Localization of Unsaturation in Fatty Acids by Identification of 2, 4-Dinitrophenylhydrazones Derived from Ozonides by Thin-Layer Chromatography

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ABSTRACT

The ozonides of unsaturated fatty acids may be reduced with 2,4-dinitrophenylhydrazine and the resulting aldehydes reacted with an excess of this same reagent to give the 2,4-dinitrophenylhydrazones of the aldehyde and aldoester fragments. The 2,4DNP of the aliphatic aldehydes can be separated by several thin-layer chromatography systems and are identifiable on the basis of their behavior in these systems. The methods described can be utilized for both qualitative identification as to the location of the double bond and for quantitative measurements of various positional isomers in some instances. All of the reactions may be performed on the plate for qualitative application, and the method can be applied to fatty acids in various forms.

As combinations of chromatographic techniques now make it relatively easy to isolate fatty acids in pure form in small quantities, it becomes possible to further characterize these fatty acids. The location of double bonds in unsaturated fatty acids has recently been accomplished by gas-liquid chromatography (GLC) of reductive ozonolysis products (2,3). These works also illustrated that when ozonolysis was conducted under low temperature conditions such as were employed, very few side products resulted. Since the reduction of an ozonide of phenanthrene by 2,4-dinitrophenylhydrazine and phenylhydrazine and the formation of the hydrazones has been reported (5) it was apparent that this might be extended to fatty acids and the derivatives identified by thin-layer chromatography (TLC) or GLC. This report presents progress in developing such a procedure and in the examination of the products of these reactions in order to make the procedure quantitative.

EXPERIMENTAL METHODS

Ozonolysis

One milligram of the methyl ester of the fatty acid was dissolved in 2 ml of methylene chloride or n-pentane, in a glass stoppered graduated test tube or centrifuge tube. This was brought to approximately −75C in an acetone dry ice bath and the effluent from an ozone generator was bubbled through the solution, the ozonolysis was conducted in a functioning hood. After the solution turns blue it was removed from the ice bath and warmed until the solid material went into solution. During this warming, ozone was being bubbled through the solution. It was then replaced in the ice bath and ozone bubbled through until a blue color was again detectable. This whole ozonolysis procedure took approximately 5–10 min. The ozone and methylene chloride or n-pentane were then removed by warming the solution to room temperature and then taken to dryness under a brisk flow of nitrogen.

Reduction and Formation of the 2,4-Dinitrophenylhydrazones

The reagent used consisted of 4.0 g of 2,4-dinitrophenylhydrazine dissolved in 40 ml of concentrated sulfuric acid. The amount of 30 ml of water was added slowly with stirring; 100 ml of 95% ethanol was added. The reagent was stable when stored in the dark. One tenth of a milliliter of this reagent was used for each double bond per milligram of fatty acid methyl ester. This provided sufficient excess to obtain quantitative conversion of the ozonide to the hydrazone. This amount of the reagent was added directly to the dry ozonide and the tube was allowed to sit at room temperature for a few minutes. The reactions to this point may be summarized by using methyl oleate as an example. Methyl oleate would have undergone the following reactions at this point:

$$CH_3(CH_2)_7CH = CH(CH_2)_7 - COOCH_3 + O_3 \rightarrow CH_3(CH_2)_7 - CHO_3CH(CH_2)_7 - COOCH_3$$

$$+$$

$$H_2NHNC_6H_3(NO_2)_2$$

$$\downarrow$$

$$CH_3(CH_2)_7HC = NHNC_6H_3(NO_2)_2$$

$$+$$

$$(NO_2)_2C_6H_3NHN = CH(CH_2)_7COOCH_3$$

$$+$$

reduced 2,4-dinitrophenylhydrazine and water

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Cleanup of Reaction Mixture Subsequent To TLC

Procedure A—for Quantitative Work. Five milliliters of chloroform and 2 ml of distilled water were added to the reaction mixture; the tube was shaken and centrifuged, and the upper water layer was removed by suction. This washing procedure was repeated two more times. Anhydrous sodium sulfate was added, the tube shaken and aliquots taken for chromatography.

Procedure B—for Qualitative Work. One milliliter of petroleum ether (bp-50-65C) was added and the tube shaken. Aliquots were taken directly from this extract and put on the thin-layer plates.

Separation of Products by Thin-Layer Chromatography

The qualitative separation and identification of products was performed using micro or macro methods. The basic difference in chromatography was that 6.66 × 6.66 cm plates, 0.25 mm thick were used for micro work while 20 × 20 cm plates, 0.5 mm thick were used for macro work.

Procedure A. Two stationary phases, n-undecane and silicone oil were used and both were satisfactory. The undecane had greater resolving power than the silicone oil. Silica gel G plates were impregnated by developing in a 10% solution of undecane or silicone oil in petroleum ether. They were then removed, allowed to dry a few minutes and the cleaned products of the ozonolysis and reduction were then placed on the plate. Usually 5 μl of the solution was added to the small plate and 10 to the large plates. The plates were usually developed with 25% water—75% methanol by volume. This mixture was very satisfactory for separating the aldehydes that would originate from fatty acids of the linoleate or oleate series (6 and 9 carbons). However, the longer chain aldehydes required a less polar mixture in order to move them from the origin on the nonpolar stationary phase. For instance, if the mixture is methyl oleate and methyl petroselineate, an 80% methanol, 20% water solution was used to separate the 9 and 11 carbon aldehyde 2,4-dinitrophenylhydrazones. After developing, the plate was allowed to dry. The spots were observed under ultraviolet light and marked with a hypodermic needle or similar object. The spots can also be made visible by spraying with 5N sodium hydroxide solution. Standard 2,4-dinitrophenylhydrazones were run at the same time that the unknown was run and the spots identified from the standards.

Procedure B. This procedure was similar to that described in detail by Urbach (4). Chromatoplates 6.66 × 6.66 cm or 20 × 20 cm of Kieselgur G 0.24 mm and 0.5 mm thick, respectively, were utilized. The plates were impregnated by developing in 10% 2-phenoxyethanol in ethanol. Development was accomplished with n-heptane. Repeated development was sometimes used, but was not always necessary.

Procedure C. The reaction products were first separated on a plate using dimethyl formamide on aluminum oxide to separate *syn* and *antio* form. The *syn* form was eluted and rechromatographed, using the phenoxyethanol system of Urbach described under procedure B. Chromatoplates 20 × 20 cm, of aluminum oxide, 0.5 mm thick, were utilized. The plates were impregnated by developing in 10% dimethyl formamide in ethanol. The chloroform extract of the reaction mixture representing 15 to 50 μg of original fatty acid was plated in four bands at the origin for development. The plates were developed with n-heptane two or three times. The need for repeated development was ascertained by visual inspection. The spot representing the *syn* form was placed in an elution tube and the 2,4-DNP eluted with 1 ml of acetone three times. This extract is taken nearly to dryness and then placed on phenoxyethanol impregnated plates and developed as outlined in procedure B.

Quantitative Determination of 2,4-Dinitrophenylhydrazones of the Straight Chain Aldehydes

The spots from the large plates may be measured quantitatively. The spots are scraped off and placed into eluting tubes. When the undecane system was used the undecane is removed by extracting twice with 2 ml of petroleum ether. The 2,4-dinitrophenylhydrazones are eluted with chloroform, usually 2 extractions with 2 ml suffices. The volume is reduced to 1 ml and the optical density read in the spectrophotometer, at 356 mμ, using semimicro cuvettes.

Conducting all Reactions on the Plate

Approximately 5 to 40 μg of the material to be investigated was placed on a Kieselgur G plate. The ozonolysis was performed by placing the plate in a chamber and saturating the chamber with the effluent from the ozone generator. The plate was allowed to remain in the chamber for 5 min. After removing the

TABLE I

R_f Values of 2,4-Dinitrophenylhydrazones of 3–9 Carbon n-Aliphatic Aldehydes[a]

	No. of carbons in aliphatic aldehyde of 2,4-dinitrophenylhydrazone						
	3	4	5	6	7	8	9
Lower band	0.63	0.54	0.40	0.27	0.18	0.10	0.05
Upper band	0.72	0.67	0.54	0.43	0.33	0.22	0.14

[a] The thin-layer plate is 20 × 20 cm, Silica gel G, 0.5 mm thick, stationary phase undecane, developing phase 25% water—75% methanol.

plate, one drop of 2,4-dinitrophenylhydrazine reagent was placed on the spot where the methyl ester or other material to be investigated was placed. The plate was dried on a hot plate for approximately 1 min. The plate was placed upside down in the ethanol solution containing 10% of the stationary phase (phenoxyethanol) and this was allowed to ascend to the spots (they were clearly visible). The plate was removed, ethanol removed by gently heating on a hot plate under a stream of nitrogen and the plate then developed in the normal way with heptane.

RESULTS AND DISCUSSION

Ozonolysis

The yield of the combination reactions was not as high if the ozonolysis solution was not warmed until all crystals dissolve at least once during the ozonolysis. The reason for this was not investigated. However, a reduction in yield was noted, and a solution of 1 mg of methyl oleate in 2 ml of methylene chloride was cloudy at −75 to −78C. The inference is that the crystallized methyl ester was not completely ozonized.

Separation of Products by Thin-Layer Chromatography

The greatest problem encountered in this area was the separation of each pure 2,4-dinitrophenylhydrazone into two spots. A detailed account of observations on this phenomena is to be published in another paper (5). These observations indicated that the 2,4-DNP formed from an aldehyde or unsymmetrical ketone were a mixture of *syn* and *antio* form. That repeated crystallization gave a product that was pure *syn* form. When this crystal was dissolved in a solvent such as acetone, petroleum ether, methanol or ethanol, it did not racemize and was pure by chromatography. However, if the crystals were dissolved in a solvent as chloroform, immediate racemization took place and the two forms were apparent when chromatographed. Also, addition of a drop of hydrochloric acid to the other solutions caused racemization. Therefore, the only way that one isomer was obtained from products of fatty acids was by crystallization and purification of the *syn* form. This required a large sample and may not be quantitative.

Table I gives the R_f values obtained from the homologous series of normal aliphatic aldehydes using the undecane system. It is apparent that derivatives of each of the adjacent members of the homologous series overlap. However, using this system, there is no overlap between every other member. This is important, since it allows for quantitative separation of the products of acids such as vaccenic acid and oleic acid. The silicone system resolves the two isomers to approximately the same extent. The phenoxyethanol system resolved the isomers even more and there was an overlap between adjacent member of the homologous series of aliphatic aldehydes. The amount of material in the *antio* form was approximately 25% in most reaction mixtures.

Figure 1 is a photograph of a chromatoplate illustrating separation of the products resulting from ozonolysis and treatment of the ozonide with 2,4-DNP reagent of methyl vaccenate, methyl oleate and a mixture of these two compounds using the undecane system. This chromatoplate illustrates the separation of the heptanal and nonanal 2,4-DNP. It also shows that the load limit of this system is very small as indicated by trailing in A′, B′ and C′ where products from 20 μg of each acid were applied to the plate.

Quantitative Determination of 2,4-Dinitrophenylhydrazones

The recovery of the 2,4-DNPH derivative of n-pentanal was studied and the recovery of this derivative from the plate after chromatography was approximately 91%. Recovery

TABLE II

Quantitative Analysis of a Mixture of Methyl Esters of Fatty Acids Using the Undecane Chromatographic System

	Percent analysis of mixture			
	By GLC of methyl esters		By optical density of 2 dinitrophenylhydrazones	
	16:1	18:1	16:1	18:1
Sample 1	49.0 ± .3	51.0 ± .3	51.0 ± 3.8	49.0 ± 2.7
Sample 2	49.0 ± .3	51.0 ± .3	46.7 ± 1.5	53.3 ± 1.3
Average	49.0 ± .3	51.0 ± .3	48.9 ± 2.9	51.1 ± 2.1

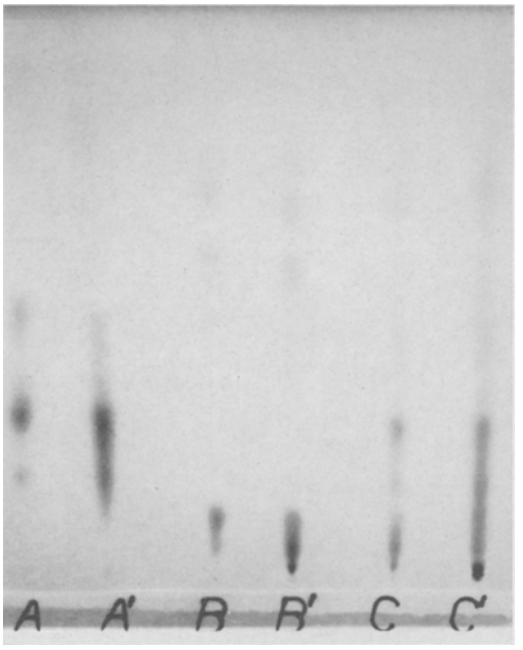

FIG. 1. Chromatoplate of the 2,4-DNP that result from methyl vaccenate and methyl oleate. Plate is 20 × 20 cm, Silica gel G, 0.5 mm thick, stationary phase undecane, developing phase 25% water—75% methanol.

A represents products from 10 μg methyl vaccenate, spots from bottom represent *antio* and *syn* form of 2,4-DNP of heptanal and the *antio* and *syn* form of 2,4-DNP of methyl undecanoate-11-al. *A'* represents products from 20 μg of methyl vaccenate. *B* represents products from 10 μg methyl oleate, spots from bottom represent *antio* and *syn* form of 2,4-DNP of nonanal and the antio and syn form of 2,4-DNP of methyl nonanate-9-al. *B'* represents products from 20 μg methyl oleate. *C* represents products from 10 μg methyl vaccenate and 10 μg of methyl oleate. *C'* represents products from 20 μg methyl vaccenate and 20 μg of methyl oleate.

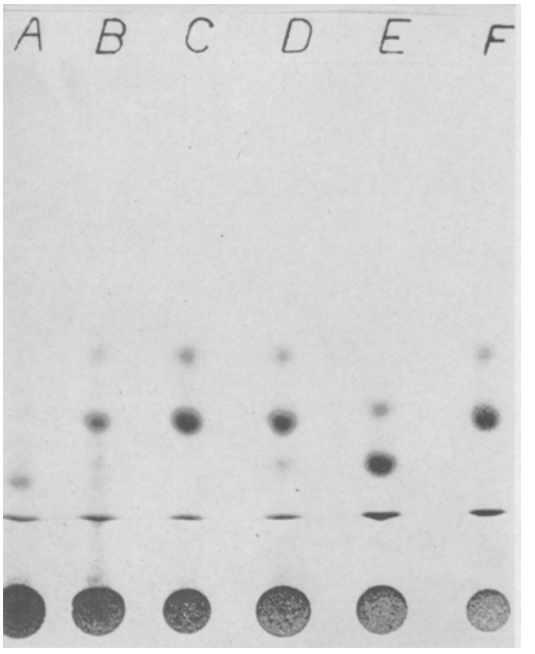

FIG. 2. Chromatoplate where ozonolysis, reduction and formation of 2,4-dinitrophenylhydrazones was conducted on the thin-layer plate. The plate is 20 × 20 cm, Kieselgur G, 0.50 mm thick, stationary phase 2-phenoxyethanol, plate developed one time with heptane. *A*, 80 μg of stearyl and palymityl, eicosatetrayl lecithin (made available by Gosta Arvidson). The site of the reaction is the round dark spot (samples *A-F*). The first thin black spot indicates the border of the 2-phenoxyethanol (stationary phase) and the spot is actually composed of the 2,4-DNP of the aldoester fragment and in the case of *A* it contains the di-2,4-DNP of malonaldehyde. The next spot represents the syn isomer of 2,4-DNP of hexaldehyde. Traces of 2,4-DNP of heptaldehyde are also present. B-40 μg of monoglyceride (14:0-5.0%, 14:1-4.4%, 16:0-5.5%, 16:1-17.3%, 18:0-4%, 18:1-57.5% and 18.2-6.2%). Spots corresponding to 6, 7 and 9 carbon aldehyde derivatives are apparent. C-40 μg of diglyceride (18:1-100%) spots for the *syn* and *antio* form of the 9 carbon aldehyde derivative are present. D-40 μg triglyceride (14:1-3.5%, 16:1-13.2%, 18:1-80.9% and 18:2-2.2%. Spots representing the 7 and 9 carbon aldehyde derivatives are apparent. E-40 μg methyl cis vaccenate—spots representing the *syn* and *antio* forms of the 7 carbon aldehyde derivates are seen. F-40 μg methyl oleate-spots representing the two isomers of the 9 carbon aldehyde derivatives are apparent.

experiments with methyl oleate carried through the entire experimental procedure with the 2,4-DNPH of a n-nonanal being the product measured gave yields of 89–92%. These data would indicate that the ozonolysis, reduction, and cleanup procedures must yield almost 100%, with the greatest loss being in the extraction of the derivative from the Silica gel G.

