seed catalyzed the release of 6.4 to 9.6 μ-moles of free fatty acids per milligram of protein when blended with olive oil and phosphate buffer and shaken for 20 min at 43 C. A 20 fold purification was achieved by differential centrifugation of an ammonium hydroxide extract of the acetone powder. Results from Sephadex G-200 chromatography and polyacrylamide gel electrophoresis suggested that the lipase activity was associated with a molecule of molecular weight greater than 200,000. Free fatty acids, 1,2- and 1,3-diglycerides, monoglycerides and glycerol were found in the digestion products. With most substrates the 1,2- to 1,3-diglyceride ratio was approximately 2:1 and monoglycerides tended to accumulate. Analysis of the digestion products from synthetic triglycerides of known structure indicated that both primary and secondary ester positions of the triglyceride molecule were hydrolyzed and that considerable isomerization of 1,2-diglyceride to 1,3-diglyceride occurred. The monoglyceride was consistently lower than the 1,2-diglyceride and in the majority of cases also lower than the 1,3-diglyceride in the fatty acid originally present in the 2 position of the triglyceride. No fatty acid preference was observed.

INTRODUCTION

INTEREST IN *Vernonia anthelmintica* seed has been stimulated by the identification of the principal fatty acid in the seed oil as vernolic acid; *cis*-12, 13-epoxy-*cis*-9-octadecenoic acid. This occurs primarily as the simple triglyceride trivernolin, which can be recovered in good yield by wet-flaking the seed in the cold with petroleum ether and one crystallization at –20 C of the petroleum ether extract (1). Krewson et al. (2) found that grinding and incubation of the seed prior to extraction led to the formation of free fatty acid and 1,3-divernolin, suggesting the presence of a lipase specific for the 2 position of the triglyceride. Such an enzyme would be of value in studies of triglyceride structure.

The purpose of this investigation was to isolate the lipase in *V. anthelmintica* seed and to test positional specificity with synthetic substrates.

METHODS

Five batches of *Vernonia anthelmintica* seed were obtained (C. F. Krewson, USDA Eastern Regional Research Laboratory, Philadelphia, Pa.). The seed was stored at –2C. The racemic triglycerides employed in specificity studies were synthesized in this laboratory (3). Purity of the glycerides was ascertained by GLC, TLC, and lipolysis with pancreatic lipase. The commercial olive oil used in routine assays was purified by passage through neutral alumina (4). The solvents referred to as pentane and hexane were Skellysolve F and Skellysolve B, redistilled before use.

Preparation of Acetone Powder

In the procedure most commonly employed, 10- to 50-g portions of ground seed were stirred in a beaker with 3 to 4 volumes of acetone. The acetone was filtered with suction through Whatman No. 1 paper. A light colored, low density solid which decanted with the acetone was collected on the filter, while considerable dark, fibrous residue remained in the beaker. On restirring the contents of the beaker with three additional portions of acetone, decreasing amounts of the lighter solid could be decanted. The tan precipitate was broken up with a spatula and washed by stirring and decantation with several successive portions of acetone, leaving the heavier particles, generally darker in appearance, behind. The acetone powder collected on the filter was air dried and stored in a refrigerator.

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Digestion Conditions

The acetone powder or enzyme solution to be assayed, sufficient 0.1 M phosphate buffer (pH 7.5) to make a total volume of 15 ml, and 0.2 ml of olive oil were added to a small Waring Blendor. After blending for 45 sec, the emulsion was immediately decanted into a 50-ml Erlenmeyer flask and agitated in a water bath at 43 C for 19 min. At the end of the digestion period the reaction was stopped by the addition of 0.5 ml of 20% sulfuric acid and the contents of the flask were decanted into a porcelain casserole and extracted by the silica gel procedure (5) using 250 ml of either ethyl ether or chloroform-methanol (90:10), as solvent. An aliquot representing one fourth or one half of the sample was titrated to the thymol blue end point with 0.05 N alcoholic potassium hydroxide to measure free fatty acids. The remainder was evaporated to dryness at 40 C to 50 C on a flash evaporator prior to TLC.

High-melting solid substrates were dissolved in small quantities of warm pentane and blended cautiously with buffer and enzyme until the substrate was distributed throughout the solution. The mixture was then emulsified by blending at high speed and processed in the usual manner. Controls were run by using acetone powders from seed which was autoclaved prior to grinding, by boiling the enzyme preparation prior to digestion, or by omitting enzyme from the digestion mixture.

Digestion Products

The products of all digestions were monitored by TLC. In specificity trials, the digestion products were isolated by preparative TLC, esterified by refluxing with 2% sulfuric acid in methanol, and estimated by GLC using DEGS and SE-30 columns in an Aerograph Model 600D gas chromatograph equipped with a flame ionization detector. All operations subsequent to placing the material on the thin-layer plate were performed quantitatively, so that in addition to determining the fatty acid composition of each glyceride type, the relative amounts of glyceride in the various TLC bands could be compared.

Thin-Layer Chromatography

For qualitative work glass plates were coated with 0.25 mm layers of silica gel G (Merck), while 0.5-mm layers were employed in preparative TLC. Two solvent systems were used: hexane-ethyl ether-acetic acid (60:40:2), or chloroform-acetone-acetic acid (95:5:0.4). Lipid components on the plates were visualized with iodine fumes, bromthymol blue or 2,7-dichlorofluorescein.

Protein

The modification of the Lowry procedure described by Litwack (6) was followed to measure protein N in enzyme solutions.

RESULTS AND DISCUSSION

Preliminary Observations

Very little lipolytic activity was observed when an aqueous extract or suspensions of acetone powder were shaken with a pre-emulsified olive oil suspension as is frequently done in pancreatic lipase digestions. When the acetone powder was blended with distilled water in the presence of olive oil and then shaken, the rate of lipolysis was greatly increased. Blending with 0.1 M phosphate buffer at pH 7 resulted in an additional fourfold increase and provided a simple system in which to assess enzyme activity.

In digestions using phosphate buffer, maximum free fatty acid release occurred between pH 7.5 and 8.0. No differences in activity were observed when the incubation temperature was varied from 37 C to 43 C. Above 48 C activity declined, although the enzyme still demonstrated some activity at 80 C. Of the additives commonly employed in pancreatic lipase digestions, only bile salts increased free fatty acid release, and the effect was small.

Individual acetone powder preparations varied in activity. The nitrogen contents and activities of several powders are shown in Table I. Of five batches of seed tested, only the Iowa, a poor quality seed, differed appreciably from the others. Nitrogen contents of the most active fractions varied from 6.93% to 9.30%, while the specific activities (μ moles free fatty acid/min/mg protein) varied from 0.20 to 0.44. Some data on successive decantations are shown to illustrate that the most readily decantable material was most active and also highest in specific activity on a protein basis.

In physical appearance and lipolytic activity acetone powders prepared by blending ground seed (vs. stirring) and powders prepared at –2 C (vs. room temperature) resembled those shown in Table I. Refrigerated powders were quite stable. Several samples were held for more than a year without losing activity.

Purification

Phosphate buffer extracts of the acetone powder were higher in specific activity (1.1 to 1.5) than the acetone powder, but yields were low. Ammonium hydroxide extracted over twice as much activity as phosphate buffer although specific activity was only slightly higher

than that of the starting material. Also, ammonium hydroxide solutions were more stable than water, salt, or phosphate buffer solutions containing enzyme. Irreversible inactivation of proteolytic enzymes is postulated and a preliminary treatment with ammonium hydroxide was successfully employed in subsequent purification trials.

A number of extracts were chromatographed on 300 × 2.5 (ID) mm Sephadex G-200 columns, using dilute phosphate buffer (pH 7.0) or 0.01 M ammonium hydroxide solutions as eluants. With crude extracts two protein peaks were observed and lipase activity was associated with the forefront of the largest peak, which eluted immediately after one void volume. An eightfold increase in specific activity over the crude extract was achieved for the fractions containing the most activity.

When samples previously purified by the centrifugation procedure described below were chromatographed on Sephadex G-200, lipase activity coincided with a single rapidly moving peak and only slight increases in specific activity were obtained. These elution volumes indicated that lipolytic activity is associated with a molecule of molecular weight greater than 200,000.

TABLE I

Comparison of Lipolytic Activity of Acetone Powders Prepared From Different Batches of *Vernonia anthelmintica* Seed.

Seed source	Acetone powder description	Protein[a] %N	Free fatty acids μ moles	Specific[b] activity
Pakistan	lightest material	8.74	197	0.33
Pakistan	lightest material	9.30	205	0.30
Iowa	1st & 2nd extraction	5.51	48	0.20
Nebraska	1st extraction	7.36	160	0.37
	2nd extraction	7.41	151	0.35
	3rd & 4th extraction	5.18	56	0.26
Oklahoma	1st & 2nd extraction	6.93	167	0.44
	3rd extraction	6.60	114	0.33
	4th extraction	5.12	57	0.27

[a] By micro-Kjeldahl.

[b] μ moles free fatty acids/min/mg protein.

A number of enzyme preparations were analyzed by polyacrylamide slab or polyacrylamide disc electrophoresis. Five or six bands could be detected in crude extracts of the acetone powder. It was not possible to relate lipolytic activity to any band. However, the only visible stain in high specific activity (low protein) samples was stained material in the sample slot, another indication that the lipase is associated with a large molecule.

TABLE II

Products From the Lipolysis of Synthetic Triglycerides by Purified *Vernonia anthelmintica* Seed Lipase

Substrate[a]		Residual Triglyceride	Diglycerides 1,2	Diglycerides 1,3	Mono-glyceride	Free fatty acids	Glycerol[b]
POP Fatty acid	16:0	66	54	79	91	70	
composition (M%)	18:1	34	46	21	9	30	
Relative moles of glyceride[c]		2086	324	160	430	1600	86
Diglycerides	16:0-18:1		298	67			
	16:0-16:0		26	93			
OPO Fatty acid	16:0	33	47	26	16	36	
composition (M%)	18:1	67	53	74	84	64	
Relative moles of glyceride[c]		1056	320	171	509	1375	–44
Diglycerides	16:0-18:1		301	89			
	18:1-18:1		19	82			
OSS Fatty acid	18:0	64	80	66	72	62	
composition (M%)	18:1	36	20	34	28	38	
Relative moles of glyceride[c]		2566	389	216	323	1462	72
Diglycerides	18:0-18:1		156	147			
	18:0-18:0		233	69			
PSS Fatty acid	16:0	34	21	31	32	41	
composition (M%)	18:0	66	79	69	68	59	
Relative moles of glyceride[c]		1635	262	89	614	1683	35
Diglycerides	16:0-18:0		110	55			
	18:0-18:0		152	34			

[a] POP = *rac* glycerol-2-oleate-1,3-dipalmitate; OPO = *rac* glycerol-2-palmitate-1,3-dioleate; OSS = *rac* glycerol-1-oleate-2,3-distearate; PSS = *rac* glycerol-1-palmitate-2,3-distearate

[b] Determined by difference; 1/3 (free fatty acids—diglyceride—2 × monoglyceride)

[c] On basis of 1000 moles of triglyceride hydrolyzed; diglyceride + monoglyceride + glycerol = 1000

Differential Centrifugation

A 10- to 20-fold increase in specific activity was obtained by the centrifugation procedure illustrated in Fig. 1. In the preparation shown, the final pellet, representing 1/100 of the initial protein, had a specific activity about 30 times that of the initial starting material. The digestions described in the next section were performed with enzyme purified in this manner.

Specificity

The products resulting from lipolysis of two symmetrical and two unsymmetrical synthetic triglycerides with *Vernonia* seed lipase are shown in Table II. As described under Methods, the fatty acid composition and relative moles of glyceride were obtained from the fatty acids determined by GLC of the lipolysis products separated by TLC. The diglyceride values were calculated from the above data, assuming no fatty acid interchange between molecules.

Several points may be noted with respect to the POP (*rac* glycerol-2-oleate-1,3-dipalmitate) and OPO (*rac*-glycerol-2-palmitate-1,3-dioleate) digestions: 1) The similarity of products suggests that the enzyme does not differentiate between 16:0 and 18:1. 2) Initial hydrolysis must occur at both primary and secondary positions of the triglyceride to account for the amounts of mixed 1,2- and simple 1,3-diglycerides found. 3) The ratio of 1,2- to 1,3-diglyceride is approximately 2:1. 4) The composition of the 1,2-diglyceride shows a slight excess of outside position acid, while the 1,3-diglyceride contain considerable amounts of the 2-position acid. Isomerization of 1,2-diglyceride to the 1,3-isomer, therefore, would appear to be an important aspect of the lipolysis sequence. Incubation of 1,2-dipalmitin caused an isomerization of 10.9% in the control and 23.1% with the enzyme present. 5) Monoglycerides tend to accumulate to 1,3-dipalmitin. In this type of digestion the glycerol concentration never approached that of the monoglyceride, while diglyceride was readily hydrolyzed. 6) The monoglyceride contains less of the acid occupying the 2 position of the original triglyceride than either diglyceride.

Although compatible with the symmetrical triglyceride data, the results with unsymmetrical triglyceride substrates are generally inconclusive, since the mixed 1,3-diglyceride could arise by either attack on the 2 position of the triglyceride or by isomerization of one of the possible 1,2-diglycerides. There is evidence for isomerization of 1,2-distearin with both PSS and OSS (*rac* glycerol 1-palmitate 2,3-distearate and *rac* glycerol-1-oleate 2,3-distearate).

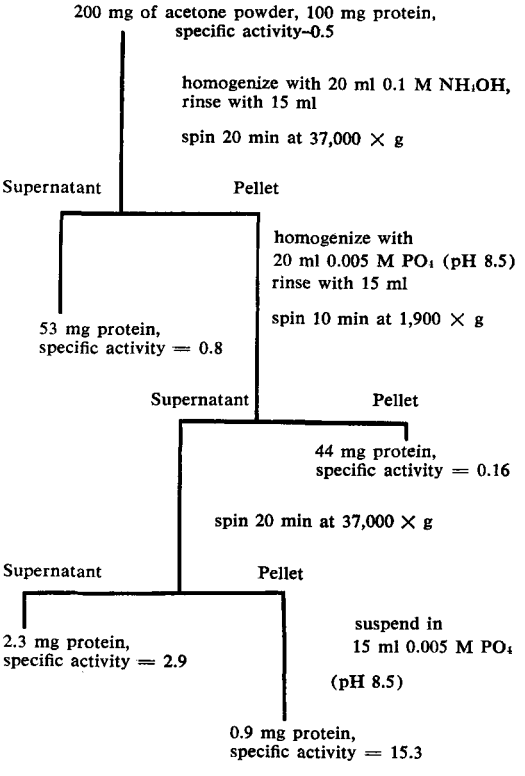

FIG. 1. Purification of Vernonia seed lipase by differential centrifugation. All operations carried out at 2 C. Specific activity = μ moles free fatty acid/min/mg protein.

With all substrates tested, the monoglyceride composition more closely resembled the composition of the 1,3-diglyceride than that of the 1,2-diglyceride, and in the majority of digestions it was lower than either diglyceride in the acid originally present in the 2 position. One might postulate that monoglycerides were formed by hydrolysis of 1,2-diglycerides with a preferential attack on the 2 position. However, random hydrolysis of 1,3-diglyceride with a slight preference for the acid transferred during isomerization or an intermolecular preference for one particular diglyceride species are equally plausible explanations. Therefore, the only firm conclusions which may be drawn are that hydrolysis of triglyceride occurs at both the 1 (3) and 2 positions, that isomerization of 1,2-diglyceride to 1,3-diglyceride occurs, and that the monoglyceride formed contains little of the acid originally present in the 2 position of the triglyceride.

The four digestions for which results are given were carried out with purified enzyme;

other digestions with acetone powders gave similar patterns of digestion products. During preliminary trials many enzyme preparations were assayed and monitored by TLC; in only one instance, which could not be reproduced, did visual observations of the digestions products on TLC disclose a predominance of 1,3-diglyceride over 1,2-diglyceride. The enzyme preparation does not, therefore, appear to be primarily specific for the 2 position of the triglyceride molecule.

ACKNOWLEDGMENT

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Glyceryl Ethers in Insects: Identification of Alkyl and Alk-1-enyl Glyceryl Ether Phospholipids[1]

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ABSTRACT

Alkyl and alk-1-enyl glyceryl ethers have been identified in the phospholipids of three insect species, the American cockroach (*Periplaneta americana*), the tobacco budworm (*Heliothis virescens*), and the boll weevil (*Anthonomus grandis*). Glyceryl ethers were not detected in the neutral lipids. The ethers were found in the phospholipid fraction of whole insects and in isolated fat body tissue. The ether content varied among the three insect species, and fluctuated during various developmental stages. Gas liquid chromatographic analysis of the alkyl glyceryl ethers and aldehydes derived from the alk-1-enyl glyceryl ethers of the cockroach and budworm showed striking differences in chain length. However, the hydrocarbon side-chain of the two ether fractions were similar in length for each species. Preliminary evidence indicates that 1-^{14}C-acetate can be incorporated into alkyl ethers but not into alk-1-enyl ethers of *Heliothis* pupae.

INTRODUCTION

SEVERAL FACTORS CONTRIBUTE to the scarcity of information about glyceryl ethers of insects, but the major one is that glyceryl ethers comprise but a small percentage of the phospholipids of most animals, and are usually not found in the neutral lipids. A few studies have been published which mentioned the occurrence of ether-containing lipids in insects. However, these generally dealt with the phospholipids, and merely noted the presence of ether-containing lipids, primarily plasmalogen, as minor components.

Recently published reviews of the literature reveal no extensive studies of glyceryl ethers in insects (1-3). Hack et al. (4) described the phospholipid patterns of about 15 species representing five insect orders. Ethanolamine plasmalogen was detected in American cockroach nymphs after the first instar, and its concentration increased until the adult stage was reached. Similar changes in plasmalogens were noted in fruit fly and mealworm larvae. Small quantities of phosphatidylcholine plasmalogen were found in dragonfly nymphs (5). Siakotos and Zoller (6) reported the isolation of plasmalogen from tissues of the American cockroach. Later Hodgson (7) showed that plasmalogens were a minor component of the tobacco hornworm moth, and very small amounts were also found in the immature stages. Kamienski et al. (8) reported that ethanolamine phosphatides occurred primarily in the form of plasmalogen in the mealworm. Crone and Bridges (9) were able to identify both plasmalogens (1.3%) and a glycerol ether phosphatide (1%) as components of phospholipids of the house fly. Plasmalogens of both phosphatidylcholine and phosphatidylethanolamine were also known to occur in silkworms (10), mosquitoes (11) and other insects. However, no structural characterizations were attempted in any of these studies.

The identification and partial characterization of alkyl and alk-1-enyl glyceryl ethers[4] in three insect species using the recently developed method of Wood and Snyder (12) are reported in this paper.

MATERIALS AND METHODS

Insects

Periplaneta americana (L.) was reared on Moorman's Mintrate chick mash and water ad lib. Adult males and females were collected from the stock colony and frozen before lipid extraction and analysis. Fat body tissue was dissected from adult females on crushed ice under CO_2-gas anaesthesia.

Anthonomus grandis Boheman, the boll weevil, was reared individually from egg to adult in vials containing a semidefined diet by procedures previously described (13), except that the diet was not autoclaved. The larvae

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[4] In this paper, alkyl ethers refer to those compounds that do not have unsaturation in the 1 position of the hydrocarbon chain attached through an ether linkage to glycerol, although unsaturation may occur elsewhere in the chain. Alk-1-enyl ethers, commonly referred to as vinyl ethers, have a double bond in the 1 position of the hydrocarbon chain, and also may be unsaturated elsewhere in the hydrocarbon chain.

and pupae were taken from the rearing vials, washed with water to remove uneaten diet and frass, blotted dry, and stored frozen at –20 C before lipid extraction. Late-stage pupae were taken from the diet vials and allowed to transform into adults on moistened filter paper in Petri dishes.

Heliothis virescens (F.), the tobacco budworm, was reared from the first instar larval stage to the pupal stage singly in plastic containers on a semidefined diet as described by Berger (14). Larvae and pupae were removed from the diet, washed and blotted dry. The larvae were not fed for 8 hr to minimize gut content.

Late-stage pupae were transferred to fine sawdust in screen covered paper cartons until emergence of the moths. The moths were not fed, but were collected and frozen within 24 hr of emergence.

To check for incorporation of ^{14}C from acetate into the glyceryl ethers, a group of 6-day-old *H. virescens* pupae was anaesthetized in CO_2 gas for about 1 min and each was injected with an aqueous solution of sodium 1-^{14}C-acetate. Each pupa received exactly 1 μliter containing 1 μCi of ^{14}C. The pupae were kept at room temperature for 4 hr and killed by freezing.

Chemicals and Standards

The preparation and properties of the isomeric glyceryl monoethers used as standards have been described (15). Other standards for thin-layer chromatography (TLC) were obtained from the Hormel Institute (Austin, Minnesota). Silicic acid for separating neutral and phospholipids (325 mesh) was a product of Bio-Rad Laboratories (Richmond, California). All other chemicals were reagent grade and were used without further purification. All solvents except diethyl ether were glass distilled and were obtained from Burdick and Jackson Laboratories, Inc. (Muskegon, Michigan). Diethyl ether (Mallinckrodt, St. Louis, Missouri) was used fresh from 1-lb cans opened daily.

Lipid Isolation and Subfractionation

Whole larvae, pupae, adults, dissected fat body and samples of the diets of the three insects were weighed, and were homogenized in sufficient ice cold water to form a thick slurry for about 1 min. This was done to pulverize the tissues and cuticle as finely as possible before lipid extraction. The homogenates were immediately frozen in lyophilization vessels in liquid nitrogen. After lyophilization, the dry sample weight was recorded, and a total lipid extract[5] of the dry material was prepared with chloroform-methanol (2:1) by the Folch technique (16). The extract was dialyzed overnight against double distilled water. The resulting chloroform phase was washed with water, reduced to dryness under vacuum, and weighed to obtain total lipid weight. The lipids were then made up to 40 mg/ml in chloroform solution. Duplicate aliquots were separated into neutral and phospholipids by silicic acid chromatography (12) and the percentage of each fraction was recorded. A known quantity (3-5 mg) of each fraction was then reduced with $LiAlH_4$ and quantitatively analyzed for alkyl and alk-1-enyl glyceryl ethers according to the procedure of Wood and Snyder (12).

Thin-Layer Chromatography of Glyceryl Ethers

Chromatoplates were prepared and developed as described previously (12). Samples were analyzed in duplicate along with three aliquots of the standard, 1-octadecoxy-2, 3-propanediol. Quantitative photodensitometry was employed according to the procedure of Privett et al. (17). A few milligrams of each of the phospholipid alkyl and alk-1-enyl glyceryl ethers were isolated by TLC for analysis by gas-liquid chromatography (GLC). The developed TLC plates were sprayed lightly with 0.2% rhodamine 6G in 95% ethanol, and the two glyceryl ether bands, outlined under ultraviolet light, were scraped into fritted glass funnels. The ethers were eluted from the adsorbent with chloroform-methanol (2:1, v/v).

For ^{14}C-acetate incorporation studies, the phospholipids were isolated and their glyceryl ethers were separated by TLC as detailed above. Preliminary area scans of radioactivity in each major lipid fraction were performed by visualizing the spots in iodine vapor, scraping each spot into a scintillation vial, and measuring its radioactivity in a liquid scintillation counter. Zonal ^{14}C profiles were then made on a second set of TLC plates by employing procedures described by Snyder (18). Radioactivity in each zone was determined by the automated system of Snyder and Smith (19).

Derivative Preparation

The 1-alkyl glyceryl ethers were converted to the isopropylidene derivatives (20) and an-

[5] The tissues were finely ground in water and shell frozen in liquid nitrogen. The dry tissue was extremely light and fluffy and occupied a rather large volume in the lyophilization vessel. It was extracted in chloroform-methanol with constant shaking for about 1 hr., and the extraction repeated twice more with fresh chloroform-methanol. The weighed amount of lipid obtained was the same as obtained when extracting fresh tissue in other experimental work with the same species.

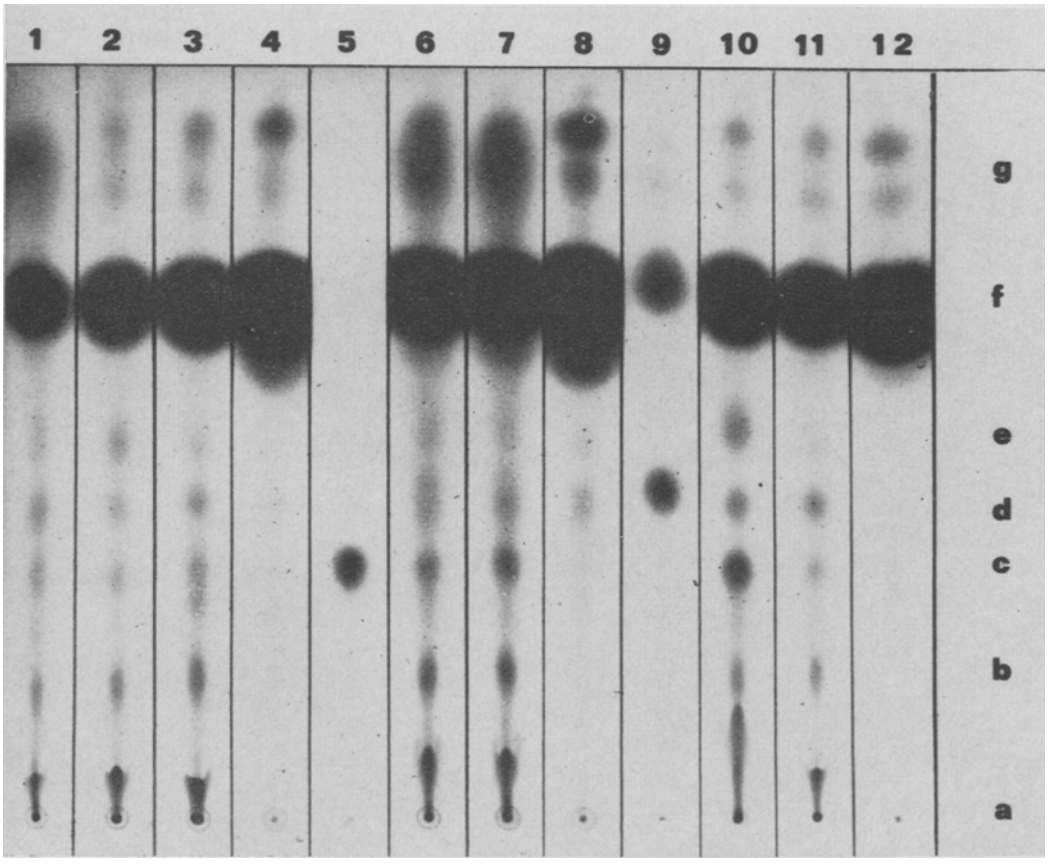

FIG. 1. TLC separation of $LiAlH_4$-reduced neutral and phospholipids of three insect species. Lane 1-3, phospholipids of mature larvae, pupae and adults of *Heliothis virescens*. Lane 4 neutral lipids of *H. virescens*. Lane 6 and 7 phospholipids of pupae and adults of *Anthonomus grandis*, lane 8, neutral lipids of pupal *A. gradis*. Lane 10 and 11, phospholipids of adult female *Periplaneta americana*, and isolated fat body of female *P. americana*, lane 12, neutral lipids of fat body. Standards are in lane 5, alkyl glyceryl ether, and lane 9 alk-1-enyl glyceryl ether. Areas identified by letters are hydrogenolysis products of sphingomyelin, a and b; alkyl glyceryl ethers, c; alk-1-enyl glyceryl ethers, d; fatty alcohols, f; unidentified components, e and g. Origin at a, solvent front at line beneath lane numbers.

alyzed by TLC and GLC before and after catalytic hydrogenation over platinum oxide. The alk-1-enyl glyceryl ethers were hydrolyzed in 90% acetic acid containing 0.5 N HCl and 0.0015 N $HgCl_2$ according to the procedure of Pietruszko and Gray (21). The resulting aldehydes were isolated by TLC and their percentage composition was determined by GLC.

Gas-Liquid Chromatography

An Aerograph Model 600D gas chromatograph equipped with a flame ionization detector was used for the analyses. The derivatives were separated on a 5 ft x ⅛ in.-glass column packed with 15% ethylene glycol succinate methyl silicone polymer (EGSS-X) coated on 100-120 mesh Gas-Chrom P (Applied Science Laboratories, State College, Pennsylvania). The helium carrier gas flow rate was 20-30 ml/min. Air and hydrogen were adjusted to give maximum detector sensitivity. Aldehydes and isopropylidene derivatives were analyzed at column temperatures of 160 and 210 C, respectively. The injector temperature was 265 C.

RESULTS AND DISCUSSION

Identification of Glyceryl Ethers by TLC

A typical TLC plate of reduced neutral and phospholipid samples for each insect species is shown in Fig. 1. Both the alkyl glyceryl

TABLE I

Quantitative Densitometric Analysis of the Alk-1-enyl and Alkyl Glyceryl Ethers in Three Insect Species

Lipid Source	Lipid Type[a]	% NL or PL	% Glyceryl Ether[b]		% Total Ether	Corrected Lipid Ether %[c]
			Alk-1-enyl	Alkyl		
Tobacco Budworm *(Heliothis virescens)*						
Penultimate instar larva	NL	92.7	0	0	0	0
	PL	7.3	4.90	0.52	5.42	16.26
Last instar larva	NL	92.3	0	T	T	T
	PL	7.7	0.74	0.06	0.80	2.40
Pupa, 3 days old	NL	93.8	0	T	T	T
	PL	6.2	0.07	0.11	0.18	0.54
Pupa, 8 days old	NL	96.4	0	0	0	0
	PL	3.6	1.91	0.35	2.26	6.78
Adult, unfed	NL	94.7	0	0	0	0
	PL	5.3	0.17	0.13	0.30	0.90
Boll Weevil *(Anthonomus grandis)*						
Pupa	NL	77.9	T	0	T	T
	PL	22.1	0.12	0.20	0.32	0.96
Adult, unfed	NL	71.8	0	T	T	T
	PL	28.2	0.31	1.85	2.16	6.48
American Cockroach *(Periplaneta americana)*						
Male, adults	NL	64.5	0	T	—	—
	PL	35.5	0.56	0.96	1.52	4.56
Female, adults	NL	73.8	0	T	—	—
	PL	26.2	0.48	0.97	1.45	4.35
Female, fat body	NL	97.8	0	0	0	0
	PL	2.2	1.71	1.93	3.64	10.92

[a] NL—neutral lipid; PL—phospholipid; T—trace of lipid detected.

[b] Expressed as percentage of total lipids in fraction.