The absorption maximum of the 3–9 carbon straight chain aldehydes was at 358 mμ. The absorption maximum of the 2,4-DNPH of methyl n-nonanoate al was also 358 mμ.

The results of quantitative analysis of a mixture of methyl oleate and methyl palmitoleate are presented in Table II. The values for samples 1 and 2 are triplicate determinations of the 9 carbon aldehyde and 7 carbon aldehyde derivatives obtained from methyl oleate and methyl palmitoleate, respectively.

A sample of methyl eicosatrienoate (95%) and methyl docosatrienoate (5%) isolated from EFA deficient pig testis lipids was examined by this quantitative procedure. The results show that 84.4% of the material was 5,8,11 eicosatrienote and 7,10,13 docosatrienoate and while 15.6% was 7,10,13 eicosatrienoate and 9,12,15 docosatrienoate. While Privett et al. (2) did not give actual percentage composition, they examined methyl eicosatrienoate from liver lipids of EFA deficient rats by the reductive ozonolysis procedure and found that the 5,8,11 eicosatrienoate was the major component and 7,10,13 eicosatrionate the minor component.

Quantitative analysis of a mixture of 28.5% methyl petroselinate, 31.3% methyl oleate and 40.2% methyl vaccenate using the combination of dimethyl formamide and the phenoxyethanol chromatographic systems gave the following results by spectrophotometric analysis of separated 2,4-dinitrophenylhydrazones: 29.2 ± 0.2% methyl petroselinate, 30.1 ± 0.6% methyl oleate and 40.6 ± 0.5% methyl vaccenate. This method in which the analysis is conducted with the isolated *syn* forms appears to be precise and accurate for this combination of fatty acids.

Results of Conducting all Reactions on the Plate

Figure 2 illustrates the application of this technique to 4 different classes of lipids. The sensitivity of the method is shown by the fact that spots for hexanal and heptanal derivatives are seen in the monoglyceride preparation (B) where the GLC data indicates that hexadecenoic and octodecadienoic acids were present. These acids are probably 9-hexadecenoic acid and 9,12-octadecadienoic acid and therefore yield the 6 and 7 carbon aldehyde derivatives. The chromatoplate pictured in Figure 2 is 20 × 20 cm. However, small plates have been used very successfully, and the total analysis can be conducted in 20 to 30 min.

Advantages and Disadvantages of the Methods Described

The main advantages of the methods are: (1) it does not require a GLC apparatus; (2) it does not require a hydrogen reduction procedure; (3) the products are very stable; and (4) extremely small samples may be utilized for the analysis. The major disadvantages are: (1) the results are not clear cut when the fatty acids yield aldehydes of adjacent members of the homologous series due to overlap of the *syn* and *antio* form of the 2,4-dinitrophenylhydrazone of these aldehydes; and (2) the derivatives of short chain (4 carbon and less) are not separated from aldoester products by the chromatographic procedures developed.

ACKNOWLEDGMENTS

Laboratory facilities provided by B. Borgstrom. Ozone generator constructed by Leo Aido. Technical assistance by Mrs. Ulla-Britt Carlson. Photographic work by Miss Ingrid Ekval.

Supported in part by USPAS research grants No. 6338 and 05302 (Met.) and a research career program award No. 18,411 from the Arthritis and Metabolic Disease Institute.

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[Received Sept. 24, 1965]

X-Ray Diffraction Powder Studies of Some Dithiol Diesters of Long Chain Acids

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ABSTRACT

X-ray diffraction powder data are reported for 25 mono- and dithiol diesters of straight chain aliphatic acids where the acid portion of the molecule consists of one of the following acids: octanoic, decanoic, dodecanoic, tetradecanoic, hexadecanoic or octadecanoic acids, and where the thiol portion consists of one of the following: 2-mercaptoethane, 1,2-ethanedithiol, 1,3-propanedithiol, 1,4-butanedithiol or 1,5-pentanedithiol. The individual compounds can be identified and distinguished by the long spacing data. The compounds crystallize in tilted monomolecular layers.

INTRODUCTION

A RECENT PUBLICATION (1) describes the synthesis of monothiol and dithiol diesters of long chain acids. Many of these compounds are solid crystalline materials whose melting points are above room temperature. These compounds are suitable for a study by X-ray diffraction. This paper reports X-ray studies of 25 mono- and dithiol diesters of straight chain aliphatic acids containing an even number of carbon atoms, namely, octanoic, decanoic, dodecanoic, tetradecanoic, hexadecanoic, and octadecanoic acids. The general formula of the mono diesters is $R\text{-}\overset{O}{\overset{\|}{C}}\text{-}O\text{-}(CH_2)\text{-}S\text{-}\overset{O}{\overset{\|}{C}}\text{-}R$ and of the dithiol diesters is $R\text{-}\overset{O}{\overset{\|}{C}}S\text{-}(CH_2)_n\text{-}S\text{-}\overset{O}{\overset{\|}{C}}\text{-}R$ where n varies between two to five.

EXPERIMENTAL

The synthesis and the purification of the compounds used in this study were described elsewhere (1). Most of these compounds were crystallized from acetone, hexane and chloroform at room temperature. X-ray diffraction measurements were made with a General Electric XRD-3 direct recording unit, using nickel-filtered CuKα radiation ($\lambda = 1.5405$ Å), 1° beam slit, 0.1° detector slit, medium resolution soller slit, scanning speed 2° /min, chart speed 60 in./hr. The X-ray data listed in Table I were obtained from unground samples. Thin layers measuring approximately 0.5 in. by 1.0 in. were firmly pressed on a glass slide to insure adherence. The long spacings listed in Table I are the average of several orders taken from oriented samples. The first few orders were often not included because of the limited accuracy with which they could be measured. Complete X-ray powder data were obtained from ground (unoriented) samples but are not included in this paper due to the large amount of space that would be required to describe them. Shown in Figure 1 are the long spacings of the compounds studied plotted against the number of carbon atoms in each of the individual acid portions of the molecule.

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RESULTS AND DISCUSSION

All compounds investigated can be identified and distinguished on the basis of the X-ray diffraction long spacing data. The compounds investigated gave X-ray diffraction patterns which contained a large number of relatively sharp diffraction peaks. This is characteristic of the highly crystalline nature of these compounds. The same crystalline forms appeared irrespective of the solvent used, namely, hexane, acetone, or chloroform. Three compounds, namely, 1,2-ethanedithiol didodecanoate, 1,4-butanedithiol didecanoate and 1,4-buthanedithiol didodecanoate, always crystallized in two polymorphic forms. The number of orders used in the calculation of each long spacing is given in Table I.

A plot of the total number of carbon atoms in each acid chain (y) against the long spacings (x) of the 2-mercaptoethanol diesters (Fig. 1) showed that the long spacing values fall in a straight line whose equation as determined by the method of least squares is $y = 0.444143x - 2.82$. This would indicate that the 2-mercaptoethanol diesters studied crystallize in the same polymorphic form. A plot of the total number of carbon atoms in each acid chain (y) against the long spacings (x) of the 1,2 ethanedithiol diesters (Fig. 1) showed that the long spacing values fell on two straight lines. The values of the dioctanoate, didecanoate, and the smaller long spacing

TABLE I

Long Spacings of Mono- and Dithiol Diesters

Compound	Total atoms in chain[a]	Long Spacing Å	No. of orders used	Crystallized form
2-Mercaptoethanol didecanoate	24	28.85 ± .0279	8	A
2-Mercaptoethanol didodecanoate	28	33.34 ± .0114	14	CHA
2-Mercaptoethanol ditetradecanoate	32	37.88 ± .0179	10	CHA
2-Mercaptoethanol dihexadecanoate	36	42.40 ± .0184	15	CHA
2-Mercaptoethanol dioctadecanoate	40	46.84 ± .1023	6	CHA
1,2-Ethanedithiol dioctanoate	20	23.70 ± .0203	6	CHA
1,2-Ethanedithiol didecanoate	24	28.46 ± .0206	8	CHA
1,2-Ethanedithiol didodecanoate	28	33.04 ± .0199	8	CHA
		33.92 ± .0183	10	
1,2-Ethanedithiol ditetradecanoate	32	38.40 ± .0099	15	CHA
1,2-Ethanedithiol dihexadecanoate	36	42.92 ± .0179	14	CHA
1,2-Ethanedithiol dioctadecanoate	40	47.40 ± .0420	12	CHA
1,3-Propanedithiol didecanoate	25	28.12 ± .0350	7	CHA
1,3-Propanedithiol didodecanoate	29	32.28 ± .0106	14	CHA
1,3-Propanedithiol ditetradecanoate	33	36.47 ± .0108	18	A
1,3-Propanedithiol dihexadecanoate	37	40.72 ± .0159	15	CHA
1,4-Butanedithiol dioctanoate	22	26.28 ± .0115	9	CHA
1,4-Butanedithiol didecanoate	26	30.78 ± .0297	5	CHA
		31.48 ± .0878	3	
1,4-Butanedithiol didodecanoate	30	35.24 ± .0316	5	CHA
		35.96 ± .0153	17	
1,4-Butanedithiol ditetradecanoate	34	40.52 ± .0105	11	CHA
1,4-Butanedithiol dihexadecanoate	38	45.03 ± .0124	18	CHA
1,5-Pentanedithiol didecanoate	27	25.61 ± .0986	4	CHA
1,5-Pentanedithiol didodecanoate	31	29.14 ± .0127	10	CHA
1,5-Pentanedithiol ditetradecanoate	35	32.74 ± .0165	10	CHA
1,5-Pentanedithiol dihexadecanoate	39	36.26 ± .0328	11	CHA
1,5-Pentanedithiol dioctadecanoate	43	39.76 ± .0397	5	CHA

[a] Hydrogen and carbonyl oxygen atoms not included.
H = *n* Hexane. A = Acetone. C = Chloroform.

value of one polymorphic form of the didodecanoate fell on a line whose equation as determined by the method of least squares is y = 0.428212x − 2.1612, whereas the larger long spacing value of the other polymorphic form of the didodecanoate and the values of the ditetradecanoate, dihexadecanoate, and the dioctadecanoate fell on a line whose equation as determined by the method of least squares is y = 0.444838x − 3.0871. The data indicate that a change in molecular packing (2) takes place when the number of carbon atoms in the acid chain is greater than 12 for this series.

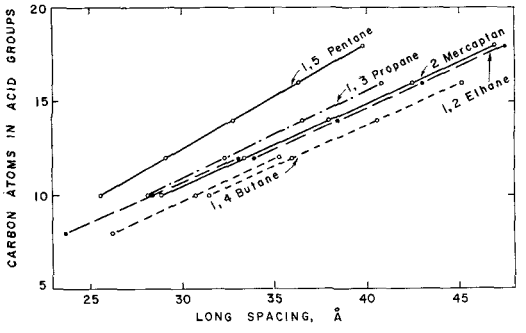

FIG. 1

The relative amounts of the two polymorphic forms found in the X-ray spectra of 1,2-ethanedithiol didodecanoate appeared to be a function of the solvent's polarity. The higher the polarity of the solvent the higher the relative amount of the polymorphic form exhibiting the larger value of the long spacing was present in the spectra.

When the total number of carbon atoms in each acid chain (y) was plotted against the long spacing values (x) of the 1,3-propanedithiol diesters (Fig. 1), their values fell on a straight line whose equation as determined by the method of least squares is y = 0.476292x − 3.3844. Only one polymorphic form was observed. A similar plot of the long spacings of the 1,4-butanedithiol diesters (Fig. 1) showed that the long spacings values fell on two straight lines. The long spacing values of the dioctanoate and the smaller long spacing values of the didecanoate and the didodecanoate, both of which showed the presence of two polymorphic forms fell on a line whose least squares equation is y = 0.446425x − 3.7365, whereas the larger long spacing values of the other polymorphic forms of the didecanoate and the didodecanoate and those of the ditetradecanoate and the dihexadecanoate fell on a

TABLE II

Least Squares Equations for Mono- and Dithiol Diesters Y (carbon atoms in each side chain) = B x (long spacing) + A

Series	B	A
2-Mercaptoethanol diesters	0.444143	−2.82
1,2-Ethanedithiol diesters (low members)	0.428212	−2.1612
1,2-Ethanedithiol diesters (high members)	0.444838	−3.0871
1,3-Propanedithiol diesters	0.476292	−3.3844
1,4-Butanedithiol diesters (low members)	0.446425	−3.7365
1,4-Butanedithiol diesters (high members)	0.442375	−3.9208
1.5-Pentanedithiol diesters	0.564639	−4.4637

straight line whose least squares is y = 0.442375x − 3.9208. A change in molecular packing (2) is indicated when the acid chain contains 10 to 12 carbon atoms in this series. A similar plot of the 1,5 pentanedithiol diesters (Fig. 1), gave a straight line whose least squares equation is y = 0.564639x − 4.4637. Only one polymorphic form was observed in this series. The above results are summarized in Table II.

Given in Table III are the results of the least squares analysis for each series studied where the y parameter was taken as the total number of atoms in the chain (Table I). The limits about B, the linear regression coefficient, and A, the y intercept given in Table III are the 95% confidence limits. Also given in Table III are the reciprocals of the linear regression coefficients in units of angstrom units per additional carbon atom. The average increments in long spacings per additional carbon atom were all less than the maximum calculated increment for a carbon atom, 1.306 Å; therefore, these compounds crystallize in tilted mono- molecular layers.

Examination of Table III shows that significant overlap exists in the 95% confidence ranges in both the B and A parameters of the low members of 1,2-ethanedithiol diester and the lower members of the 1,4-butanedithiol diester series. Therefore the packing in the solid state of these two series is probably isomorphic. Examination of Table III shows that significant overlap exists in the 95% confidence ranges in both the B and A parameters of the high members of 1,2-ethanedithiol diester and the high members of the 1,4-butanedithiol diester series. Therefore the packing of these two series is also probably isomorphic.

Examination of Table III shows that the linear regression coefficient B for the 2-mercaptoethanol diester series is almost identical to those of the high members of both the 1,2-ethane and the 1,4-butanedithiol diester series. A study of the 95% confidence limits also shows considerable overlap. In contrast the y intercepts A of the three series shows only insignificant overlap. However, the covalent radius for sulfur is 1.04 Å, whereas the covalent radius for oxygen is only 0.74 Å (3). The difference in their covalent diameter is therefore 0.60 Å. If one assumes that this difference is equal to the difference in the longest crystallographic axis that one would expect between the same dithiol and monothiol derivative and dividing by 1.306 Å to obtain for the difference in the A parameter one gets a figure of 0.46 Å. If one subtracts this value of 0.46 Å algebraically from the A parameter of the 2-mercaptoethanol diester series and assumes that the 95% confidence limits are relatively unchanged, one now gets considerable overlap in the 95% confidence ranges of the A parameters of these three series. This would lead one to suspect that these three series crystallize isomorphically. This, however, cannot be said with certainty without further study.

Normally an alternation in long spacing between members in a homologous series containing even and odd numbers of carbon atoms is characteristic of long chain compounds (4). Examination of Table III shows that the curves resulting from the 1,3-propanedithiol

TABLE III

Least Squares Equation and 95% Confidence Limits for Mono- and Dithiol Diesters y (total atoms in chain) = B × (long spacing) + A

Series	B	A	$\frac{1}{B}$ (Å per additional carbon atom)
2-Mercaptoethanol diesters	0.888287 ± 0.004775	−1.6310 ± 0.183242	1.126
1,2-Ethanedithiol diesters (low members)	0.856425 ± 0.041177	−0.3225 ± 1.1807	1.168
1,2-Ethanedithiol diesters (high members)	0.889676 ± 0.003883	−2.1742 ± 0.1588	1.124
1,3-Propanedithiol diesters	0.952585 ± 0.010439	−1.7689 ± 0.3628	1.050
1,4-Butanedithiol diesters (low members)	0.892850 ± 0.011746	−1.4730 ± 0.3644	1.120
1,5-Pentanedithiol diesters	1.1293 ± 0.004054	−1.9281 ± 0.1338	0.886

diesters and the 1,5-pentanedithiol diesters are too far removed from the other series to be the same crystalline forms; in fact, too far removed from each other to be the same crystalline forms.