[c] Multiplication of the total ether percentage by 3 is necessary because the glyceryl ethers determined in the free form (nonesterified) represents approximately one third of the total weight of the molecule from which they were derived.

ethers (area c) and the alk-1-enyl glyceryl ethers (area d) are present in each species. The ethers are confined almost exclusively to the phospholipid fraction. Occasionally a trace of an interfering compound ran in the area of alk-1-enyl ethers in the boll weevil neutral lipids (lane 8), but its chromatographic behavior indicated it was not a glyceryl ether.

No glyceryl ethers appear in the two neutral lipid samples of tobacco budworm or cockroach (lanes 4 and 12, respectively). Other than noting their possible trace occurrence, no further characterization of the ethers in the neutral lipid fraction was made. Phospholipid glyceryl ethers were present during all stages of metamorphic development examined in the tobacco budworm (lanes 1-3), boll weevil (lanes 6-7), and adults of the American cockroach (lanes 10-11). Glyceryl ethers were also found in the isolated fat body tissue of the cockroach in addition to the whole insect (lane 11).

Hydrogenolysis products other than glyceryl ethers can also be seen in Fig. 1. Hydrogenolysis products of sphingomyelin are among the more polar materials remaining in regions a and b. The large spot at area f consists of fatty alcohols, and in the neutral lipid fractions (lanes 4, 8, and 12) sterols are also present. Areas e and g have not been identified, but the latter probably consists of hydrocarbons. The unidentified regions are not unique to insect lipids since they have previously been found in approximately the same amounts in a number of rat tissues (12).

Quantitative Analysis

The percentage composition of neutral and phospholipids, and the results of the quantitative analyses of the alkyl and alk-1-enyl glyceryl ethers are given in Table I. The values for the ethers are presented along with total ether percentage and corrected lipid ether percentage for each of the species. The corrected lipid ether percentages were obtained by multiplying the total ether percentage by three. This was necessary since the glyceryl ethers as determined in the nonesterified form represent approximately one third of the total weight of the molecule from which they were derived.

The data in Table I clearly indicate that the glyceryl ether content in the phospholipid fraction varies among each species, and during successive developmental stages. In *Heliothis,*

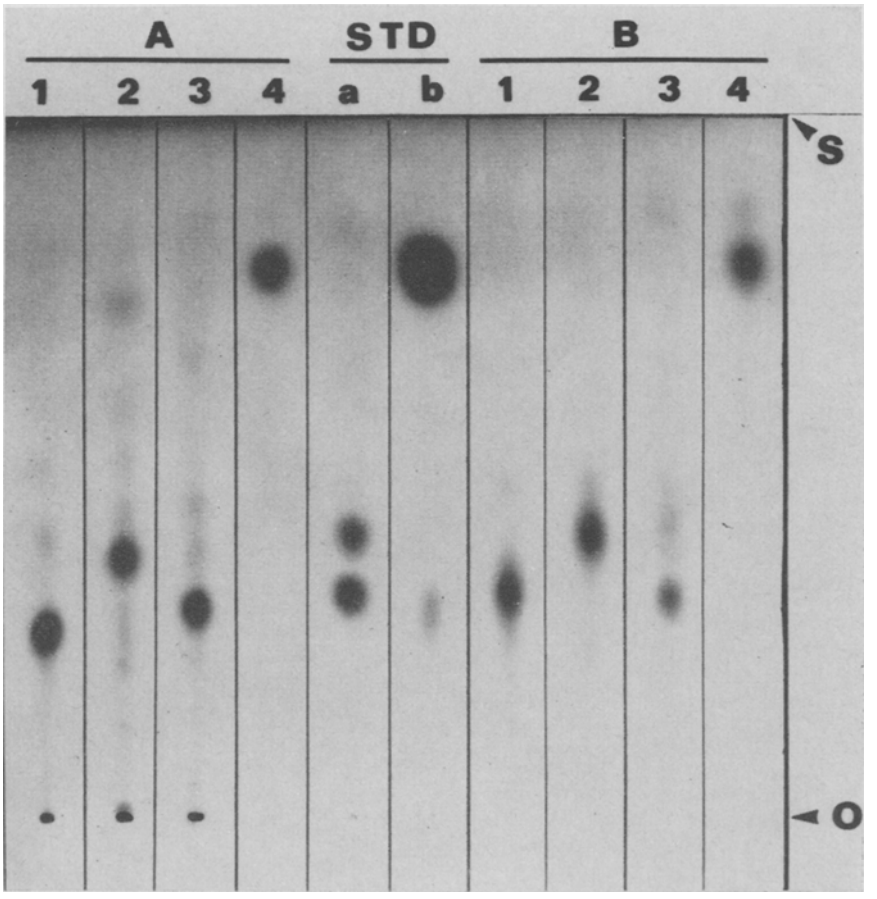

FIG. 2. TLC behavior of glyceryl ethers and derivatives of *Periplaneta americana* (Group A), and *Heliothis virescens* (Group B). The numbered lanes are 1, alkyl glyceryl ethers; 2, alk-1-enyl glyceryl ethers; 3, alk-1-enyl glyceryl ethers of lane 2 after hydrogenation; 4, isopropylidine derivatives of the alkyl glyceryl ethers of lane 1. The derivatives were first purified by TLC before being separated in above chromatogram. Standards are a, alkyl glyceryl ether (lower spot) and alk-1-enyl glyceryl ether (upper spot); b, isopropylidine. O, origin; S, solvent front.

alk-1-enyl ethers drop drastically from the penultimate to the last larval instar and again at pupal transformation, but show a buildup later in the pupal period and another sharp decrease when eclosion of the adult takes place. The alkyl ethers, by contrast, are much lower, and show a large decrease taking place between the penultimate and last larval instar.

In the American cockroach (Table I), the alkyl ethers predominated over alk-1-enyl ethers. The concentrations of each type of ether were nearly identical in whole insects of both sexes, although fewer total ethers by weight occurred in isolated fat body from female roaches. The percentage of the two ether types were higher in the fat body phospholipids of females than in total roach phospholipids. Earlier work on the phospholipids of American cockroaches (4) established that alk-1-enyl phosphatide was absent in the egg and first nymphal instar but appeared in the second nymphal instar and continued to increase in quantity throughout the preadult stages.

The adult boll weevil contained ten times as much alkyl ethers as pupae. The alk-1-enyl ethers were about three times higher in adults. Analyses of both types of glyceryl ethers were also made of boll weevil eggs and larvae. Although alkyl and alk-1-enyl glyceryl ethers were easily detected in both of these immature stages, quantitative densitometric analysis was not made owing to the occurrence of a pink compound at the same Rf as the glyceryl ethers. This unknown compound was also detected in

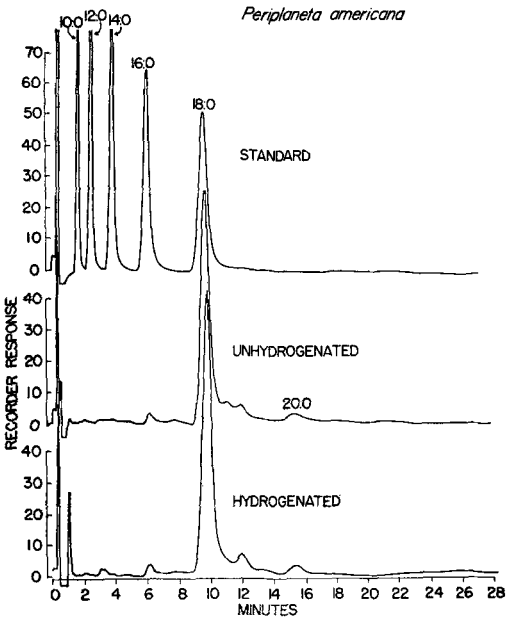

FIG. 3. GLC separation of standard, unhydrogenated and hydrogenated alkyl glyceryl ethers of *Periplaneta americana* as the isopropylidine derivatives.

the insect's diet, and possibly occurred in the unabsorbed gut contents. It was not present in the nonfeeding pupal stage and was not detected in the unfed adults.

TABLE II

GLC Analysis of Alkyl Glyceryl Ether Isopropylidene Derivatives and Alk-1-enyl Glyceryl Ether Aldehydes of Two Insect Species[a]

Chain length[b] of ether-linked hydrocarbons	% Alkyl glyceryl ether		% Alk-1-enyl glyceryl ether	
	Heliothis	*Periplaneta*	*Heliothis*	*Periplaneta*
16:0	8.2	2.5	15.9	5.4
17:0	T	T	0.9	5.6
18:0	43.5	79.4	34.6	67.3
18:1	T	8.0	2.1	19.5
19:0	2.4	6.4	2.8	2.1
20:0	31.2	3.6	35.8	....
20:1	7.2	....	....	....
21:0	....	....	4.6	....
22:0	T	....	1.9	....
Unknown	7.7	....	1.4	....

[a] Analyses were made on 5 ft × 1/8 in.-glass columns packed with 15% EGSS-X. Isopropylidene derivatives were chromatographed at 210 C and aldehydes were chromatographed at 160 C.

[b] Identification of even carbon numbered chains up to C_{20} are based on co-chromatography with known standards at two or more temperatures and logarithmic plots of retention time versus carbon number. Identification of odd carbon numbered chains are based solely on logarithmic plots of retention times versus carbon number, and is therefore considered tentative.

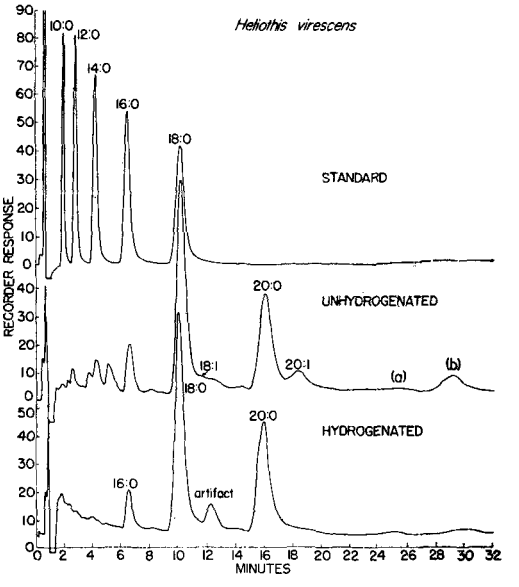

FIG. 4. GLC separation of the standard, unhydrogenated and hydrogenated alkyl glyceryl ether derivatives of *Heliothis virescens* as the isopropylidine derivatives.

TLC Behavior of Alkyl and Alk-1-enyl Glyceryl Ether Derivatives

Figure 2 shows the purity of the alkyl and alk-1-enyl glyceryl ethers (lanes 1 and 2, respectively) isolated by TLC from the $LiAlH_4$ reduced phospholipids of the American cockroach adults (A) and the tobacco budworm pupae (B). After hydrogenation, the alk-1-enyl glyceryl ethers of both species migrated with the alkyl glyceryl ethers as expected (lane 3). Isopropylidene derivatives were prepared from the alkyl glyceryl ethers (lane 1). Because of what appeared to be an incomplete reaction, the isopropylidene derivatives were cleaned up by TLC and are shown in lane 4. Thus, the TLC behavior of hydrogenated and isopropylidene derivatives as shown in Fig. 2 is further confirmation of the identity of the glyceryl ethers of *Periplaneta* and *Heliothis*. The boll weevil phospholipid glyceryl ethers were not further characterized as derivatives because of insufficient material.

Analysis of the Alkyl Glyceryl Ethers by GLC

The alkyl glyceryl ethers isolated from the phospholipids of the American cockroach and the tobacco budworm were analyzed by GLC as their isopropylidene derivatives. Chromatogram tracings of hydrogenated and unhydrogenated derivatives along with standards are shown in Fig. 3 and 4 for each species. Quan-

titative calculations of these particular analyses are summarized in Table II. It is evident from these results that the hydrocarbon moieties are distinctly different from each other in these two species. The side chain in *Periplaneta* consists almost exclusively of 18 carbons, and almost all (79%) is saturated. Smaller quantities of 16:0, 20:0, and a component tentatively identified as 19:0 are also present.

Glyceryl ether analysis of *Heliothis* showed a very different spectrum of chain lengths. Two major chain lengths were evident, 18:0 was the predominant one, and also both saturated and unsaturated 20-carbon components occurred, which together constituted nearly 40% of the total. A sizeable 16:0 component totaling about 8% also was found. The peaks before 16:0 on the unhydrogenated chromatograms were not included in the percentage calculation since they disappeared after hydrogenation. The peak marked (a) on the *Heliothis* chromatogram fell on a log plot as 22:0, but it was very wide and its retention time could not be measured accurately. The peak marked (b), which disappeared after hydrogenation, remains unidentified. The peak occurring on the hydrogenated *Heliothis* chromatogram between 18:0 and 20:0 appears to be an artifact.

Analysis of Alk-1-enyl Ether Composition

The alk-1-enyl ether fractions from *Periplaneta* and *Heliothis* were hydrolyzed with acid, and the aldehydes thus formed were purified by TLC and analyzed by GLC. The results are given in Table II. The hydrocarbon side chain of the alk-1-enyl ethers is very similar to that described above for the alkyl ethers of each species. In *Heliothis*, three major components were found, 16:0, 18:0, and 20:0, with corresponding minor unsaturated chains, whereas in *Periplaneta*, as with the alkyl ether chains, the 18-carbon saturated and mono-unsaturated hydrocarbons were the principal constituents. The similarity between the alkyl and alk-1-enyl ether fractions of either species suggests that the two ether pools are related. It is possible that one fraction serves as precursor for biosynthesis of the other. Evidence from experiments with the terrestrial slug *Arion ater* suggests that alk-1-enyl ethers were formed by dehydrogenation of alkyl ethers (22).

Incorporation of ^{14}C From Labeled Acetate Into Alkyl Glyceryl Ethers in *Heliothis virescens*

Phospholipids from *H. virescens* pupae injected with 1-^{14}C-acetate were isolated and reduced with $LiAlH_4$. Preliminary area scans showed that most of the radioactivity was present in the alcohol fraction. Thus the corresponding original phospholipid fatty acids had incorporated radioactivity from the injected labeled acetate. However, a small amount of radioactivity was associated with the alkyl ethers. Detailed zonal scanning and radioassay of 2-mm zones confirmed the area scanning (Fig. 5). Small peaks at 0.5 to 3 cm corresponding to area a to b in Fig. 1 contain only minor amounts of isotope. Peak 1 at 4 cm coincides exactly with the chromatographic position of the alkyl glyceryl ethers, and the large peak 2 at 8 cm matches the alcohols. Alk-1-enyl glyceryl ethers (which migrate to 6 cm), on the other hand, contain little significant radioactivity above background. From these results apparently small amounts of alkyl ether are synthesized from acetate, but no incorporation takes place in alk-1-enyl ethers during the 4-hr postinjection period used in these studies. Calculations of the areas under each of the radioactive peaks in Fig. 5 were made with the following results: glyceryl alkyl ethers, 13.1%; alcohols, 55.6%; all other components together totaled 31.3%.

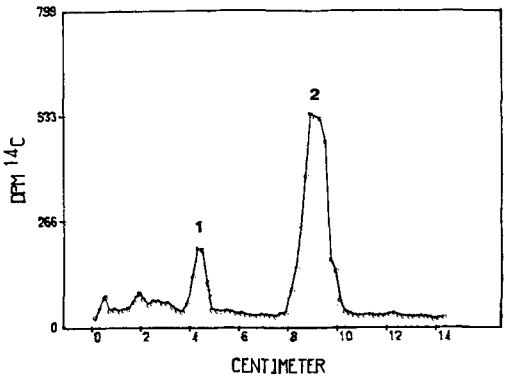

FIG. 5. Zonal profile of ^{14}C radioactivity of $LiAlH_4$ reduced phospholipids 4 hr after injection of 6-day-old *Heliothis virescens* pupae with 1-^{14}C-acetate. Peak 1 consists of the alkyl glyceryl ethers and peak 2 of alcohols. Other minor peaks at 0.5 to 3 cm correspond to area a and b in Fig. 1.

Acetate is known to be incorporated into both alkyl and alk-1-enyl glyceryl ethers in other biological systems (3). However, the significance of incorporation into alkyl ethers of *Heliothis* and not into the alk-1-enyl ethers suggests that if alkyl ethers give rise to alk-1-enyl ethers, the process is very slow in insects. Although we found some ether-containing lipids in the diets fed these three species, the present results with labeled acetate imply that diet is

not the only source of the ether-bonded hydrocarbon side chain. Incorporation studies were not carried out with the boll weevil or American cockroach.

In this paper we report the presence and identification of alkyl and alk-1-enyl glyceryl ethers in three phylogenetically different insect species. The ethers were found in the phospholipid fraction of all stages examined, but did not occur in significant amounts in the neutral lipids. They were also found in isolated fat body tissue of the cockroach.

We further show that the ether content varies between species and during development, but the significance of the variations is not presently understood. The identifications are further substantiated by TLC behavior of hydrogenated and isopropylidene derivatives. Analyses of the ether bonded hydrocarbon side chain by GLC further characterizes the ethers by delineating the chain length for two of the species (cockroach and budworm).

We have also shown with some preliminary experiments using 1-^{14}C-acetate that the budworm incorporates label into the alkyl ethers, suggesting the possibility of biosynthesis of glyceryl ethers in this and other insects.

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Studies on the Preparation and Analysis of Glyceryl Ether Derivatives and the Isolation and Reductive Ozonolysis of Unsaturated Glyceryl Ethers[1]

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ABSTRACT

Glyceryl ethers were identified and quantified by the GLC analysis of their alkyl iodide (special conditions described), acetonide and allyl alkyl ether derivatives. The acetoxy-mercuri-methoxy derivatives of unsaturated glyceryl ethers were separated from saturated glyceryl ethers by TLC and the fractions were analyzed as alkyl iodides. Monoiodides from saturated glyceryl ethers and diiodides from unsaturated glyceryl ethers were also separated by TLC and then analyzed. Allyl alkyl ethers had small retention volumes and these derivatives were separated into pure fractions by preparative GLC. Ozonides were reduced either to alcohols with lithium aluminum hydride or to aldehydes with dimethyl sulfide. The alcohols were converted to mono- and diiodides which were separated by TLC and analyzed by GLC. The recovery of n-C_6 and n-C_7 monoiodides was not quantitative. Diiodides and aldehydes were both identified and quantified by GLC. Ozonolysis data suggest that the 16:1 glyceryl ethers from dogfish liver oil contain 7,8 and 9,10 isomers and the 18:1 glyceryl ethers contain 9,10 and 11,12 isomers.

INTRODUCTION

SEVERAL METHODS have been developed for the isolation and analysis of glyceryl ethers. Pure glyceryl ethers are generally isolated from unsaponifiable lipids by column (1-3) or TLC (4-6). Unsaturated glyceryl ethers are separated from saturated glyceryl ethers by column chromatography of acetoxy-mercuri-methoxy adducts of glyceryl ether acetonides (2) or TLC of free glyceryl ethers on silver ion impregnated plates (7). Glyceryl ethers have been identified and analyzed by the GLC separation of diacetate (8), dimethoxy (1), trimethyl silyl or trifluoroacetyl (7), acetonide (2), alkoxy glycolaldehyde (4) and alkyl iodide (5) derivatives. These procedures are discussed in a recent review by Mangold and Baumann (9). Olefinic bonds in glyceryl ethers have been located by chromic acid (10) or permanganate-periodate (2) oxidation followed by the GLC analysis of methyl ester and α-carboxymethoxy ester fragments. In the present investigation, glyceryl ethers were identified and quantified by the GLC analysis of their alkyl iodide, acetonide and allyl alkyl ether derivatives and olefinic bonds were located by several reductive ozonolysis methods (Fig. 1).

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

MATERIALS AND METHODS

Materials

Materials included were tetradecanol, hexadecanol, octadecanol, 9-hexadecenol and 9-octadecenol, prepared by reducing the corresponding methyl esters (Hormel Institute, Austin, Minn.) with lithium aluminum hydride; 1,6-hexanediol, 1,9-nonanediol and methanesulfonyl chloride (Matheson Coleman and Bell, Cincinnati, Ohio); 1-iodohexane, 1-iodooctane, 1-iododecane, 1,3-diiodopropane and 1,5-diiodopentane (Eastman Organic Chemicals Rochester, N. Y.); 1,4-diiodobutane (Aldrich Chemical Co. Milwaukee, Wis.); selachyl alcohol from dogfish liver oil (supplied by W. Chalmers, Western Chemical Industries Ltd. Vancouver, Canada); and pentane (spectrograde) and hexane (technical grade) (Phillips Petroleum Co. Bartlesville, Okla.). Hexane was refluxed with potassium permanganate-sulfuric acid, washed until neutral, dried over magnesium sulfate and redistilled.

Purification of Glyceryl Ethers

Commercial selachyl alcohol (1 g) was refluxed with 40 ml of 0.5 *N* alcoholic potassium hydroxide for 3 hr under nitrogen. The non-saponifiable fraction was extracted three times with 50 ml of chloroform. The chloroform phase was washed first with distilled water then water saturated sodium chloride and finally dried over magnesium sulfate. Glyceryl ethers were concentrated by evaporation and applied to TLC plates (Silica Gel G, Brinkman Instruments Inc., Westbury, N. Y.) from a disposable pipette fitted with a wick of cotton which had been extracted previously with

chloroform-methanol. The glyceryl ethers formed a narrow streak at the origin. Plates were developed with hexane-ether (50:50, v/v) and fractions were made visible by spraying with 2',7'-dichlorofluorescein. The glyceryl ether band was scraped from the plate and the glyceryl ethers were extracted three times with 20 ml of concentrated hydrochloric acid-methanol (5:95, v/v). The extract was concentrated to approximately 10 ml and diluted with chloroform. The chloroform phase was washed with water and the marking dye was extracted with 1% potassium carbonate. The chloroform solution was then washed with saturated sodium chloride, dried over magnesium sulfate and concentrated. Glyceryl ethers purified in this manner contained no cholesterol and gave only one spot on a TLC plate developed with hexane-ether-acetic acid (90:10:1, v/v/v). The absence of free fatty acids and monoglycerides was confirmed by infrared analysis (Beckman IR-5) which showed no carbonyl absorption in the 1730-1750 cm^{-1} region.

Alkyl Iodides

Alkyl iodides were synthesized from glyceryl ethers by the method of Guyer et al (5) except that the hydriodolysis mixture was refluxed for only 2 to 3 hr. In small scale preparations (approximately 1 mg of glyceryl ether) alkyl iodides were extracted with pentane rather than hexane and all washing steps were eliminated. The pentane solution was filtered through anhydrous sodium sulfate and concentrated.

Since the direct addition of hydriodic acid to lithium aluminum alcoholates at room temperature produced a violent reaction, the alcoholates were cooled to –70 C and 5 ml of 57% hydriodic acid was added cautiously and with stirring. The flask was warmed to room temperature and was then refluxed for 3 hr. The flask was cooled in an ice bath and a saturated solution of sodium chloride was added. Alkyl iodides were extracted with hexane and the hexane solution was washed successively with water, 5% potassium carbonate, 5% sodium thiosulfate and saturated sodium chloride. The hexane solution was dried over magnesium sulfate and concentrated.

Alkyl iodides were identified by TLC on Silica Gel G. The plate was developed with hexane. R_f values were 0.92 for all monoiodides, 0.69 for long-chain diiodides prepared from unsaturated glyceryl ethers and 0.58 for short-chain diiodides prepared from lithium aluminum alchoholates. Monoiodide and diiodide fractions were readily separated and purified by preparative TLC.

Acetonides

Acetonides were synthesized from glyceryl ethers by the method of Hanahan et al. (2).

Allyl Alkyl Ethers

Allyl alkyl ethers were synthesized from glyceryl ethers by the method of McInnes et al. (11) for the synthesis of allyl esters from monoglycerides. A preliminary TLC analysis indicated that allyl ethers were partially cleaved to alcohols by catalytic hydrogenation.

Separation of Saturated and Unsaturated Glyceryl Ethers

The acetoxy-mercuri-methoxy derivatives of the unsaturated glyceryl ethers were prepared by the method of Mangold and Kammereck (12) except that solid mercuric acetate was removed by filtration before the chloroform solution was washed with water. The mercury adduct, approximately 30 mg dissolved in chloroform, was applied to a TLC plate as a streak and the plate was developed with ether. The saturated glyceryl ether band was made visible with 2',7'-dichlorofluorescein and the mercury adduct of the unsaturated glyceryl ether fraction was made visible with S-diphenylcarbazone. Bands were scraped from the plate and glyceryl ethers were extracted by the method described previously for the purification of glyceryl ethers. The S-diphenylcarbazone marker was not extracted from hydrochloric acid-methanol when the glyceryl ethers were extracted with chloroform. Chloroform was removed under the vacuum attained with a water pump and traces of water were removed with a high vacuum pump. The glyceryl ether fractions were stored under dry nitrogen.

Reductive Ozonolysis

Ozone generated in a Welsbach T-408 generator was bubbled through 50 ml of pentane until a blue color developed. The pentane solution was maintained at –70 C with a dry-ice-acetone bath. Glyceryl ethers, 25-50 mg dissolved in pentane, were cooled until a precipitate formed. The mixture was warmed slightly and the precipitate dissolved. The glyceryl ether solution was then added to the ozone in pentane solution. The flask was flushed with dry nitrogen and excess ozone was removed as judged by the disappearance of the blue color.

The metal hydride reduction of ozonides was first described by Sousa and Bluhm (13). In our study, approximately 1 g of lithium aluminum hydride was mixed with 50 ml of anhydrous ether. Excess metal hydride settled out with 10 ml of supernate was added slowly

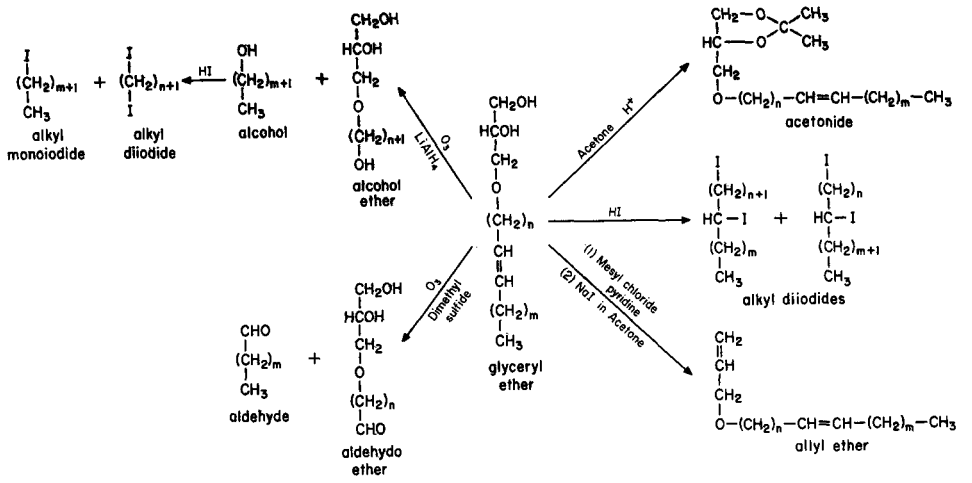

FIG. 1. Derivatives prepared from glyceryl ethers and their reductive ozonolysis products.

with a syringe to the ozonide which was cooled in the dry ice-acetone bath. This mixture was warmed slowly to room temperature and pentane and ether were evaporated in a stream of dry nitrogen. Ether (20 ml) was added to the flask and the mixture was refluxed for 3 hr. Ether was removed from the lithium aluminum alcoholate residue when the flask was warmed in a water bath. In small scale reductive ozonolysis, 1 mg of a glyceryl ether mixture was added to 2 ml of pentane saturated with ozone and the ozonides were prepared as described above. The ozonide were reduced with 5 ml of ether saturated with lithium aluminum hydride and the lithium aluminum alcoholate residue was concentrated as described above.

Ozonides were reduced to aldehydes and aldehydo ethers with dimethyl sulfide by the method described by Ramachandran et al (14).

Gas-Liquid Chromatography

Derivatives were identified and analyzed with Aerograph 200 and 1200 chromatographs equipped with flame ionization detectors and an Aerograph 350-B chromatograph equipped with thermal conductivity detectors. A 10-ft. stainless steel column, 1/8 in. O.D. (flame ionization) or 1/4 in. O.D. (thermal conductivity) containing 20% ethylene glycol succinate polyester on 80-100 mesh Gas Chrom P (Applied Science Laboratories Inc., State College, Pa.), was used for chromatographic separations. Specific operating conditions for analytical GLC are described in the text.

RESULTS AND DISCUSSION

GLC Analysis of Glyceryl Ether Derivatives

The GLC tracings for alkyl iodides prepared for reference compounds and purified

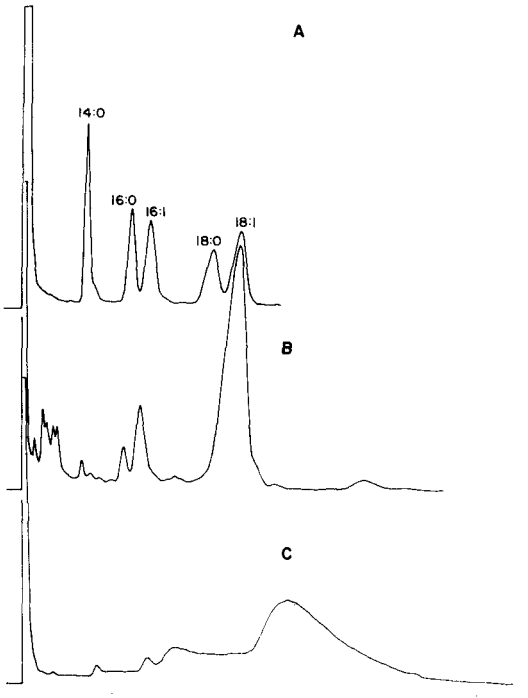

FIG. 2. GLC analysis of alkyl iodides synthesized from a tetradecanol, hexadecanol, 9-hexadecenol, octadecanol and 9-octadecenol mixture (A), and selachyl alcohol (B and C). Tracings were obtained with an Aerograph 350-B. Helium was the carrier gas and the flowrate was 60 ml/min. In A and B, the operating temperatures were: injector 280 C, column 185 C, detector 285 C. In C, the injector was 162 C and other operating conditions were the same as A and B. The solvent peak represents the full-scale deflection of the detector. Retention volume data at these operating conditions are summarized in Table I.

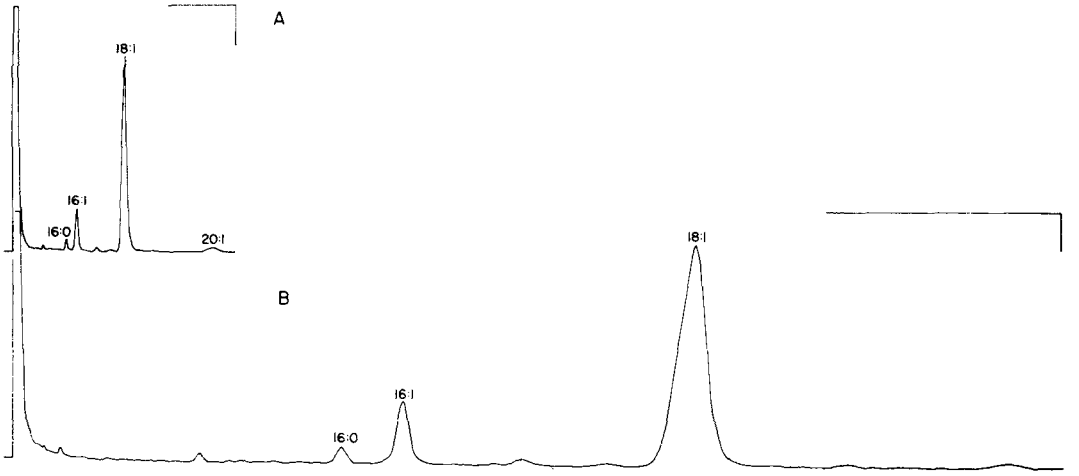

FIG. 3. GLC analysis of allyl ether (A) and acetonide (B) derivatives synthesized from selachyl alcohol. Tracings were obtained with an Aerograph 200. The operating temperatures were: injector 260 C, column 185 C, detector 220 C. Helium was the carrier gas and the flow-rate was 30 ml/min. The solvent peak represents the full-scale deflection of the detector. Retention volume data at these operating conditions are summarized in Table I.

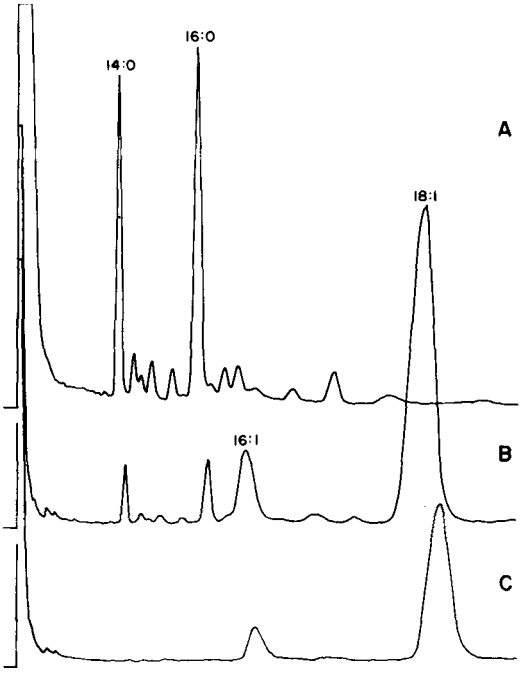

FIG. 4. GLC analysis of alkyl iodides synthesized from selachyl alcohol (B), and the saturated (A) and unsaturated (C) glyceryl ether fractions isolated by TLC of mercuri-adducts. Tracings were obtained with an Aerograph 200 equipped with a flame ionization detector. Operating temperatures were: injector 320 C, column 185 C, detector 280 C. Helium was the carrier gas and the flow-rate was 30 ml/min. The solvent peak represents the full-scale deflection of the detector.

selachyl alcohol are presented in Fig. 2. Unsaturated glyceryl ethers add hydriodic acid across their double bonds and form alkyl diiodides (Fig. 1) which are readily separated from alkyl monoiodides by TLC. The secondary iodide undergoes thermal dehydrohalogenation during GLC analysis and the derivatives are chromatographed as alkenyl iodides (5). Both the rate and extent of thermal dehydrohalogenation are affected by the temperature at the injection port and the sample size. Hanahan (15) found extensive trailing with alkyl diiodides on GLC. We observed similar trailing when the temperature of the Aerograph 350-B injector was lowered to 162 C (Fig. 2 C). Trailing was also found when alkyl diiodides containing secondary iodide groups were injected directly on the column of the Aerograph 200 instrument. We positioned the tip of the syringe just beyond the injector septum to facilitate dehydrohalogenation. When iodides from selachyl alcohol were analyzed with the thermal conductivity detector, the major peak sometimes had a minor shoulder on the trailing edge (Fig. 2 B). This shoulder was included with the major peak when the concentration of this component was estimated from the peak area. The shoulder was not observed when a smaller sample was chromatographed and analyzed with the flame ionization detector (see Fig. 4 B). Dehydrohalogenation is nonspecific and several positional isomers of the alkenyl iodides are formed (15,16); however, these isomers are not sep-

arated by the GLC procedure used in this study and the concentration of unsaturated glyceryl ethers which contain one double bond may be estimated from the area of the alkenyl iodide peaks. Dehydrohalogenation of alkyl iodides from glyceryl ethers which contain two or more double bonds yields positional isomers which are separated into several peaks by GLC (5) and these minor components cannot be estimated as alkenyl iodides.

Retention volumes, V_R, and relative retention volumes, r, were calculated for the alkyl iodide derivatives. In calculating r, the V_R of an iodide derivative was divided by the V_R of the 16:0 alkyl iodide which was used as the reference compound. Since log r is a linear function of the number of carbon atoms, n, in a homologous series the data were summarized by linear regression equations (Table I).

Peak areas in GLC tracings were measured by triangulation or weighing an aluminum foil pattern of the peak. The quantitative analysis of standard iodide mixtures synthesized from saturated and unsaturated fatty alcohols indicated that correction factors (Table II) were required to convert peak areas to composition in mole per cent (thermal conductivity) and weight per cent (flame ionization). The correction factors reported in this table may only apply to the instruments and operating conditions used in the present study although similar correction factors are obtained for 14:0, 16:0 and 18:1 alkyl iodides from thermal conductivity data in a previous study where correction factors were ignored (5). When the small correction factors obtained in this study and other studies with the thermal conductivity detector are compared with the large correction factors obtained with the flame ionization detector, it is apparent that the thermal conductivity detector is preferred for quantitation.

Gas-liquid chromatographic tracings for allyl alkyl ether and acetonide derivatives prepared from purified selachyl alcohol are presented in Fig. 3. V_R and log r data for these derivatives are summarized in Table I. The 16:0 allyl alkyl ether[2] and the 16:0 acetonide[2] derivatives were used as reference compounds when r was calculated for the derivatives series. The acetonide derivatives had large retention volumes compared to the allyl ether derivatives (Table I). Large retention volumes are characteristic of cyclic compounds and may be related to their high dipole moments (17). Allyl ethers were the most suitable derivatives for separation by preparative GLC since these derivatives had small retention volumes. Since standard mixtures of allyl ethers and acetonides were not available, it was assumed that the peak areas for these derivatives were proportional to composition in weight per cent when the derivatives were analyzed with a flame ionization detector.

The composition of purified selachyl alcohol

TABLE I

Relative Retention Volumes for Glyceryl Ether Derivatives and Cleavage Products

Series	log r[a]	Reference compound	V_R[b]
A. Glyceryl ether			
Alkyl iodide			
Saturated	0.124n - 1.988	$C_{16:0}$	758(185 C)
Monoene	0.119n - 1.830	$C_{16:0}$	758(185 C)
Allyl ether			
Saturated	0.111n - 1.760	$C_{16:0}$	137(185 C)
Monoene	0.110n - 1.681	$C_{16:0}$	137(185 C)
Acetonide			
Saturated[c]	0.126n - 2.014	$C_{16:0}$	1110(185 C)
Monoene	0.102n - 1.550	$C_{16:0}$	1110(185 C)
B. Cleavage product			
Alkyl monoiodide	0.170n - 1.543	C_9	901(122 C)
Alkyl diiodide	0.133n - 1.199	C_9	1170(200 C)
Aldehyde	0.253n - 2.279	C_9	554(110 C)
Aldehyde	0.187n - 1.684	C_9	188(145 C)

[a] The term n in the regression equation represents the number of C atoms in the alkoxy group in the parent glyceryl ether (Series A) or the number of C atoms in the cleavage product (Series B).

[b] Retention volume in milliliters for the reference compound at the temperature specified in parentheses. Alkyl iodides from the glyceryl ethers and the alkyl monoiodide cleavage products were analyzed with a thermal conductivity detector. Other derivatives were analyzed with a flame ionization detector.

[c] Only acetonides from 14:0 and 16:0 glyceryl ethers were available for analysis.

TABLE II

Correction Factors[a] for the Quantitative Analysis of Long-Chain Alkyl and Alkenyl Iodides

Iodide	RMR[b]	RWR[c]
14:0	0.93	0.48
16:0	0.94	0.69
16:1	1.16	0.87
18:0	1.00	1.00
18:1	1.18	1.16
20:0	1.19	—
20:1	1.27	1.57

[a] Peak area X correction factor yields corrected peak area.

[b] Relative mole response obtained with thermal conductivity detector (A-350-B).

[c] Relative weight response obtained with flame ionization detector (A-200).

[2] The carbon number: double bond nomenclature refers to the alkoxy group in the glyceryl ether and not the number of carbon atoms in the acetonide or the number of carbon atoms and double bonds in the allyl alkyl ether.

TABLE III

Composition of Purified Selachyl Alcohol Estimated From the Analysis of Alkyl Iodide, Allyl Alkyl Ether and Acetonide Derivatives[a]

Glyceryl ether	Alkyl iodide[b] I	II	III	Allyl Alkyl ether[c] mole %	Acetonide[c]	Mean
A			0.3			trace
14:0	1.0	0.8	0.4	1.1	1.0	0.9
B			0.9			trace
16:0	3.5	2.9	1.5	2.6	2.7	2.6
16:1	12.7	10.6	7.5	12.6	11.5	10.9
17:1	1.4	trace	1.4	1.7	1.4	1.2
18:0	trace	trace	1.8	0.6	trace	0.5
18:1	77.7	81.6	81.0	77.0	82.2	79.6
20:1	3.8	4.0	5.3	4.3	1.1	4.3[d]

[a] Operating conditions are described in Fig. 2-4.

[b] Preparations I and II were analyzed with a thermal conductivity detector: preparation III was analyzed with a flame ionization detector.

[c] Analyzed with a flame ionization detector. Composition in mole per cent calculated from peak area divided by molecular weight.

[d] The 20:1 acetonide was not included in mean.

was estimated from analyses of alkyl iodide, allyl alkyl ether, and acetonide derivatives (Table III). Standard deviations for replicate tracings varied from 0.1 to 0.7 for minor components and from 0.3 to 2.1 for the major 16:1 and 18:1 components. Standard deviations were similar for the different derivatives. The three derivatives gave similar data for all components except the 20:1 glyceryl ether even though peak areas for allyl alkyl ethers and acetonides were not corrected for relative response. The low recovery of the 20:1 acetonide is unexplained although it is difficult to estimate the composition of a minor component with a large V_R. A comparison of the data for the 16:1 and 18:1 glyceryl ether components indicates that alkyl iodide derivatives may be used to estimate the composition of unsaturated glyceryl ethers.

Separation of Saturated and Unsaturated Glyceryl Ethers

Saturated and unsaturated glyceryl ether fractions were obtained by the TLC separation of saturated glyceryl ethers and the mercuri-adducts of unsaturated glyceryl ethers. GLC tracings of alkyl iodides prepared from purified selachyl alcohol, the saturated glyceryl ether fraction and the unsaturated glyceryl ether fraction are presented in Fig. 4. Monoiodides from saturated glyceryl ethers and diiodides from unsaturated glyceryl ethers were also separated by TLC of the alkyl iodide mixture. The TLC separation of mono- and diiodide fraction is important only in the identification of components and the estimation of composition. The diiodide fraction cannot be used in structural studies since the addition of hydriodic acid to the double bond and the elimination of

TABLE IV

Composition of Saturated and Unsaturated Glyceryl Ether Fractions[a]

Glyceryl ether	Saturated Hg-Adduct	Monoiodide from TLC	Unsaturated Hg-Adduct[b] I	II	Diiodide from TLC	Total Calculated[c]	Mean[c]
			mole %				
14:0	17.2	17.7				0.8	0.9
16:0	49.1	68.2				2.6	2.6
16:1			12.3	12.8	9.7	11.1	10.9
17:1				1.9	1.1	1.0	1.2
18:0	11.2	14.1				0.6	0.5
18:1	22.4		83.5	80.6	85.3	79.8	79.6
20:1			4.2	4.7	3.8	4.1	4.3
14:0/16:0	0.35	0.26				0.31	0.35
16:1/18:1			0.15	0.16	0.11	0.14	0.14

[a] Operating conditions are described in Fig. 2. All glyceryl ether fractions were analyzed as alkyl iodides.

[b] The unsaturated glyceryl ether fraction was analyzed before (I) and after (II) hydrogenation.

[c] Calculated from the mean composition of saturated and unsaturated glyceryl ether fractions.

[d] Data from Table III.

hydriodic acid from the double bond are both nonspecific reactions.

The analyses of glyceryl ether fractions separated as alkyl iodides and mercuri-adducts are compared in Table IV. Alkenyl iodide derivatives of diiodides were not identified in the monoiodide fraction by GLC when this fraction was isolated by TLC (Table IV). Unsaturated glyceryl ethers in the saturated glyceryl ether fraction separated from the mercuri-adducts by TLC varied from a trace (Fig. 4 A) to as much as 22.4 mole per cent of the saturated fraction (Table IV). The unsaturated component represented less than 1 mole per cent of the unsaturated glyceryl ethers in the original mixture since the total saturated glyceryl ether fraction accounted for only 4 mole per cent of the original mixture (Table III). Mole ratios for the major saturated and unsaturated glyceryl ether components, 14:0/16:0 and 16:1/18:1, were similar in the total mixture and the fractions separated by TLC (Table IV). The composition of selachyl alcohol was calculated from the composition of the saturated and unsaturated glyceryl ether fractions and was similar to the composition obtained by the analysis of the total glyceryl ether mixture (Table IV).

Reductive Ozonolysis of Unsaturated Glyceryl Ethers

Reference monoiodides were synthesized from short-chain alcohols. Reference diiodides were synthesized from short-chain α,ω-alkane diols and reference aldehydes were synthesized by the reductive ozonolysis of 1-alkenes and the methyl esters of unsaturated fatty acids. The reference compounds were readily identified by GLC on EGS columns. The n-C_6 and n-C_7 aldehydes were separated from the solvent peak when the column temperature was in the 110-120 C region. The n-C_{11} aldehyde and dimethyl sulfoxide peaks overlapped in this region but the two compounds were separated at 145 C. V_R and log r data are summarized in Table I.

Quantitation was studied by the reductive ozonolysis of known mixtures of unsaturated methyl esters. Aldehydes were obtained in the expected ratios and these derivatives were used both for the identification and quantitative analysis of the terminal methyl fragments of the unsaturated glyceryl ethers. Monoiodides and diiodides were separated by TLC prior to GLC analysis. This TLC isolation step eliminated minor unidentified peaks from the GLC tracings. The recovery of n-C_6 and n-C_7 monoiodides was lower than theoretical and was variable. The relative concentrations of these derivatives were not improved by continuous solvent extraction or the analysis of the alcohols before their conversion to alkyl iodides. Monoiodides were used, therefore, only for the identification of cleavage products. Diiodides gave the expected ratios and these derivatives were used both for the identification and quantitative analysis of the terminal ether fragments of the unsaturated glyceryl ethers.

TABLE V

Composition of Aldehyde and Diiodide Fragments Obtained From Selachyl Alcohol and Allyl Ethers

Fragment	Selachyl alcohol[a]	Allyl ether[b] 16:1 mole %[c]	Allyl ether[b] 18:1
Aldehyde			
C_8	1.5		2.8
C_7	17.7	93.6	9.4(10.2)
C_8	3.6		2.4
C_9	70.6	6.4	82.4(89.8)
C_{10}	2.3		2.2
C_{11}	4.2		1.7
Diiodide			
C_6	0.3		
C_7	1.3	6.0	1.8
C_8	1.5		3.9
C_9	89.9	94.0	86.9(92.1)
C_{11}	6.4		7.4(7.8)
C_{13}	0.5		

[a] Aldehydes represent mean data from four ozonides. Diiodides were obtained from one ozonide and purified by TLC. The relative composition of C_9 and C_{11} diiodides was similar for other preparations which were not purified by TLC.

[b] Aldehydes represent mean data from two ozonides. Diiodides represent mean data from 2 (16:1) and 3 (18:1) ozonides. Data in parentheses are calculated by omitting minor components.

[c] Composition in mole per cent calculated from peak area divided by molecular weight.

Ozonolysis artifacts were sometimes identified in the monoiodide, diiodide and aldehyde fractions. These artifacts generally contained fewer carbon atoms than the expected product. Thus small amounts of n-C_8, n-C_7 and n-C_6 aldehydes were found after the reductive ozonolysis of pure methyl oleate. The artifacts were always recovered in decreasing yields so that $C_8 > C_7 > C_6$. In one experiment with pure methyl oleate, the sum of the ozonolysis artifacts, $C_8 + C_7 + C_6$, was 6 mole per cent of the aldehyde mixture and the expected n-C_9 aldehyde accounted for 94 mole per cent of the aldehyde mixture. The artifacts were generally obtained in lower yields and did not affect quantitation with known mixtures of unsaturated methyl esters (14).

The relative composition of cleavage products from selachyl alcohol is presented in Table V. The n-C_6 and n-C_{10} aldehydes probably represented ozonolysis artifacts. The n-C_8 alde-

hyde may have included both an artifact and the cleavage product from the 17:1 glyceryl ether. The major n-C_7 and n-C_9 aldehydes were assigned to the major 16:1 and 18:1 glyceryl ethers. However, the C_7/C_9 aldehyde ratio, 0.25, was much greater than the 16:1/18:1glyceryl ether ratio, 0.14 (Table III). While this discrepancy may be explained in part by an n-C_7 artifact from the 18:1 glyceryl ether, only an atypical cleavage pattern yielding an abnormally large n-C_7 artifact would completely explain the low ratio. The occurrence of positional isomers was, therefore, investigated by the reductive ozonolysis of specific glyceryl ether derivatives.

Allyl ethers were separated into four fractions by preparative GLC on a 10-ft column, 3/8 in. O.D., which contained 20% EGS on 80-100 mesh Gas Chrom P. The column temperature was 200 C and the helium flow rate was 200 ml/min. Analytical GLC showed that the first fraction contained 14:0, 16:0 and 16:1 allyl ethers. The second fraction contained only the 17:1 allyl ether. The third fraction contained 18:0 and 18:1 allyl ethers and the fourth fraction contained 18:1 and 20:1 allyl ethers. The 18:1 allyl ether accounted for 21.6 mole per cent of the allyl ethers in the fourth fraction.

The 16:1 fraction yielded n-C_7 and n-C_9 aldehydes and similar amounts of the corresponding n-C_9 and n-C_7 diiodides (Table V). These data suggested that the 16:1 glyceryl ether was a mixture of 7,8 and 9,10 isomers. The 18:1 fraction yielded significant amounts of the n-C_7 and n-C_9 aldehydes and similar amounts of the corresponding n-C_{11} and n-C_9 diiodides (Table V). Ozonolysis artifacts were also identified in these fractions. It is unlikely that the n-C_7 aldehyde was an artifact since this explanation of the data requires an unusual cleavage reaction where the C_7 artifact is much greater than the C_8 artifact. The n-C_{11} diiodide could represent an unknown product with the same R_f and V_R as the n-C_{11} diiodide. However, this explanation is unlikely since the relative concentrations of n-C_7 aldehyde and n-C_{11} diiodide which were obtained by different reductive ozonolysis methods are similar. The n-C_9 and n-C_{11} diiodides from the reductive ozonolysis of the 18:1 allyl ether fraction were recently quantified as 6.4 and 93.6 mole per cent respectively of the diiodide mixture by GLC on Apiezon L and the peaks were identified by their mass spectra. The n-C_7 and n-C_9 diiodide peaks from the 16:1 allyl ether fraction were separated on Apiezon L and the n-C_9 diiodide identified by mass spectra. Splitting patterns for diiodides will be discussed in a subsequent paper.

Reductive ozonolysis data confirm previous studies by other cleavage methods (2,10) which have shown that the 9,10 isomer is a major component of both the 16:1 and 18:1 glyceryl ethers. However, the reductive ozonolysis data suggest that the 16:1 glyceryl ether contains a small amount, 6 mole per cent, of the 7,8 positional isomer and the 18:1 glyceryl ether contains a small amount, 7.8 to 10.2 mole per cent of the 11,12 positional isomer. A number of isomers of monoethylenic fatty acids have been described in marine lipids (18) including dogfish liver oil (19). Minor components in the 16:1 and 18:1 glyceryl ethers may arise from the conversion of these fatty acids to alcohols and the incorporation of the alcohols into glyceryl ethers (20,21).

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Suitability of *Geotrichum candidum* Lipase for the Stereospecific Analysis of Some Triglycerides[1,2,3]

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ABSTRACT

A procedure is described for determining the stereospecific structure of triacid triglycerides containing oleic acid. The method utilizes the unique specificity of the lipase system from *Geotricum candidum* for hydrolyzing fatty acids which contain *cis*-9-unsaturation.

The procedure involves a pancreatic lipase hydrolysis of 10-20 mg of substrate to determine the fatty acids in the β-position and the incubation of another 50 mg of triglyceride with *G. candidum* lipase to obtain diglyceride for further treatment. The α,α- and α,β-diglycerides are collected separately, converted to phenyl phosphatides and treated with phospholipase A. The analysis of the monoglycerides, produced with pancreatic lipase and the analysis of the β-lysophosphatides allows the determination of the ratios of the original 2 position oleic acid *sn*-triglycerides while the analysis of the α-lysophosphatides aids in the determination of the original *sn*-triglycerides which contained oleic acid in the 3 position. The 1 position oleic acid *sn*-triglycerides are calculated by difference.

Racemic and enantiomeric triglycerides containing palmitic, stearic and oleic acids were synthesized and used to establish the limits of the new method. The good agreement between the actual and observed values for a mixture of isomers indicates that the procedure will be useful in the analysis of triacid triglycerides which contain oleic acid. The application of the procedure to triacid triglycerides which contain other unsaturated fatty acids is discussed.

INTRODUCTION

THE COMPLETE STEREOSPECIFIC analysis of a natural triglyceride mixture is an awesome task. In addition to the difficulty of separating a fat into triglyceride species, one is eventually faced with the unenviable chore of determining the proportions of enantiomeric triglycerides in mixtures of molecules which do not behave as expected. The most common criterion used to distinguish antipode from racemate is not applicable to triglycerides composed of fatty acids of similar chain length since these isomers do not appreciably rotate the plane of polarized light.

Because of this apparent lack of optical rotation, Schlenk proposed that these glycerides be called cryptoactive triglycerides (1) and suggested that other physico-chemical approaches such as x-ray diffraction, piezoelectric effects and mixed melting point diagrams be used to ascertain the presence or absence of optical isomerism. Using one or another of these methods Schlenk has been able to detect the presence of enantiomer or racemate in a large number of triglycerides.

In addition to these physico-chemical procedures, enzymatic analyses are available which can be utilized to assess the presence or absence of enantiomeric triglycerides. The best known methods are the stereospecific analyses developed by Brockerhoff (2,3) and the procedure utilizing diglyceride kinase reported recently by Lands et al. (4). Morris (5) has described a procedure which utilizes both enzymatic and physico-chemical approaches. Thus, it is theo-

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[2]Presented in part at the AOCS Meeting, Washington, D.C., April, 1968.

[3]The numbering used in this manuscript is according to the rules for "stereospecific numbering" as outlined in the IUPAC-IUB Commission on Biochemical Nomenclature (CBN), The Nomenclature of Lipids, European J. Biochem. 2 (1967) 127-131. In this system, the glycerol molecule is viewed in a Fischer projection formula with the secondary hydroxyl (or derived group) to the left and the hydrogen on the asymmetric carbon to the right. Then, the carbinol group at the top is position 1 and the group at the bottom is position 3. When stereospecific numbering is indicated the numbering is prefixed with *sn;* where the racemic glyceride is intended by the formula, the numbering is prefixed with *rac.* Also in this paper, an abbreviated representation of triglyceride nomenclature is used and when stereospecific numbering is intended, the first fatty acid depicted is in position 1 while the last fatty acid mentioned is in position 3. For example, *sn*-PSO is used as an abbreviation for *sn*-glyceryl-1-palmitate-2-stearate-3-oleate. In the abbreviated formula, *rac*-PSO, palmitic and oleic acids are considered to occupy the 1 and 3 positions equally. Where the Greek letters α and β are employed, no knowledge of optical isomerism is intended; thus, in these instances racemates, enantiomers or partial racemates could be indicated.

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retically possible with the methods presently available to obtain a complete stereospecific description of many mixtures of triglycerides. However, there is a special case of the triacid triglyceride mixture where the physico-chemical methods alone cannot be effectively used and for which the enzymatic procedures must be modified and developed to a greater degree.

In a triacid triglyceride mixture containing palmitic, stearic and oleic acids, for example, exhaustion of all preparative techniques would undoubtedly still leave the possibility of the presence of the six isomers, *sn*-glyceryl-1-palmitate-2-oleate-3-stearate (*sn*-POS), *sn*-glyceryl-1-stearate-2-oleate-3-palmitate (*sn*-SOP), *sn*-glyceryl-1-oleate-2-palmitate-3-stearate (*sn*-OPS), *sn*-glyceryl-1-stearate-2-palmitate-3-oleate (*sn*-SPO), *sn*-glyceryl-1-palmitate-2-stearate-3-oleate (*sn*-PSO) and *sn*-glyceryl-1-oleate-2-stearate-3-palmitate (*sn*-OSP). It is unlikely that any of the existing physico-chemical methods could accurately determine the correct proportions of each isomer when the mixture contained different quantities of some or all of the isomers.

Brockerhoff has reported on this problem (6) and concluded that the analysis of a triacid triglyceride mixture was unsolvable with present techniques. Lands and Slakey (7) and Jensen et al. (8) correctly surmised that the problem could be overcome and outlined procedures that theoretically would allow the complete analysis of the mixture. The procedure suggested by Jensen et al. (8) involves the use of a lipase system from *Geotrichum candidum* which is highly specific for fatty acids containing a *cis*-9-unsaturation (9-11) and is therefore only applicable to triglycerides containing an unsaturated fatty acid. However, the procedure is amenable to immediate testing.

In this paper the applicability of *G. candidum* lipase in stereospecific analyses is assessed and a procedure for determining the configurational isomers in triacid triglyceride combinations containing palmitic, stearic and oleic acids is developed.

MATERIALS AND METHODS

Lipases

The purified pancreatic lipase used in this study was purchased from Worthington Biochemical Corporation and contained 100 units/mg. Phospholipase A was *Crotalus atrox* venom purchased from the Ross Allen Reptile Institute. In some experiments, the venom from *Ancistrodon p. piscivorus* was employed. The *G. candidum* lipase was a gift from J. A. Alford of the USDA, Beltsville, Maryland and was prepared as described in his publication (12).

Synthetic Glycerides

The glycerides *rac*-PPO, *rac*-POS, *rac*-PSO, *rac*-SPO, *sn*-PSO, *sn*-OSP and *sn*-OOP were prepared according to established procedures as previously described (13,14). The cryptoactive triglycerides were prepared from D-acetone glycerol, which was synthesized by procedures outlined by Baer (15). The *sn*-OSP was made by initially synthesizing *sn*-glyceryl-3-palmitate and subsequently *sn*-glyceryl-1-oleate-3-palmitate prior to acylating the 2-position with stearoyl chloride while the *sn*-PSO was prepared via the intermediates *sn*-glyceryl-3-oleate and *sn*-glyceryl-1-palmitate-3-oleate. The *sn*-OOP was a byproduct formed during the synthesis of *sn*-glyceryl-1-oleate-3-palmitate. As judged by gas-liquid and thin-layer chromatography the purity of these triglycerides was in excess of 99%. Correctness of position was ascertained with the aid of pancreatic lipase and phospholipase A as described below. In addition, the purity of some of the starting materials for the cryptoactive glycerides was determined by comparison of published and observed specific rotations and melting points (14). With the limitations of the methods in mind, the positioning within the glycerides is considered to approach 99%.

Enzymatic Procedures

Preparation of Emulsions. Enough substrate was weighed into a 50-ml beaker so that the final emulsion contained 25 mg/ml. The fat was melted by heating gently on a steam bath, sufficient 1% gum arabic solution added and the contents emulsified by insonation. Such emulsions were found to be stable for at least one week when kept in a refrigerator at 12 C.

Pancreatic Lipase Analysis. One half to one milliliter of the emulsified substrate was mixed with 7.5 ml of 0.25 M tris buffer (pH 8.4) and 0.5 ml of 0.1 M $CaCl_2$. The mixture was allowed to equilibrate by shaking in a water bath at 37 C prior to the addition of 1 ml of tris buffer containing 1 mg (100 units) of purified pancreatic lipase. When the digestion had proceeded for 1 min, 0.5 ml of 20% sulfuric acid was added and the lipids were extracted with 250 ml of chloroform-methanol (90:10) as previously described (16). The majority of the solvent was removed by rotary evaporation at reduced pressure and the concentrated lipid was separated by preparative thin-layer chromatography on 500-μ thick layers of silica gel G using petroleum ether (35-45 C)-ethyl ether-

acetic acid (90:30:1) as the developing solvent and brief exposure to iodine vapors to visualize the resulting bands. The monoglyceride fraction was scraped into a 50-ml flask, converted to esters by H_2SO_4 catalyzed methanolysis and analyzed by GLC. In addition a small portion of the original substrate was esterified and also analyzed by GLC and the fatty acid compositions of the primary and secondary positions determined (17).

Geotrichum candidum Lipase Procedure. Two milliliters of the emulsion were placed in a 25-ml beaker and mixed with 7.5 ml of distilled water and 0.5 ml of 0.1 M $CaCl_2$; a small magnet was placed in the beaker and the contents allowed to equilibrate with stirring at 37 C in a Sargent Recording pH Stat (E. H. Sargent & Co., Model S-30240) set at pH 8.5. Ten or 15 mg of *G. candidum* lipase was added to the incubation beaker. The digestion was allowed to proceed for 15 min during which time the pH was kept constant at 8.5 by the automatic addition of 0.09N NaOH. At the end of this period the mixture was quickly transferred to a separatory funnel and diluted with 25 ml of distilled water. Fifty milliliters of petroleum ether 35-45C-ethyl ether (1:1) was added, the contents agitated and the organic and water phases allowed to separate. The interfacial fluff which contained most of the calcium soaps of the fatty acids was discarded with the water layer. The organic phase was dried with Na_2SO_4, filtered and the lipid concentrated under reduced pressure at less than 35 C. The lipids were separated by preparative thin-layer chromatography on 500-μ layers of Silica Gel G containing 5% by weight of boric acid (18). A developing solvent of chloroform-acetone (96:4) was used to separate the α,α'- from the α,β-diglycerides. The diglycerides were visualized by brief exposure to iodine vapors and individually isolated by scraping the bands and extracting the lipid from the adsorbent with 50 ml of chloroform-methanol (90:10). Solvent was removed from the glycerides under reduced pressure at less than 35 C and the diglycerides processed as described below. The α,β-diglycerides were usually contaminated with small amounts of free fatty acids; however, the presence of these did not appear to interfere with the subsequent steps and analyses.