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[Received July 27, 1965]

Gas-Liquid Chromatographic Analysis of Cyclopropene Fatty Acids[1]

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ABSTRACT

A gas-liquid chromatographic method is described for the quantitative estimation of cyclopropene fatty acids as their methyl mercaptan derivatives. This method estimates individual cyclopropene acids as well as normal and cyclopropane acids. Nine seed oils were analyzed for their cyclopropene fatty acid content.

Evidence was obtained for the presence of a cyclopropene fatty acid of shorter chain length than malvalic in *Althaea rosea cav* and one with a higher chain length than sterculic in *Bombacopsis glabra* seed oil. This method is less accurate for cottonseed oil than for the other oils tested because of the appearance of some unsymmetrical peaks of unknown origin.

The mercaptan derivatives of the cyclopropene acids may be isolated by silver ion thin-layer chromatography.

Small amounts of cyclopropane fatty acids were found in a number of the oils analyzed for cyclopropene fatty acids.

INTRODUCTION

At present there are two methods in general use for the estimation of cyclopropene fatty acids in vegetable oils; a spectrophotometric method based on the Halphen test (1) and hydrogen bromide titration at elevated temperature (2). The present authors have had very limited success with the spectrophotometric method. The hydrogen bromide method has given reproducible results, but like the modified Halphen test, it estimates only the total cyclopropene fatty acid content and not the individual acids.

Application of gas-liquid chromatography (GLC) to the analysis of cyclopropene fatty acids has been hindered by their extreme instability at high temperatures. Currently, the analysis of these compounds by GLC is carried out after hydrogenation to the heat stable cyclopropane derivative (3–5). Unfortunately, the hydrogenation often gives rise to a number of side products, making it necessary to correct the results in order to obtain absolute values.

[1] Presented at the AOCS meeting, Chicago, October 1964.

The demonstration of the addition reaction of mercaptans to cyclopropene compounds by Kircher (6) has suggested a new approach to the analysis of these compounds. This report describes a quantitative GLC method for the analysis of cyclopropene fatty acids based on the methyl mercaptan addition product, and a TLC method for the isolation of these derivatives.

EXPERIMENTAL

Procedure

Oils are extracted from crushed seeds with petroleum ether (bp 30–60). The solvent is evaporated under vacuum in a rotary evaporator. The fatty acid methyl esters are prepared by transmethylation of 100 mg of the oils in 5 ml of methanol containing 1% sodium methoxide as catalyst. The reaction is allowed to proceed at room temperature with stirring for 24 hr. After adding 10 ml water the methyl esters are extraced with petroleum ether. The extracts are washed free of the alkaline catalyst with distilled water, dried over anhydrous sodium sulfate, and the solvent evaporated in a stream of nitrogen.

The method of Kircher (6) with the following modifications is used to prepare the mercaptan derivatives of the fatty acid esters:

Ten or 15 mg of the fatty acid methyl esters are treated with a 100% excess (0.25–0.5 ml) of a 12.5% (w/v) solution of methyl mercaptan (Eastman Kodak Company) in benzene and held at room temperature for 5 hr. The solvent and excess mercaptan are removed in a stream of nitrogen and the products analyzed directly by GLC. It is essential to use fresh mercaptan solutions each time in order to obtain quantitative results.

Gas-Liquid Chromatography

A. For the quantitative determination of cyclopropene acids only: If it is desired to know only the kind and amounts of the cyclopropene acids in the oil one may use an Apiezon L column. A 45 cm × 0.4 cm column of 3% Apiezon L on 60–80 mesh Gas Chrom RZ (Applied Science Labs, Inc., State College, Pa.) at 220 with a flame ionization detector and a helium flow rate of 50–60 ml/min was found to give optimum results.

Under these conditions the C_{18} normal acids elute in about 4 to 5 min, the malvalate derivative in about 8 min, and the sterculate derivatives in about 12 min. Similar columns with SE-30 give comparable results.

B. For the quantitative determination of all the acids present: Since under these conditions the nonpolar Apiezon column separates the fatty acid esters by carbon number only, it is necessary to use a polar column for the determination of the normal acids. 20% diethylene glycol succinate (DEGS) on Gas Chrom RZ in 90 cm × 0.4 cm column packed as described by Wood et al. (7) is used. The helium flow rate of about 50 ml/min and a flame ionization detector were found to be best.

The elution time for the malvalate derivative is about 22 min and for the sterculate about 28 min.

Thin-Layer Chromatography

Preparative thin-layer chromatography is carried out with Adsorbosil-1 (Applied Science Labs, Inc., State College, Pa).

The amount of 50 g of Adsorbosil-1 is dissolved in 71 ml of a 12% solution of silver nitrate and applied at 0.75 mm thickness on 8 × 8 in. glass plates. The plates are allowed to dry in air for 30 min and then activated at 110C for 2 hr. The activated plates are cooled and stored in a desiccator in the dark. In order to separate mercaptan derivatives from other fatty acid esters a solvent system consisting of 10% ethyl ether in hexane is used. The derivatives separate from other fatty acid esters, staying near the base line well behind methyl linoleate.

Identification of the Peaks of Malvalate and Sterculate Derivatives

The relative retention times of malvalic and sterculic acids were determined by comparison of the GLC graphs of methyl mercaptan treated fatty acid methyl esters of *Sterculia foetida, Hibiscus syriacus,* and *Gossypium hirsutum* seed oils. *Sterculia foetida* seed oil has been reported to contain a higher percentage of sterculic than malvalic acid, whereas *Hibiscus syriacus* seed oil and *Gossypium hirsutum* seed oil contain a higher percentage of malvalic than sterculic acid (3,8).

A sterculic acid concentrate was prepared by the urea adduct method as described by Kircher (6). The product contained about 94% of sterculate and about 4% of malvalate as determined by the present GLC method.

Tests for Quantitative Response

The quantitative efficacy of the method was tested by 3 procedures:

1. Due to the unavailability of pure cyclopropene fatty acids, quantitative capability of this method could not be tested with absolute standards. However, the methyl mercaptan derivatives of their methyl esters can be prepared pure by TLC. The linearity of the response of the detector to the mercaptan derivatives in mixtures with other fatty acid esters was tested by making quantitative mixtures of the derivatives with methyl stearate and methyl palmitate. The ratio of the weight percentage to area percentage was compared.
2. Known mixtures of corn oil with *Sterculia foetida* seed oil were made and the fatty acids were analyzed by the method described under Procedure. The theoretical and the determined values were compared.
3. The GLC method was compared to the HBr method (2) by analyses of *Sterculia foetida* oil and cyclopropene acids isolated from *Sterculia foetida* oil by the urea adduct technique.

Cyclopropene Fatty Acid Content of Some Seed Oils

A number of seed oils were analyzed for their cyclopropene fatty acid content by the described procedure.

RESULTS AND DISCUSSION

The exact nature of the reactions between the cyclopropene ring and methyl mercaptan is not clear. They can undergo either a free radical or nucleophilic addition. The reaction may be represented as in Figure 1. The $-SCH_3$ group can be added to either the 9- or 10-position, and the saturated ring may be either *cis* or *trans* configuration.

Under the experimental conditions described, the absence of any additional peaks on gas chromatograms indicated that no side reactions occurred. However, if the reaction is carried

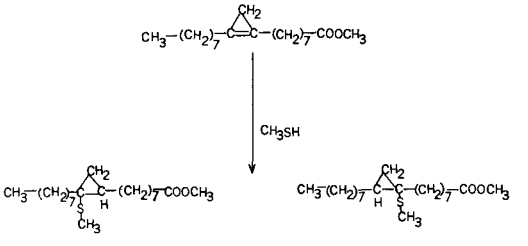

FIG. 1. Methyl mercaptan addition products of methyl sterculate.

TABLE I

Equivalent Chain Length of Methyl Mercaptan Derivatives of Cyclopropene Fatty Acid Methyl Esters

Compound	Stationary phase: Apiezon L 220C	Stationary phase: DEGS 210C
CH_3SH derivative of malvalate	20.38	23.90
CH_3SH derivative of sterculate	21.38	24.90
CH_3SH derivative of the unknown cyclopropene acid in *Bombacopsis glabra*	22.38	25.90
CH_3SH derivative of the unknown cyclopropene acid in *Althaea rosea cav*	18.90	——
Dihydrosterculate	18.80	19.40
Methyl stearate	18.00	18.00

out above 35C, methyl linoleate forms an addition product which will give a peak on GLC between the malvalate and sterculate derivatives.

The addition products do not have the disagreeable mercaptan odor. On GLC columns operated up to 240C, the derivative showed no sign of decomposition. The stability of the derivatives provides a method of purifying cyclopropene containing compounds for structural analysis.

The methyl mercaptan addition products have very long retention times due to the presence of $-SH_3$ groups. In DEGS columns, the mercaptan derivative of malvalate has a retention time of 4–5 times that of methyl stearate. On polar columns the retention times of the mercaptan derivatives have about half that of the nonpolar columns. Nonpolar columns permit the use of higher temperatures which further reduces the overall retention time. Apiezon L columns used in this study permitted the elution of the mercaptan derivative of sterculic acid in about 12 min at 222C. Note, however, that the use of Apiezon L under the described conditions, although giving a clear cut analysis of cyclopropene acids, separates normal fatty acids by carbon number only.

The equivalent chain length of the various fatty acid derivatives are given in Table I. On DEGS columns all these derivatives have a higher retention time than on the Apiezon L column.

The GLC behavior of methyl mercaptan treated fatty acid methyl esters of *Sterculia foetida, Hibiscus syriacus* and *Gossypium hirsutum* seed oils is shown in Figure 2. The identity of the peaks may be ascertained by comparison of the retention items of the methyl mercaptan treated fatty acid esters of *Gossypium hirsutum* and *Hibiscus syriacus* seed oils, both of which have been shown to contain relatively greater amounts of malvalic than sterculic acid (3,8). The peaks are symmetrical so that quantitation by triangulation is possible.

The GLC behavior of the methyl mercaptan treated *Sterculia foetida* seed oil fatty acid

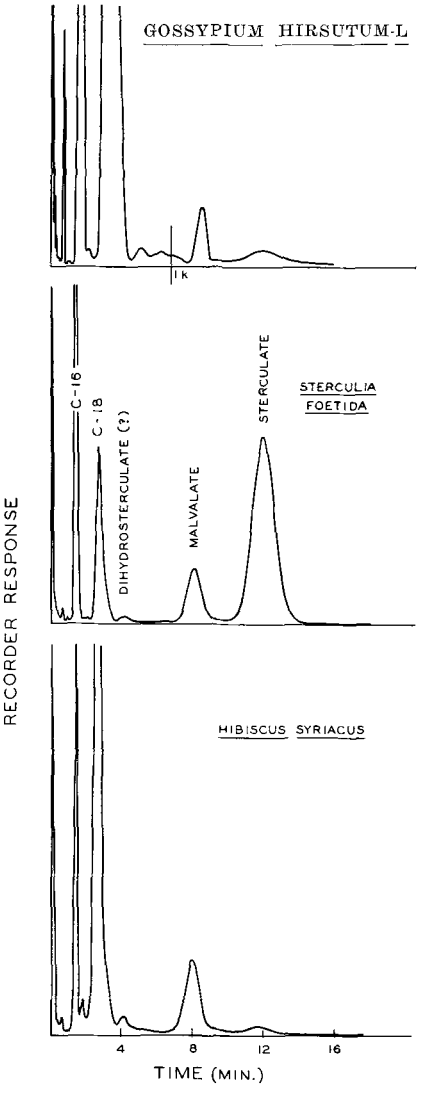

FIG. 2. GLC separation of methyl mercaptan treated fatty acid methyl esters of *Gossypium hirsutum, Sterculia foetida* and *Hibiscus syriacus* seed oils. Column: 45 × 0.4 cm glass column packed with 3% Apiezon L on Gas Chrom RZ (60–80 mesh) at 220C. Flow rate 60 ml of helium per minute. All runs except part of the *Gossypium hirsutum* were made at 3K attenuation.

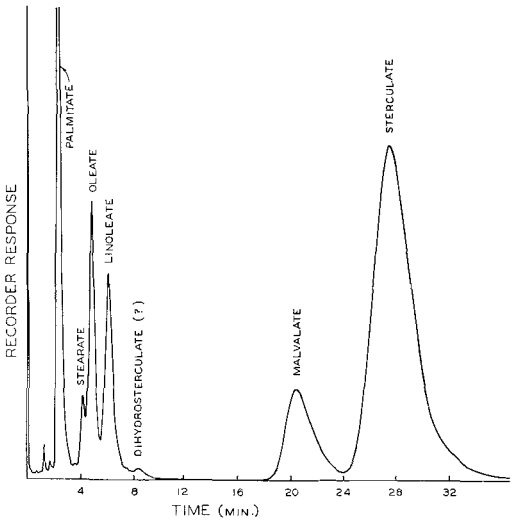

FIG. 3. GLC separation of methyl mercaptan treated fatty acid methyl esters of *Sterculia foetida* seed oil on DEGS column. 90 × 0.4 cm copper column, 20% DEGS on Gas Chrom RZ (60–80 mesh) at 210C, flow rate: 60 ml/min of helium. Flame ionization detector.

esters on DEGS columns is given in Figure 3. Note the long retention time and skewed shape of the mercaptan derivatives. The unsymmetrical nature of the peak may be due to the positional and geometric isomers formed during the reaction. In spite of these difficulties the DEGS column may be used for the complete fatty acid analysis of oils containing cyclopropene fatty acids if one takes the time required for the elution and measures the areas represented by the mercaptan derivatives by planimeter or disc integrator. Thus one may obtain a rapid estimation of the cyclopropene acids by the use of the Apiezon L column and a more time consuming complete analysis by the DEGS column.

Cyclopropene fatty acid contents of a number of seed oils as determined by the Apiezon L column are given in Table II. Malvalic and sterculic acids appear to be common constituents of all the samples analyzed. Dijkstra and Duin (9) have reported the presence of an 18 carbon cyclopropene acid in kapok seed oil and have given it the name "Bombacic acid." The present work shows that there are two cyclopropene fatty acids in kapok seed oil; one has a retention time on GLC as sterculic acid and the other a retention time of malvalic.

The analysis of methyl mercaptan treated fatty acid methyl esters of *Althaea rosea cav* shows, besides malvalic and a small amount of sterculic acids, a major peak with a shorter retention time than either of these. That this is probably a cyclopropene acid is indicated by the fact that the GLC of the methyl esters produced the characteristic decomposition products of cyclopropene fatty acids. Also, on silver ion thin-layer chromatography of the mercaptan treated fatty acid methyl esters developed with ether: hexane (10:90), the acid stayed just above the origin. This shows that this peak is due to a mercaptan derivative. In the case of *Bombacopsis glabra* seed oil analysis, a peak appeared after the sterculate derivative which again appears to be due to cyclopropene fatty acid.

TABLE II

Cyclopropene Fatty Acid Contents of Various Seed Oils as Determined by GLC

Species	Common name	Cyclopropene acid content		
		Malvalic	Sterculic	Unknown
		Percent		
Sterculia foetida	Hazel sterculia	10.33	53.22	—
Hibiscus syriacus	Rose of Sharon	13.55	2.25	—
Hibiscus esculentus L.	Okra	0.50	Trace[a]	—
Bombax malabaricum	Kapok	6.01	6.77	—
Tilia platyphilla	Lime	2.39	0.87	—
Althaea rosea cav	Hollyhock	4.46	Trace[a]	12.23
Lavatera trimestris	Herb tree mallow	6.10	0.30	—
Bombacopsis glabra	A. Robyns	3.09	34.45	5.50
Gossypium hirsutum L.[b]	Cotton	1.58	0.84	—

[a] Less than 0.25%.

[b] Calculated without taking into consideration the unsymmetrical peaks.

Sterculia foetida seeds were obtained from the Director, Bureau of Plant Industry, Manila, The Philippines. *Althaea rosea cav* seeds were purchased from Vaughan's Seed Company, Chicago, Illinois, *Bombax malabaricum* seeds from E. A. Menninger, Stuart, Florida. Oil of *Hibiscus syriacus* and *Lavatera trimestris* seeds was a gift from E. L. Skau, Southern Regional Research Laboratory, New Orleans, La. *Bombacopsis glabra* seeds were a gift from G. G. Shone, North Staffordshire College of Technology, Stoke on Trent, England. *Tilia platyphilla* seeds were purchased from F. W. Schumaker, Sandwich, Mass. *Gossypium hirsutum L.* and *Hibiscus esculentus L.* seeds were obtained from local markets.

TABLE III

Linearity of Recorder Response Between Fatty Acid Methyl Esters and Methyl Mercaptan Derivatives

Sample	Components	Calculated	Found	Weight % area %
		%	%	
Standard mixture 1	Palmitate	44.84	44.90	0.997
	Stearate	24.05	23.69	1.015
	CPA esters[a]	31.11	31.41	0.990
Standard mixture 2	Palmitate	38.03	37.33	1.018
	Stearate	45.91	46.67	0.979
	CPA esters[a]	16.06	16.00	1.003

[a] Methyl mercaptan derivatives of cyclopropene fatty acid methyl esters: calculated as methyl sterculate.

Linearity of the recorder response between the fatty acid methyl esters and the methyl mercaptan derivatives are given in Table III. The weight per cent/area per cent for the mercaptan derivative is found to be one. This is not surprising when considering the fact that the flame ionization detector is insensitive to sulfur dioxide.

The quantitative capability of the present method as compared to the HBr procedure is shown in Table IV. The GLC analysis agrees fairly well with the hydrogen bromide titration method. The sample of cyclopropene acids prepared by the urea adduct method contained a considerably higher ratio of sterculate to malvalate compared to the original sample, showing that the sterculate preferentially forms the adduct under the conditions used. Thus, the composition of the urea adducts cannot be used as a measure of the cyclopropene composition of the original oil. If malvalic acid is present in only small amounts, there is a possibility that it may not be detected in the final concentrate.

The quantitative capability of the method was further tested by preparing standard mixtures of corn oil and *Sterculia foetida* seed oil and analyzing the mixtures for their cyclopropene acid content. The results are given in

TABLE IV

Comparison of GLC and Hydrogen Bromide Quantitative Methods for Cyclopropene Fatty Acids

Sample	HBr	GLC	
		Malvalic	Sterculic
	%	%	%
Sterculia foetida seed oil	61.67[a]	10.33	53.22
Cyclopropene fatty acid purified from *Sterculia foetida* seed oil by urea adduct method	96.74[a]	3.27	94.69

[a] Calculated as sterculic acid.

TABLE V

GLC Analysis of Mixtures of *Sterculia foetida* Seed Oil with Corn Oil

Mixture	Cyclopropene fatty acid content		Deviation
	Calculated[a]	Found	
	%	%	
Number 1	2.89	3.04	+0.15
Number 2	19.17	19.31	+0.14
Number 3	29.62	30.68	+1.06
Number 4	40.60	41.08	+0.48

[a] Based on the value for *Sterculia foetida* seed oil sample as 64.72%.

Table V. The close agreement gives confidence that the method is quantitative.

Cottonseed oil presents problems in applying this method. Some irregular peaks appear after the C_{18} and before the malvalate. In certain cases an incomplete reaction was also noticed. Harris et al. (10) had similar difficulty in applying the HBr method to cottonseed oil. They overcame this by treating the oil with alumina. We applied this technique but it did not remove the irregular peaks. The effect was found in fresh oil extracted from both normal and glandless seeds as well as in refined commercial products.

Presence of small amounts of cyclopropene fatty acids have been reported to be present in *Hibiscus syriacus* seed oil (3). In the present study a minor constituent acid was found in a number of other seed oils which contain cyclopropene fatty acids. On silver ion TLC, this acid moves with the saturated. However, it does not fit the graph representing log of retention time against carbon number of straight chain saturated fatty acid esters (11). The TLC and GLC behavior of the methyl ester of synthetic *cis* 9,10-methylene octadecanoic acid was compared. They were found to behave identically. It is tentatively assumed, therefore, that it is a 19 carbon cyclopropane acid. The content of the cyclopropane acid in various seed oils is given in Table VI. However, if the biosynthesis of cyclopropene fatty acids is by desaturation of the corresponding analog (3,12), one should expect to

TABLE VI

Cyclopropane Fatty Acid Content of Various Seed Oils Which Contain Cyclopropene Fatty Acids

Species	Cyclopropane fatty acid content
	%
Sterculia foetida	0.43
Hibiscus syriacus	1.03
Hibiscus esculentus L.	1.50
Bombax malabaricum	2.55
Tilia platyphilla	1.30
Bombacopsis glabra	2.53

find dihydromalvalic acid as well as dihydrosterculic. We were not able to detect any cyclopropane acid corresponding to malvalic acid.

ACKNOWLEDGMENTS

Cis 9,10-methylene octadecanoic acid provided by R. D. Wood.

This work is taken from a dissertation to be submitted to the Graduate College of Texas A&M University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Supported in part by a grant from the National Institutes of Health (AM-06011).

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[Received Aug. 31, 1965]

Preparation and Properties of Various Salt Forms of Plant Phosphatidyl Inositols[1]

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ABSTRACT

The Ca and Mg content of flax and corn phosphatidyl inositol fractions has been determined. Procedures were devised to prepare various salt forms of phosphatidyl inositol. The divalent cations were exchanged for monovalent ions (Na or K) on chelating resin columns. With the Folch wash procedure the Na or K forms of phosphatidyl inositol were completely converted to the Ca form. The nature of the metal ion associated with the phosphatidyl inositol had a striking influence on the solubility properties of the lipid. The differences in mobility on silicic acid columns of the various salt forms were utilized to free the phosphatidyl inositol from nitrogenous contaminants.

THE CATIONS that are associated with naturally-occurring phospholipids have received little attention, although they exert great influence on solubility properties of the phospholipids and may be of importance in transport phenomena, lipoprotein binding and enzyme activity. The chromatographic behaviors of phosphatidyl serine (1,2) and phosphatidyl inositol diphosphate (3) have been shown to depend upon the type of metal ion combined with the lipid. In this paper the preparation of various salt forms of phosphatidyl inositol and their properties will be discussed.

Phosphatidyl inositol (PI) fractions prepared from plant sources have a high ash content. Flax PI upon ignition gave a 9.4% ash and corn PI 10.0%. Investigation of this ash revealed that magnesium and calcium were the major cations present. The determination of the amounts of Ca and Mg present in the PI fractions was complicated by interference from phosphorus, but a procedure was adapted from a method used by Malmstadt and Hadjiioannou (4,5) to determine Ca and Mg in plant leaves. In this method phosphate was removed by precipitation with zirconium nitrate.

The Ca and Mg of the plant lipids have been exchanged for Na or K by passing the lipids through a chelating resin column in the Na or K form. An organic solvent system (chloroform:methanol:water — 5:4:1, v/v/v) was used to ensure the solubility of the PI fractions. The chelating resin removed 97% of the Ca and Mg and replaced it with Na or K.

The Na form of PI was easily converted to a calcium salt by the washing procedure of Folch et al. (5). Calcium acetate was added to the upper layer of the chloroform-methanol-water (8:4:3, v/v/v) solvent system and the lipid to the lower layer. After the lower layer had been washed twice with fresh portions of upper phase containing no Ca, the lipid was isolated from the lower phase. The molar ratios of phosphorus to calcium in these lipids were always approximately 2 to 1, the same as the P/Ca+Mg ratio observed in the PI fractions isolated from corn and flax phosphatides.

With the Folch procedure the replacement of Ca and Mg by Na was only partially complete. Increasing the ratio of Na to Ca and Mg did increase the displacement. When the concentration of Na in the upper phase was increased from 4 meq of Na per milliequivalent of Ca and Mg to 16 meq of Na, the recovery of the original Ca and Mg dropped from 64% to 31%. The chelating resin columns, however, were much more efficient for this replacement step.

The solubility properties of the salts of PI varied greatly. The Na form of PI dissolved easily in water at the level of 2.5%, but the Ca form gave a gel-like suspension at one-half this concentration. If PI (Na salt) was partitioned between ether and water, 94% of the phospholipid was recovered from the aqueous layer. However, with PI (Ca salt) 75% was found in the ether layer under the same experimental conditions.

The type of cation associated with a lipid also determines its chromatographic mobility. Rathbone (2) noted that the Ca and Mg forms of phosphatidyl serine were eluted from silicic acid-Hyflo Super-Cel columns with chloroform-methanol (4:1,v/v), whereas the Na or K forms were eluted with chloroform:methanol (3:2,v/v). Hendrickson and Ballou (3) found that the Ca and Mg salt of phosphatidyl

[1] Presented in part at AOCS symposium, Pomona College, Claremont, Calif., August 1965.

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inositol diphosphate preceded the Na salt on a DEAE-cellulose column with a gradient elution of ammonium acetate in chloroform-methanol-water. This tendency for the Ca and Mg salts to be eluted with less polar solvents than the Na or K forms has been observed with phosphatidyl inositol. When flax PI (Na salt) was chromatographed on a silicic acid column, the major PI fraction was eluted with chloroform-methanol (3:1,v/v). However, with PI (Ca salt) the bulk of the lipid was eluted with chloroform-methanol (7:1,v/v).

The preparation by silicic acid column chromatography of PI free of nitrogenous contaminants has been found to be very difficult from certain sources such as yeast (7) and brain (3). Phosphatidyl inositol fractions from plant sources presented the same problem, but the different chromatographic mobilities of the various salt forms on silicic acid provided a method by which essentially nitrogen-free material could be obtained. Corn PI (Ca and Mg salt) was converted to PI (K salt) with a chelating resin column (K^+ form), and the K was exchanged for Ca by the Folch procedure. The PI (Ca salt) eluted from silicic acid by chloroform-methanol (7:1,v/v) was changed to PI (Na salt) with another chelating resin column (Na^+ form). This PI (Na salt) was chromatographed on silicic acid, and a low nitrogen fraction (N 0.08%) was obtained upon elution with chloroform:methanol (3:1,v/v).

EXPERIMENTAL AND RESULTS

Analytical Methods

Total nitrogen was determined by the micro-Kjeldahl method and total phosphorus by the procedure of Harris and Popat (8). The phytosphingosine content of the samples was determined as long-chain base nitrogen (LCB-N) by the method of Lauter and Trams (9). The dinitrophenylation procedure of Long and Staples (10) was used to estimate ethanolamine and serine.

Ca and Mg Determinations

The automatic titration method of Malmstadt and Hadjiioannou (4,5) was modified for the determination of Ca and Mg in lipid samples with high phosphorus content. The samples were hydrolyzed by the perchloric acid-nitric acid digestion procedure of Harris and Popat (8). Phosphates were removed by precipitation with zirconium nitrate. Interfering heavy metals were extracted as diethyldithiocarbamate complexes with carbon tetrachloride. Triethanolamine was added immediately before the titration to mask any traces of iron, manganese, copper or aluminum remaining in the samples.

The Sargent-Malmstadt Spectro-Electro Titrator was used with a Sargent Automatic Constant Rate Buret. The filter wheel on the titrator was set at 650 mμ. The constant rate buret, assembled with the 10 ml tube, delivered titrant at the rate of 1 ml/min. Ten milliliter beakers were used as sample holders for the automatic titrations. The indicators were prepared and the disodium dihydrogen ethylenediamine tetraacetate dihydrate (EDTA) titrant was standardized as indicated by Malmstadt and Hadjiioannou (4,5).

The lipid sample (approx. 10 mg) was weighed into a phosphorus digestion tube, and 1 ml of perchloric acid and 4–5 drops of concentrated nitric acid were added. The sample was heated very carefully over a low flame until nitric acid fumes appeared. After the digestion mixture became clear, 3–4 more drops of nitric acid were added. The heating was continued until the solution was colorless. Water (0.5 ml) was added, and the hydrolysis mixture was heated for another 30 min. The hydrolyzed sample was transferred to a 50 ml beaker with a Pasteur pipet. The digestion tube was washed four times with 2 ml portions of deionized water. The beaker was placed on a sand bath heated by a hot plate, and the perchloric acid mixture was evaporated to dryness below its boiling point. The residue was taken up in 2 ml of 1 N hydrochloric acid and again evaporated to dryness. This residue was dissolved and transferred to a centrifuge tube in 8 ml of deionized water.

The amount of zirconium nitrate added was critical, because too little did not precipitate all the phosphate and too much interfered with the sharp color change needed for automatic termination of the titration. For a 10 mg sample containing approximately 3.5% phosphorus, 0.2 ml of 1% zirconium nitrate was added. The sample was thoroughly mixed, and the tube was placed in boiling water for 3 min to aid flocculation of the precipitate. The tube was cooled and centrifuged 3 min. The zirconium nitrate addition was repeated with 0.02 ml increments until no further precipitate was observed.

The supernatant was transferred to a 30 ml separatory funnel. The precipitate was washed with 1 ml of deionized water; the wash was added to the separatory funnel. The complexing agent (0.2 ml of 1% sodium diethyldithiocarbamate) was added, and the solution was

extracted three times with 10 ml portions of carbon tetrachloride. The upper aqueous layer was divided into two portions, and these samples were placed in 10 ml beakers.

One sample was used to determine the total Ca+Mg. A buffer, 0.5 ml of an ammonium hydroxide-ammonium chloride solution (4,5), was added to the sample. The beaker was inserted into the titrator, and stirring was started before 2 drops of triethanolamine and 1 drop of the total Ca and Mg indicator (Eriochrome Black T, G. Frederick Smith Co.) were added. The sample was titrated to the automatic endpoint with the standard EDTA solution. The color change was from wine-red to blue.

The other aliquot was analyzed for Ca. The pH of the sample was adjusted to 13 with 1 N sodium hydroxide. Two drops of ethanolamine and 1 drop of calcium indicator (Calcon, J. T. Baker and Co.) were thoroughly mixed with the sample. The titration with EDTA was automatically terminated when the color of the solution changed from pink to blue. The Mg present was calculated by the difference between the Ca content and the total Ca+Mg.

Preparation of Crude PI Fractions

Flax PI was prepared from linseed oil phosphatides as described previously (11). Corn crude PI was isolated from corn germ oil phosphatides by the same procedure except that lipophytin, a polyphosphate fraction, was removed before the countercurrent extraction of the inositol lipids (12). The crude PI fractions thus obtained were characterized by analyses and by chromatography. Typical data for nitrogen, phosphorus and long-chain base nitrogen in flax and corn PI fractions are listed in Table I.

The percentages of Ca and Mg are given in the same table. The most abundant divalent cation in these fractions was Mg, particularly in corn where only a trace of Ca was found.

Ethanolamine, serine, choline and phytosphingosine were identified as hydrolysis products when 10 mg samples of flax or corn crude PI were hydrolyzed with 0.5 ml of 6 N hydrochloric acid at 100C for 6 hr. Ethanolamine-containing lipids accounted for approximately 50% and serine-containing compounds 12% of the total nitrogen in a flax crude PI fraction. Phytosphingolipids contributed another 11%.

TABLE I

Analyses of Flax and Corn Crude PI Fractions

Sample	N %	P %	LCB-N %	Ca %	Mg %
Flax PI	0.44	3.56	0.08	0.27	0.99
Corn PI	0.68	3.30	0.17	0.09	1.15

The nitrogenous compounds in the crude PI fractions were identified by chromatographing the intact lipids on formylated paper according to the method of Hörhammer, Wagner and Richter (13). Standards and samples (100 μg) were spotted on the paper with a micropipet. The paper was run for 18 hr in a descending system with the upper layer of *n*-butanol-acetic acid-water (4:1:5,v/v/v). After the chromatogram had been dipped in Rhodamine 6G (0.0001% in 0.25 M dipotassium hydrogen phosphate) (14), the spots were observed under ultraviolet light and then visualized by dipping in Nile Blue (0.05% in 0.1M sulfuric acid). When flax or corn PI (Ca and Mg salt) were chromatographed on formylated paper, an intense PI spot appeared at an R_f of 0.46. The major contaminant had an R_f of 0.80. This is the region of phosphatidyl ethanolamine and phosphatidyl choline which are not separated by this solvent system. Trace amounts of phosphatidyl serine (R_f 0.74) and phytoglycolipid (R_f 0.15–0.25) were observed.