In some digestions, the total lipids produced during the lipolysis were estimated. In these cases, the digestion mixture was extracted with chloroform-methanol (90:10) as described (16) and all the fractions resulting from separation by preparative thin-layer chromatography were extracted from the adsorbent, the solvent removed, the glycerides converted to methyl esters by base catalysis (19) and these analyzed by GLC. A known amount of methyl heptadecanoate was added to each fraction to facilitate quantitation.

Synthesis of Phosphatidyl Phenols. The procedure described here is a modification of that reported by Brockerhoff (2). The diglycerides, contained in a stoppered flask were cooled for 15 min by placing them at –25 C. To the cooled diglycerides, the following reagents were added: 1 ml of absolute chloroform, 0.2 ml of pyridine and 0.1 ml of phenyl dichlorophosphate (K & K Laboratories). The pyridine was distilled from barium oxide and the chloroform was washed with water to remove alcohol, dried with calcium chloride and distilled. The synthesis was allowed to proceed for 4 hr at room temperature in stoppered flasks. After 4 hr, a few drops of distilled water were added to each flask, the contents swirled and allowed to stand an additional 10 min. Then the phenyl phosphatides were transferred to a separatory funnel with 60 ml of chloroform-methanol (1:1), 25 ml of distilled water were added, the contents were vigorously shaken and the phases allowed to separate. The lower layer, containing the phosphatides, was transferred to a 50-ml flask fitted with a standard taper joint and the solvents were removed under reduced pressure at less than 40 C. An easily cleaned all-glass and teflon system was employed in these evaporations as bumping often occurred, and repeated rinses of the apparatus with chloroform-methanol (1:1) were sometimes necessary to prevent losses of material. The evaporation was considered complete when no solvent could be seen and the odor of pyridine could not be detected. The phosphatides, without further purification, were then incubated with phospholipase A as described below.

Phospholipase A Analysis. Twenty milligrams of phospholipase A was transferred to a flask containing the phosphatidyl phenols with the aid of 7 ml of anhydrous ethyl ether (Mallinckrodt). Two milliliters of 0.2M (pH 7.5) HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, Calbiochem) and 2 drops of 0.1M $CaCl_2$ were added, the flask stoppered and placed in a 25 C water bath with shaking. After 10 hr, 25 ml of benzene and 1 ml of a chloroform solution containing a known amount of pentadecanoic acid were added and the solvents removed under reduced pressure at less than 40 C. As above, bumping often occurred and repeated rinsings with chloroform-methanol (1:1) were required to pre-

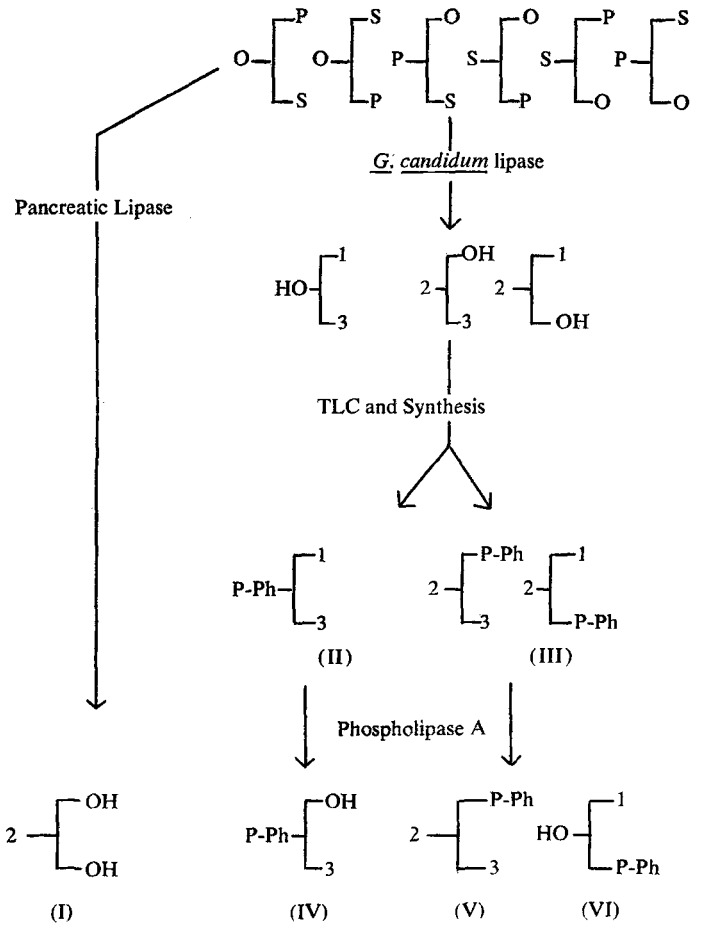

FIG. 1. Stereospecific analysis of a triacid triglyceride mixture. P, O, and S represent palmitic, oleic and stearic acids esterified to glycerol. P-Ph represents phosphoryl phenol. The numbers 1, 2 or 3 represent fatty acids esterified to the glycerol molecule at those positions.

vent losses of material. When all of the water was removed, sufficient chloroform-methanol (1:1) was added to facilitate streaking the material on a TLC plate coated with a 500-μ layer of Silica gel G. A special 20 × 25 cm plate was employed for these preparative separations so that the solvent front could run a distance of ca. 22 cm. The plate was developed in a solvent system consisting of chloroform-methanol-acetic acid (90:10:2); the separated free fatty acids, nonhydrolyzed phosphatide and lysophosphatide were visualized by exposure to iodine vapors and scraped into 50-ml flasks containing a known amount of methyl heptadecanoate. Fifteen milliliters of absolute methanol and 5 drops of concentrated sulfuric acid were added to the adsorbent containing the separated fraction. A boiling chip was added and the contents refluxed for 3 hr. After the contents had cooled the methanol phase was diluted with water and extracted once with 25 ml of petroleum ether. The petroleum ether was dried with Na_2SO_4 and the methyl esters concentrated to a volume suitable for analysis by GLC.

Gas Liquid Chromatography

Methyl esters were separated on 10 ft. by 0.25 in. columns containing 18% DEGS coated on Anakrom ABS 70/80 mesh (Analabs). Columns were operated at 195 C in a Barber-Colman model 5000 Selecta System employing dual flame detectors. A Disc Integrator attached to the recorder was used to quantitate the areas under the peaks.

General Procedure

Each of the triglycerides was analyzed separately in duplicate and a mixture containing 12.5 mg each of *sn*-PSO, *rac*-PSO, *rac*-SPO and *rac*-POS was run in quintuplicate. The 1,3-*sn*-DG intermediates for the *sn*-PSO and *sn*-OSP were converted to β-phosphatidyl phenols and analyzed with phospholipase A. Controls containing no substrate were carried through the entire analytical procedure. Additional runs with *G. candidum* were carried out on several substrates to determine amounts and fatty acid compositions of diglyceride formed and the extent of acyl migration. The relative proportions of fractions within a sample were calculated with the aid of the methyl heptadecanoate as an internal standard. The pentadecanoic acid standard was used to ascertain relative proportions between samples.

The mixture of triacid triglycerides was treated as outlined schematically in Fig. 1. In the first step, the triglyceride mixture was incubated with pancreatic lipase; the monoglycerides (I) produced were isolated and analyzed to estimate the fatty acid constituents of the 2 position. Another portion of the triglyceride mixture was partially hydrolyzed with *G. candidum* lipase and the resulting diglycerides separated into α,α'-diglycerides and α,β-diglycerides. The two diglyceride fractions were converted into phosphatidyl phenols (II and III) and incubated with phospholipase A. The β-lysophosphatide fraction (IV) was used to aid in the calculation of the proportions of *sn*-POS and *sn*-SOP and the α-lysophosphatide (VI) was employed to aid in estimating the amounts of *sn*-PSO and *sn*-SPO. The remaining glycerides are estimated by difference. Per cent *sn*-POS = (A) x (B); % *sn*-SOP = (A) x (C) or (A) – % *sn*-POS; % *sn*-PSO = (D) x (E); % *sn*-SPO = (D) x (F) or (D) – % *sn*-PSO; % *sn*-OPS = (G) – % *sn*-SPO; and % *sn*-OSP = (H) – % *sn*-PSO. A = % 18:1 in 2 position, % 18:1 in monoglycerides from pancreatic lipolysis; B = % 18:0 in β-lysophosphatide; C = % 16:0 in β-lysophosphatide; D = % 18:1 in 3 position = (X) x (Y), where X = % phosphorous in 3 position of original α-phosphatide, % hydrolysis of α-phosphatide, and Y = % 18:1 in 1 and 3 positions = (100) – (A); E = % 16:0 in α-lysophosphatide; F = % 18:0 in α-lysophosphatide; G = % 16:0 in 2 position = % 16:0 in monoglycerides from pancreatic lipolysis; and H = % 18:0 in 2 position = % 18:0 in monoglycerides from pancreatic lipolysis.

The per cent of oleic acid in position 3 (D) can also be estimated from the ratio of α-lysophosphatide to the total phosphatide; however, the accuracy of this value then depends on equal rates of hydrolysis by *G. candidum* of oleic acid from the α- and β-positions.

TABLE I

Fatty Acid Composition of the Products Resulting From the Stereospecific Analysis of Some Diglycerides[a]

Diglyceride	Free fatty acid		Lysophosphatide	
	Mole %			
	16:0	18:1	16:0	18:1
sn-glyceryl-l-Palmitate-3-Oleate	97.8	2.2	trace	>99.9
sn-glyceryl-l-Oleate-3-Palmitate	trace	>99.9	99.3	0.7
rac-glyceryl-l-Oleate-3-Palmitate	51.8	48.2	49.1	50.9

[a] Fifty milligrams of the diglycerides were converted to the β-phenyl phosphatides and these were reacted with phopholipase A.

RESULTS AND DISCUSSION

Purity of Starting Materials

The purity of the cryptoactive triglycerides was deduced from the purity of the original D-acetone glycerol and 3-*sn*-monoglycerides (14). In addition, the 1,3-*sn*-diglyceride intermediates used in the synthesis of the enantiomeric triglycerides were converted to phosphatides by reaction with phenyl dichlorophosphate and incubated with phospholipase A. As shown by De Haas and Van Deenen (20), and confirmed in this paper, phospholipase A will hydrolyze only the 1-*sn*-position of a β-phosphatide, thus allowing the stereochemical discrimination between α positions of a glyceride. The results of this analysis are shown in Table I. When *sn*-glyceryl-l-palmitate-3-oleate was subjected to the analysis, the lysophosphatide consisted of >99% oleic acid whereas palmitic acid was the predominate acid in this fraction when the enantiomer *sn*-glyceryl-l-oleate-3-palmitate was examined. For comparison the results of the analysis of *rac*-glyceryl-l-oleate-3-palmitate are also reported in Table I, and as expected, both free fatty acid and lysophosphatide fractions contained essentially equal amounts of both acids. These results confirm previous observations regarding the specificity of phospholipase A and illustrate clearly that the acyl groups were placed within the glyceride molecule as expected.

Preliminary Experiments

It was necessary to determine the suitability of the *G. candidum* lipase and to establish conditions which would maximize the yield and purity of the diglyceride products resulting from lipolysis before the procedure could be

TABLE II

Effect of Digestion Time or TLC Adsorbent on Extent of Acyl Migration and Amount of Monoglyceride Formed During Digestion of Racemic SPO[a]

Conditions	αβ-Diglyceride found as % of total diglycerides	Monoglyceride found as % of partial glycerides
Digested for 15 min	96.0	8.2
Digested for 30 min	91.5	48.6
Separated on Silica gel G	89.8[b]	—
Separated on Boric acid Impregnated Silica Gel G	94.4[b]	—

[a] Digested with 10 mg of *Geotrichum candidum* lipase at 37 C with pH constant at 8.5.

[b] Digested for 15 min.

applied to a synthetic mixture. After several trials it was concluded that a 15-min digestion period was preferable and that boric acid impregnated TLC plates were necessary to minimize acyl migration during isolation of the diglyceride fractions (Table II). The longer periods of digestion not only gave higher yields of the incorrect isomer but also produced considerable quantities of monoglyceride. In Table III it can be seen that a major percentage of the glyceride products was diglyceride of the correct isomer when a 15-minute-incubation period was used. For a 50-mg sample the diglyceride product usually amounted to ca. 20 μmoles, which, as shown below in the results of the individual triglycerides and of the mixture, was sufficient for subsequent analysis.

In both Table II and III, it is noted that monoglyceride was found during the digestion of *rac*-SPO. If the *G. candidum* lipase were absolutely specific for oleic acid, no monoglyceride should have been observed. Undoubtedly acids other than oleic were hydrolyzed by the lipase as has been shown previously (10,11); consequently, it was important to ascertain to what extent the diglyceride fractions reflected the hydrolysis of acids other than oleic. The fatty acid composition of the diglycerides produced by the action of *G. candidum* lipase on several triglycerides are reported

TABLE III

Glyceride Products Found After Digestion of Racemic Glyceryl-1-Stearate-2-Palmitate-3-Oleate With *Geotrichum candidum* Lipase[a]

Glyceride	Mole %
Residual Triglyceride	46.7
α,α-Diglyceride	1.4
α,β-Diglyceride	43.7
Monoglyceride	8.2

[a] Fifty milligrams of substrate incubated for 15 min with 10 mg of enzyme at 37 C with pH constant at 8.5.

TABLE IV

Mole % of Fatty Acids Found in the Diglycerides Resulting from *G. candidum* Lipase Digestion of Various Substrates[a]

Substrate	Diglyceride	Theory[b]	Observed		
		16:0	16:0	18:0	18:1
rac-SPO	α,β	50.0	49.8	50.2	trace
rac-POS	α,α	50.0	52.4	47.6	trace
rac-PSO	α,β	50.0	48.7	51.3	trace
sn-PSO	α,β	50.0	45.9	54.1	trace
sn-OSP	α,β	50.0	48.9	51.1	trace
rac-PPO	α,β	100.0	100.0	—	—
sn-OOP	α,α	50.0	51.6	—	48.4
sn-OOP	α,β	50.0	50.3	—	49.7

[a] Substrates were digested with 15 mg of enzyme for 15 min at 37 C and with pH constant at 8.5.

[b] Calculated with the assumption that only oleic acid is hydrolyzed from the triglyceride.

in Table IV. The enzyme's high degree of specificity for oleic acid is confirmed. Also, it is clear that the diglyceride composition is highly predictable since the conversion of triglyceride to diglyceride is accompanied by a loss of oleic acid. These results are in agreement with those reported previously (11) and demonstrate that the *G. candidum* lipase possesses an important prerequisite of enzymes employed in stereochemical analyses of triglycerides; namely, the ability to produce diglycerides which are representative of the original triglycerides.

A point, although not pertinent to this study, concerns the possibility that the observed hydrolysis of saturated fatty acids with *G. candidum* lipase (10,11) is primarily the result of the conversion of diglyceride to monoglyceride. The absence of oleic acid in the diglycerides derived from monooleate triglycerides and the accumulation of monoglycerides in these same digestions can be interpreted to support this hypothesis.

Separation of Products of Phospholipase A Hydrolysis

The phospholipids in this study were incubated with phospholipase A as the neutral compounds rather than as the triethylammonium salts employed in the Brockerhoff analysis (2), consequently a different solvent system was necessary to separate the incubation products. Although several solvent systems gave successful results the most consistent separations were achieved with a chloroform-methanol-acetic acid (90:10:2) system. A reproduction of the separation achieved on a preparative TLC plate developed in this system is shown in Fig. 2. The free fatty acids possessed an R_f of 0.68, the phosphatide an R_f of 0.40, and the lysophosphatide had an R_f of 0.08. Running with

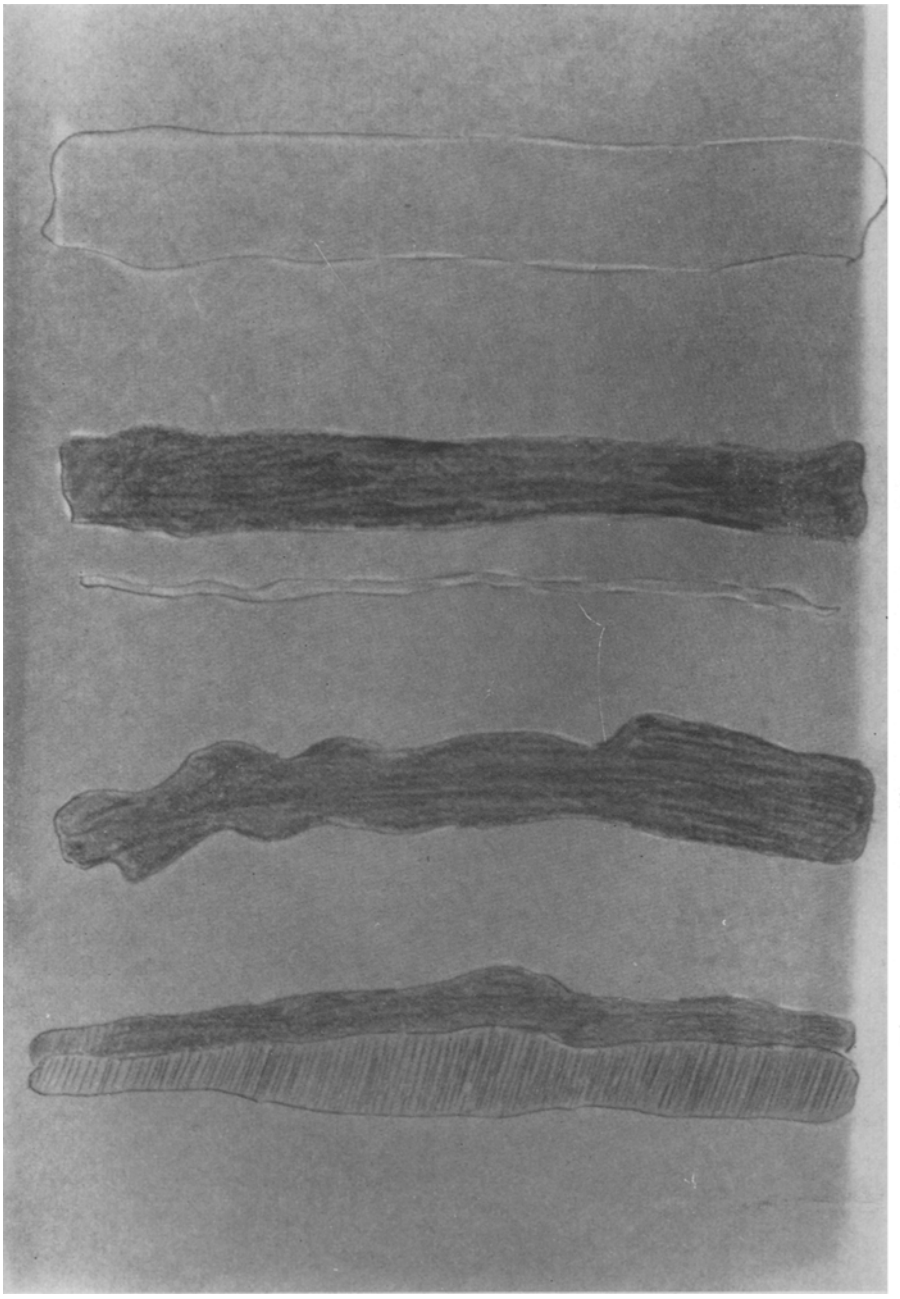

FIG. 2. Separation of phosphatides by thin-layer chromatography; solvent system chloroform-methanol-acetic acid (90:10:2).

and below the lyso compound was the protein fraction with an R_f of ca. 0.02; the presence of this material in the lysophosphatide did not appear to interfere with subsequent analyses. Unreacted phenyl phosphate also ran in this fraction, while unreacted diglyceride, when present, appeared at the solvent front. Other unknown materials appearing below the free fatty acid and at the solvent front apparently were not of lipid origin as they did not yield products discernible in gas-liquid chromatography of methyl esters.

TABLE V

Fatty Acid Composition of the Original Triglyceride and Some of the Fractions Resulting From Enzymatic Analysis of Racemic SPO[a]

Fraction analyzed	Theoretical 16:0	18:0	18:1	Observed 16:0	18:0	18:1
	Mole %					
Triglyceride	33.3	33.3	33.3	33.7	33.5	32.8
Monoglyceride[b]	100.0	0.0	0.0	99.0	0.5	0.5
α-Phosphatide[c]	50.0	50.0	0.0	48.9	50.9	0.2
α-Lysophosphatide[c]	0.0	100.0	0.0	3.8	96.2	trace

[a] The triglyceride was analyzed as described in materials and methods.

[b] Derived from pancreatic lipase analysis.

[c] Derived from phospholipase A digestion of phenyl phosphatides synthesized from the diglycerides resulting from the *G. candidum* lipase incubation. Phospholipase A digestion was 48.1%. Theory = 50%.

TABLE VII

Fatty Acid Composition of the Original Triglyceride and Some of the Fractions Resulting From Enzymatic Analysis of Racemic POS[a]

Fraction analyzed	Theoretical 16:0	18:0	18:1	Observed 16:0	18:0	18:1
	Mole %					
Triglyceride	33.3	33.3	33.3	33.8	33.1	33.1
Monoglyceride[b]	0.0	0.0	100.0	trace	trace	>99.9
β-Phosphatide[c,d]	—	—	—	trace	trace	—
β-Lysophosphatide[c]	50.0	50.0	0.0	52.4	47.6	trace

[a] The triglyceride was analyzed as described in materials and methods.

[b] Derived from pancreatic lipase analysis.

[c] Derived from phospholipase A digestion of phenyl phosphatides synthesized from the diglycerides resulting from the *G. candidum* lipase incubation.

[d] Theoretically no unreacted phosphatide should remain; however, digestion was not complete in some cases thus traces of fatty acids were found in this fraction. Phospholipase A digestion was 98.5%. Theory = 100%.

Analysis of Triglycerides

The results from the enzymatic analysis of the synthetic triglycerides *rac*-SOP, *rac*-PSO, *rac*-POS, *sn*-PSO and *sn*-OSP are summarized in Tables V-VIII. In all but one case the results closely approximated theoretical values. Several points are worthy of note regarding the purity of the substrates and the suitability of the enzymes involved in the analyses.

The excellent purity of the triglycerides is reflected in the analysis of the original triglycerides and the monoglycerides derived from the pancreatic lipolyses. For example, in Table V, pancreatic lipolysis of *rac*-SPO gave 99% palmitic acid in the monoglyceride. By difference, of course, almost all of the oleic and stearic acids must have been associated with the α-positions. All of the glycerides gave comparable results (Tables V-VIII) when examined with pancreatic lipase.

The virtual absence of the oleic acid in all of the phosphatides confirmed the specificity of *G. candidum* lipase in the conversion of triacid triglycerides containing oleic acid to completely saturated diglycerides and the confirmed specificity enabled the following predictions. When diglycerides derived from racemic triacid triglycerides containing oleic acid in the α positions were converted to phosphatides and incubated with phospholipase A, both 1- and 3-*sn*-phosphatides should have been present and since only the 3-*sn*-phosphatide (L-α-phospholipid) is hydrolyzed (21,2) equal moles of lyso- and unreacted phosphatide were expected. On the other hand, since phospholipase A has been shown, above and elsewhere (20), to hydrolyze the stereospecifically numbered 1 position of the β-phosphatide, triglycerides containing oleic acid solely in the 2 position should have been converted to products that were all hydrolyzed by phospholipase A leaving only lysophosphatide and free fatty acid. These predictions were borne out and are noted as the per cent hydrolysis during phospholipase A digestion. These values, listed in the footnotes of the tables approached the theoretical 50% for *rac*-SPO and *rac*-PSO (Tables V and VI) and are very close to the theoretical 100% for *rac*-POS (Table VII).

In the case of the enantiomeric triglycerides (Table VIII) similar reasoning led to the predictions that the product derived from *sn*-PSO was entirely converted to lyso compound while the mirror image triglyceride, *sn*-OSP yielded a product which was not attacked by phospholipase A. As can be seen in Table VIII, the per cent hydrolysis for the 3-phospholipid derived from the *sn*-PSO was 98.4%, very close to the theoretical 100%; however, the results with the antipode was an unexpected 13%.

TABLE VI

Fatty Acid Composition of the Original Triglyceride and Some of the Fractions Resulting From Enzymatic Analysis of Racemic PSO[a]

Fraction analyzed	Theoretical 16:0	18:0	18:1	Observed 16:0	18:0	18:1
	Mole %					
Triglyceride	33.3	33.3	33.3	33.7	33.4	32.9
Monoglyceride[b]	0.0	100.0	0.0	0.6	98.8	0.5
α-Phosphatide[c]	50.0	50.0	0.0	51.8	48.2	0.0
α-Lysophosphatide[c]	100.0	0.0	0.0	95.8	4.2	0.0

[a] The triglyceride was analyzed as described in materials and methods.

[b] Derived from pancreatic lipase analysis.

[c] Derived from phospholipase A digestion of phenyl phosphatides synthesized from the diglycerides resulting from the *G. candidum* lipase incubation. Phospholipase A digestion was 54.7%. Theory = 50%.

TABLE VIII

Fatty Acid Composition of the Original Triglyceride and Some of the Fractions Resulting From Enzymatic Analysis of Two Cryptoactive Triglycerides[a]

Fraction	Triglyceride Substrate	Theory			Observed		
		16:0	18:0	18:1	16:0	18:0	18:1
		Mole %					
Triglyceride	*sn*-PSO	33.3	33.3	33.3	33.1	33.3	33.6
Monoglyceride[b]	*sn*-PSO	0.0	100.0	0.0	0.3	99.4	0.3
α-Phosphatide[c,d]	*sn*-PSO	0.0	0.0	0.0	trace	trace	0.0
α-Lysophosphatide	*sn*-PSO	100.0	0.0	0.0	98.8	1.2	0.0
Triglyceride	*sn*-OSP	33.3	33.3	33.3	32.7	34.2	33.1
Monoglyceride[b]	*sn*-OSP	0.0	100.0	0.0	0.5	99.5	trace
α-Phosphatide[c]	*sn*-OSP	50.0	50.0	0.0	52.4	47.6	0.0
α-Lysophosphatide[c,e]	*sn*-OSP	0.0	0.0	0.0	41.3	58.7	0.0

[a] The triglyceride was analyzed as described in materials and methods.

[b] Derived from pancreatic lipase analysis.

[c] Derived from phospholipase A digestion of phenyl phosphatides synthesized from the diglycerides resulting from the *G. candidum* lipase incubation.

[d] Theoretically no unreacted phosphatide should remain; however, the digestion proceeded to only 98.4%. Theory = 100%.

[e] Theoretically no lyso compound should be formed; however, there was 13% apparent digestion. Theory = 0%.

Since the synthesis of both enantiomers involved starting materials of comparable purity, identical synthetic sequences and the same purification procedures, it is unlikely that the *sn*-OSP was partially racemized. In these runs with the cryptoactive triglycerides, the venom from *Ancistrodon p. piscivorus* was employed. Contamination of the enzyme preparation with other lipase(s) may account for the anomalous results. In any case, neither the *Ancistrodon p. piscivorus* nor the *sn*-OSP were employed in further analyses.

Except for the equivocal results with *sn*-OSP, the positional specificity (20-21) of the phospholipase A was also verified as can be seen by noting the fatty acid compositions of the lysophosphatides (Tables V-VIII). In Table V, for example, the lysophosphatide eventually derived from *rac*-SPO contained 96.2% stearic acid, while the lysophosphatide derived from *rac*-PSO (Table VI) yielded 95.8% palmitic acid. When *sn*-PSO was the original substrate the analytical sequence eventually yielded a lysophosphatide which contained primarily palmitic acid (Table VIII). Predictions based on the stereospecific action of phospholipase A on β-phospholipids, confirmed above (Table I), were also borne out as palmitic and stearic acids were observed in approximately equal molar quantities in the lysophosphatide resulting from the analysis of *rac*-POS.

In accordance with the rules outlined in the methods section above, the data in Tables V to VIII can be used to estimate the glyceride species present in each analysis and thus offer a partially independent evaluation of the purity of the glycerides. When the calculations were applied to the data in Table V it was observed that the triglyceride contained 46.1% *sn*-SPO and 52.9% *sn*-OSP; values which are close to those expected for a racemic mixture of SPO. Similar values were obtained when the isomer percentages were calculated for the data in Tables VI and VII. Analogous calculations employing the data for *sn*-PSO in Table VIII gave an estimate of 96.9% as *sn*-PSO and 2.5% as OSP.

Despite these positive results, it was clear that calculations of this type can lead to ambiguous results if any of the 1-*sn*-phosphatide were hydrolyzed as may have occurred to yield the data observed for *sn*-OSP in Table VIII. For example, considerably more *sn*-SPO (7.6%) is predicted, with the aid of the lysophosphatide values in Table VIII than can be accounted for by the composition of the monoglycerides produced by pancreatic lipolysis. Nevertheless, these results (Tables V-VIII), by and large, can be interpreted to establish that the enzymes involved are suitable for the stereospecific analysis of triacid triglycerides containing oleic acid and that the synthetic triglycerides examined, except possibly for the *sn*-OSP, can be used to simulate a natural triacid triglyceride mixture to test the analytical scheme proposed.

The results from the analysis of the triacid triglyceride mixture are shown in Table IX. The agreement between theoretical and observed values was very good for all of the fractions analyzed. The per cent of oleic acid estimated to occupy the 2 position was closer to

TABLE IX

Fatty Acid Composition and Relative Molar Ratios of Some of the Fractions Resulting From the Stereospecific Analysis of a Triacid Triglyceride Mixture[a]

Fraction analyzed	Theory			Observed			Relative Molar Ratio	
	16:0	18:0	18:1	16:0	18:0	18:1	Theory	Observed
	Mole %							
Monoglyceride[b]	25.0	50.0	25.0	25.4	48.7	25.9	1	—
β-Lysophosphatide[c]	50.0	50.0	0.0	52.2	47.8	0.0	1	1.04
α-Lysophosphatide[d]	75.0	25.0	0.0	73.9	26.1	0.0	2	1.87
α-Phosphatide[d]	50.0	50.0	0.0	51.0	49.0	0.0	1	1.00

[a] Analyzed as described in Materials and Methods. Results are averages of 5 runs of equal mixtures of *rac*-POS, *rac*-PSO, *rac*-SPO and *sn*-PSO.

[b] Derived from pancreatic analysis.

[c] Derived from phospholipase A digestion of the β-phosphatide synthesized from the α,α-diglyceride fraction resulting from *G. candidum* lipolysis.

[d] Derived from phospholipase A digestion of the α-phosphatide synthesized from the α,β-diglyceride fraction resulting from *G. candidum* lipolysis.

theoretical when the monoglyceride value (25.9) was used than when the ratio of β-lysophosphatide to total phosphatide (26.6) was employed. This may reflect a faster rate of hydrolysis by *G. candidum* lipase of 2 position oleic acid which has been noted previously (11). However, the preferential loss of α,β-diglycerides or α-phosphatides or acyl migration of diglycerides may also have contributed to this result.

Since the oleate value for the 2 position is employed directly or indirectly in all the formulas (See Materials and Methods), the pancreatic lipolysis data were used in the determination of the isomer proportions. In addition to values contained in Table IX, two values, X and Y, not shown directly in the table were needed to calculate the isomer ratios in this mixture of triglycerides. The value X, 65.2%, which is an estimate of the per cent hydrolysis of the α-phosphatide, was obtained from the ratio of α-lysophosphatide to total α-phosphatide and the value Y, 74.1%, which is an estimate of the oleic acid present in both 1 and 3 positions, was obtained by subtracting the oleic acid content in the monoglyceride from 100%. With these values the per cent of oleic acid in position 3 (D) can be calculated. The other terms, A-C and E-H are drawn directly from Table IX.

TABLE X

Comparison of Triacid Triglyceride Isomers Observed to Those Actually Present in a Mixture Subjected to Enzymatic Analysis[a,b]

Isomer	Calculation	Actual	Observed	Standard Deviation
		Mole %		
sn-POS	(A) × (B)	12.5	12.39	1.44
sn-SOP	(A) × (C)	12.5	13.51	1.44
sn-PSO	(D) × (E)	37.5	35.62	2.53
sn-SPO	(D) × (F)	12.5	12.58	2.53
sn-OPS	(G) × (F)	12.5	12.82	2.53
sn-OSP	(H) — % *sn*-PSO	12.5	13.08	2.53

[a] Analyzed as described in Materials and Methods.

[b] Values were derived from the results shown in Table IX as discussed in the text: A = % 18:1 in 2 position; B = % 18:0 in β-lysophosphatide; C = % 16:0 in β-lysophosphatide; D = % 18:1 in 3 position; E = % 16:0 in α-lysophosphatide; F = % 18:0 in α-lysophosphatide; G = % 16:0 in 2 position; and H = % 18:0 in 2 position.

The result of these calculations is displayed in Table X; also shown are the actual isomer compositions and the formulas used in the calculations. None of the observed isomer percentages was significantly different from the actual value. The larger standard deviation for the four isomers containing α-position oleate reflects the error involved in determining the per cent hydrolysis of α-phosphatide in addition to the errors in the determination of the 2 position oleic acid content and the fatty acid compositions of the lysophosphatide fractions.

The good agreement between actual and observed values indicates that this procedure will be useful to investigators interested in elucidating the stereochemical structure of natural triglycerides. In principle the procedure is also applicable to purified dioleate triglycerides. In this case only α,α-diglycerides resulting from *G. candidum* lipase hydrolysis, are converted to phosphatides and the ratio of oleic acid in the lysophosphatide resulting from phospholipase A hydrolysis yields the per cent of the *sn*-glyceryl-1-acyl-2,3-dioleate isomer and the monoglycerides from pancreatic lipase analysis will give the per cent of the symmetrical dioleate glyceride present.

Since the lipase from *G. candidum* has been shown (11) to also preferentially hydrolyze palmitoleic and linoleic acids, triglycerides containing these acids should also be amenable to

analysis. The presence of three of these unsaturated fatty acids in the same molecule requires separation of diglycerides or phosphatide derivatives by silver nitrate chromatography.

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Pollen Lipids Attractive to Honeybees

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ABSTRACT

Two substances attractive to honeybees have been isolated from mixed pollens. One is a pigment which was identified as a lutein ester, the other is a free fatty acid which chromatographed was identified as a C_{18} straight-chain dienoic or trienoic acid.

INTRODUCTION

HONEYBEES MAKE DISTINCT preferential selections among the flowers from which they gather nectar and pollen. The differential visitation of flowers for nectar is regulated by the sugar contents of the nectar and by its aroma and volume. Among the flowers which offer pollen, some species are more attractive to bees than others. Different pollens may differ considerably in chemical composition and nutritional value, but at present little is known about the factors which attract foraging bees to pollen sources and elicit pollen collection behavior.

Taber (1) reported that pollen extracted by hexane or ethyl ether was no longer collected by honeybees, but that they readily gathered cellulose powder to hich a small amount of extract was added. Hügel (2) reported that the material attractive to bees was a steroid or a mixture of steroids.

In our attempts to identify the attractive substance in pollen, we have not been able to detect the attractive steroid substance found by Hügel (2), but we found that two other substances were attractive to bees: a carotenoid ester and a free fatty acid. The purpose of this paper is to describe these substances.

EXPERIMENTAL PROCEDURE

Materials

Pollen mixtures used in this study were collected by honeybees during the summer of 1966 at the Central Experimental Farm, Ottawa, Canada. The pollens were mostly from clovers (*Melilotus, Trifolium, Lotus,* etc.). They were obtained by mechanical trapping devices attached to the entrances of beehives. The traps were emptied daily and the pollen harvested was stored in a freezer at –20C.

The fatty acid methyl esters used for TLC and GLC standardization were purchased from The Hormel Institute and Applied Science Laboratories; the sterols from Distillation Products Inc.

All solvents were reagent grade and were redistilled before use.

Liquid Extraction

Pollen samples (250 g) were homogenized for 2 min in a Waring Blendor in 2 vol of hexane. After standing for 30 min, the homogenate was filtered and the residue was re-extracted twice with 2 vol of hexane. The pooled extracts were concentrated to 100 ml.

Column Chromatography

The hexane extract was fractionated by column chromatography, using a 1.9 × 15 cm silicic acid (Bio Rad HA, 325 mesh, Bio-Rad, Richmond, Cal.) column (3). The extract (5 ml) was transferred directly onto the column and elution was started with 50 ml of hexane, and continued with 50 ml each of 2%, 5% 10% and 50% diethyl ether in hexane. After removal of solvent in vacuo, the lipid residue was taken up in chloroform and assayed for attractiveness to bees.

For preparative chromatography, a 5-cm I.D. glass column filled to a height of 16 cm with silicic acid powder (Mallinckrodt Chemical Works, St. Louis) was loaded with 50 ml of hexane extract. Five fractions were collected using the following elution sequence: 100 ml hexane, 300 and 200 ml benzene, and 200 and 280 ml 50% ether in hexane.

Countercurrent Distribution

The fraction containing the major pigment was hydrolyzed in 15% ethanolic KOH and subjected to a 127-transfer counter-current fractionation (E-C Apparatus Company, Swarthmore, Pa.). The solvent system was hexane-benzene-87% methanol (1:1:1.5) developed by Curl (4) for the separation of carotenoids into monols plus hydrocarbons, diols, diol epoxides and polyols.

Thin-Layer Chromatography

Thin-layer chromatography was performed on standard 20 × 20 cm chromatoplates coated

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with a 250 μ layer of Silica Gel G. The solvent system hexane-diethyl ether-acetic acid (90:10:1) was used for the separation of pollen lipids into classes and also for the purification of fatty acid methyl esters (5). Spots were located by exposure to iodine vapors or by spraying with 20% aqueous perchloric acid or dichlorofluorescein.

Pollen carotenoids were best separated by TLC using the solvent system hexane-diethyl ether-acetone (60:30:20) (6).

A non-colored fraction which was attractive to bees was collected from column chromatography and further purified by preparative TLC with the solvent system chloroform-methanol (99:1). The different zones were scraped off the plate, and eluted with 5 ml of a mixture of chloroform-methanol (1:1), and methylated for GLC analysis.

The fatty acid methyl esters were separated according to their degree of unsaturation on silver nitrate chromatoplates. These were prepared by spraying standard Silica Gel plates with a 20% silver nitrate solution in 50% aqueous acetone and heating at 100C for 30 min immediately before use. They were developed in the solvent system hexane-diethyl ether-acetic acid (70:30:1). Spots were visualized with dichlorofluorescein. Each zone was scraped off, as above, assayed for attractiveness to bees, and investigated further by GLC.

Gas-Liquid Chromatography

Fractions obtained by column chromatography and TLC were methylated in methanolic boron trifluoride, using 1 ml of reagent (14% methanolic boron trifluoride, Applied Science Laboratories) and 1 ml of methanol per 10 mg of sample. Methyl esters were extracted with hexane and purified by TLC. Their separation by GLC was performed on a 6 ft × 3/16 in. column of 5% DEGS on 80/100 mesh Gas Chrom Q. The instrument was calibrated with authentic methyl esters.

Methyl esters of fatty acids were also separated according to their chain length on a 6 ft × 3/16 in. column of 3% XE 60 silicone (supplied by D. Turner, Toronto) on Gas Chrom Q, 80/100 mesh, at 150C.

Ozonolysis

A 5-mg sample was dissolved in 2 ml hexane and treated with ozone at –65C until complete reaction, as demonstrated by TLC. Solvent was evaporated and the ozonides were separated by TLC.

Hydrogenation

Fatty acid methyl esters (1 mg), as separated by silver nitrate Silica Gel TLC, were hydrogenated in 4 ml hexane in the presence of 16 mg Adam's catalyst (platinum oxide) for 1 hr with 1 lb hydrogen pressure. The products were analyzed by GLC.

Other Methods

The major carotenoid fraction from the silicic acid column was hydrolyzed in 15% ethanolic KOH at 90C for 1 hr, in order to determine the nature of the pigment. The hydrolysis product was extracted with ether and purified by TLC. Its spectral absorptions were measured in various solvents with a Bausch & Lomb Spectronic 502 spectrophotometer. The carotenoid was also partitioned between hexane and 90% methanol (7) before and after hydrolysis and tested for epoxide by the hydrochloric acid-diethyl ether color test (8).

Bioassay

The method of testing fractions for attractiveness to honeybees was an adaptation of a method used by Shearer and Boch (9). In a flight room, the bees were given the choice between 4, 6, or 8 dishes placed on a 1 × 1 m turntable. The dishes contained Avicel cellulose powder to which the various fractions were adsorbed by allowing the solvent to evaporate. Appropriate controls and replications were included. The table rotated at ¼ turn per minute and the bees foraging on each dish were counted at 1 min intervals over a period not exceeding 1 hr. The temperature in the flight room was maintained at 20-25C by air conditioning which also provided a slight breeze. The room was illuminated by banks of "daylight-white" fluorescent tubes.

Bioassays were run after the bees had spent more than 2 weeks in the flight room where, except during tests, they foraged from a mixture of soybean flour and dried brewer's yeast (3:1). This mixture had a creamy gray color. It failed to attract bees when pollen extract mixed with Avicel was available.

RESULTS AND DISCUSSION

More than one factor is apparently involved in the attractiveness of pollen to bees. One factor appears to be the color of pollen or the nature of its pigmentation. Another factor seems to be an olfactory attractant.

The Color Factor

Fractionation of pollen extract with mixtures of ether in hexane by column chromatography

TABLE I
Fractionation of Pollen Extract by Column Chromatography

Fraction	Eluent[a]	Color[b]	Attractiveness[c] A	Attractiveness[c] B	Main Components (determined by TLC)
1	100% H	+		+	Hydrocarbons
2	2% E in H	+	+	+	Triglycerides
3	5% E in H	++++	+++	+	Triglycerides, pigments, and sterols
4	10% E in H	+	+	+	Sterols and glycerides
5	50% E in H		+++	++++	Fatty acids and traces of sterols

[a] The fractions were eluted with various mixtures of ether (E) in hexane (H).

[b] A deep reddish yellow pigment was present predominantly in fraction 3. Relatively little color was present in fractions 1, 2 and 4.

[c] The fractions were tested for attractiveness to bees (A) before and (B) after adding riboflavin to make all fractions equally colored.

gave five fractions, two of which, No. 3 and 5, were attractive to bees (Table I). Fraction 3 contained a yellow colored pigment, while fraction 5 was not colored. When the pigment was removed from fraction 3 by separation on TLC, the attractiveness to bees disappeared. This indicated that the pigment was an attractive factor. We tested the pigment alone, as purified on TLC, and also other yellow compounds such as vegetable color for margarine, ginger and turmeric extracts, mineral colors and riboflavin, and we found that all were attractive to bees. When all five fractions were made equally yellow by adding appropriate amounts of riboflavin, the bees showed a preference for fraction 5, confirming that the color of the pigment was the attractive factor in fraction 3.

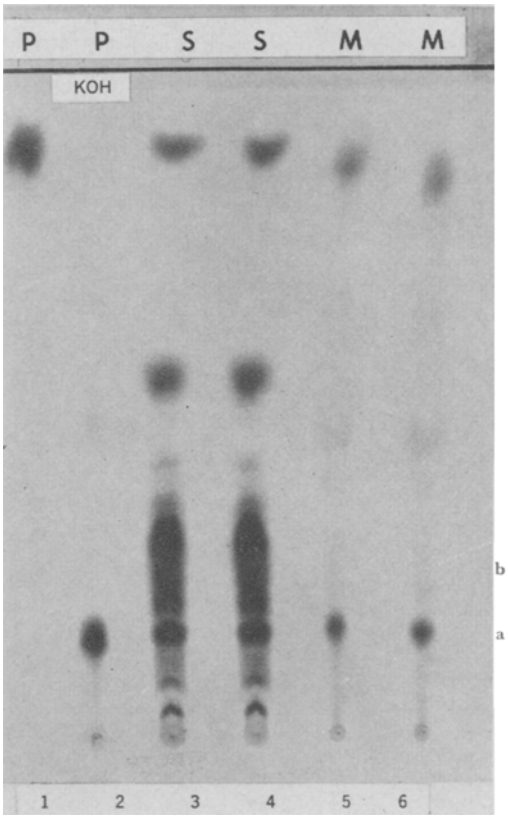

FIG. 1. TLC separation of pigments Solvent system—hexane-diethyl ether-acetone (60:30:20); a, lutein; b, chlorophyll; c, pigment ester. 1, pollen pigment; 2, hydrolyzed pollen pigment; 3 and 4, spinach pigments; 5 and 6, commercial leaf xanthophyll.

The pigment found in the extract was characterized by chromatographic and spectrophotometric methods (10). The presence of an ester was first established by alkaline hydrolysis of the intact pigment and by TLC analysis of the products, as shown in Fig. 1. Partition of the pigment between hexane and 90% methanol showed that it was epiphasic before hydrolysis, indicating that it was an acylated polar carotenoid.

Countercurrent distribution of the hydrolyzed pigment demonstrated that it was a xanthophyll (diol), the observed N_{100} value (tube number of the peak of a 100-transfer run) being 65, which was in good agreement with the N_{100} value of 70 reported by Curl and Bailey (8) for a diol. This excluded the possibility of a monol, a diol monoepoxide or diepoxide, or a polyol, which have N_{100} values of 96, 42, and 10 respectively. Moreover, the hydrochloric acid-ether color test was negative and indicated that the pigment was not an epoxide.

Figure 1 shows that the xanthophyll is a lutein, because of its chromatographic similarity with spinach lutein identified by Hirayama (6). Moreover, its spectral absorptions at 438 and 466 mμ in hexane, and 448 and 478 mμ in chloroform were close to those reported by Lepage and Sims (10) for wheat flour lutein,

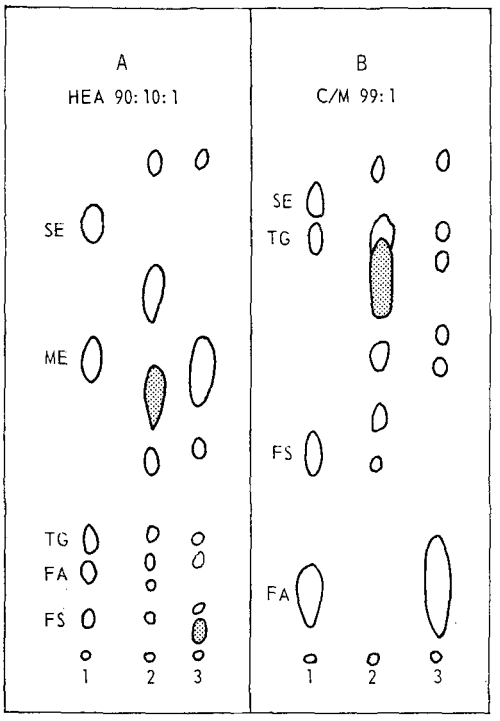

FIG. 2. TLC separation of pollen lipids. Spots were revealed with iodine vapors. A1, standard mixture (free sterols, free fatty acids, triglycerides, fatty acid methyl esters, sterol esters); A2, pollen extract before BF_3 methanolysis; A3, pollen extract after BF_3 methanolysis; B1, standard mixture; B2, column fraction 3; B3, column fraction 5. Dotted spots indicate location of pigments.

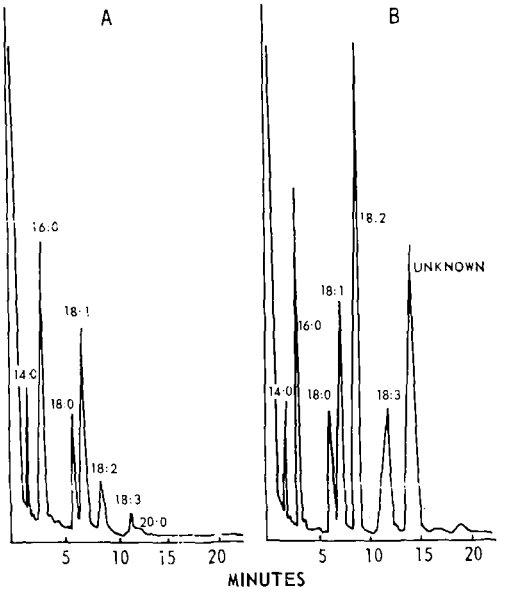

FIG. 3. GLC separation of methyl esters of fatty acids (A) from column fraction 3 and (B) from column fraction 5 which contained the unknown fatty acid. Separation on 5% DEGS at 170C.

and by Goodwin (11) for translutein. Zeaxanthin, which has a lower R_f value on TLC and absorbs at longer wavelengths than lutein, was not present in detectable amounts.

The Acidic Factor

The presence of a second attractive factor in pollen was demonstrated by column chromatography and bioassay as shown in Table I. Fractionation of fraction 5 by TLC, using the chloroform-methanol (99:1) solvent system, showed that the attractiveness was in the free fatty acid zone (Fig. 2B). When the free fatty acid zone was completely freed of sterols it remained attractive, while the sterols alone were not attractive. The free fatty acids were methylated with boron trifluoride in methanol and the products purified by TLC (Fig. 2A). It was found that the methyl esters were attractive.

The fatty acids, when analyzed by GLC as methyl esters (Fig. 3A), were myristic, palmitic, oleic, linoleic and linolenic acids. The fatty acids found in column fractions 2, 3, 4 and 5 were similar except that fraction 5 contained, in addition, an unusual fatty acid (Fig. 3B).

Bee-collected pollen, stored at –20C for 4-16 mon. contained ca. 20% moisture and 12% total lipids. For this study, however, the pollen was not dried prior to extraction, and the lipids were only incompletely extracted. The extract from a 1 kg pollen sample contained 39.7 g lipids. Following column chromatography, 755 mg were recovered from the free fatty acid zone on TLC, and GLC peak area measurements indicated that linoleic, linolenic and the unusual acid were 284, 67 and 203 mg, respectively.

It appears that it is this unknown fatty acid which rendered column fractions 5 attractive to bees. Its equivalent chain length (ECL) was 21.0 on a 5% DEGS column, but on a nonpolar 3% XE 60 column, its ECL was only 18.5, which excluded the possibility of a C_{20} or C_{21} fatty acid.

The unknown fatty acid methyl ester behaved as a dienoate (Fig. 4A) on silver nitrate Silica Gel chromatoplates and migrated together with methyl linoleate, although GLC analysis indicated that more of the unknown ester was in the lower half of the dienoate

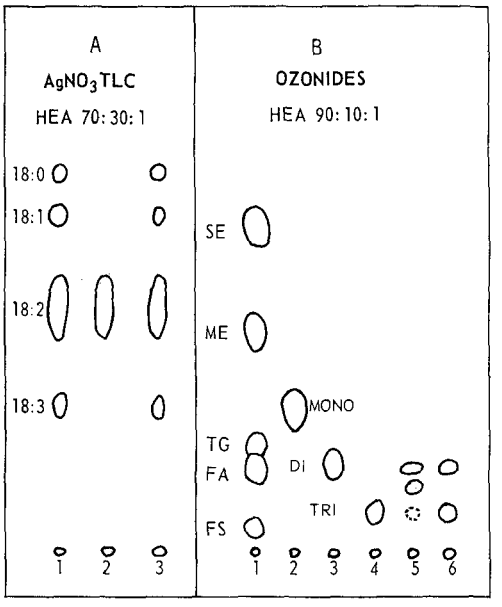

FIG. 4. TLC separation of fatty acids from column fraction 5; (A) methyl esters separated according to their degree of unsaturation, and (B) ozonides separated according to their degree of ozonization. A1, standard mixture of methyl esters; A2, methyl linoleate; A3, methyl esters of FA from column fraction 5 (see B3 in Fig. 2); B1, standard mixture (free sterols, fatty acids, triglycerides, methyl esters and sterol esters); B2, mono-ozonide from methyl oleate; B3, di-ozonide from methyl linoleate; B4, tri-ozonide from methyl linolenate; B5, ozonides from methyl linoleate and from the methyl ester of the unknown fatty acid, after 20 min ozonolysis; B6, after 40 min ozonolysis.

zone than in the upper half. All attempts to separate the two esters by using various TLC solvent systems have been unsuccessful.

Hydrogenation of the TLC fraction containing methyl linoleate combined with the unknown methyl ester gave a single product which had the same retention times on the two GLC columns as methyl stearate. This indicates that the unknown fatty acid is a straight-chain unsaturated C_{18} acid.

The TLC fraction containing the unknown methyl esters was further investigated by TLC analysis of its ozonides (Fig. 4B). When pure oleate, linoleate and linolenate were treated with ozone, the product separated on chromatoplates into three well defined spots which corresponded to the mono-, di-, and tri-ozonide respectively from oleate, linoleate and linolenate. When the TLC fraction containing the unknown methyl ester was subjected to the same treatment with ozone, it yielded two ozonides (Fig. 4B), of which the top spot corresponded to the di-ozonide derived from the linoleate, and a second spot of lower R_f value which, on further treatment with ozone, gave rise to an ozonide with the R_f value of a triozonide. As the unknown methyl ester migrated as a dienoate on silver nitrate Silica Gel plates (Fig. 4A), it seemed unlikely that it was a trienoate unless it contained a *trans* $\alpha\beta$ unsaturation which could allow it to migrate as observed (12). The infrared spectrum of the linoleate-unknown mixture supported this hypothesis.

Further attempts are being made to separate the unknown fatty acid from linoleic acid and to determine its chemical structure.

ACKNOWLEDGMENTS

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Novel Fatty Acids From the Royal Jelly of Honeybees (*Apis mellifera*, L.)

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ABSTRACT

Three new compounds isolated from the royal jelly of honeybees (*Apis mellifera*, L.) have been identified as 8-hydroxyoctanoic acid, 3-hydroxydecanoic acid and a dextrorotatory isomer of 3,10-dihydroxydecanoic acid.

INTRODUCTION

SECRETIONS OF THE MANDIBULAR glands of honeybees (*Apis mellifera*, L.) contain complex mixtures of unusual fatty acids. In the case of the queen honeybee, the major component of the mixture is the physiologically active 9-keto-*trans*-2-decenoic acid, which serves as a sex pheromone, and a control pheromone important in regulating the behavior of worker bees (1-3). The mandibular gland secretion of the worker honeybee occurs in the larval food substance, royal jelly. Under certain conditions it is possible to collect large amounts of royal jelly and to examine its chemical constitution in detail. The lipids of royal jelly were shown by Townsend and Lucas in 1941 (4) to consist principally of free fatty acids. Butenandt and Rembold (5) isolated from royal jelly 10-hydroxy-*trans*-2-decenoic acid, which is now often termed "royal jelly acid," and which has been the subject of a number of studies (6-9). Several other aliphatic hydroxy-acids, dicarboxylic acids and aromatic acids have more recently been isolated from royal jelly (8,10,11).

In a previous communication (11), we reported two new crystalline fatty acids from royal jelly. The present paper describes the identification of these compounds as well as an additional previously unidentified acid.

METHODS AND MATERIALS

New samples of components 5 and 7 described in our previous publication (11) were isolated in the following manner. Selective esterification of crude acids yielded a methyl ester fraction greatly enriched in saturated acids. Chromatography of this fraction on silicic acid (Unisil, Clarkson Chemical Co.) using petroleum ether (bp 30-60 C) and ethyl ether provided several fractions. Those fractions eluting with 0-20% ethyl ether contained the esters of dicarboxylic acids. The fractions eluted with 25-30% ether were rich in component 5, while those eluted with 50% ether were rich in component 7. These two components were isolated by preparative gas-liquid chromatography. We used an F & M model 500 instrument, having a 183 × 0.62 cm column packed with 10% EGSS-X on Diatoport S (Applied Science Laboratories) equipped with a thermal conductivity detector and operated at 180 C. Infrared spectra were determined with a Perkin Elmer Infracord. Optical rotary dispersion curves were determined with a Cary 60 spectropolarimeter. Mass spectra were determined using an Atlas CH-4 mass spectrometer equipped with a gas chromatographic inlet system (12). Synthetic 8-hydroxyoctanoic acid was prepared by the method of Smissman et al. (9). The method of Hauser and Karnovsky (13) was used to isolate D-3-hydroxydecanoic acid from *Pseudomonas aeruginosa* rhamnolipid prepared in our laboratory. Hydroxy acid esters were converted to the silyl ethers by treatment with bis-(trimethylsilyl)acetamide (14).

RESULTS

8-Hydroxyoctanoic Acid

The crystalline acid isolated previously (11), which had a melting point of 61-63 C, and was designated component 7, was converted to the methyl ester. The mass spectrum showed considerable similarity to that of methyl 8-hydroxyoctadecanoate (15) in the lower m/e region. Methyl 8-hydroxyoctadecanoate has a strong peak at m/e 173 resulting from cleavage between carbons 8 and 9. The spectrum of component 7 lacks this peak, indicating that the hydroxyl must be terminal or near terminal. On the basis of the gas-liquid chromatographic behavior of the methyl ester, the compound was considered to be 8-hydroxyoctanoic acid. The methyl ester of synthetic 8-hydroxy octanoic acid had identical gas-liquid chromatographic behavior with the isolated compound on either DEGS or SE-30 liquid phases, and the mass spectra of the two com-

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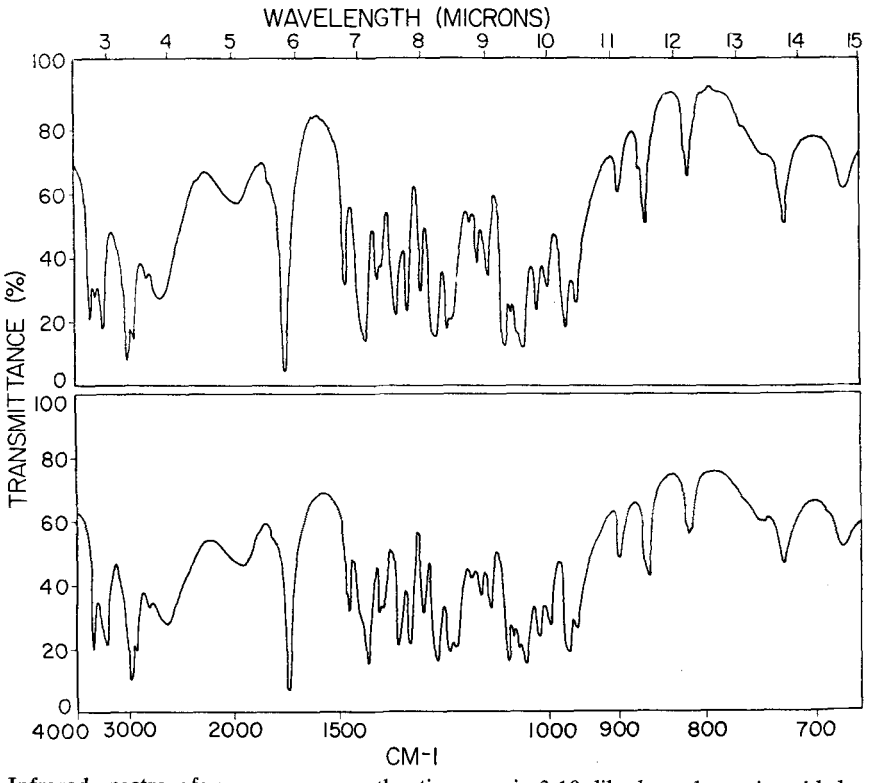

FIG. 1. Infrared spectra of: upper curve, authentic racemic 3,10-dihydroxydecanoic acid; lower curve, isolated dihydroxy acid.

pounds were identical. No parent ion was observed, but prominent ions of m/e 144 (M-30), 129, 124, 101, 96, 87, 74, 55 and 43 were observed. The spectra of the trimethylsilyl derivatives were much more definitive. No parent ion was evident, but a prominent peak at m/e 231 represents loss of a methyl group from the trimethylsilyl group. This behavior is typical of these derivatives (16). Peaks for the loss of methanol (m/e 199) and methanol plus water (m/e 181) from the m/e 231 ion were present, as well as prominent metastable peaks corresponding to these transitions. An intense peak at m/e 103 probably resulted from cleavage of the terminal methylene carbon bearing the silyl protecting group. The spectra of the derivatives of the authentic and isolated compounds are identical in all respects, including the positions of metastable peaks. Component 7 is therefore 8-hydroxyoctanoic acid.