Preparation of PI (Na or K Salt)

The conversion from PI (Ca and Mg salt) to PI (Na or K salt) was accomplished by the use of a chelating resin. The resin (Chelex 100, 50–100 mesh, BioRad Laboratories) was prepared in the proper ionic form by the following procedure. It was treated twice with two volumes of 2 N hydrochloric acid and washed thoroughly with deionized water. The resin was then recycled three times with two volumes of 2 N sodium hydroxide (or potassium hydroxide according to the cation desired) and washed with deionized water until the pH was approximately 12. The pH of the resin was adjusted to 8.0 by repeated additions of 2 N acetic acid. For greater solubility of the lipids the solvent phase for the resin was changed to chloroform-methanol-

TABLE II

Analyses of Crude PI Fraction before and after Chelating Resin Column

	Before chelating resin column	After chelating resin column	Recovery %
Weight	14.1 g	12.5 g	89
Nitrogen	0.44%	0.35%	71
Phosphorus	3.56%	3.39%	84
Ca+Mg (expressed as Mg)	1.15%	0.04%	3

water (5:4:1,v/v/v) by pouring this solvent system through the resin on a Buchner funnel. The pH adjustment and the change of solvents were done always immediately before preparation of a column. When the chelating resin was poured into a column, the resin particles in this mesh size tended to float in this solvent system but could be held in place with a perforated porcelain disc or glass wool plug.

In a typical fractionation flax PI (Ca and Mg salt) (14.1 g) was dissolved in 50 ml of chloroform-methanol-water (5:4:1,v/v/v) and added to a chelating resin column (38 cm by 55 cm; total volume of 624 cc). The column was eluted slowly with 1.8 liters of the same solvent system. Table II shows the analyses of the crude PI before and after it passed through the chelating resin.

Interconversion of Salt Forms of PI by Folch Procedure

PI (Na salt) to PI (Ca salt). Flax PI (Na salt) (147 mg) was dissolved in 36 ml of lower phase of the Folch chloroform-methanol-water (8:4:3,v/v/v) system. Calcium acetate monohydrate (21.5 mg) was dissolved in 24 ml of the upper layer (5×10^{-3} M). Calcium acetate was selected rather than magnesium acetate, because the calcium salt was less soluble in organic solvents. The layers were shaken for 1 min and separated by centrifugation. The lower layer was removed and washed twice with 24 ml portions of upper phase which contained no calcium. The lipid (120 mg) was recovered from the lower layer. The percentage of Ca was 1.99% which was very close to the 1.90% Ca+Mg (expressed as Ca) content of the native PI isolated from flax.

PI (Na salt) to PI (Cu salt). Other divalent cations can be substituted for the monovalent with the Folch procedure. The copper salt formed very readily. PI (Na salt) (1.000 g) was dissolved in 240 ml of the lower phase of the chloroform-methanol-water (8:4:3,v/v/v) system. $CuCl_2.2H_2O$ (0.1387 g) was dissolved in 160 ml (0.01N) upper layer. When the two layers were equilibrated, the green copper color immediately transferred to the lower phase. The emulsion problem was not as great as in the Ca and Na exchanges. Only short centrifugations were required after equilibration and the washings. A pale green lipid (0.804 g) was recovered from the lower phase.

PI (Ca and Mg salt) to PI (Ca salt). A high concentration of Ca did not completely replace the Mg in the native Ca and Mg salt of PI. If a chloroform-methanol-water distribution was made using the same concentrations of lipid and calcium acetate as previously, the percentage of Ca salt in the PI (Ca and Mg salt) was increased from 14 to 82.

PI (Ca and Mg salt) to PI (Na salt). Thirty milliliters of lower phase containing 252 mg of flax PI (Ca+Mg, as Mg 1.15%) was equilibrated with 20 ml of upper phase in which 58 mg of NaCl (5×10^{-2} N) was dissolved. After centrifugation and evaporation 225 mg of lipid was found in the lower layer. The Ca+Mg of the recovered lipid was 0.82% (as Mg). If the concentration of the NaCl in the upper layer was increased to 20×10^{-2} N, more Ca and Mg were displaced, but the lipid still contained 0.42% Ca+Mg (as Mg).

Silicic Acid Chromatography. Corn PI (Ca and Mg salt) was converted to the K salt with a chelating resin column (K^+ form). The K was exchanged for Ca in the usual Folch system.

Mallinkrodt silicic acid (100–200 mesh) was prepared for use in column chromatography by washing with methanol and decanting the fines. Immediately before use the silicic acid had been activated at 140C for 12 hr. The silicic acid (450 g) was slurried into the column with chloroform. The column measured 4.8 cm by 57 cm for a total volume of 1033 cc

TABLE III

Chromatography of Corn PI (Ca salt) on Silicic Acid Column

Fraction	ml	Weight, mg	N %	P %	Ca %
PI (Ca salt) (Starting material)		5426	0.68	2.99	1.84
C[a]	3600				
CM 7:1 I	395	642 (12)[b]	0.26 (4)	0.98 (4)	0.82 (5)
CM 7:1 II	980	2599 (48)	0.41 (29)	3.27 (52)	2.38 (63)
CM 7:1 III	2220	303 (6)	0.44 (4)	3.10 (6)	1.68 (5)
CM 4:1	3500	564 (10)	0.60 (9)	3.30 (11)	1.74 (10)
CM 3:1	3600	227 (4)	0.59 (4)	2.78 (4)	1.42 (3)
CM 1:4	3500	500 (9)	1.12 (15)	2.94 (9)	0.85 (4)
		(89)	(65)	(86)	(90)

[a] C and M indicate chloroform and methanol respectively. The ratios are by volume.
[b] Parentheses indicate per cent recovery.

TABLE IV
Chromatography of Corn PI (Na salt) on Silicic Acid Column

Fraction	ml	Weight, mg	N %	P %
PI (Na salt) (starting material)		1506	0.36	3.29
CM 7:1[a]	1420	217 (14)[b]	0.40 (16)	1.83 (8)
CM 4:1	1875	132 (9)	0.26 (6)	2.84 (8)
CM 3:1 II	560	195 (13)	0.12 (4)	3.32 (13)
II	865	365 (24)	0.08 (5)	3.40 (25)
CM 7:3	1450	123 (8)	0.24 (6)	3.18 (8)
CM 1:4 I	350	296 (20)	0.70 (38)	3.45 (21)
II	1600	190 (13)	0.58 (20)	2.42 (9)
		(101)	(95)	(92)

[a] CM indicates chloroform-methanol mixtures; the ratios are by volume.
[b] Parentheses indicate per cent recovery.

and holdup volume of 910 cc. The corn PI (Ca salt) (5.426 g) was dissolved in 25 ml of chloroform and added to the column. Table III shows the milliliters of each solvent system passed through the column and the analyses of the eluted fractions.

The PI (Ca salt) in the chloroform-methanol fraction, CM 7:1 II-Table III, was converted to PI (Na salt) with a chelating resin column (Na^+ form). This PI (Na salt) (1.506 g) was chromatographed on a column of 180 g of silicic acid (1.4 cm by 66.5 cm; total volume of 410 cc). The weight, nitrogen and phosphorus data of the fractions from this column are given in Table IV.

Essentially pure PI with very slight contamination with nitrogenous lipids was obtained in fraction CM 3:1 II-Table IV. The homogenity of this fraction was checked by chromatography on formylated paper. PI was the major lipid observed with only traces of slower moving materials.

ACKNOWLEDGMENTS

Supported in part by a research grant (NB-00574) from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, USPHS.

Linseed oil phosphatides donated by Minnesota Linseed Oil Company and corn germ oil phosphatides by Refining Unincorporated.

Technical assistance by Mrs. Eun Kyung Kim, Mrs. Nancie McCabe and Mrs. Diana Madden.

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[Received Aug. 6, 1965]

The Isolation and Partial Characterization of Gangliosides and Ceramide Polyhexosides from the Lens of the Human Eye

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ABSTRACT

The first isolation of glycolipids from the lens of the human eye is described. Neutral (ceramide polyhexosides) and acidic (gangliosides) glycolipids were separated by column chromatography and further resolved by thin-layer chromatography. The components were methanolyzed, converted to trimethyl silyl ethers and the ratios of the components determined. Two types of monosialogangliosides were found. The most abundant ganglioside contained long chain base/fatty acid/glucose/galactose/neuraminic acid in the ratio 1/1/1/2/1. The ratio of components of the minor ganglioside fraction was 1/1/1/1/1. Dihydrosphingosine was the major base and the major fatty acids were palmitate and nervonate. The ceramide polyhexosides all had a glucose/galactose molor ratio of 1/1 and the mixture of ceramide polyhexosides had a dihydrosphingosine/sphingosine molar ratio of 7.85. The fatty acids ranged from C_{10} to C_{25} with both odd and even carbon chains and were saturated or monounsaturated with palmitate, oleate, and nervonate predominating.

INTRODUCTION

RECENT ADVANCES in techniques of analyzing lipids, especially the use of column, thin-layer and gas-liquid chromatography, permit the separation, characterization and quantification of lipid classes from complex lipid mixtures such as tissue extracts. They also frequently indicate the presence of new lipids. The micro character of these methods has made possible the analysis of very small samples such as the lipids of a single lens. Such analyses have shown that the composition of human lenticular lipids is significantly different from that of cattle and rabbits (1,2), the two most commonly used species for ophthalmic research.

Our initial investigations of human lenticular lipids indicated the presence of gangliosides and neutral glycolipids (1,3). Although a variety of glycolipids have been found in other tissues, none have been reported previously in the lens. This paper presents details on the isolation and characterization of the glycolipids present in the lens of the human eye.

MATERIALS AND METHODS

Tissue Specimens

A total of 120 cataractous human lenses were the starting tissue for this study. Cataractous lenses are more available than normal lenses, can be obtained after surgery, and do not appreciably differ from normal lenses in lipid composition (1). The lenses were stored in clean dry vials at −20C until a sufficient quantity was accumulated.

Extraction

Groups of 20 lenses each were homogenized in an all-glass Potter-Elvehjem type tissue grinder (Kontes Glass Co., Vineland, New Jersey) with 200 ml of chloroform/methanol 2/1 (v/v) saturated with water. The extracts were then filtered through a sintered glass filter of medium porosity. The residue was re-extracted twice with 100 ml of the same solvent and filtered. The combined filtrates from each group of lenses were evaporated to dryness under reduced pressure in a rotary flash evaporator (Buchler Instruments, Fort Lee, New Jersey) with prior purging of the system with nitrogen. Total lipid content was determined by the procedure of Rouser et al. (8).

Thin-Layer Chromatography

An examination of the lipid classes was made by two-dimensional TLC (9). Aliquots (200 μg) of the total lipid extract from a single human lens were applied to 20 × 20 cm glass plates coated with Silica Gel Plain (Research Specialties) containing 10% magnesium silicate. The plates were then developed in the first dimension with chloroform/methanol/water 65/25/4 (v/v), dried in air for 10 min, developed in the second dimension with n-butanol/acetic acid/water 60/20/20 (v/v). After drying, the plates were sprayed with one of the following reagents: 1) sulfuric acid-

potassium dichromate (10), a general spray reagent for the localization of lipid classes; 2) the specific phospholipid reagent of Dittmer and Lester (11); 3) an α-naphthol reagent (12) for the detection of glycolipids; and 4) ninhydrin reagent (0.1% in n-butanol) for compounds containing amino groups.

Isolation of the Gangliosides

The first step in the procedure was cellulose column chromatography (13). Six separate columns (2.5 I.D. × 20 cm) were used with extracts from 20 lenses (approximately 94 mg) applied to each. The major lipid fraction was eluted first with 10 column volumes (about 800 ml) of chloroform/methanol 9/1 saturated with water. The gangliosides plus water soluble nonlipid material was then eluted with 10 column volumes of methanol/water 9/1.

Gangliosides were obtained from the latter fraction by passing it through a column (2.5 × 20 cm) of diethylaminoethyl (DEAE) cellulose (8). Three columns were required to handle the material obtained from the above cellulose columns, i.e., about 120 mg of ganglioside fraction per DEAE column. Samples were applied to the DEAE columns in methanol. Water soluble nonlipids were eluted with 10 column volumes (about 750 ml) of methanol and the mixture of gangliosides free of other lipids was eluted with 10 column volumes of glacial acetic acid.

Partial Characterization of Mixed Gangliosides

Gas-liquid chromatography (GLC) was used to identify the products obtained after methanolysis of the mixed gangliosides (14). In this procedure methanolysis is carried out with anhydrous 0.5 N methanolic hydrochloric acid and glucose, galactose, and neuraminic acid are analyzed as their trimethylsilyl (TMS) derivatives. The lipid bases were determined by the method of Gaver and Sweeley (15) with which methanolysis is carried out in aqueous 1.0 N methanolic hydrochloric acid (10 M with respect to water) and the bases are analyzed as their TMS derivatives. The fatty acids were analyzed as their methyl esters (16).

Resolution of the Gangliosides by Preparative TLC

In order to obtain individual gangliosides, 1 mg of mixed gangliosides from DEAE cellulose columns was separated by TLC with n-propanol/water 70/30 as solvent. Spots were located by spraying with water. While still moist, the individual spots of gangliosides were scraped from the plate with a razor blade, transferred to small beakers and 10 ml of methanol added. After stirring, the mixture was passed through a small sintered glass filter of fine porosity to remove the adsorbent. The eluates were then evaporated to dryness under a stream of nitrogen, subjected to methanolysis as described above and analyzed by GLC.

Isolation of Neutral Glycolipids

After cellulose column chromatography to remove gangliosides, a series of columns (DEAE, TEAE and Florisil) was used to recover a total neutral glycolipid fraction free of other lipids. Finally TLC was used for separation and recovery of individual neutral glycolipids. As a first step, the chloroform/methanol 9/1 eluates from the 6 cellulose columns were evaporated to dryness, dissolved in chloroform and pooled into 2 samples of about 165 mg lipid each. Fifty grams of DEAE slurried in glacial acetic acid was then packed into each of two columns (4.5 cm I.D.) to a height of 20 cm. The columns were washed with one liter portions each of the following solvents in succession: methanol, chloroform/methanol 1/1, chloroform/methanol 9/1 and finally, chloroform. After the sample was applied to the column, the neutral glycolipids together with phospholipids were eluted with one liter of chloroform/methanol 1/1 followed by 5 liters of methanol. Similar fractions from the two columns were combined for further separation.

Phosphatidyl ethanolamine (PE) was removed from the mixture by triethylaminoethyl (TEAE) cellulose column chromatography (8). One hundred grams of TEAE (Cellex T, Bio-Rad Laboratories, Richmond, Calif.) was put into the acetate form by washing it as a slurry first with 2.5 liters of glacial acetic acid followed by 2.5 liters of methanol. The washed TEAE was resuspended in methanol and packed into a tube (8.5 cm I.D.) to a height of 12.5 cm. The column was then washed with 2 liters of methanol followed by 2 liters of chloroform/methanol 1/1. The pooled polar lipid fractions from the two DEAE columns were dissolved in a small volume of chloroform/methanol 1/1 and applied to the column. Elution was begun with 1500 ml (approximately 5 bed volumes) of the same solvent followed by 2400 ml (approximately 8 bed volumes) of methanol. These two fractions containing neutral glycolipids, lecithin, and sphingomyelin were combined for further separation. The

column was cleared of PE with 1500 ml of glacial acetic acid.

The separation of mixed neutral glycolipids from lecithin and sphingomyelin was made with a Florisil column. Water-washed, heat activated Florisil (60/100 mesh) was slurried in methanol containing 5% redistilled, 2,2-dimethoxypropane (DMP) and packed into a 4.5 cm (I.D.) column to a height of 12 cm (17). The sample was applied to the column in a small volume of methanol. The neutral glycolipids were eluted with 500 ml of methanol containing 5% DMP and lecithin and sphingomyelin eluted with 600 ml chloroform/methanol 2/1 saturated with water. The mixed glycolipids were then partially characterized by GLC with the previously mentioned procedures.