TABLE I

Fatty Acids Isolated from Royal Jelly

Dicarboxylic Acids	Reference
$HOOC(CH_2)_8COOH$	8
$HOOC(CH_2)_7COOH$	10[a]
$HOOC(CH_2)_6COOH$	10[a]
$HOOC(CH_2)_5COOH$	10[a]
$HOOC(CH_2)_6CH{=}CHCOOH$	8
Monohydroxy Acids	
$HOCH_2(CH_2)_6CH{=}CHCOOH$	5
$HOCH_2(CH_2)_8COOH$	11
$HOCH_2(CH_2)_8COOH$	present work
$CH_3(CH_2)_6CHOHCH_2COOH$	present work
HO ⟨⬡⟩ CH_2COOH	16
$CH_3CHOH(CH_2)_7COOH$	17[b]
Dihydroxy Acids	
$HOCH_2(CH_2)_6CHOHCH_2COOH$	present work

[a] The dicarboxylic acids were identified only by their retention times on gas chromatography.

[b] The evidence (18) presented for this compound was indirect and inconclusive. We have never observed the presence of the compound in our royal jelly samples.

3-Hydroxydecanoic Acid

In a previous communication (11) we discussed an unknown acid which we had presumed to be suberic acid as reported by Pain et al. (10). It later became clear that component 5 was a mixture, and contained a hydroxy fatty acid. The hydroxyl group was resistant to acetylation, a property we have observed to be typical of β-hydroxy acids. This

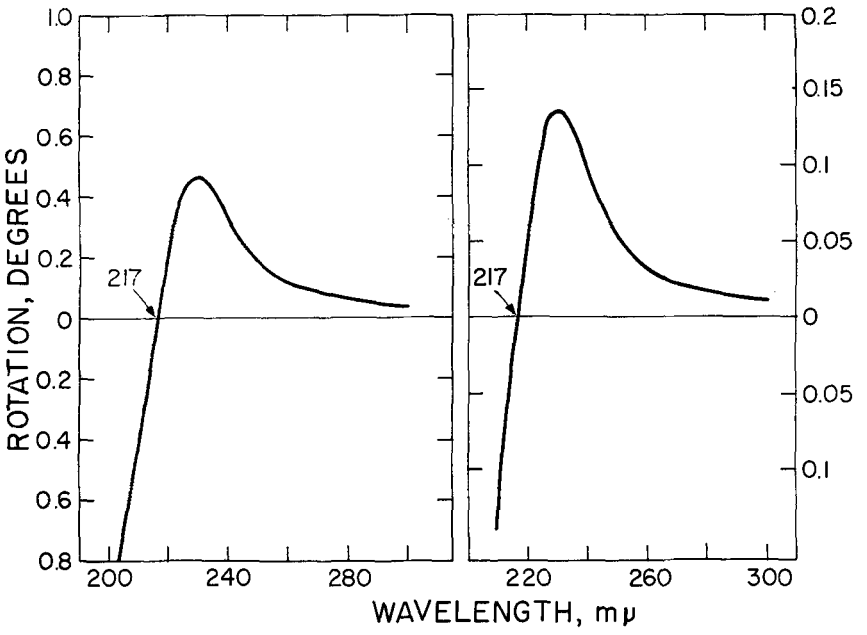

FIG. 2. Optical rotatory dispersion curves of 3, 10-dihydroxydecanoic acid from royal jelly (left), and D-β-hydroxydecanoic acid from *Pseudomonas rhamnolipid* (right). The 3,10-dihydroxy acid was determined in methanol at a concentration of 232 μmoles/ml. The β-hydroxydecanoic acid concentration was 102 μmoles/ml in methanol.

hydroxy acid was isolated as its methyl ester by preparative gas-liquid chromatography. Its chromatographic properties were identical to those of methyl 3-hydroxydecanoate ((11) Table I and II). Furthermore, mass spectrometry of the methyl ester gave a very intense peak at m/e 103 and essentially no peaks corresponding to larger ions. This characteristic of 3-hydroxyacid methyl esters (14) because of the facile cleavage between carbons 3 and 4. The mass spectrum of the trimethylsilyl derivative of the methyl ester gave unambiguous proof of the structure. No parent ion was observed, but an intense peak at M-15 (m/e 259) was present (16). Prominent peaks at m/e 201 and 175 represent cleavages at either side of carbon atom 3 with charge retention on the silyl-bearing fragment. The spectra of the trimethylsilyl derivative of authentic methyl-3-hydroxydecanoate and the isolated compound were identical. Component 5, therefore, contains 3-hydroxydecanoic acid. Unfortunately insufficient material was isolated for a determination of the optical rotation.

3, 10-Dihydroxydecanoic Acid

The dihydroxy acid $C_{10}H_{20}O_4$ described in the earlier publication (11) was converted to the methyl ester and examined in the mass spectrometer. The compound gave an unusually large peak at m/e 103, characteristic of methyl esters of 3-hydroxy acids (15). As in the case of the 3-hydroxydecanoic acid ester, this ion appeared to predominate to the extent that no peaks of significant intensity with m/e ratios higher than 103 were observed. The mass spectrum thus confirmed the previous assignment of one of the hydroxy groups to the 3 position, but failed to give information about the location of the other.

Smissman et al. (9), in a study of the synthesis of the royal jelly acids, prepared the DL-3,10-dihydroxydecanoic acid as an intermediate. Although the melting point of this racemic mixture (88-89 C) was quite different from that of the isolated compound (101.5-102 C) a sample of the compound was obtained from Smissman for comparison. The infrared curves of the two compounds proved to be identical in nearly every detail (Fig. 1). It seemed likely, therefore, that the isolated acid was one of the optical isomers of 3,10-dihydroxydecanoic acid.

The optical rotatory dispersion behavior of the isolated dihydroxydecanoic acid was observed and compared with that of D-3-hydroxydecanoic acid from the rhamnolipid of *Pseudomonas aeruginosa*. The curves are shown in Fig. 2. The dihydroxy acid from royal jelly is,

therefore, the dextrorotatory isomer of 3,10-dihydroxydecanoic acid.

DISCUSSION

Two fatty acids isolated in crystalline form from the royal jelly of honeybees have been identified as 8-hydroxydecanoic acid and the dextrorotatory isomer of 3,10-dihydroxydecanoic acid. A third acid was isolated as its methyl ester and shown to be 3-hydroxydecanoic acid. The various acids which have been characterized so far from royal jelly are shown in Table I.

The occurrence of the 3,10-compound is of considerable interest, since this may be either a precursor or metabolite of the major royal jelly acid, 10-hydroxy-2-decenoic acid. Essentially no information is available on the biosynthesis and metabolism of the royal jelly acids.

Several other minor components of the royal jelly fatty acid mixture (11) remain unidentified. We have examined mass spectra of several of these, but have failed to obtain any definitive information about these compounds. It seems likely that characterization of the minor components will necessitate isolation of substantial quantities of the esters by preparative gas chromatography.

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Triglyceride Sub-Classes of Various Dog Adipose Tissue Sites[1,2]

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ABSTRACT

Lipids were extracted from four dog adipose tissue sites, perirenal, pericardial, inguinal subcutaneous, and mesenteric. The triglycerides were purified by thin-layer chromatography and the sub-classes separated by silver nitrate-silica gel plates. Gas chromatography was used to delineate the fatty acid composition of each sub-class. The major sub-class was 011, followed by 012, 001, 111 and 112 in that order (0 = saturated fatty acid; 1 = one double bond; 2 = two double bonds). The other components were present in minor amounts. The pericardial triglyceride had more 012 while the perirenal adipose tissue contained less 011 and more 001 than other sites. The other sub-classes were essentially similar.

The distribution, based on 100%, of each saturated fatty acid was not the same in all sub-classes, although the over-all average was similar in each site.

INTRODUCTION

RECENT ADVANCES in methodology have enabled the investigator to distinguish double bond sub-classes of triglycerides with relative ease. Barrett et al. (1) were able to accomplish this task by utilizing silver-nitrate-impregnated silica gel on thin-layer plates. This technique has been applied to triglycerides of adipose tissue in various sites and species (2-4).

This study was undertaken to survey the triglyceride structures of various adipose sites in a dog fed a general meat diet ad lib.

METHODS

A 20-kg male mongrel dog was procured from the Hahnemann Medical College animal colony. This animal had been in the colony for at least 1 month and had been fed ad lib. Thrivo or Ken-L-Ration. The dog was anesthetized with Nembutal and exsanguinated. A sample of adipose tissue from perirenal, pericardial, inguinal subcutaneous and mesenteric sites was excised, weighed and immediately homogenized in a Waring Blender with 20 vol of chloroform-methanol (2:1). The material was filtered into a separatory funnel, overlayed with 0.2 vol of physiological saline and allowed to stand overnight. The lower chloroform phase was removed, dried with anhydrous magnesium sulfate, and evaporated to a small volume in a Buchler rotary evaporator under vacuum. The solution was transferred to a screw top tube and evaporated to dryness under nitrogen. The residue was dissolved in an appropriate amount of hexane, sealed with a teflon lined cap, and stored at –20C until further work-up.

An anti-oxidant was added to the homogenates and stored material, 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline, Santoquin (K and K Laboratories, Inc.).

Silica gel G thin-layer plates (0.5 mm thick, 20 × 20 cm) were prepared to separate the triglycerides from the other lipid classes. A solvent system of hexane-ethyl ether-acetic acid (78:20:2) was used in a chamber lined with filter paper to insure saturation. The total lipid extract was applied 1.5 cm from the base across the plate in an amount equivalent to 100 mg with a Pelick-Radin applicator (Applied Science, Inc.) and the solvent allowed to rise to a line cut into the gel 18 cm from the bottom. The plate was sprayed with 0.02% 2′,7′-dichlorofluoroscein (pH 7) to visualize the various bands, which were identified by standards obtained from Applied Science, Inc.

The triglyceride band was scraped from the plate and eluted from the silica gel with 5% methanol in ether. Fluoroscein dye was removed on a small Unisil column (Clarkson Co.). The procedure was repeated with sufficient plates to prepare 50-100 mg of triglyceride from each tissue. Triglycerides were dissolved in hexane and stored at –20C with the addition of Santoquin.

An aliquot of the triglyceride solution was taken to determine its fatty acid composition. The hexane solution was placed in a screw top tube, evaporated to dryness under nitrogen, and

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[2]Presented at the AOCS Fall Meeting, Philadelphia, 1966.

TABLE I
Fatty Acid Composition of Various Dog Adipose Tissue Sites

Adipose tissue site	Fatty acid composition (Mole %)							
	14:0[a]	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Mesenteric	3.0	21.0	5.7	8.6	46.6	13.7	0.9	0.2
Subcutaneous	3.1	21.9	6.4	6.9	45.9	13.9	1.1	0.3
Pericardial	3.1	22.8	4.7	10.3	45.4	12.9	0.5	....
Perirenal	1.6	20.8	4.9	11.0	44.3	15.6	1.3	0.2

[a] Fatty acid carbon length: Number of double bonds.

0.2 ml of concentrated sulfuric acid, 0.2 ml 2,2-dimethoxypropane, and 5 ml methanol were added to the dried material. The tube was closed with a teflon-lined cap and heated at 40C for 2 hr. The methyl esters were extracted by addition of 10 ml of cold distilled water and 5 ml of hexane. The mixture was shaken and the hexane removed with a Pasteur pipette. Two more 5-ml additions of hexane were used and the hexane extracts pooled, dried with sodium sulfate, and evaporated under nitrogen. The residue was dissolved in an appropriate amount of hexane and analyzed by GLC.

A Beckman model GC-4 or Perkin-Elmer Model 800 instrument was used with a 6 ft × ⅛-in. stainless steel column washed with methanol followed by 5% dichlorodimethylsilane in chloroform. The column was packed with 10% diethylene glycol succinate polymer on silanized Chromsorb W, 80-100 mesh (Applied Science, Inc.). The column was maintained at 180C, isothermal, with a flow rate of 40 ml/min of helium. Hydrogen (from a hydrogen generator, Milton-Roy Co.) at 30 ml/min and air at 250 ml/min were used in a flame ionization detector. The inlet was heated at 250C and the detector compartment at 275C. The injection volume was 0.5 μl or 1.0 μl.

Triglyceride sub-classes were separated on 25% silver nitrate-silica gel H (wt/wt) thin-layer plates (0.5 mm). A wedge was cut into the gel, the apex being 4 cm wide and each leg being constructed so that it exited 10 cm from the bottom of the plate when measured from the side. A blank plate was covered with filter paper which was held in place by thread. Strips of polypropylene (3 mm thick) were placed on three sides of the silver nitrate plate, which was scraped clean of silica gel on the edges. Then the filter paper plate was placed over the silver nitrate one and onto the polypropylene strips, and clamped into place with spring clips. The paper was placed to face the gel and was separated from it by approximately 2.5 mm.

This arrangement was used since the "sandwich" construction without filter paper made the development time lengthy, even though the close approximation of the cover plate should have facilitated rapid attainment of equilibrium. The "sandwich' was placed in a tank and developed in a solvent system of ethyl ether-benzene (10:90) to a height of 18 cm. The plate was remoed, dried under nitrogen, and run in a second system consisting of benzene-petroleum ether (b.p.36-52C), (80:20), also in a "sandwich" configuration. The plate was dried and sprayed with 0.02% 2',7'-dichlorofluoroscein to ascertain the bands under ultraviolet light.

The use of a wedge conferred the advantage of spreading the bands with the largest concentration across the top part of the plate in a semicircle while those with a small amount were spread the least, near the origin. This type of geometry also assisted in separation of bands of small concentration adjacent to ones of high concentration. The double development produced three bands of triglycerides containing one double bond.

The usable range of concentration, applied to the origin of the wedge with this type of material, was 5-25 mg. The most satisfactory amount, with respect to separation and assay, was approximately 15 mg.

The bands were scraped from the plate and eluted with 5% methanol in ether if the triglyceride carbon number was to be determined, otherwise, the silica gel was placed directly in the methylation mixture with a measured quantity of heptadecanoic acid as internal standard and assayed for fatty acid composition, as detailed previously.

The gas-liquid chromatographic apparatus was calibrated against metabolism study section (NIH) standard mixtures, A, B, C, and D, testing linearity and mass sensitivity of the detector as well as resolving power of the column. The analyses checked within 1.5% (actual percentage composition) of stated values. The area under each peak was calculated by measuring the height and width at half height. The mole per cent of each peak was computed.

TABLE II

Silver Nitrate Thin-Layer Chromatographic Run of Dog Subcutaneous Adipose Tissue Triglyceride

Band	Fatty acid Concentration (mμM)	Major triglyceride sub-class	Fatty acid composition in mole %							
			14:0[a]	16:0	16:1	18:0	18:1	18:2	18:3	20:4
1	118	....	8.0	39.2	7.9	16.7	28.2	....	....	....
2	255	000	10.8	65.8	....	23.4	....	....	....	....
3	242	001	8.1	39.6	....	20.1	32.2	....	....	....
4	1227	001	2.5	39.9	3.1	22.4	32.1	....	....	....
5	1269	001	7.7	41.6	4.8	11.7	34.2	....	....	....
6	456	011	7.7	29.5	8.6	6.5	47.8	....	....	....
7	4346	011	2.8	25.2	8.3	8.7	52.6	2.4	....	....
8	731	002	3.8	21.7	6.3	7.9	40.9	17.3	0.6	....
9	2287	111	0.7	3.1	11.7	0.9	79.5	3.5	0.4	....
10	2478	012	....	19.9	6.1	9.9	34.0	30.2	....	....
11	463	012	4.4	13.6	10.9	2.7	35.4	33.1	....	....
12	2009	112	....	0.4	6.3	....	59.1	34.3	....	....
13	144	112	....	8.7	25.7	5.0	23.0	37.5	....	....
14	595	022	....	15.1	1.3	32.9	4.0	45.3	1.6	....
15	541	122	....	7.8	0.5	2.9	33.9	46.6	8.4	....
16	445	014	....	11.1	5.5	2.3	28.9	17.4	12.3	22.5
17	38	....	....	28.5	8.8	5.3	36.9	20.4	....	....
18	694	222	....	....	4.1	....	....	92.5	3.5	....
19	....	....	....	....	....	....	....	....	....	....

[a] Carbon number: Double bond content.

Duplicate runs on silver nitrate thin-layer chromatography showed a relative deviation of about 10% for the major components, while the average absolute per cent difference was 1.2%.

RESULTS

Table I lists the fatty acid composition, in mole per cent, of the mesenteric, subcutaneous, pericardial and perirenal adipose tissue triglyceride. Oleate was highest in concentration followed by palmitate, which was half as large. Linoleate was next in level and somewhat lower was stearate; myristate and palmitoleate were minor components. The values were similar among the different sites and no distinguishing trend was discernible.

Tables II through V demonstrate typical separations of triglycerides from each site. The larger area above the least polar triglyceride band was analyzed for components eluting with the retention time of the standard fatty acids found in triglycerides. These are designated band 1. Spaces between visible bands were also assayed since spreading into these areas was found from adjacent large components. For example, in Table II these non-fluorescing bands were 1, 11, 13 and 17.

TABLE III

Silver Nitrate Thin-Layer Chromatographic Run of Dog Mesenteric Adipose Tissue Triglyceride

Band	Fatty acid concentration (mμM)	Major triglyceride sub-class	Fatty acid composition in mole %							
			14:0[a]	16:0	16:1	18:0	18:1	18:2	18:3	20:4
1	19	....	....	100.00	....	....	....	....	....	....
2	249	000	13.9	56.8	5.1	21.8	2.4	....	....	....
3	118	001	8.6	39.3	10.5	15.6	26.1	....	....	....
4	680	001	2.7	37.8	3.5	24.7	31.4	....	....	....
5	1217	001	10.6	41.6	4.7	11.3	31.9	....	....	....
6	362	011	....	22.9	10.2	10.4	56.5	....	....	....
7	3207	011	2.6	23.1	7.3	8.6	55.8	2.6	....	....
8	627	002	1.9	26.6	7.3	7.3	37.6	19.3	....	....
9	1675	111	....	1.9	10.2	1.4	83.8	2.6	....	....
10	1822	012	....	18.3	7.1	8.5	35.5	30.6	....	....
11	342	012	....	11.4	15.0	2.0	41.4	30.1	0.2	....
12	1021	112	....	....	7.8	....	57.0	35.2	....	....
13	186	112	....	6.5	31.3	3.0	25.4	33.2	0.7	....
14	243	022	....	20.7	3.4	10.6	3.5	58.7	3.1	....
15	104	....	....	16.9	10.7	4.9	18.8	38.6	10.0	....
16	342	....	....	7.0	5.3	2.4	29.9	46.4	9.1	....
17	66	....	....	15.9	15.3	5.4	34.2	13.9	15.4	....
18	158	....	....	15.0	10.2	5.6	25.8	19.3	15.2	9.0
19	78	....	....	16.9	17.1	4.6	34.9	26.6	....	....

[a] Carbon number: Double bond content.

TABLE IV

Silver Nitrate Thin-Layer Chromatographic Run of Dog Pericardial Adipose Tissue Triglyceride

Band	Fatty acid concentration (mμM)	Major triglyceride sub-class	Fatty acid composition in mole %							
			14:0[a]	16:0	16:1	18:0	18:1	18:2	18:3	20:4
1	39	....	....	62.6	....	37.4	....	....	....	....
2	80	000	21.0	39.0	....	27.7	12.2	....	....	....
3	102	001	17.1	40.6	....	22.0	20.2	....	....	....
4	1090	001	6.2	41.2	3.7	18.6	30.3	....	....	....
5	1643	001	13.1	38.9	5.5	10.4	32.2	....	....	....
6	968	011	6.4	25.2	6.8	11.1	50.5	....	....	....
7	5387	011	2.2	22.1	6.7	10.9	54.7	3.5	....	....
8	588	002	4.5	25.6	7.2	13.0	32.5	17.2	....	....
9	3638	111	....	3.0	6.6	1.6	84.5	4.3	....	....
10	4220	012	1.3	17.3	6.1	10.3	35.5	29.5	....	....
11	743	012	....	13.0	8.6	2.9	38.8	36.7	....	....
12	2425	112	....	1.3	5.1	0.6	58.3	34.7	....	....
13	346	112	....	9.1	18.6	8.3	28.8	35.2	....	....
14	639	022	....	20.5	1.5	14.5	3.2	60.3	....	....
15	251	....	....	23.0	....	12.6	17.0	47.4	....	....
16	588	....	1.9	8.2	2.5	4.8	31.2	42.8	8.6	....
17	240	....	1.4	17.2	4.6	8.4	36.6	15.9	15.9	....
18	226	....	....	20.1	3.9	14.2	30.3	19.9	11.6	....
19	273	....	....	18.5	3.9	8.4	34.4	29.1	5.8	....
20	38	....	9.7	43.8	2.9	43.7	....	....	....	....

[a] Carbon number: Double bond content.

The sequence of double bond structures was the same as reported extensively by Gunstone and Padley (5) for silver nitrate-impregnated silica gel. The number of double bonds in each fatty acid was represented by 0, 1, 2, 3, and 4. No attempt was made to assign a position on the glycerol moiety. The observed pattern was 000 as the least polar lipid (at the top of the thin-layer plate), followed by three bands of 001; next was the major species 011 and a minor constituent 002. The next two components were 111 and 012 succeeded by 112 and so on, in the expected sequence. A few bands at the bottom of a plate could not be defined as a single type of structure and were probably mixtures of several sub-classes, or minor impurities, such as lower glycerides, free fatty acids or undetected polyunsaturated acids.

The author would like to propose that the numerical listing of double bonds be given preference over an alphabetic one such as S

TABLE V

Silver Nitrate Thin-Layer Chromatographic Run of Dog Perirenal Adipose Tissue Triglyceride

Band	Fatty acid concentration (mμM)	Major triglyceride sub-class	Fatty acid composition in mole %							
			14:0[a]	16:0	16:1	18:0	18:1	18:2	18:3	20:4
1	65	....	11.0	39.3	....	28.7	21.0	....	....	....
2	867	000	12.1	59.0	....	26.3	2.7	....	....	....
3	279	001	9.6	43.1	....	21.8	25.5	....	....	....
4	2355	001	4.1	38.4	3.3	21.0	33.2	....	....	....
5	2555	001	8.7	40.2	4.9	12.6	33.6	....	....	....
6	618	011	6.5	22.5	7.2	9.2	54.7	....	....	....
7	3541	011	2.2	20.3	5.5	12.2	56.0	3.8	....	....
8	1389	002	5.9	27.5	5.9	9.8	28.3	22.6	....	....
9	3292	111	....	2.0	8.3	1.4	84.9	3.4	....	....
10	4271	012	2.0	18.3	6.6	9.8	36.6	26.7	....	....
11	587	112	3.8	9.9	9.9	2.9	41.0	32.6	....	....
12	2781	112	....	0.7	5.3	0.1	61.5	32.4	....	....
13	290	112	2.1	8.1	20.1	4.4	34.0	31.5	....	....
14	782	022	1.7	19.6	2.4	13.0	3.9	57.1	2.4	....
15	285	....	7.3	22.3	7.7	8.5	17.4	32.4	4.4	....
16	759	....	1.5	8.4	3.8	4.2	30.8	43.5	7.8	....
17	197	....	3.9	15.6	7.4	9.9	27.4	27.2	8.6	....
18	220	....	3.4	12.0	6.0	6.9	40.7	4.9	17.4	8.7
19	273	....	....	17.7	4.2	10.6	21.5	24.6	10.3	11.1
20	197	....	....	12.0	4.2	7.5	35.2	20.2	8.7	12.1
21	140	....	....	18.7	3.8	9.9	25.6	26.6	5.2	10.3
22	43	....	....	37.9	....	62.1	....	....	....	....

[a] Carbon number: Double bond content.

for saturated, M for monoene, D for diene, etc. (6) or U_1 for monoene, U_2 for diene, etc. (4), or O for monoene, L for diene (3).

While three bands of 001 were clearly present, over-lapping occurred to varying degrees, depending on the particular plate. In general, the first band contained more myristate than the second, while the second 001 had more stearate and less myristate than the third band.

The solvent system also produced a large amount of overlapping into the 002 sub-class, although it gave some separation of the 112 sub-class into 18:1, 18:1, 18:2 and 16:1, 18:1, 18:2. The latter component was more polar and moved slightly behind the former, which was the major substance in this sub-class. Also resolved into two fairly pure sub-classes were those triglycerides containing three double bonds, i.e., 111 and 012.

Table VI contains data showing the saturated fatty acid composition in the sub-classes of various adipose tissues. The percentages of saturated fatty acids were summed and equated to 100% and the individual percentages calculated. The 022 band was not included in the calculation of the averages since it exhibited a lower myristate and higher stearate than the other sub-classes. The over-all averages for each fatty acid were identical in the different tissues. The palmitate value was remarkably constant while the myristate and stearate varied inversely.

In Table VII the double bond sub-classes in the four adipose tissues investigated are found, displayed as their percentage distribution. The three 001 bands were summed and a single percentage is shown. Also collected into one value were the ill-defined components at the bottom of the thin-layer plates. The data was qualitatively similar for the various sites. The major sub-class was 011, followed by 012, 001, 111, and 112 in that order. The others were present in minor amounts. Each sub-class was corrected by subtracting any overlapping components.

The pericardial triglyceride had more 012 while the perirenal adipose tissue was observed to contain less 011 and more 001 than other sites. The other sub-classes were essentially similar in the various fat depots, and their differences did not exceed the experimental variation.

DISCUSSION

Data on dog adipose tissue triglyceride sub-classes is nonexistent in the literature. Some information is available for rat and human tissue. Kaunitz et al. (3) reported results taken from human autopsy material. There was considerable variation among the individuals, as might be expected from this type of source material. To obtain some estimate of mean values I averaged all those for perirenal adipose tissue sub-classes in their paper. The result was 000, 3.3%; 001, 18.0%; 011, 33.3%; 002, 4.2%; 111, 16.4%; 012, 15.3%; 112, 7.8%; 022, 1.2%. Privett et al. (2) investigated various rat tissues using different types of fat in the diet as well as one group which was fat-free. They used the epididymal fat to represent depot triglycerides.

When the perirenal results in this paper are compared to those of Kaunitz et al. (3) it can be seen that 011 in humans was appreciably higher while the other sub-classes approximate one another.

TABLE VI

Saturated Fatty Acid Composition of Double Bond Sub-Classes in Various Adipose Tissue Sites (Mole %) Based on 100%

	000	001	001	001	011	002	012	022	Average
Subcutaneous adipose tissue									
14:0	11	12	3	13	18	11	tr[a]	tr	10[b]
16:0	66	59	62	68	68	65	67	32	65
18:0	23	30	35	19	15	24	32	69	25
Pericardial adipose tissue									
14:0	27	21	9	18	6	10	5	tr	14[b]
16:0	50	51	62	53	63	59	60	59	57
18:0	35	28	28	14	31	30	36	41	29
Mesenteric adipose tissue									
14:0	14	14	4	17	8	5	tr	tr	9[b]
16:0	57	62	58	66	67	74	68	66	65
18:0	22	25	38	19	25	20	32	34	26
Perirenal adipose tissue									
14:0	12	13	7	14	6	14	7	5	10[b]
16:0	59	52	61	65	59	64	63	57	60
18:0	29	29	33	21	35	23	34	38	29

[a] tr = trace.

[b] The average value does not include the 022 band.

TABLE VII

Percent of Total Triglyceride Based on Unsaturation Classes in Various Adipose Tissue Sites in Dog

Sub-classes[a]	Mesenteric	Sub-cutaneous	Pericardial	Perirenal
000	1.5	3.1	0.9	2.7
001	16.3	13.6	11.6	19.7
011	29.5	28.6	28.2	23.3
002	3.6	2.1	1.3	3.7
111	13.8	12.4	15.5	13.6
012	17.5	16.0	21.1	17.6
112	10.6	11.7	11.8	10.4
022	2.4	6.2	3.6	2.7
OTHERS	4.9	6.2	6.0	6.3

[a] 0—Saturated fatty acid; 1—One double bond; 2—Two double bonds.

Values represent an average of duplicates. Sub-class fatty acid position is not assigned.

In the various dog adipose sites the subclasses exhibited a moderately uniform distribution, the most marked difference being in the perirenal profile. This could be due to differences in innervation, volume of the vascular bed and concentration or specificity of various enzymes among many possibilities (7). It has been pointed out that generally the mesenteric and omental areas are the most active with respect to fatty acid metabolism, followed closely by other internal depots, while the subcutaneous adipose tissue triglyceride is less active (7).

An interesting observation was made by Hollenberg (4). He fractionated rat fat pad triglycerides on silver nitrate-silica gel and measured the fatty acid composition. He recalculated the saturated fatty acid distribution in various bands on the basis of 100%. The sub-classes were 000, 001, 011 + 002, 012, 022. All except the 022 band showed a similar pattern, 14:0, 7%; 16:0, 80%; 18:0, 13% with the 4 double bond group having the highest 18:0, being 17%. Kaunitz et al. (3) also published this type of data. I have averaged their perirenal adipose tissue results which were 14:0, 15%; 16:0, 64%; 18:0, 19%. In the data shown here, the average values were uniform from tissue to tissue, being overall 14:0, 11%; 16:0, 62%; 18:0, 27%. A comparison of this data from dog, rat and human shows similarities as well as differences. Rat had a higher 16:0 while dog exhibited a larger 18:0 and human a somewhat increased 14:0. The 022 band in the dog, in most sites, had the greatest 18:0, in agreement with Hollenberg's results in the rat. The subcutaneous data for the 022 band in Table VI was different, the 18:0 being higher than 16:0. Whether this will be found in other dogs remains for further experimentation. The three 001 bands did not have the same saturated fatty acid distribution.

There did appear to be a general trend for the saturated fatty acids to have a similar distribution in most of the double bond subclasses. The average value would lead to this conclusion, but one cannot automatically assume all bands have the same profile.

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Acetoacetate Metabolism of Rats Fed High Fat or Restricted Calorie Diets[1]

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ABSTRACTS

Ethyl-^{14}C-acetoacetate was used to trace oxidation and metabolism of acetoacetate when rats were fed a high fat diet (80% of total calories from beef tallow or corn oil, carbohydrate free), a high carbohydrate diet (2% corn oil) or a high carbohydrate diet with restriction of calories to one half of ad lib. consumption for two weeks. The rate of expiration of $^{14}CO_2$ in all groups of animals did not differ significantly and was not related to plasma concentration of acetoacetate. The high fat diets slightly enhanced the oxidation of acetoacetate to $^{14}CO_2$ over a 3 hr period compared to other diets. Incorporation of acetoacetate into fatty acids did not differ significantly among groups. Rats fed the high carbohydrate diet ad lib. incorporated into liver cholesterol more acetoacetate than did any other group, but dietary unsaturated fat resulted in greater incorporation of acetoacetate into cholesterol than saturated fat. High calorie and high beef tallow groups were ketonemic but the low concentration of plasma acetoacetate in rats fed a high corn oil diet indicates that unsaturated fatty acids are not ketogenic. The data show that utilization of acetoacetate is not significantly reduced in a ketonemic condition and support the premise that overproduction of ketone bodies is the cause of ketonemia. Rats appeared to be normal during the two-week period when no carbohydrate was included in the diet.

INTRODUCTION

THE ONSET of ketosis is known to depend on a number of factors, among them the level of fat in the diet. Dekaban and Mizel (1) reported that if the dietary fat is increased to over 80% of the total calorie intake, significant changes occur in the blood ketone body level in young children. Mayes (2), however, found that ketonemia of rats, initiated by starvation, was markedly reduced by consumption of approximately 6 g of butter fat per day; less than 6 g/day did not reduce ketonemia. He established a calorie deficiency hypothesis (3) to explain ketosis. Freund (4), found that once maximum starvation ketosis has developed, a high fat diet no longer had an anti-ketotic effect. Sinclair (5) has suggested that unsaturated fatty acids should be antiketogenic.

Extrahepatic tissues can oxidize ketone bodies and use them as fuel of respiration. Nelson et al. (6) and Wick and Drury (7) have demonstrated that the utilization of acetoacetate increases with rising blood concentration until the maximum is reached, after which the utilization rate becomes constant. Wick and Drury (7) also pointed out that the upper limit of utilization was reached when the oxidation of acetoacetate was about 90% of the O_2 consumed by the rabbit. During ketosis the turnover rate of infused acetoacetate in the pregnant ovine was directly proportional to the plasma acetoacetate concentration until a maximum concentration of about 10 mg/100 ml (0.17 mM/liter) was obtained [total ketone body expressed as acetoacetate would be about 20 mg/100 ml (0.35 mM/liter)] (8).

Ketosis has been attributed to overproduction of ketone bodies or to underutilization by the peripheral tissues. The former of these two possibilities is supported by the work of Sauer (9) and Bergman and Kon (10). Overproduction is considered to be the result of oxidation of fatty acids derived from a high fat diet (1) or from mobilization of depot fat (9,10).

Underutilization of ketone bodies has been observed in diabetic ketotic rats (11). Foster (12) has suggested that the control of acetoacetate metabolism is located at the site of regulation of acetoacetate synthesizing enzymes and mediated by insulin. Foster's data indicate that increased acetoacetate synthesis during starvation was not initiated by changes in fatty acid oxidation, fatty acid synthesis, increased free fatty acid concentration in blood, nor by increased acetyl-CoA concentration in the liver.

The present study contributes evidence to support the concept that underutilization of ketone bodies is not a causative factor in ketosis of semi-starvation or consumption of a high fat diet and differentiates between the

[1]Presented at the AOCS Meeting, Chicago, October, 1967.

TABLE I
Composition of Diets (Wt %)

	Group 1 control	Group 2 ½ calories	Group 3 high beef tallow	Group 4 high corn oil
Casein[a], vitamin-free	15	30	26	32.5
Beef tallow[b]	0	0	51.6	0
Corn oil[c]	2	4	3.4	43.8
Corn starch[a]	73	46	0	0
Cellulose[a]	4	8	8.9	11.1
Salt mixture[a], Jones and Foster	4	8	6.7	8.4
Vitamin mixture[d]	2	4	3.4	4.2

[a] General Biochemicals, Inc., Chagrin Falls, Ohio.

[b] Beef kidney depot fat obtained from the department of Animal Science, CSU, and rendered by grinding, heating and filtering.

[c] Mazola, obtained on local retail market.

[d] Vitamin mixture in cellulose provided in mg/kg ration: thiamin HCl 50, riboflavin 50, pyridoxine HCl 50, pantothenate-Ca 200, niacin 200, folic acid 20, biotin 2, vitamin B_{12} 0.3, menadione 40, calciferol 1.0, vitamin A acetate 30, α-tocopherol 1,000, p-aminobenzoic acid 2,000, inositol 2,000, choline Cl 20,000.

ketotic effects of saturated and unsaturated dietary lipids.

EXPERIMENTAL PROCEDURES

Animals, Diets and Design of Experiment

Forty female rats, (Sprague-Dawley derived, Laboratory Suppliers, Atlanta, Georgia) 3 months old, weighing 140 to 190 g were distributed into four groups of 10 rats. Animals were housed in individual wirebottom cages. Group 1 received a high carbohydrate diet (Table I) and served as the control group. Rats in group 2 were restricted in calorie intake to one half the ad lib. consumption of group 1. The diet contained doubled quantities of corn oil, casein, vitamin and salt mixtures so that only carbohydrate was reduced. Group 3 and group 4 received a carbohydrate-free diet high in beef tallow or corn oil. It was necessary to increase the solid ingredients in the high corn oil diet to obtain a palatable product (Table I). All diets were stored in the refrigerator. Food intake and weight gain of individual animals were recorded weekly.

At the end of a two-week feeding period, each rat was given an intraperitoneal injection of a 3-5 μc of ethyl-3-^{14}C-acetoacetate (3.7 mc/mM, Nuclear-Chicago Corp.) in 0.3-0.5 ml of physiological saline solution (0.9%). The ethyl-acetoacetate was not hydrolyzed prior to injection. Immediately after injection, the animal was placed for 3 hr in a respiration chamber for the collection of CO_2. Collection was made by suction of CO_2-free air through the chamber and into NaOH solution. At the end of each half hour interval, the NaOH solution in the traps was changed. After 3 hr the rat was anesthetized with Na-pentobarbital, the liver rapidly excised, weighed, wrapped in aluminum foil and stored frozen (–15 C) until analyzed. A blood sample was extracted by heart puncture, collected in a heparin coated tube, and chilled in an ice bath. After the blood sample was centrifuged, the plasma was transferred to a vial and held in an ice bath until assayed for acetoacetate within 2 hr after collection.

Chemical and Radiochemical Analyses

Liver lipids were extracted by the method of Folch et al. (13). A sample of the chloroform-methanol (C-M) extract was dried and weighed to determine total fat. Total lipid ^{14}C was determined by counting a dried sample in a Beckman Scintillation System (efficiency, 92.7%) in a toluene solution containing 0.05 g/liter 1,4-Di-2-(5-phenyloxazolyl)-benzene and 4 g/liter 2,5-diphenyloxazole. Samples of the C-M solution were saponified and extracted twice with petroleum ether for the precipitation of cholesterol as digitonide for counting (14) and for determination of total cholesterol by the Zak, et al. method (15). The concentration of acetoacetate in plasma was determined by the nitroprusside reaction as adapted by Schilke and Johnson (16). Thirteen minutes was the time required for maximum color development instead of 18 min as described by the authors. Fatty acid composition of serum lipids was determined for those rats for which sufficient serum was available. Serum lipids were extracted in the same way as liver lipids. Samples of the lipid extract were dried under N_2 and methylated by heating with BF_3-methanol reagent at 60 C for 45 min. (17). Methyl esters were separated by gas chromatography using an F + M Model 5750 with a flame ionization detector equipped with a stainless steel column 6 ft × 1/8 in. packed with 6% diethylene glycol succinate on chromosorb W, 60-80 mesh. Helium was the carrier gas (50 p sig) and the temperature was programmed from 140-215 C at 2°/min.

Samples of the NaOH-CO_2 solution were pipetted onto a piece of filter paper (Whatman No. 1) which was put in a scintillation counting vial and scintillation solution was added for counting. Standard acetoacetate was counted to determine dose in counts per minute and quenching by the filter paper and NaOH solution.

RESULTS AND DISCUSSION

Body weight and the results of analyses of the liver lipids are shown in Table II. Weight

TABLE II
Body Weight and Liver Lipids of Rats Fed Diets Varying in Fat and Calorie Concentration

Diet	Body Weight		Liver			
	Initial	Final	Weight	Total Lipid		Cholesterol
	g	g	g	mg	%	mg
High CHO[a]	195[b] ±5.13	210 ±6.78	7.6 ±0.36	357 ±26.5	4.6 ±0.25	18.7 ±0.68
High CHO—½ cal.	198 ±8.38	152 ±5.60	4.3 ±0.20	225 ±25.5	5.2 ±0.45	11.1 ±0.59
High beef tallow	194 ±9.52	222 ±9.60	8.2 ±0.31	725 ±79.4	8.8 ±0.82	21.6 ±1.37
High corn oil	198 ±7.60	216 ±6.51	7.9 ±0.24	503 ±40.8	6.4 ±0.51	24.7 ±2.92

[a] High CHO = high carbohydrate, 2% corn oil. High CHO—½ cal. = food intake limited to one half of ad lib. consumption of high carbohydrate diet. High beef tallow = about 82% of calories as beef tallow, no carbohydrate. High corn oil = about 75% of calories as corn oil, no carbohydrate.

[b] Mean of 8 to 10 values ± standard error of the mean.

gains were similar for animals fed the various diets except for the animals restricted in calories. The latter group lost from 31 to 69 g in body weight. The groups consuming high beef tallow or high corn oil diets had larger livers and larger amounts of fat in the liver (8.78 or 6.35% vs. 4.65%) than those fed a high carbohydrate diet. The liver weight of calorie-restricted rats was only about half the liver weight of the control group.

The liver cholesterol level of calorie-restricted animals was significantly lower ($P<0.001$) than in other groups. Both high fat fed groups had higher cholesterol levels than the control group, but only the cholesterol level of rats fed beef tallow was significantly greater. The large standard error of the mean of the corn oil group probably accounts for its lack of significant difference from the control group. Comparison of high corn oil fed animals with high beef tallow fed animals shows that liver cholesterol of corn oil fed rats was greater but the difference was not statistically significant.

Fatty acid composition of the serum lipids was related to composition of the Dietary fat (Table III). When calorie intake was inadequate the proportions of palmitate and oleate decreased while stearate and arachidonate increased. High beef tallow diet supplied excess stearate and oleate, resulting in higher proportions of those fatty acids in serum. Linoleate was reduced in this group, but arachidonate was maintained in approximately the same concentration as it was in rats fed high carbohydrate. The high corn oil diet supplied excess linoleate which resulted in increased linoleate and arachidonate in serum accompanied by reduction of oleate. Fatty acid composition of serum lipids did not appear to bear any relationship to acetoacetate metabolism.

Acetoacetate

The plasma acetoacetate values expressed as millimoles per liter of plasma are presented in Table IV. The mean concentration of plasma acetoacetate of calorie-restricted rats was about twice that of the control group. This value is similar to the data of Weinhouse and Millington (18) who used liver slices prepared from fasted rats and fed rats. There is no statistical difference in acetoacetate level among all groups, although the concentration of acetoacetate of rats fed beef tallow is almost 2½-fold higher than in rats fed high carbohydrate and 2-fold higher than for calorie-restricted animals. The probability value for high carbohydrate rats (mean = 0.34 mM/liter) versus the high beef tallow group (mean = 0.85 mM/liter) is <0.10 but > 0.05.

Note that the mean concentration of acetoacetate of corn oil fed rats was half that of the level of beef tallow fed rats. Although

TABLE III
Fatty Acid Composition of Serum Total Lipids (Per Cent of Total Fatty Acids)

Diet	No. Samples	Fatty acid 16:0	18:0	18:1	18.2	20.4
High CHO[a]	4	18.9 ±2.01	19.35 ±1.10	22.87 ±3.17	16.34 ±1.54	23.33 ±0.87
High CHO—½ cal.	2	13.37	25.48	15.42	16.90	28.84
High beef tallow	5	15.01 ±2.51	25.69 ±2.21	27.27 ±3.63	11.28 ±0.74	20.74 ±3.38
High corn oil	8	16.54 ±0.49	24.26 ±0.96	8.96 ±0.28	23.69 ±1.15	26.62 ±0.93

[a] Same as Table II.

TABLE IV

Plasma Acetoacetate Concentration and Incorporation of Ethyl-3-^{14}C-Acetoacetate Into Expired CO_2, Liver Cholesterol and Total Lipids of Rats Fed Diets Varying in Fat and Calorie Concentration

Diet	Plasma Aceto-acetate	$^{14}CO_2$[a]	Liver Cholesterol TA[b]	Liver Cholesterol SA[c]	Liver Total Lipid TA[b]	Liver Total Lipid SA[c]
	mM/liter		x 10^{-3}		x 10^{-3}	
High CHO[d]	0.34[e] ±0.14	40.57 ±2.61	0.104 ±0.014	5.82 ±0.89	2.32 ±0.35	6.76 ±1.02
High CHO—½ cal.	0.61 ±0.12	45.01 ±1.81	0.050 ±0.007	4.53 ±0.65	0.268 ±0.05	1.22 ±0.21
High beef tallow	0.85 ±0.22	47.59 ±3.77	0.050 ±0.007	2.29 ±0.26	0.224 ±0.02	0.33 ±0.04
High corn oil	0.44 ±0.14	48.64 ±2.82	0.078 ±0.008	3.52 ±0.48	0.276 ±0.02	0.582 ±0.07

[a] Per cent of injected dose expired in 3 hr.

[b] TA = Total activity (per cent of injected dose).

[c] SA = Specific activity (TA/mg of compound).

[d] Same as Table II.

[e] Mean ± standard error of the mean. Acetoacetate data are from 4 to 10 observations. All other data are from 8-10 observations.

there is a large difference between the corn oil group and the beef tallow group, no statistical difference exists probably due to the small number of observations in the beef tallow group.

In all groups, the plasma acetoacetate level was relatively low. None of these groups can be considered as having the condition of ketosis. Although no significant differences existed among them, the values of beef tallow and calorie-restricted groups apparently approach ketonemia as indicated by comparing serum acetoacetate concentration with the control group.

CO_2 and $^{14}CO_2$

The values of ^{14}C in CO_2 after intraperitoneal administration of labeled acetoacetate to rats are shown in Table IV. The values indicate per cent of injected ^{14}C expired in 3 hrs. The rate of expiration of $^{14}CO_2$ is shown in Figure 1. The rate of excretion of $^{14}CO_2$ in all groups of animals did not differ. The maximal specific activity for each rat was reached with ½ to 1 hr after the injection of the labeled acetoacetate. This indicates that oxidation of a large portion of the injected ^{14}C to CO_2 took place during the first hour after the injection of the ^{14}C-acetoacetate.

The total output of CO_2 was not notably affected by the diets. The cumulative effect of diet upon $^{14}CO_2$ expired differed. The corn oil diet caused a significant increase ($P<0.05$) in the amount of $^{14}CO_2$ expired in 3 hr (40.57% vs. 48.64% of the injected dose). Restricted-calorie and high fat groups did not differ significantly from control, from each other or from corn oil groups. This indicates that the corn oil significantly enhanced the oxidation of acetoacetate to $^{14}CO_2$; it is probable that beef tallow had the same effect, but the standard error of the mean was greater so the difference from control animals was not significant.

In a study of the pregnant ovine by Bergman et al. (19), it was shown that about half of the injected acetoacetate was oxidized to CO_2. The present data show that 40-48% of the injected acetoacetate was oxidized to $^{14}CO_2$ in 3 hrs. The oxidation of acetoacetate to CO_2 was not related to plasma concentration since the highest expiration in 3 hrs was associated with a low mean plasma acetoacetate concentration (high corn oil group).

Incorporation of Labeled Acetoacetate Into Lipid

The values for ^{14}C in liver lipid and cholesterol are shown in Table IV. The values reported indicate net incorporation within the 3-hr period of exposure to ^{14}C-acetoacetate. Total activity (TA) is per cent of injected dose in the liver and specific activity (SA) is total activity per milligram of total lipid or cholesterol.

The values for total activity of total lipid of all modified diet groups were not different from each other but they were about 9 times lower than the control group. Beef tallow caused a decrease of approximately 20-fold in specific activity of total lipid in comparison to the control diet. Corn oil and calorie-restricted diets caused a 12-fold and 6-fold decrease in speci-

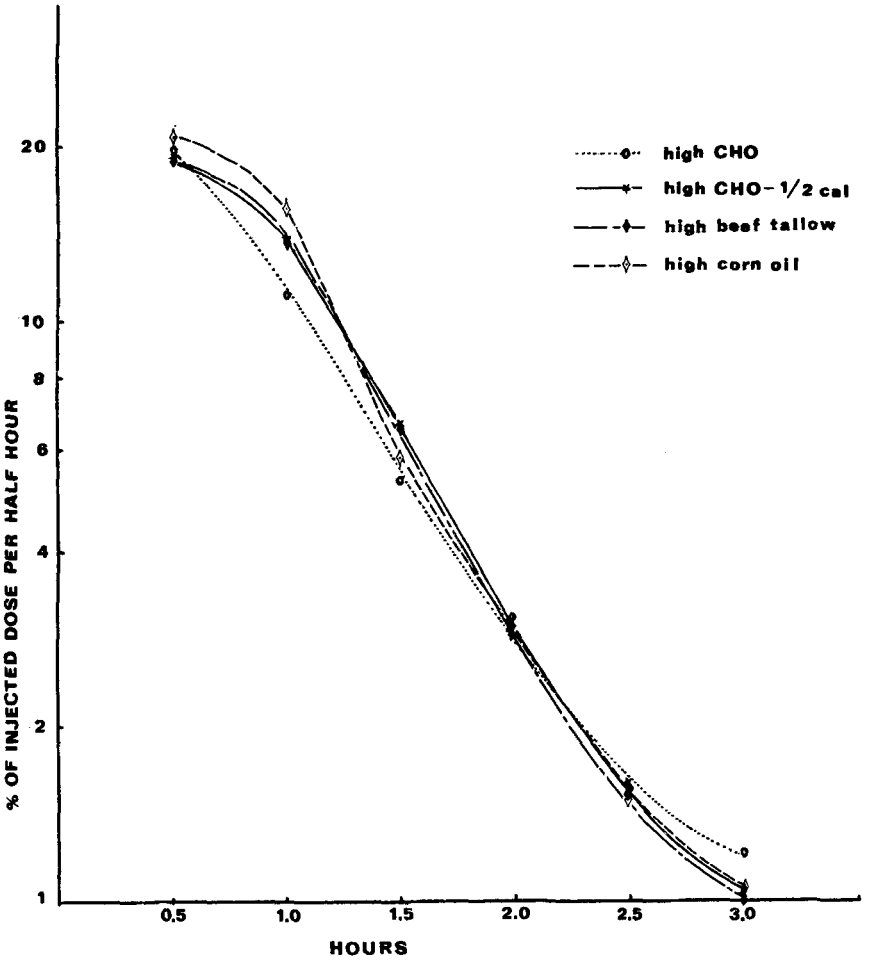

FIG. 1. Expiration of $^{14}CO_2$ by rats fed high carbohydrate (5% fat calories), high carbohydrate (one half calorie), high beef tallow (83% fat calories), or high corn oil (75% fat calories) diets and intraperitoneally injected with ethyl-acetoacetate-3-^{14}C.

fic activity, respectively. There appears to be no difference between response to the saturated compared to the unsaturated types of fat in total incorporation of acetoacetate into fatty acids. The calorie-restricted diet had the same effect as the high fat diet probably because calorie restriction results in utilization of body fat for energy.

The effect of dietary fat upon incorporation of acetoacetate into cholesterol in liver differed between types of fat. There was no significant decrease in total activity due to corn oil diet but the difference in specific activity was significant ($P<0.025$). Total activity of beef tallow and calorie-restricted groups were both reduced to about half of that of the control group. The specific activity of cholesterol of the beef tallow group was reduced 2½ times and there was no decrease for the calorie-restricted group. There was a significant difference ($P<0.025$) between beef tallow and corn oil for total activity of cholesterol but no significant difference for specific activity. The fact that TA of cholesterol was the same for the calorie-restricted, high beef tallow and high corn oil diets and the SA differed can be explained by the difference in amount of cholesterol in the liver.

In general, the data indicate that high fat diets lower liver fatty acid synthesis from acetoacetate. The data also indicate that high beef tallow and calorie-restricted diets decreased incorporation of acetoacetate into cholesterol compared to high carbohydrate controls. High corn oil diet also decreased this incorporation but not to a significant degree.

Although in rats fed high fat diets acetoacetate contributed less to the synthesis of fat and cholesterol than in those on the high carbohydrate diet, there was a difference between the effects of the two fats studied. Comparison of the effect of beef tallow and corn oil on the utilization of acetoacetate shows that corn oil promoted the incorporation of acetoacetate into cholesterol more than beef tallow. This increased incorporation perhaps can be explained by the theory of Sinclair (5) that unsaturated fatty acids split off acetyl-CoA and propionyl-CoA. In both hepatic and extrahepatic tissue the propionyl-CoA can form succinyl-CoA via carboxylation to methylmalonyl-CoA and subsequent rearrangement (20). In extrahepatic tissue the succinyl-CoA can contribute its CoA to convert acetoacetate into acetoacetyl-CoA. Acetoacetyl-CoA can form mevalonate via the intermediate formation of β-hydroxy-β-methyglutaryl-CoA (HMG-CoA). The very low percentage of the ^{14}C-acetoacetate incorporated into cholesterol, regardless of diet is probably the result of acetoacetate mobilization out of the liver, activation in peripheral tissue and finally incorporation into cholesterol either in hepatic or extrahepatic tissue.

From the data on ^{14}C-acetoacetate incorporation into $^{14}CO_2$ and lipids it is shown that ketosis induced by diet is likely to be caused by the overproduction of ketone bodies in the liver and not the underutilization of ketone bodies by the peripheral tissues. Furthermore, it is apparent that dietary lipid containing a high concentration of polyunsaturated fatty acids is not ketogenic. Thus, the kind of fat, as suggested by Sinclair, not the amount, determines ketogenicity.

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Synthesis, Purification and Characterization of 7-Ketocholesterol and Epimeric 7-Hydroxycholesterols

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ABSTRACT

Various methods were assessed for the synthesis of 7-ketocholesterol and epimeric 7-hydroxycholesterols. Upon the oxidation of cholesteryl acetate with t-butyl chromate, the resulting ketosterol acetate crystallized from methanol consisted of about 25% unoxidized cholesteryl acetate. After the sterol acetates were hydrolyzed in an aqueous K_2CO_3 medium, preparative TLC was used to fractionate the ketone from cholesterol.

Of all the reducing agents employed, only $LiAlH_4$ reduced completely the purified 7-ketocholesterol to 7-hydroxycholesterols without side reaction products and ketone contamination. Yields of 21.3 mg of 7α-hydroxycholesterol and 72.6 mg of 7β-hydroxycholesterol were obtained by preparative TLC of a diol mixture prepared by the $LiAlH_4$-reduction of 100 mg of 7-ketocholesterol. To accomplish the preparative TLC separation of diol bands without overlapping, a double development of a chromatoplate with ethyl ether-cyclohexane (90:10, v/v) and ethyl ether was essential.

Data on the melting point, optical rotation and infrared spectra, as well as TLC and GLC characteristics, were obtained for purified 7-ketocholesterol and epimeric 7-hydroxycholesterols.

INTRODUCTION

CHOLESTEROL, in the presence of oxygen, can be oxidized under a variety of conditions (1,2,3,4). The major reaction products have been identified as 7-ketocholesterol, 7α-hydroxycholesterol and 7β-hydroxycholesterol (1,2,5). These oxidation products have been detected in the unsaponifiable fraction of biological matter by numerous researchers. Haslewood (6) was the first to isolate an autoxidation product, 7α-hydroxycholesterol, from the unsaponifiable fraction of ox liver. Later, MacPhillamy (7) isolated the 7β-hydroxycholesterol from hog liver. Both of the isomers of 7-hydroxycholesterols have been obtained from pregnant mare serum (8). Recently, Chicoye et al. (9) found that the unsaponifiable matter of dehydrated egg yolk, which had previously been irradiated by energy from a fluorescent lamp or sunlight, consisted of large amounts of 7-ketocholesterol, 7α-hydroxycholesterol and 7β-hydroxycholesterol.

The present report describes procedures for the preparation of purified 7-ketocholesterol, 7α-hydroxycholesterol and 7β-hydroxycholesterol and the characterization of these compounds.

EXPERIMENTAL PROCEDURES

Synthesis of 7-Ketocholesterol

Cholesteryl acetate (mp 115 C) was oxidized with t-butyl chromate to 7-ketocholesteryl acetate by a method similar to that of Ruiz (10). The ester yield was 67% (mp 156 C). The crude ester was hydrolyzed according to the procedure of Barnett et al. (11) by treating the ketoacetate in methanol with K_2CO_3 at 25 C. Purified 7-ketocholesterol was obtained by preparative TLC as described in a following section.

Synthesis of Epimeric 7-Hydroxycholesterols

A mixture of epimeric 7-hydroxycholesterols was prepared by reducing 7-ketocholesterol with $LiAlH_4$. Residual moisture in 310 mg of pure 7-ketocholesterol was removed by adding 25 ml of dry benzene and distilling off the solvent under reduced pressure to dryness. The residue was dissolved in 25 ml of dry ethyl ether previously distilled over $LiAlH_4$. The steroid ethereal solution was added gradually to a solution containing 155 mg of $LiAlH_4$ in 25 ml of dry ethyl ether. The mixture was refluxed for 12 hr with protection from air moisture by a desiccant trap. Excess $LiAlH_4$ was destroyed by the addition of a solution consisting of 5 ml of acetone and 20 ml of ethyl ether. The mixture was acidified to about pH 2.5 with 5N H_2SO_4. After adding 50 ml of water, the two phases were allowed to separate. Upon removal of the ether phase, the aqueous phase was extracted with 50 ml of

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ethyl ether. The combined ether extract was washed with water to neutrality. After removal of the ether, the residue was dissolved in chloroform for application to preparative chromatoplates.

Preparative Thin-Layer Chromatography

A slurry of silica gel (without binder) for spreading on 20 × 20-cm glass plates was prepared by mixing 1 part of Adsorbosil-2 (Applied Science Laboratories, Inc.) with 1½ parts of water. A uniform slurry layer with a thickness of 1 mm was applied to each plate with a Desaga-Brinkmann applicator. The chromatoplates were activated in an oven at 110 C for 1 hr. A continuous steroid zone (2 cm from the bottom of the plate) was formed by applying a steroid solution with a capillary applicator. This applicator was composed of 40 capillary melting point tubes (1.5 mm apart) between two plastic plates. The steroid solutions were placed in a small chromatography trough to facilitate capillary filling. Each plate was loaded with as much as 100 mg of crude 7-ketocholesterol. On the other hand, only 20 mg of crude 7-hydroxycholesterol were applied to each plate. The plates, spotted with 7-ketocholesterol, were developed once with ethyl ether. To obtain distinct bands of the 7α- and 7β-hydroxycholesterols without overlays, the plates were developed twice with two different solvent systems. The plates were developed first with a solvent system consisting of ethyl ether-cyclohexane (90:10, v/v). After solvent evaporation at 25 C, the plates were developed again with ethyl ether. The plates were dried at 25 C. The 7-ketocholesterol bands were detected under UV light. For visualization of the separate diol bands, spraying of the chromatoplate with 0.2% alcoholic dichlorofluorescein (DCF) was used. For the collection of adsorbent powder in a sintered glass funnel, a plastic sweeping head was attached to the funnel through a glass tube inlet. The bottom stem of the funnel was inserted into a suction flask. Under reduced pressure, the powder was swept onto the sintered glass disc. The steroid on the adsorbent was eluted with chloroform-methanol (50:50, v/v). Solvents were removed under reduced pressure.

When 7-ketocholesterol residue was subjected to analytical TLC, only one spot was observed. The melting point of the ketone was 167-169 C. The ketone, crystallized subsequently from methanol, had a melting point of 172 C.

After the 7α-hydroxycholesterol (containing DCF dye) residue was dissolved in 10 ml of methanol, 75 ml of water was gradually added with stirring to precipitate out the steroid, yet keep the dye in solution. After filtering out the crystals, they were washed with 15% methanol. The white residue was dissolved in hot methanol and, upon cooling, long needles were formed (mp 186 C). When the 7β-hydroxycholesterol fraction was watered out as outlined above, a gelatinous precipitate with a small amount of dye resulted. The dye was removed by dissolving the precipitate with ether and washing the ether solution with water. The β-isomer was crystallized from methanol (mp 174 C). When each of the purified hydroxycholesterols were subjected to analytical TLC (0.25 mm adsorbent thickness) with ether as the developing agent, only one spot was noted. Instead of the water washing of steroid solutions, rechromatographing of the steroid fractions on additional TLC plates was used as an alternate method for further purification and dye removal. The chromatographic procedure was the same as outlined above, except that ether was used as the developing solvent. The dye remained near the origin of the developed plates. Band position was determined by applying 50% H_2SO_4 to a confined center zone with precautions for preventing acid contamination. Elution from the TLC adsorbent and steroid crystallization were carried out as outlined above.

Analytical Thin-Layer Chromatography

Analytical TLC plates with 0.25 mm Adsorbosil-2 layers were prepared as outlined above. Various solvent systems were used. Visualization of spots was accomplished by spraying 50% H_2SO_4 on the plates and heating at 110 C for various inspection times. R_c is defined as the ratio of the distance of the sample spot from the origin/distance of the cholesterol spot from the origin.

Infrared Spectroscopy

Infrared spectra of purified steroids were obtained with a Beckman IR-4 spectrophotometer. The steroids were dissolved either in CCl_4 or $CHCl_3$ to produce 1% solutions.

Gas Chromatography

Gas chromatographic separations were carried out in a glass column (6 ft long and 4 mm ID) packed with either 1% SE-30, 1% QF-1 or 1% NGS on Gas Chrom Q (silane-treated support), 80/100 mesh. The Barber-Colman Model 10 gas chromatograph was used with a hydrogen flame ionization detector. The following conditions were maintained: nitrogen

carrier gas flow rate, 95-120 ml/min; column temperature, 212 C; flash evaporator temperature, 280 C. A 10 μl Hamilton syringe was used to inject a suitable volume (usually 2 μl) of steroid-containing solvent. Relative retention times were calculated relative to cholestane.

RESULTS AND DISCUSSION

Synthesis and Purification of 7-Ketocholesterol

According to Ruiz (10), oxidation of cholesteryl acetate with t-butyl chromate resulted in a 66% yield of ketosterol acetate (mp 158 C). Following the procedure of Ruiz (10), we obtained a 67% yield of crude 7-ketocholesteryl acetate (mp 156 C). TLC indicated that a considerable amount of cholesteryl acetate (about 25%) was present in the crude ketoacetate. Cold hydrolysis (25 C) of 7-ketocholesteryl acetate with K_2CO_3 was used instead of hot saponification to avoid extensive degradation of the 7-ketocholesterol. A large amount of 3,5-cholestadiene-7-one was formed along with numerous other degradation compounds when 7-ketocholesteryl acetate was treated with a hot alkaline solution. After hydrolysis of the ester, crystallization of the resulting 7-ketocholesterol from methanol did not eliminate cholesterol contamination. Preparative TLC was used to separate the impurities from the 7-ketone, which upon recrystallization from methanol, had a melting point of 172 C. A melting point of 7-ketocholesterol has been reported by Fieser and Fieser (12) to be 157 C and by Bergström and Wintersteiner (5) to be 170-172 C. In our study, only one GLC peak and TLC spot was obtained with the purified 7-keto compound.