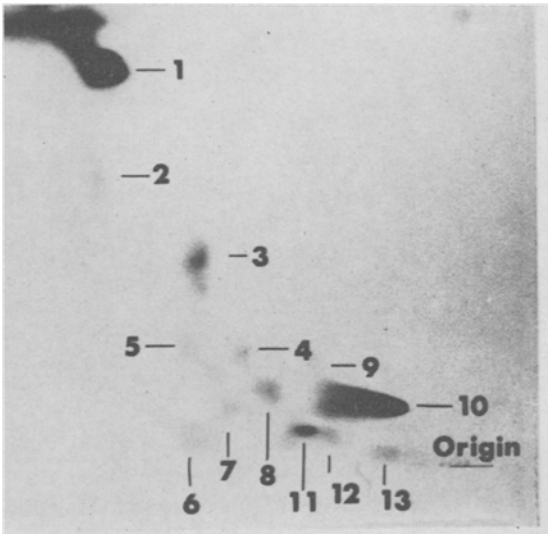
Isolation of Neutral Glycolipids by Preparative TLC

Individual components of the mixed neutral glycolipids were obtained by one dimensional TLC. Approximately 800 μg of material was applied to each of 3 plates (100 μg/individual spot) and developed in chloroform/methanol/water 65/25/4. Plates were sprayed with water to locate the spots and the bands of individual components were scraped from the plates with a razor blade, transferred to test tubes (15 × 125 mm equipped with screw caps) and dried in a vacuum desiccator over KOH pellets. Without prior elution of the compounds, methanolysis was carried out with 0.5 N anhydrous methanolic hydrochloric acid or aqueous 1.0 N methanolic hydrochloric acid as described above. After methanolysis of the adsorbed glycolipids, the adsorbent was removed by filtration through a Pasteur pipette whose tapered end contained a tightly packed plug of glass wool. Flow through this disposable filter can be facilitated, if necessary, by positive pressure applied with a rubber bulb. Fines will pass through the glass wool plug if it is not tightly packed, but these can be removed by re-passage of the filtrate through the filter when the latter contains a small cake of adsorbent. The filtrate is then analyzed for carbohydrates and fatty acids by GLC as described above.

RESULTS

Two-Dimensional TLC

The components of the lenticular lipid mixture are shown in Figure 1. In an earlier study (2) a small number of components were detected by one dimensional TLC. Additional components are detected by two-dimensional TLC combined with special spray reagents. Five of the spots were found to be ninhydrin-positive. Two of these were identified as phosphatidyl serine (PS) and phosphatidyl ethanolamine (PE) by chromatographic characteristics. The remaining components occurred in the area of the chromatogram in which



Spot No.	Tent. Identification	Reagent
1	Neutral Lipids	C, AN
2	Fatty Acids	C
3	PE + Glycolipid	C, N, AN, P
4	Glycolipid	C, AN
5	Glycolipid	C, AN
6	Phosphatidic Acid	C, P
7	PS	C, N, P
8	PI	C, P
9	Lecithin	C, P
10	Sphingomyelin	C, P
11	Glycolipid	C, N, AN
12	Glycolipid	C, N, AN
13	Glycolipid	C, N, AN

1st Dimension: C/M/H_2O 65/25/4
2nd Dimension: BuOH/HAc/H_2O 60/20/20
Silica Gel Plain + 10% $MgSiO_3$

FIG. 1. *Human lenticular lipids by two dimensional thin-layer chromatography.* Abbreviations: PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PI, phosphatidyl inositol; C, charring with potassium dichromate-sulfuric agent; An, α-naphthol; N, ninhydrin; P, phospholipid spray.

gangliosides are found. Since gangliosides are ninhydrin-positive under some conditions, these components were tentatively identified as gangliosides. Further evidence of the identity of these compounds was obtained after spraying with the α-naphthol reagent. The spots in the ganglioside area were positive to this carbohydrate detection reagent. In addition, several other α-naphthol positive spots were evident and tentatively identified as neutral glycolipids. The major α-naphthol positive component had the chromatographic properties of a synthetic sample of ceramide dihexoside and overlapped the PE spot.

Identification of Gangliosides

Recoveries from cellulose columns averaged 94% since the samples contained a small amount of protein that was not removed before application to the cellulose columns. Higher recoveries were obtained with an extract of 40 pooled human lenses and with 6 replications of bovine lenticular lipids, all of which were filtered prior to chromatography (recoveries were 99.8% with the human sample and averaged 99.6% with the bovine samples). However, it was deemed advisable to omit the filtration step to ensure that this procedure, plus the additional evaporation to dryness required, did not lead to the partial destruction or loss of any of the compounds.

A total of 30.84 mg of gangliosides was obtained from three DEAE columns (10.25, 10.27 and 10.32 mg, respectively). The percent recovery of the total sample from these columns is not meaningful because the nonlipid fraction undergoes a considerable increase in weight as a result of the ion exchange leading to recoveries in excess of 100%. However, the reproducibility obtained with the three columns is evidence of satisfactory performance.

The GLC analysis of the mixed gangliosides showed the carbohydrates to be galactose and glucose in a molar ratio of 1.56:1.00 as calculated from the peak areas. Hexosamine was not present since acetylation after methanolysis (required for GLC analysis of hexosamine) did not lead to the appearance of a peak for N-acetyl hexosamine. The neuraminic acid/glucose molar ratio was 0.94.

The lipid base was found to be predominantly dihydrosphingosine, the molar ratio dihydrosphingosine/sphingosine being 2.66 calculated from peak areas. There was no evidence for the occurrence of longer or shorter chain analogs of sphingosine. From the fatty acid composition data shown in Table I it can be seen that palmitate and nervonate predominate. In this regard gangliosides are very similar to human lenticular sphingomyelin (2).

The mixture of gangliosides was resolved by TLC with n-propanol/water 70/30 into 3 major areas, each composed of two spots which were analyzed together (Fig. 2). The pair of spots with lowest R_f (Fraction 1) comprised the major portion of the mixture. This fraction contained galactose and glucose in a molar ratio of 2:1. If the 2 spots differed in carbohydrate composition, an even ratio could not have been obtained. The slight difference in R_f value for the pair of spots is probably due to differences in base and fatty acid composition.

The other 2 fractions contained galactose and glucose in a 1:1 molar ratio. All three fractions contained neuraminic acid in a 1:1 molar ratio either with respect to glucose or to ceramide. Thus each of these three major components are monosialogangliosides.

Identification of Ceramide Polyhexosides

The neutral glycolipids upon GLC analysis were found to contain galactose and glucose in a 1:1 molar ratio. As in the case of the gangliosides, the base structures were dihydrosphingosine and sphingosine, with the former predominating in a 7.85:1.00 molar ratio. The fatty acid composition was similar to that of the gangliosides with palmitate and nervonate predominating (Table I).

TABLE I

Fatty Acid Composition of Human Lenticular Gangliosides and Neutral Glycolipids

Fatty acid	% Composition	
	Ganglioside	CPH
10:00	—	0.6
12:0	—	1.0
13:0	—	0.3
14:0	1.5	1.79
14:1	0.3	0.9
15:0	0.4	0.8
15:1	Trace	Trace
16:0	20.9	15.7
16:1	1.2	1.7
17:0	Trace	Trace
17:1	Trace	Trace
18:0	6.1	3.6
18:1	9.3	12.4
19:1	—	1.1
20:0	Trace	2.4
20:1	Trace	0.6
21:0	—	2.4
21:1	—	4.9
22:0	3.4	2.1
22:1	3.4	7.8
23:0	0.4	Trace
23:1	1.3	8.4
24:0	9.2	8.1
24:1	42.5	15.0
25:0	Trace	Trace
25:1	Trace	3.9
Unknown		4.8

TLC (Fig. 3) revealed a series of well resolved spots that appeared to occur in pairs, the pair having the highest R_f value being the most abundant. Each spot was found to have galactose and glucose in a 1:1 ratio. Further study is required to completely characterize these paired spots.

DISCUSSION

The lens of the eye is an ellipsoid structure situated behind the iris. Although it is an important part of the visual system, it lacks both blood vessels and nerves. Its cellular architecture is very simple and consists of two types of cells: *epithelial cells* that cover its anterior face in a sheet that is one cell in thickness and *lens fibers* that lie beneath the epithelium and are orientated in an antero-posterior direction. The whole structure is contained within an acellular *capsule* of collagenous material.

There are several characteristics of the lens fibers that make them unique among living cells. Those that occupy the central portion of the tissue or *lens nucleus* are formed during the fetal period. Shortly after birth they begin to lose both their nuclei and mitochondria and remain throughout life as transparent cylinders. Despite the lack of these subcellular particles, the nuclear lens fibers have metabolic activity, presumably from cytoplasmic or microsomal enzyme systems. Growth and formation of lens fibers occur throughout life by the differentiation of epithelial cells at the lens equator. Perhaps the most striking feature of the lens fibers is their size. Those occupying the central portion reach a length of over 5 mm or roughly the antero-posterior thickness of the lens at that point.

Histochemical studies have shown that the intercellular membranes of the lens fibers are strongly sudanophilic, indicating that lipoidal material is localized at this site (18). Such findings suggest that one of the major functional roles of the lenticular lipids is as structural components of the cellular membranes. Available data from other organs indicates that polar lipids occur almost exclusively as components of membranes of subcellular particles. Failure to demonstrate fatty acid oxidation in the lens is in keeping with a structural rather than a metabolic role for lipids and indicates a great stability of lenticular membranes. Thus, it appears likely that the sudanophilic areas of the lens are indeed the membranes of the lens fibers.

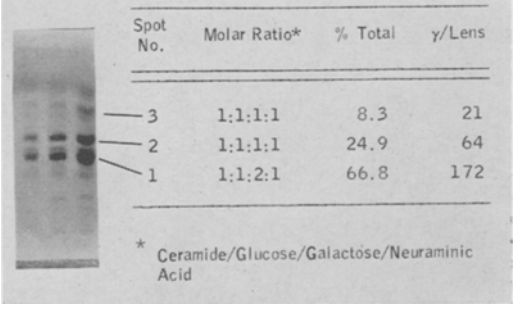


FIG. 2. Composition of human lenticular gangliosides.

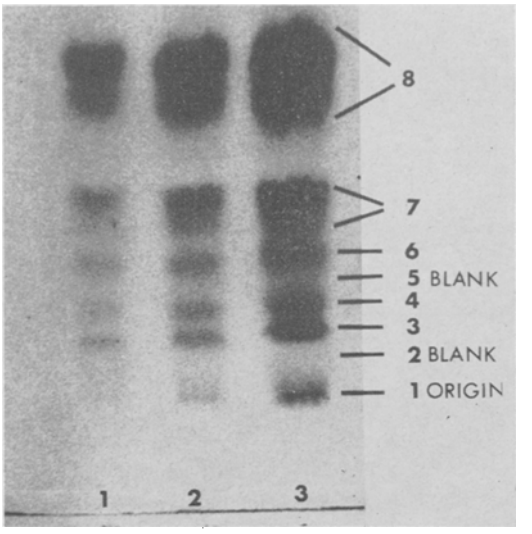


FIG. 3. Thin-layer chromatogram of the neutral glycolipids from the human lens. The plate was developed in chloroform/methanol/water 65/25/4. Spots were located with the charring technique. Sample applications represent approximately 50, 100, and 200 μg. Numbers refer to the fractions taken for carbohydrate analysis.

Dihydrosphingosine is the major lipid base in gangliosides of the lens. A high level of dihydrosphingosine has been reported for sphingolipids of spinal cord in contrast to brain where sphingosine predominates (19). Lenticular gangliosides do not contain hexosamine and in this regard, as well as in their fatty acid composition, they differ from the major brain gangliosides. Lenticular gangliosides resemble the hematosides of equine erythrocytes (4) and the major gangliosides of human spleen (7).

The nomenclature of the gangliosides has not as yet been standardized. It seems desirable to name the lenticular gangliosides as deriva-

tives of ceramide (5). Thus the names ceramide monosialotrihexoside and ceramide monosialodihexoside are proposed for the lenticular components until such time as their molecular structures have been completely defined.

The neutral glycolipids are similar to those of other tissues except in the large amount of dihydrosphingosine that they contain. Compounds with simliar carbohydrate molar ratios and fatty acid composition have been reported in human kidney for example. However, unlike this tissue in which the predominant compound is a ceramide trihexoside, the major lenticular neutral glycolipid is a ceramide dihexoside.

ACKNOWLEDGMENT

USPHS Grants NB-04277, NB-04116, and NB-01847-07 from the National Institutes of Neurological Diseases and Blindness; Special Fellowship (1-F11-BN-1328) from that Institute to the senior author; and Grant CA-03134-09 from the National Cancer Institute. Additional support was provided by grants from the Moody Foundation of Galveston, Texas and from Research to Prevent Blindness, Inc., New York City.

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[Received Aug. 31, 1965]

Evaluation of Mathematical Distribution Methods for the Determination of Triglyceride Composition[1]

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ABSTRACT

The triglyceride composition of a number of animal and vegetable fats was determined directly by means of selective argentation and lipase hydrolysis, and compared to that given by the 1,3-random,2-random method of analysis described by Vander Wal [JAOCS *37*, 18 (1960)].

Exceptions to the basic assumption of the 1,3-random,2-random method that the fatty acids in the 2-position are distributed randomly are reported.

The analyses of some fats determined by the 1,3-random,2-random method agreed closely with those determined by the direct method, but the overall results indicated that methods based on mathematical distribution patterns generally are not as precise as direct methods.

INTRODUCTION

ALTHOUGH GREAT STRIDES have been made in chromatographic techniques for the analyses and fractionation of triglycerides (1–12), methods based on mathematical distribution patterns of the fatty acids are still being widely applied for the determination of the structure of these compounds. The most widely applied method of this type is the 1,3-random, 2-random method (13–15), or slight modification thereof (16). These methods are based on the total fatty acid composition and the composition of the fatty acids distributed in the β-position of the molecule as determined via the pancreatic lipase technique (13). The agreement between the results determined by these methods and those for which analytical data are available may be very close for some fats, especially certain vegetable oils, partial for other fats and in obvious discord for still other fats.

In the present investigation the structural analysis of the triglycerides of a number of animal and vegetable fats is determined by a method based on fractionation by selective argentation and lipase hydrolysis (8) and compared to values obtained by the 1,3-random, 2-random method described by Vander Wal (17).

[1] Presented at AOCS meeting, Houston, 1965.

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EXPERIMENTAL

MATERIALS AND METHODS

Triglycerides of greater than 98% purity as determined by thin-layer chromatography (TLC) were obtained from the following sources: commercial samples of refined corn and cottonseed oil, cocoa butter, human subcutaneous and omenta fat, akee fruit (*Blighia sapida* Sapendacea), rat liver lipid of animals raised on a fat-free diet (9), and the lipids of several tissues of swine obtained fresh from a local meat packer.

Tripentadecanoin, the standard triglyceride mixture and other fatty acid esters employed as standards are products of The Hormel Institute.

The analytical method employed for the determination of triglyceride structure has been described in detail previously by the authors (8).

Gas-liquid chromatography (GLC) was carried out with an F&M Model 609 frame ionization instrument equipped with a 1/4 in. × 7 ft column packed with 10% ethylene glycol succinate polyester (EGSS-X, Applied Science Laboratories, State College, Pennsylvania) on 100–120 mesh Gas-Chrom P with a carried gas flow of 60 ml/min at 185C.

RESULTS AND DISCUSSION

The reproducibility of the method for the determination of triglyceride classes is illustrated in Table I. Previously (8), it had been shown the absolute error of the method was ± 0.7%. The standard deviation of the results in Table I is ± 0.5%. Thus, differences between experimental values of greater than 1.0 percentile units generally are significant.

The analyses of all of the samples of triglycerides are presented in Tables II and III. Since the 1,3-random-2-method provides a triglyceride analysis only in terms of saturated and unsaturated fatty acids as groups, the

TABLE I

Analysis of a Standard Triglyceride Mixture

	No. 1	No. 2	No. 3
Tripalmitin	2.4	2.4	2.4
Dipalmitomonolein	53.6	53.7	54.0
Monopalmitodiolein	38.1	38.6	38.1
Triolein	5.9	5.3	5.6

TABLE II

Structural Analysis of Natural Triglycerides

TG type 1,2,3	Corn oil	Cotton-seed	Akee	Cocoa butter	Human subcutaneous	Human omental	Rat liver
SSS	tr	0	0	0	0	1.5	2.7
SSM	1.6	4.5	4.5	0	7.8	12.3	4.2
SMS			29.3	76.3	7.4		15.0
SSD	2.7	13.5	tr	0.4	0.6	2.7	—
SDS				7.1	1.4		—
MSM	0.3	2.5	1.0	0.2	2.2	32.6	2.7
MMS	4.3		41.2	11.8	34.9		57.1
DSD	0.5	27.9	tr	tr	0.1	0.6	—
DDS	13.9				0.6		—
SMD	5.6	20.3	1.1	0.7	3.2	12.7	—
SDM	7.7		6.3	1.4	9.7		—
DSM	0.6		0	0.2	0.7		—
MMM	3.9	1.5	8.5	0.8	17.7	22.1	18.3
MMD	8.0	5.0			4.6	9.0	—
MDM	5.2				3.8		—
MDD	19.2	11.4	3.8	1.1	5.3	6.5	—
DMD	5.0						—
DDD	21.5	13.4					—

S = Saturated fatty acids, M = monoenoic fatty acids and D = dienoic fatty acids.

values in Tables II and III were recalculated in these terms for convenience in making a comparison with the 1,3-random,2-random method. The recalculated analytical data for the vegetable oils are compared to the results of the 1,3-random,2-random method in Table IV. Although the experimental values and the results obtained by the Vander Wal method agreed fairly well for corn oil and cottonseed oil triglyceride analyses, the experimental values for both cocoa butter and akee oil showed significant deviations from the 1,3-random,2-random method. For example, the values for the S_2U class are higher in both of these oils than those obtained by the 1,3-random,2-random method. Also, the experimental values for the S_1U_2 class are lower for cocoa butter and higher for akee oil than those obtained by the 1,3-random,2-random method. In the akee oil the amount of the U_3 class is lower by direct analysis than the Vander Wal calculated values. The same type of variation from the 1,3-random,2-random distribution shown here for coca butter has also been reported by Subbaram and Youngs (18).

Table V shows a comparison of the results obtained by the two methods on the triglycerides of rat liver and two samples of human fat. The experimental values for the triglyceride structure of the human tissues showed a significant disagreement with those calculated by the 1,3-random,2-random method. The

TABLE III

Structural Analysis of Swine Tissue Triglycerides

TG type 1,2,3	Mesentery	Heart	Kidney	Lung	Pancreas	Spleen	Liver
SSS	7.9	2.6	5.5	3.2	5.8	5.4	0.9
SSM	31.4	24.1	30.6	24.0	32.1	28.6	5.4
SMS	0.3	0	0	0.6	0	tr	7.3
SSD	7.5	3.6	5.4	4.9	5.8	5.4	1.6
SDS	0	tr	0.4	0	0	0	2.7
MSM	27.9	33.2	25.4	31.9	27.2	26.5	2.6
MMS	1.9	0.9	6.0	7.3	3.5	4.2	21.6
DSD	1.4	0.7	0.9	1.2	2.1	2.3	1.5
DDS	0	0.3	0	0	0	tr	3.3
SMD	0.2	0.8	0.5	0	0	0.9	7.9
SDM	0.1	0	1.2	1.4	0.9	1.1	8.2
DSM	11.1	8.6	8.8	8.1	9.7	8.7	1.6
MMM	4.1	13.5	7.6	9.0	6.0	7.4	9.9
MMD	1.6	3.1	2.5	2.4	1.7	2.1	5.3
MDM	0.8	1.9	1.3	1.3	0.9	1.2	3.6
MDD	0.4	0.9	1.2	0.5	1.1	0.9	0.8
DMD	0.8	0.5	0.3	0.5	0.5	0.6	4.5
Others	2.6	5.3	4.2	3.7	2.7	4.7	11.3

TABLE IV

Comparison of the Structural Analyses of the Triglycerides of Vegetable Oils by Direct Analyses and the 1,3-random, 2-random Methods.

TG type 1,2,3	Cocoa butter			Corn oil			Cottonseed			Akee oil		
	1	2	3	1	2	3	1	2	3	1	2	3
SSS	0	0.8	0	tr	tr	0	0	0.4	0	0	2.0	0
SUS	83.4	81.6	83.2	4.3	4.6	4.3	18.0	18.6	18.7	33.6	34.1	39.4
SSU	0.4	0.1								4.5	2.6	
SUU	13.9	16.6	15.8	32.9	32.0	33.1	50.7	48.4	49.1	48.6	45.4	46.7
USU	0.4	0.1								1.0	0.9	
UUU	1.9	0.8	1.0	62.8	63.3	62.6	31.3	32.6	32.2	12.3	15.0	13.8

1 — Blank, Verdino and Privett (8).
2 — Calculated according to Coleman or Vander Wal's 1,3-random,2-random distribution (14,15).
3 — Calculated according to Gunstone (16).
S = Saturated fatty acids and U = Unsaturated fatty acids.

greatest disagreement occurred in the analysis of the sample of subcutaneous fat. The values obtained by the 1,3-random,2-random method were low for the SU_2 class and correspondingly high for the U_3 class. The same type of disagreement has been shown previously for human fat by Subbaram and Youngs (18). Differences in the results by the two methods for the omental fat were less pronounced.

A great divergence between the results of the two methods was also observed with rat liver triglycerides. Such differences have been pointed out previously by Vander Wal (19), as well as the authors (8). The group of rats from which the fat was obtained had been maintained 12 weeks on a fat-free diet. A similar pattern of results was also obtained in rats which received either lard or corn oil in their diets, and indicated that endogenous-exogenous mixtures of triglycerides were not responsible for the variance between the two methods of analyses.

The data obtained on the swine tissue triglycerides (Table III) are condensed and compared with the values obtained by the 1,3-random,2-random method in Table VI.

Table VI shows that although the differences between the values of the two methods are not greatly different, the same triglyceride types, namely S_2U, SU_2 and U_3, always vary in the same direction. The pattern was the same in all of the tissues except the liver. The triglycerides of the liver contained predominately unsaturated fatty acids in the 2-position as opposed to saturated fatty acids in the 2-position of triglycerides of the other tissues.

A close examination of the composition of the swine tissue triglycerides (Table VII) showed that the percentage of palmitic acid esterified at the 2-position varied in the 5 tissues from 60–70% in the trisaturated glycerides to 100% in the unsaturated glycerides and, therefore, contradicts the basic assumption of the 1,3-random,2-random method that the fatty acids are randomly distributed in the 2-position. Thus, application of the pancreatic lipase technique to the total fat gives only an average value for the distribution of the fatty acids in the 2-position of all the glyceride classes.

DISCUSSION

The 1,3-random, 2-random methods of tri-

TABLE V

Comparison of the Structural Analyses of the Triglycerides of Three Animal Tissues by Direct Analysis and by the 1,3-random,2-random Methods.

TG type 1,2,3	Human *subcutaneous*			Human omental			Rat liver		
	1	2	3	1	2	3	1	2	3
SSS	0	1.6	0	1.5	1.7	0	2.7	2.1	0
SUS	8.8	12.2	18.4	15.0	16.6	16.2	15.0	20.9	37.6
SSU	8.4	5.3					4.2	4.5	
SUU	48.4	41.5	49.0	45.9	44.7	48.0	57.1	45.5	49.9
USU	3.0	4.5					2.8	2.4	
UUU	31.4	34.9	32.6	37.6	37.0	35.8	18.3	24.6	22.5

1 — Blank, Verdino and Privett (8).
2 — Calculated according to Coleman or Vander Wal's 1,3-random,2-random distribution (14,15).
3 — Calculated according to Gunstone (16).

TABLE VI

The Structural Analyses of Swine Tissue Triglycerides by Direct Analysis and the 1,3-random,2-random Method (14,15).

TG type 1,2,3	Mesentery		Heart		Kidney		Lung		Pancreas		Spleen	
	1	2	1	2	1	2	1	2	1	2	1	2
SSS	7.9	7.2	2.6	2.2	5.5	6.0	3.2	3.7	5.8	6.0	5.4	5.1
SUS	0.3	1.0	tr	0.8	0.4	1.8	0.6	1.4	0	1.3	tr	1.5
SSU	38.9	35.6	27.7	21.0	36.0	30.8	28.9	25.5	37.9	32.6	34.0	29.4
SUU	2.2	5.2	2.0	7.8	7.7	9.4	8.7	9.3	4.4	6.8	6.2	8.8
USU	40.4	44.5	42.5	49.6	35.1	39.8	41.2	44.1	39.0	44.1	37.5	42.4
UUU	10.3	6.5	25.2	18.6	15.3	12.2	17.4	16.0	12.9	9.2	16.9	12.8

1 — Blank, Verdino and Privett (8).
2 — Calculated according to Coleman or Vander Wal's 1,3-random,2-random distribution (14,15).

glyceride analyses were originally developed for the analysis of several common vegetable oils. However, in recent years, these methods have been applied to animal as well as a wide variety of vegetable oils with little proof that the distribution theory upon which they are based is valid. Although the methods of triglyceride analyses based on the 1,3-random,2-random distribution theory appears to give close agreement with experimental values on some vegetable oils, it obviously gives erroneous values on many fats as demonstrated here. Moreover, the discrepancies observed here were revealed by a method which did not give a complete determination of triglyceride structure. Had more elaborate experimental methods been applied, perhaps discrepancies would also have been revealed in the analyses of the more common vegetable oils. In this connection, it has been shown recently that distribution of the fatty acids in bayberry tallow (20), sable fish (22) and also bitter gourd triglycerides (21) varied significantly from mathematical distribution theories by direct methods of analysis. The validity of the basis for the 1,3-random,2-random theory has also been challenged by Morris (23). Thus, it is apparent that although the 1,3-random, 2-random method may still have some practical utility, it should not be applied indiscriminately.

ACKNOWLEDGMENT

Supported in part by USPHS Grant No. HE 05735 from the NIH and a grant from the Special Dairy Industry Board of the American Dairy Council.

TABLE VII

Percentage 16:0 Esterified at 2-Position in Swine Tissue Triglycerides

TG type		Mesentery	Kidney	Lung	Pancreas	Spleen
S_3	=	68.6%	61.5		69.5	73.8
S_2O_1	=	84.8%	82.5	81.3	88.7	83.7
S_1O_2	=	100%	97.1	99.7	101	98.8
SOL	=	105%	100	102	103	104
Total	=	96.7%	85.3	91.6	95.6	92.6

Assistance in the determination of triglyceride structures, David Schlichting and Francis Lightly. Akee fat provided by A. C. Ellington, Government Chemists, Kingston, Jamaica; cocoa butter sample, E. C. Hammond, Iowa State University; samples of human fats, R. J. Vander Wal, Armour & Company.

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[Received July 1, 1965]

Antigenic Properties of a Synthetic Protein Complex with Glycolipids and Related Substances[1,2]

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ABSTRACT

Erythro-sphingosine was obtained from sphingomyelin by alkaline hydrolysis. N-*p*-nitrobenzoyl-sphingosine, N-*p*-aminobenzoyl-dihydrosphingosine and dihydrosphingosine-protein were synthesized.

It was found that dihydrosphingosine-protein can produce a specific antibody which can be detected by the complement fixation test and by Ouchterlony's double diffusion method in agar. The determining factor of dihydrosphingosine may be due to the hydroxy groups at C_1 and C_3.

In the course of experimental allergic encephalomyelitis, the cross-reactivity of rabbit antisera against spinal cord, and with psychosine-protein in particular, was observed by the complement fixation test and by the Arthus reaction.

SINCE WE HAVE ISOLATED ABO-blood group specific mucolipids from human erythrocytes and Forssman active mucolipids from equine or guinea-pig kidney and spleen (1–4), the relationship of chemical structure of these mucolipids and serological activity has become of interest to us. As a first approach to the basic immunochemical study of glycolipids, we have attempted to prepare artificial antigens capable of producing by themselves the lipid-hapten specific antibodies. As already published (5), we found that psychosine, the product obtained by deacylation of cerebroside, is fully antigenic when coupled with a protein through diazotization of its N-*p*-aminobenzoyl derivative.

In the present paper, an effort has been made to elucidate whether or not a sphingosine-specific antibody can be produced by immunization with a sphingosine-protein complex. Naturally occurring *erythro*-sphingosine was obtained by alkaline hydrolysis of sphingomyelin. Acidic hydrolysis which produces the *threo* isomer of sphingosine and the *threo*- and *erythro*- *O*-methyl ethers of sphingosine was avoided (6). The demonstration of *erythro*-sphingosine product was dependent upon the fact that *erythro*, but not *threo*, dihydrosphingosine forms a crystalline tribenzoyl derivative (7). The *erythro*-sphingosine was coupled with a protein through diazotization of its N-*p*-aminobenzoyl derivative. It was found that immunization with sphingosine-protein produces sphingosine-specific antiserum in rabbits.

Joffe, Rapport and Graf (8) have found that galactocerebroside can react with antibody prepared against brain fractions in the presence of auxiliary lipids. Niedieck and Kuwert (9) have found that the hapten which is serologically active in ethanolic spinal cord extract is cerebroside, and that in the course of experimental allergic encephalomyelitis, antibodies are produced against cerebroside. Brady and Trams (10) have suggested that an autoimmune reaction, possibly directed against other lipids such as gangliosides which are predominantly located in the neuronal cell body, may be a contributing factor in the pathogenesis of demyelinating diseases. In the present paper, we have investigated the cross-reactivity of psychosine-protein, sphingosine-protein, and other sugar-proteins with antibody prepared against the rabbit spinal cord by the complement fixation test. Antibody capable of cross-reacting with psychosine-protein complex has been found in the sera of rabbits immunized with an encephalitogenic mixture of spinal cord and adjuvants. Judging from the antigenicity of psychosine-protein, one of the determinant groups of antigenicity of spinal cord may be concerned with glycolipids, particularly cerebroside. These experimental results will be presented in the present paper.

MATERIALS AND METHODS

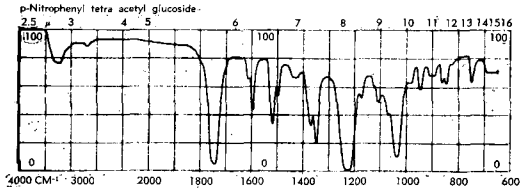
Alkaline Hydrolysis of Sphingomyelin

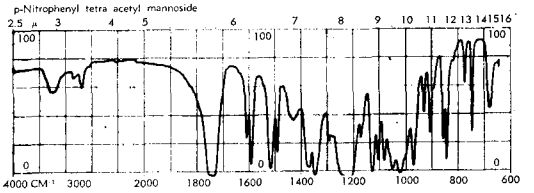
Sphingomyelin (4–5 g) obtained from bovine brain, was hydrolyzed for about 2 hr in 100 ml of 90% aqueous butanol solution containing N KOH as a final concentration under reflux

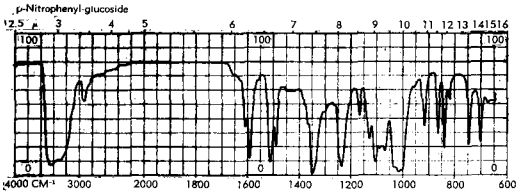
[1] Presented at the Prof. Ernst Klenk Symposium on Glycolipids and the Nervous System, AOCS Meeting, Houston, April 1965.

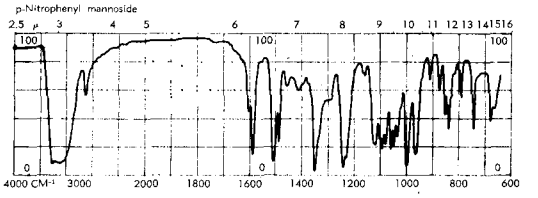
[2] Abbreviations: GVB, gelatin-veronal buffer containing 1.5×10^{-4} M $CaCl_2$ and 5×10^{-4} M $MgCl_2$, pH 7.5; SE, dihydrosphingosine—egg albumin; SS, dihydrosphingosine—Serum albumin; CE, ceramide—egg albumin; PS, psychosine—serum albumin; PE, psychosine—egg albumin; GE, glucose—egg albumin; LS, lactose—serum albumin; LE, lactose—egg albumin; Gal-NS, N-acetyl galactosamine—serum albumin; GNE, N-acetyl glucosamine—egg albumin. C^1H_{50} refers to the hemolytic unit of complement, i.e., that amount required to lyse 50% of 5.0×10^8 sensitized red cells under standard conditions (24).

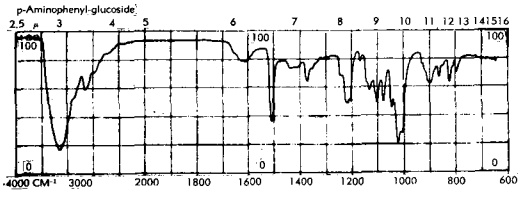
on a mantle heater (5). The hydrolysate was then washed with distilled water three times in a separatory funnel. The upper butanol solution was concentrated to dryness. The residue containing crude sphingosine was chromatographed on a silicic acid column to obtain *erythro*-sphingosine. The final yield was about 50–60% of the theoretical value.