Synthesis and Purification of 7α- and 7β-Hydroxycholesterols

The effectiveness of some common reducing agents for the conversion of 7-ketocholesterol or its ester to epimeric 7-hydroxycholesterols was assessed by TLC. Na reduction of 7-ketocholesteryl acetate, by the method of Kramli (13), brought about the formation of approximately equal amounts of the diol epimers, as estimated by TLC. However, about 50% of the initial amount of ketone was not reduced and appreciable amounts of saturated 3,7-diols were produced as reaction products. Since the R_f values of saturated 3,7-diols and the corresponding unsaturated diols on TLC were close, purification of the 7-hydroxycholesterols would be difficult. The reduction of 7-ketocholesterol by $NaBH_4$ was assessed by adding a solution containing 0.2 g of $NaBH_4$ in 1.4 ml of water to 2 g of 7-ketocholesterol in 24 ml of methanol and 8 ml of CCl_4. The reaction mixture was held at about 25 C for 2 hr. The predominant reaction product was 7β-hydroxycholesterol, but a large amount of 7-ketocholesterol was unreduced. When 7-ketocholesterol was reduced with $LiAlH_4$, as outlined in the Experimental Procedures, only the two isomers of 7-hydroxycholesterol without ketone contamination were detected on developed chromatoplates. Upon the reduction of 100 mg of purified 7-ketocholesterol by $LiAlH_4$ and the separation of the reaction products on preparative TLC, 72.6 mg of the 7β-isomer and 21.3 mg of the 7α-isomer were obtained. With each of these isomers, only one GLC peak and one TLC spot were formed. In the above mentioned preparative TLC procedure, two distinct epimer bands were obtained by the double development of chromatoplates with ethyl ether-cyclohexane (90:10, v/v) and ethyl ether as solvents.

An attempt was made to separate the α-isomer from the β-isomer by crystallization. When an ether solution of the reaction products of reduced 7-ketocholesterol was allowed to stand at about 25 C for around 18 hr, long needle-like crystals (mp 177 C) were formed. The percentages of 7α- and 7β-hydroxycholesterols in these crystals were estimated to be about 30% and 70%, respectively, by TLC and specific rotations. Wintersteiner and Ruigh (14) also observed the formation of a mixed crystal (mp 174-176 C) from ether and estimated the content of the 7α-epimer (termed 7β-epimer in their paper and other older literature) to be up to 20%. Recrystallization of the mixed crystal from methanol did not change these properties. In our study, no crystal formation could be achieved in ether when either purified epimer was present alone. The 7α-diol was crystallized out of a methanolic solution by cooling to about 5 C. On the other hand, when the 7β-diol in methanol was submitted to the same treatment, a clear gel was formed. Crystalline 7β-diol was obtained by evaporating methanol from the solution until the entire hot concentrate was transformed into a chalky mass. Purification of the β-epimer eluted from TLC adsorbent was achieved by triturating the chalky mass with a small amount of methanol and filtering to recover the particulate diol.

The melting point of 7α-hydroxycholesterol was reported as 157 C by Fieser and Fieser (12) whereas Henbest and Jones (15) obtained a melting point of 185 C ($[\alpha]^{17}_D$-86.6). Our purified α-diol ($[\alpha]^{29}_D$-83.6) had a melting

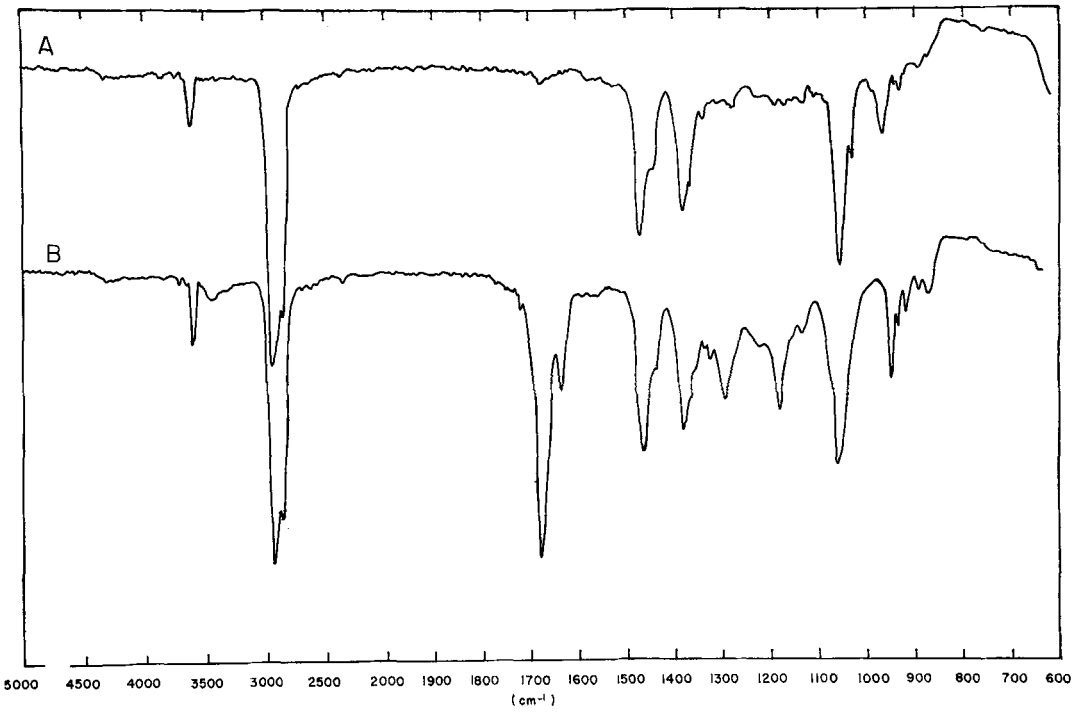

FIG. 1. Infrared spectra of cholesterol (A) and 7-ketocholesterol (B).

point of 186 C. The melting point value (174 C) of our 7β-diol ([α]$^{29}_{D}$ 5.9) was in reasonably good agreement with that (178 C) of Fieser and Fieser (12).

Characterization of 7-Keto, 7α-Hydroxy and 7β-Hydroxycholesterols

The R_c values of TLC should be valuable for identification and characterization of the 7-ketocholesterol and epimeric 7-hydroxycholesterols. The $R_c \times 100$ values of TLC for 7-ketocholesterol, 7β- and 7α-hydroxycholesterols were 69, 60 and 44, respectively, for ethyl ether as the solvent and 67, 53 and 42, respectively, for ethyl acetate-cyclohexane (60:40). Ethyl ether was by far the most effective solvent for resolving the epimeric 7-hydroxycholesterols. When TLC plates were sprayed with 50% H_2SO_4 or saturated $SbCl_3$ in chloroform, the 7-ketocholesterol spot became yellow colored only after about 10 hr at 110 C, while the 7-hydroxycholesterol spots turned blue immediately, even at room temperature. According to Claude (16), a 7-ketocholesterol spot on $SbCl_3$-sprayed TLC plate turned violet at room temperature, but Smith et al. (1) indicated no color display.

TABLE I

Gas Chromatographic Data on 7-Ketocholesterol, 7-Hydroxycholesterols and Cholesterol

Steroid	Relative retention time[a]			Steroid number
	SE-30	QF-1	NGS	SE-30
7-ketocholesterol	2.53	17.60	24.70	29.90
7α-hydroxycholesterol	4.08	b[b]	b[b]	31.34
7β-hydroxycholesterol	4.23	b[b]	b[b]	31.45
cholesterol	2.09	3.10	7.59	29.30
7-ketocholesterol (Silyl ether derivative)	5.41	.....	.....	.....
7α-hydroxycholesterol (Silyl ether derivative)	2.74	.....	.....	.....
7β-hydroxycholesterol (Silyl ether derivative)	2.78	.....	.....	.....
cholesterol (Silyl ether derivative)	2.60	.....	.....	.....

[a] Relative to cholestane.

[b] Degradation.

From the data of GLC experiments, the relative retention times (RRT) and steroid number (SN) of 7-ketocholesterol, 7-hydroxycholesterols, and cholesterol were calculated. The results are reported in Table I. With the nonselective liquid phase SE-30, almost symmetrical peaks were obtained with the sterols and their TMS derivatives. No degradation of the sterols was apparent. When a mixture of either the 7-hydroxycholesterols or their TMS

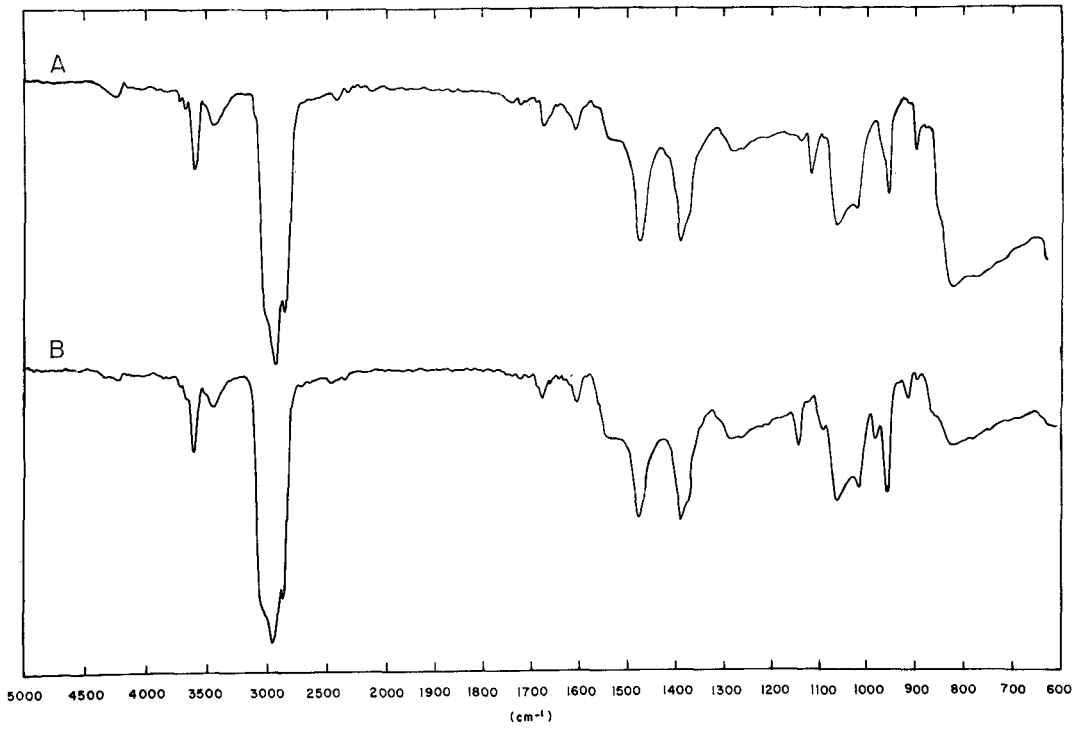

FIG. 2. Infrared spectra of a 7α-hydroxycholesterol (A) and 7β-hydroxycholesterol (B).

derivatives was applied to the SE-30 column, the epimers were not resolved into separate peaks. Considering only molecular weight, the RRT of the 7-hydroxycholesterol-TMS derivatives should have been higher than the TMS derivatives of 7-ketocholesterol. With the selective phases, QF-1 and NGS, the RRT values of 7-ketocholesterol were considerably higher than the RRT for nonselective SE-30. The diol epimers on QF-1 and NGS columns decomposed. When either of the diol isomers was applied to the NGS column, one peak with a RRT of 1.74 was formed, but thereafter extensive tailing on the chromatogram was evident. The infrared spectra of 7-ketocholesterol and cholesterol in CCl_4 and 7-hydroxycholesterols in $CHCl_3$ are presented in Fig. 1 and 2. Although, in many cases, CCl_4 is a better solvent for IR analyses than $CHCl_3$, the use of $CHCl_3$ as a solvent for the diol epimers was necessary because of their low solubility in CCl_4. In the functional group region between 5000 and 1300 cm^{-1}, the IR spectrum of each sterol had several characteristic absorption bands. With all of the sterols, a sharp peak, representing the free hydroxyl stretching, was located at 3600^{-1}. A small band at approximately 3400 cm^{-1} was observed in the spectra of the ketone and diol epimers. The intermolecular hydrogen bonding between the 7-ketone molecules may account for the small peak in the 3400 cm^{-1} region. In the case of the hydroxycholesterols, intermolecular H bonding between the $CHCl_3$ and the hydroxy compounds may cause some absorption in the same spectral region. The OH stretching peaks of the diols were visibly larger than those of 7-ketocholesterol and cholesterol. All four sterols had typical absorption bands at 1470 cm^{-1} and 1390 cm^{-1} (CH_2 and CH_3 bending, respectively). With regard to 7-ketocholesterol, a strong peak at 1690 cm^{-1} was apparent due to the conjugated C = 0 stretching. In the fingerprint region, the spectra of the 7α- and 7β-diols were significantly different. The spectrum of the α-epimer, but not the β-epimer, had a small peak at 1112 cm^{-1}. Three characteristic peaks in the spectrum of the β-epimer were located at 980, 1090, and 1140 cm^{-1}. Numerous other peaks were present with slight variation in location and intensities. The spectra of both epimers had two large bands at 1060 and 1017 cm^{-1}. The spectrum of keto compound had sharp major peaks at 1292, 1180, 1060 and 948 cm^{-1}.

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Fatty Acids in Lipids of Maturing Wheat[1]

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ABSTRACT

Two varieties of hard red winter wheat were sampled at various stages of maturity. The lipids in those samples were fractionated into free polar, free nonpolar and bound lipids. Fatty acids of those fractions were determined. Major acids present were palmitic, oleic, linoleic and linolenic. Both wheat samples showed similar qualitative, but not quantitative patterns in distribution of fatty acids during maturation. In the free polar lipid fraction, the palmitic acid content decreased with maturation while the linoleic acid content increased. The free nonpolar fractions showed a slight increase in linoleic acid; the concentration of other acids decreased slightly as the wheat matured. The bound lipid fraction showed a marked increase in linoleic acid, accompanied by decreases in the other major fatty acids, especially linolenic.

INTRODUCTION

CHANGES IN THE FATTY ACID composition of oils from seeds at various stages of maturity have been reported by many investigators. The plants studied include soybeans (1), castor beans (2), rape (3), crambe and rape (4,5), walnut (6), and Chinese tallow tree (7). In general those results showed a decrease in palmitic acid content, coupled with a gradual resumption of the typical fatty acid composition with maturation.

Although determination of the fatty acid composition of mature wheat has indicated that the major acids are palmitic, oleic and linoleic (8), there is little information on fatty acid composition of ripening cereal grain. Lindberg et al. (9) reported fatty acid compositions of total methanol extracts of barley, oats and wheat harvested in Sweden at five stages of maturity. The fatty acid composition of the wheat was found to change during ripening with the proportion of 16:0, 18:1 and 18:3 decreasing; and 18:2 increasing. The compositions of the major lipid fractions of maturing wheat have been determined by TLC (10). This examination indicated no significant or consistent changes in distribution of bound lipids (unextractable in petroleum ether, extractable in water saturated butanol). However, among the free lipids (petroleum ether extractable), free fatty acids, monoglycerides and diglycerides consistently decreased during maturation. Conversely, digalactosyl diglyceride increased during this period.

The present work reports changes in fatty acid compositions in major lipid fractions of maturing hard red winter wheat grown under field conditions in the United States.

EXPERIMENTAL PROCEDURES

Wheat Samples

Kaw and Pawnee wheat at stages of maturity from 27 days before ripe to ripe were harvested in Manhattan, Kansas, in 1961. The wheat samples were separated from the chaff and straw, and were air-dried in thin layers in the shade with the aid of electric fans, and stored in closed metal containers at 4 C until analyzed.

Determinations of moisture, ash and protein made according to AOCS methods (12) have been reported previously (13). That study found that although the two wheats were comparable in protein content, flours milled from them differed considerably in their rheological properties and oxidation requirements. Flour from Pawnee had higher oxidation requirements but required shorter mixing time than that from Kaw.

Analysis of amino acid compositions of the two wheats at three stages of maturity showed only minor differences between the varieties (14).

Lipid Extraction and Fractionation

Free lipids were extracted exhaustively with petroleum-ether (bp 35-60) in a Goldfish extractor. Residual petroleum-ether in the wheat was allowed to evaporate at room temperature, and bound lipids were extracted with water-saturated 1-butanol as described previously (15). The butanol extracts were purified by redissolving in petroleum-ether. Free lipids were fractionated into nonpolar and polar fractions on silicic acid columns (15).

[1]Cooperative investigations of Kansas Agricultural Experiment Station and Crops Research Div., ARS, USDA.

TABLE I

Weight and Composition (on a 14% Moisture Basis) of Two Wheat Varieties Harvested at Various Stages of Maturity

Pawnee				Kaw			
Days Pre-ripe	Wt 1000 kernels	Free lipids	Bound lipids	Days Pre-ripe	Wt 1000 kernels	Free lipids	Bound lipids
	mg	%	%		mg	%	%
25	6.27	1.97	1.90	27	5.14	1.85	1.94
23	9.37	2.03	1.61	25	7.54	1.76	1.85
21	11.92	2.08	1.16	23	10.56	1.63	1.48
19	15.80	1.81	1.20	21	14.30	.....	.....
17	17.08	1.78	1.37	19	17.32	.....	.....
15	20.26	1.93	1.28	17	22.30	.....	.....
12	20.77	1.86	1.43	14	23.83	.....	.....
9	20.46	1.82	1.46	11	26.04	.....	.....
6	22.57	1.73	1.32	8	28.71	1.60	1.25
3	21.54	1.69	1.37	5	30.42	1.53	1.14
0	22.78	1.77	1.22	0	27.88	1.53	1.07

Gas-Liquid Chromatography

The methyl esters were prepared by heating the lipids with 10 ml of 2% H_2SO_4 in methanol at 65 C for 2 hr. The esters were recovered by extraction with hexane. The hexane solution was washed with water, dried with anhydrous sodium sulfate, and evaporated almost to dryness under a stream of nitrogen.

The ester mixtures were analyzed by gas chromatography on a column of 7.5% diethylene-glycol succinate on 70-80 mesh Chromosorb G (Varian-Aerograph, Walnut Creek, Calif.). An argon ionization detector was used. Quantitative results with National Heart Institute Fatty Acid Standard F agreed with the stated composition data with relative error of less than 10% for all components. The quantities of esters were determined by disc integration without correction factors.

Distributions of fatty acids were computed as per cent of fatty acids recovered. The main fatty acids identified were palmitic, oleic, linoleic and linolenic. Minor components present included the fatty acids 18:0, 16:1, 17:0, 15:0 and 14:0. Tentatively identified were these minor fatty acids: 14:1, 15:1 and 16:2 with an unidentified minor peak eluted between linoleic and linolenic acids.

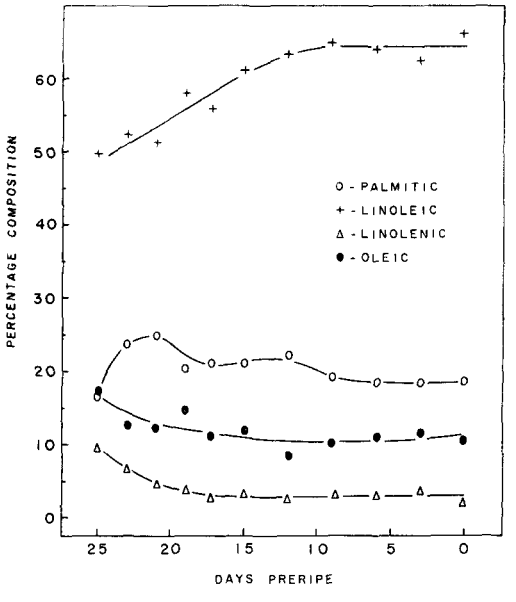

FIG. 1. Fatty acid composition of bound lipids of Pawnee wheat harvested at various stages of maturity.

RESULTS AND DISCUSSION

As is shown in Table I, the proportion of bound lipid in the wheat decreased from about 1.9% to 1.1-1.2% on maturation. This relative decrease, however, was accompanied by an increase in kernel weight, so that the amount of bound lipid per kernel increased initially but then underwent a slight decrease from the maximum in the later stages of maturation. During this period no significant or consistent changes in the proportions of lipids present in the fraction were observed by TLC analysis (10).

The fatty acid composition of the bound lipid fraction was observed to change as is indicated by Fig. 1 and Table II. The relative concentration of linoleic acid increased markedly, accompanied by decreases in palmitic, oleic and linolenic acids. Expressed on a kernel basis and considering the decrease of bound lipids in the kernel, it becomes apparent that, in Pawnee wheat, only linolenic acid actually decreases,

TABLE II

Fatty Acid Composition[a] of Lipids in Maturing Wheat

Days Pre-ripe	Free Lipids								Bound Lipids			
	Nonpolar				Polar							
	16:0	18:1	18:2	18:3	16:0	18:1	18:2	18:3	16:0	18:1	18:2	18:3
Pawnee												
25	18.1	21.7	47.1	8.9	35.4	18.2	31.7	2.5	16.6	16.7	49.8	9.9
23	17.5	24.7	46.8	8.6	25.7	14.8	46.9	7.5	23.9	12.8	52.5	6.9
21	17.2	26.4	42.8	8.0	25.8	15.1	47.5	4.7	25.0	12.5	51.2	4.8
6	12.2	23.3	53.1	7.7	.....	.....	.....	.....	18.8	11.2	64.0	3.1
3	16.6	22.8	47.5	8.0	19.9	14.3	56.4	3.6	18.6	11.8	62.5	3.9
0	15.5	23.1	47.7	8.3	17.8	12.9	61.0	3.9	18.9	10.7	66.2	2.0
Kaw												
27	18.3	14.1	51.5	13.4	54.5	12.3	3.0	1.4	24.8	11.8	41.1	10.7
25	21.9	15.3	51.1	8.8	39.2	21.1	10.1	1.9	22.6	11.6	49.1	8.0
23	19.2	17.6	52.7	8.7	37.2	21.2	24.7	2.5	24.8	11.9	54.2	5.4
8	17.8	17.6	58.3	5.7	40.5	19.3	14.6	1.6	19.7	11.5	61.6	4.5
5	17.3	18.6	55.0	6.4	39.7	19.7	22.8	1.3	19.7	12.6	60.0	4.1
0	15.5	16.4	61.6	5.8	39.5	20.6	19.8	2.1	20.0	12.1	59.5	4.7

[a] As per cent of total recovered by gas chromatography.

to 0.48 of its original amount. The amount per kernel of the other fatty acids increased on maturation. The increases, however, varied among the fatty acids: oleic 1.5, palmitic 2.7 and linoleic 3.1 times their original quantities.

The Kaw wheat showed a similar pattern, except that linolenic acid also increased to 1.4 times its initial quantities. The contents in mature wheat of the other acids were, palmitic 2.5, oleic 3.2 and linoleic 4.5 times those of the immature wheat. From this it is clear that the mature wheat contained much larger quantities of molecular species containing linoleic acid.

The relative amounts of the free lipids also decreased during maturation (Table I). However, the actual weight of free lipids in the kernels increased on maturation to 3.2 times that in immature Pawnee and 4.5 times in Kaw. The nonpolar lipids of this fraction also underwent a relative decrease as shown in Table III. Again, on a kernel basis this represented increases to 3.1 times for Pawnee and 4.2 times that in immature wheat for Kaw samples. Both relative and actual levels of free polar lipids increased to 4.5 times for Pawnee and 7.3 for Kaw.

Among the free nonpolar lipids the concentration of palmitic acid decreased in both varieties, and linolenic acid decreased in Kaw. Oleic acid remained fairly constant in both varieties; linoleic and linolenic also remained fairly constant in Pawnee. Only linoleic acid in Kaw showed a significant relative increase. All of the acids in both varieties, however, increased due to the increased amount of free nonpolar lipid in the kernel.

The greatest difference between the varieties in content and composition was observed with the free polar lipids. However, except for oleic acid which underwent a relative decrease in Pawnee and a relative increase in Kaw, all acids showed the same pattern. In both varieties the linoleic acid underwent the greatest change, increasing to 9.8 times the initial amount in Pawnee and 49.5 times the initial in Kaw. At least a portion of this increase in linoleic acid could be associated with the increase of galactolipids on maturation, observed in this fraction (10). Wheat galactolipids are known to contain large quantities of linoleic acid (17). In this respect, it would be of interest to determine the fatty acid composition of the galactolipids of these varieties to determine if they reflect the difference in composition observed for the free polar lipid fraction.

TABLE III

Lipid Fractions (as Per Cent of Total) in Petroleum-Ether Extracts

Pawnee			Kaw		
Days Pre-ripe	Nonpolar %	Polar %	Days Pre-ripe	Nonpolar %	Polar %
25	88.7	10.2	27	91.0	8.5
23	86.3	12.3	25	90.5	8.5
21	85.8	12.8	23	85.5	12.3
6	85.0	14.4	8	85.8	12.4
3	84.8	14.3	5	84.9	13.4
0	85.1	14.3	0	84.9	13.9

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Fractionation of silicic acid column chromatography by R. D. Daftary; wheat samples provided by K. F. Finney.

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SHORT COMMUNICATIONS

3,5-Di-*Tert*-Butyl-4-Hydroxytoluene (BHT) as an Artifact From Diethyl Ether

THE RECENT NOTE BY Radin et al. (1) on lipid contaminants arising from polypropylene apparatus prompts us to publish our finding of an artifact from certain brands of reagent grade anhydrous diethyl ether.

Ethyl ether was first suspected when two ether extracted flavor isolates from widely divergent natural sources were examined by combined gas chromatography-mass spectrometry and found to contain an aromatic compound with a prominent molecular ion at m/e 220, a base peak at m/e 205, and large peaks also at m/e 57 and 41. Comparison of the fragmentation patterns of the unknown with a catalog of standard spectra (2) indicated the antioxidant 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), also called Ionol, as a very likely identification. Since this compound was clearly not a natural product, we were prompted to establish the source of this interesting artifact. Examination of the mode of preparation of both flavor isolates for a common source of contamination showed that both samples were ethyl ether concentrates. To check the possibility of ether as the source of BHT, a rotary evaporator was used to concentrate 100 ml portions of four different brands of reagent grade anhydrous ether to approximately 100 μl. Gas chromatography of the ether concentrates was carried out on a packed 6 ft × 0.085 in. ID column containing 3% butanediol succinate (BDS) and .05% Igepal Co-880 on 100-120 mesh Chromosorb G and also on a 300 ft × 0.010 in ID BDS coated capillary. The packed column was used in an Aerograph Model 204 at 140 C with a carrier gas flow of 25 ml/min and a 7:1 effluent split. The capillary runs utilized an F and M Model 810 with an isothermal column temperature of 190 C. Three of the four ether concentrates (J. T. Baker Chemical Co., Allied Chemical Co., and Matheson Colman and Bell) gave peaks on both instruments with retention times the same as known BHT dissolved in freshly distilled ethyl ether. After trapping the fractions with the retention time of authentic BHT from the packed column in a glass capillary, identification was made using both mass spectrometry and infrared spectrometry. Mass spectra were consistent with those published for BHT (2), and the infrared spectro of the unknowns and authentic BHT in carbon tetrachloride solution were superimposable. There is, then, no doubt that certain brands of anhydrous ethyl ether contain BHT as an additive which is undeclared by name. While it is probably desirable to add some compound to inhibit peroxide formation in anhydrous ethyl ether, we believe that the presence and identity of this additive should be stated on the label. The fourth brand (Mallinckrodt) in our study contained the declared additive diethyldithiocarbamate and no BHT. Both BHT and diethyldithiocarbamate are easily removed by distillation.

The presence of even small amounts of BHT artifact in a sample could be a problem. Specifically, chemists conducting quantitative antioxidant analyses (4) or lipid oxidation studies would wish to avoid the use of ether containing antioxidants which may interfere. BHT gives a response with electron capture detectors (3) and has recently been shown to possess a biological effect in inhibiting growth of monkey kidney cells in tissue culture (5).

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LETTER TO THE EDITOR

Note on the Availability of Phycomyces Mutants Deficient in β-Carotene Biosynthesis

Sir: The purpose of this letter is to draw attention to the availability of Phycomyces mutants deficient in enzymes for β-carotene biosynthesis. These mutants should be most helpful in the study of still unclarified steps of β-carotene biosynthesis.

Beta-carotene is the principal pigment in wild type of Phycomyces and accounts for over 95% of the colored carotenes. Little is known about the enzymes which are involved in the dehydrogenation and cyclization steps. The dehydrogenation steps probably involve a water soluble cofactor and the cyclizations a participation of protons, whilst the carotenes themselves are water insoluble. These reaction steps therefore presumably take place at lipid-water interfaces and involve enzymes that are membrane bound. In connection with a search for the receptor pigment in the light sensitive sporangiophores of Phycomyces several β-carotene deficient mutants were isolated and partially characterized.

Similar to other organisms three main mutant blocks have been located in Phycomyces: a block decreasing the production of phytoene and resulting in a low overall concentration of carotenes (albino 5, albino 12); a failure of the dehydrogenation enzymes, resulting in the accumulation of high amounts of phytoene but low concentrations of the less saturated successors (albino 1, albino 10); and a defect of the cyclization step, leading to a replacement of β-carotene by lycopene (albino 12, R1).

Formation of artificial heterokaryons between some of the mutants and strikingly different effects of diphenylamine on some of the mutants yielded suggestive but as yet inconclusive results regarding the mechanisms of these disturbances. Heterokaryons between R1 and albino 5 and between albino 10 and albino 12 complement giving a carotene composition similar to that of wild type. The results are understandable assuming that enzymes missing in one mutant are supplied by the other. Diphenylamine (15 μg/ml medium) produces in albino 5 a carotene pattern that is a precisely scaled down version of the pattern induced by diphenylamine in wild type. Beta-carotene synthesis is suppressed by about a factor of 100 with a concomitant accumulation of the more saturated precursors. The lycopene mutants, on the other hand, are much less affected by diphenylamine than are wild type or albino 5. Lycopene is still the principal pigment in albino 12. In R1 the absorption spectrum of petroleum ether extracts shows that lycopene, ζ-carotene and phytofluene are present in about equal amounts, and lycopene itself is reduced by about a factor of 10. These observations suggest that diphenylamine interferes predominantly with the cyclizations, while dehydrogenations are relatively mildly affected. A puzzling feature is the finding that diphenylamine permits the synthesis of lycopene in the lycopene mutants but not in the wild type. Details of this work are described elsewhere (Heisenberg and Cerdá-Olmedo, in press; Meissner and Delbrück, Plant Physiol., in press).

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