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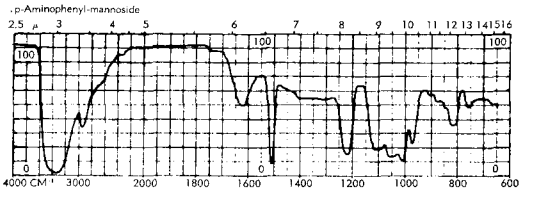

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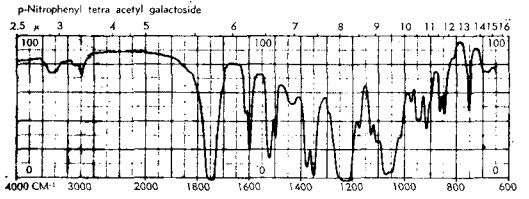

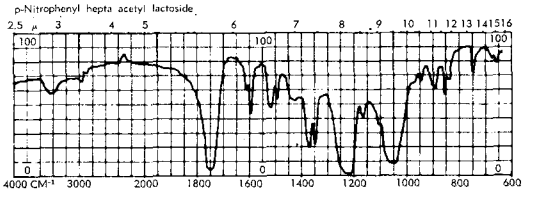

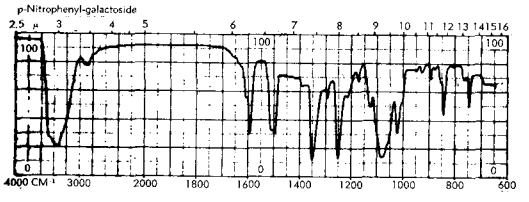

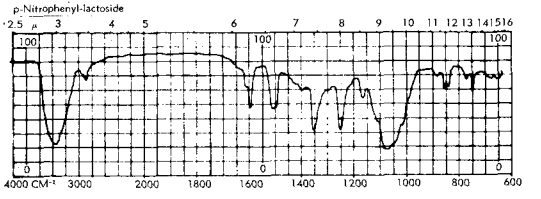

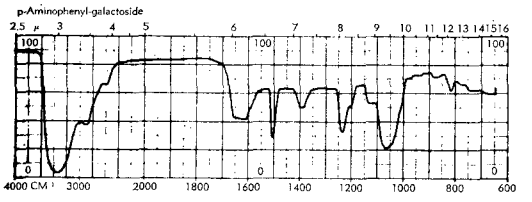

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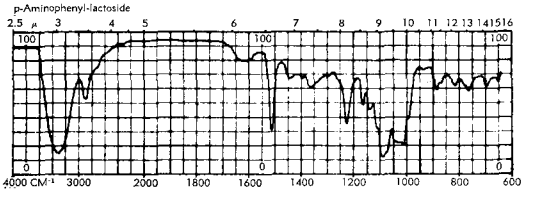

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FIG. 1. (a-d) Infrared spectra of various sugar derivatives in the synthesis of *p*-aminophenyl glycosides.

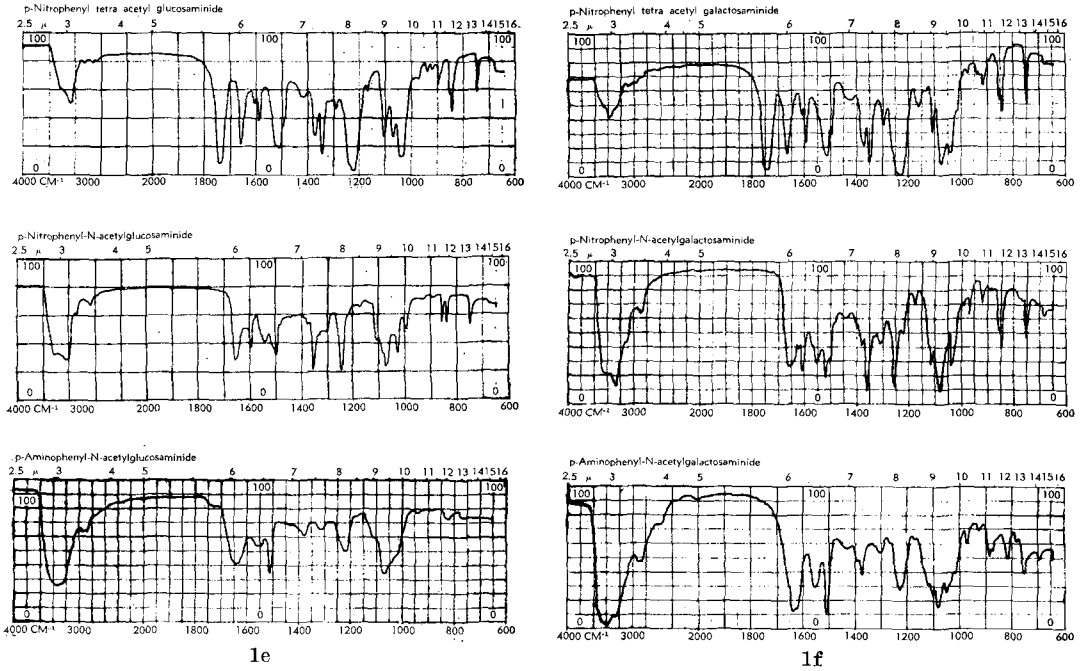

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FIG. 1. (e-f) Infrared spectra of various sugar derivatives in the synthesis of *p*-aminophenyl glycosides.

Preparation of Tribenzoyl-Dihydrosphingosine

Part of the sphingosine was used to prepare its crystalline derivative, tribenzoyl-dihydrosphingosine, to demonstrate the *erythro* configuration. The sphingosine was reduced by catalytic hydrogenation with palladium on Norit A as catalyst to form dihydrosphingosine. The dihydrosphingosine was benzoylated with benzoylchloride in pyridine solution to yield crystalline tribenzoyl-dihydrosphingosine.

Preparation of Sphingosine-Protein Complex

As already reported in the previous paper (5), the sphingosine was benzoylated with *p*-nitrobenzoylchloride in chloroform-methanol (2:1) solution by the Schotten-Baumann method. N-*p*-nitrobenzoyl-sphingosine thus obtained was reduced by catalytic hydrogenation to form N-*p*-aminobenzoyl-dihydrosphingosine. Water-insoluble N-*p*-aminobenzoyl-dihydrosphingosine was dissolved in aqueous *N* CHl-methanol mixture. The solution was cooled to 1C and mixed with $NaNO_2$ in ice-cold water. Then, the reaction mixture was reacted with crystalline bovine serum albumin and egg albumin, respectively. After dialysis against distilled water and lyophilizing, the synthetic dihydrosphingosine-serum albumin (SS) and dihydrosphingosine-egg albumin (SE) were obtained.

Preparation of Azo Protein Containing Various Sugars

Following the methods of Babers and Goebel (11) and of Westphal et al. (12), various sugars were coupled with protein through diazotization of their *p*-aminophenyl derivatives. *P*-aminophenylglycosides of glucose, galactose, mannose and lactose, and of N-acetyl-glucosamine and N-acetyl-galactosamine were all prepared and coupled to protein. The infrared spectra of the various sugar derivatives in the synthesis of *p*-aminophenyl-glycosides are shown in Figure 1. These sugar-proteins had similar patterns in ultra-violet and visible absorption spectra with absorption maxima (1) at 280 mμ due to the protein, (2) near 370–380 mμ and (3) a shoulder or maximum near 430 mμ. Maxima (2) and (3) are probably due to the diazophenyl moiety. They contained about 30 phenyl sugars per one molecule of serum albumin or egg albumin.

Preparation of Ceramide-Protein Complex

Ceramide was obtained from equine spleen. It was benzoylated with *p*-nitrobenzoylchloride in pyridine solution to get 1,3 *O*-di-*p*-nitrobenzoyl-ceramide. Then, the 1,3 *O*-di-*p*-nitrobenzoyl-ceramide was reduced by catalytic hydrogenation to obtain 1,3 *O*-di-*p*-amino-

benzoyl-dihydroceramide. The 1,3 *O*-di-*p*-aminobenzoyl derivative was coupled to protein. Infrared spectra of the ceramide derivatives obtained in the synthesis of 1,3 *O*-di-*p*-aminobenzoyl-dihydroceramide are shown in Figure 2. Chemical analysis of the ceramide derivatives is summarized as follows;

Ceramide $C_{38}H_{75}NO_3$
(as Arachidyl-N-sphingosine)

	C	H	N
Calculated	76.90	12.65	2.36
Found	76.65	12.33	2.08

mp = 80–82C

1,3 *O*-di-*p*-nitrobenzoyl-ceramide $C_{52}H_{81}N_3O_9$

Calculated	70.04	9.09	4.71
Found	68.93	8.58	4.75

mp = 84–86C

1,3 *O*-di-*p*-aminobenzoyl-dihydroceramide $C_{52}H_{87}N_3O_5$

Calculated	74.79	10.44	5.04
Found	73.75	9.84	5.02

mp = 120–125C

Preparation of Antiserum Against the Synthetic Dihydrosphingosine-Serum Albumin (SS)

White rabbits weighing between 2.5 and 3 kg were used. A saline solution containing

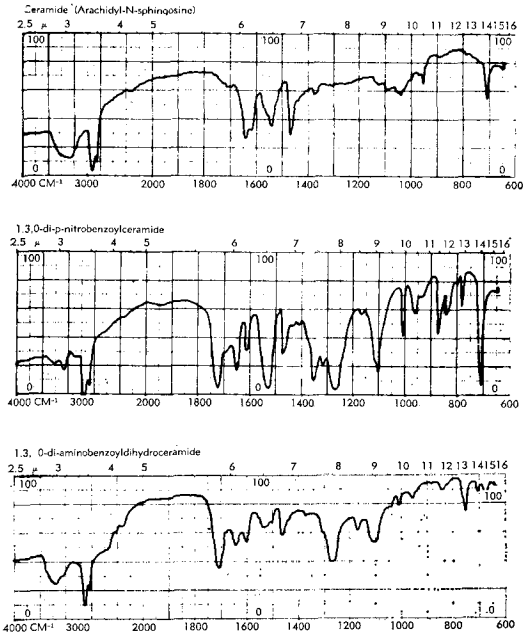

FIG. 2. Infrared spectra of ceramide derivatives.

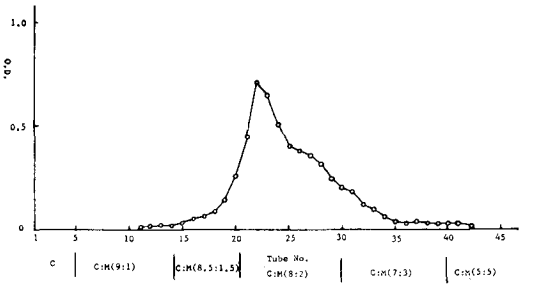

FIG. 3. Silicic acid column chromatography of crude sphingosine. Silicic acid: Hyflosupercel (2:1) = 60 gm. Crude sphingosine = 1 gm. Each tube contained 10 ml. of effluent. 0.05 ml. of aliquot of each tube was analyzed with ninhydrin reagent.

5 mg/0.5 ml of artificial antigen (SS) was injected intravenously. After a 5 hr interval, 2 ml of saline suspension containing an alum precipitate of 20 mg of SS was injected intravenously. After a 2-day interval, 20 mg of antigen as the alum precipitate was injected every other day for 2 or 3 weeks. After a 2-week interval, 4 ml of saline solution of 40 mg of antigen was finally injected intravenously as booster. The rabbits were bled from the ear artery or carotid one week after the last injection. The blood was allowed to clot at 37C for one hour and to stand at 4C overnight. The serum was collected and stored at −20C until used.

Preparation of Antibody Against Spinal Cord

A saline suspension of spinal cord (rabbit) together with Mycobacterium was emulsified with oil (Drakeol No. 6: Aracel A, 9:1 V/V) at a 1:1 ratio by volume in the Potter-Elvehjem homogenizer. Each rabbit received the emulsion intradermally in the 4 foot-pads or gluteal regions. About 2 weeks after the injection, the rabbits with complete paralysis or ataxia were bled completely by cardiac puncture.

Complement Fixation Test

The procedure developed by Kabat and Mayer (13) was used in the present experiment. Sera were inactivated at 56C for half an hour and diluted with gelatin veronal buffer containing 0.00015 M $CaCl_2$ and 0.0005 M $MgCl_2$, pH 7.5 (GVB). Various amounts of antigens were also dissolved with GVB. A checkerboard arrangement of antigen and antibody was set up for each test, all tests being carried out in test tubes. In each test, 5 $C'H_{50}$ of guinea-pig serum was used as complement. Sensitized red cell (0.2 ml) was added to 1.3 ml of total fixation volume. Fixation proceeded for 16 hr

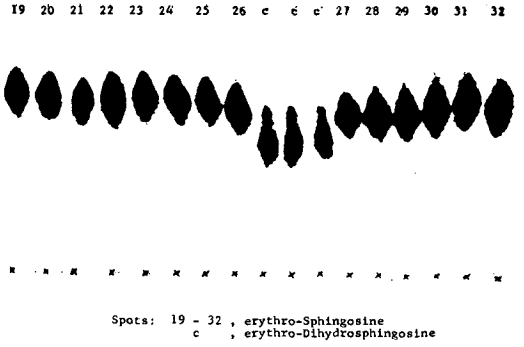

FIG. 4. Thin-layer chromatogram of aliquots from silicic acid column chromatography (Fig. 5). Plate: Kieselgel G, 0.5 mm thick, solvent, chloroform:methanol:water (65:25:4, v/v/v); ascending time: 45 min. at room temperature; sprayed with ninhydrin reagent and heated at 80C for 10 min. spots: (19–32) *erythro*-sphingosine, (c) *erythro*-dihydrosphingosine obtained by catalytic hydrogenation of *erythro*-sphingosine.

at 4C and finally 30 min at 37C with shaking. The hemolytic reaction proceeded for 60 min at 37C with shaking. The reading was given as degree of hemolysis, with "0" representing no lysis, and "4" representing complete lysis.

Qualitative Precipitin Test

The precipitin tests were carried out with the standard ring test and Ouchterlony's double diffusion in agar.

Arthus Reaction

The abdominal region of the immunized rabbits were shaved. One tenth milliliter of the saline solution containing 100 μg of various antigens was intradermally injected. Two or three hours and 24 hours after the challenge, the skin reaction was recorded as follows; negative "—", edema "$\pm \sim +$", erythema "++", hemorrhage "+++", and necrosis "++++".

FIG. 5. Thin-layer chromatogram of *erythro*-sphingosine (1) and psychosine (2). The procedure followed Fig. 6.

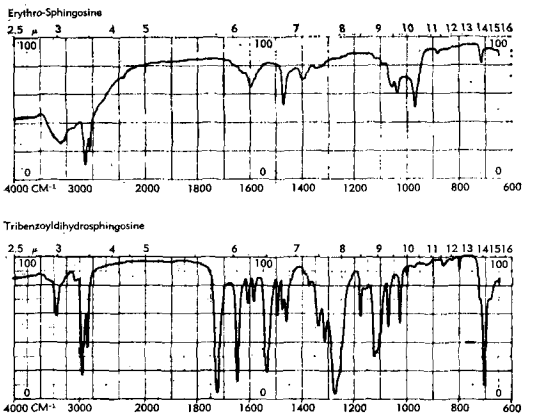

FIG. 6. Infrared spectra of *erythro*-sphingosine and tribenzoyldihydrosphingosine. Prepared as KBr pellets.

Fluorescent Antibody Techinque

The procedure developed by Goldstein, Slizys and Chase (14) was modified in the present experiment. Psychosine and spinal cord specific antibodies were used in the fluorescent antibody technique. Brain and kidney tissues from the mouse were frozen in hexane chilled to −70C. Sections were cut at 4 μ in a cryostat at −20C. The staining of the freshly frozen tissue section with fluorescent anti-psychosine or antispinal cord solution was performed without prior fixation or treatment with organic solvents.

Analytical Methods

Ultraviolet and visible absorption spectra were taken with the Hitachi UV and visible spectrophotometer (EPS-1). Infrared spec-

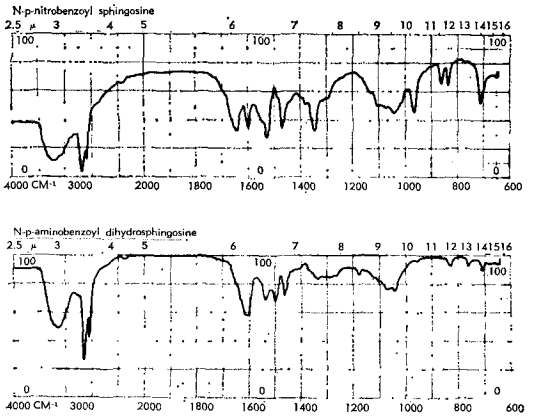

FIG. 7. Infrared spectra of N-*p*-nitrobenzoyl-sphingosine and N-*p*-aminobenzoyl-dihydrosphingosine. Examined as KBr pellets.

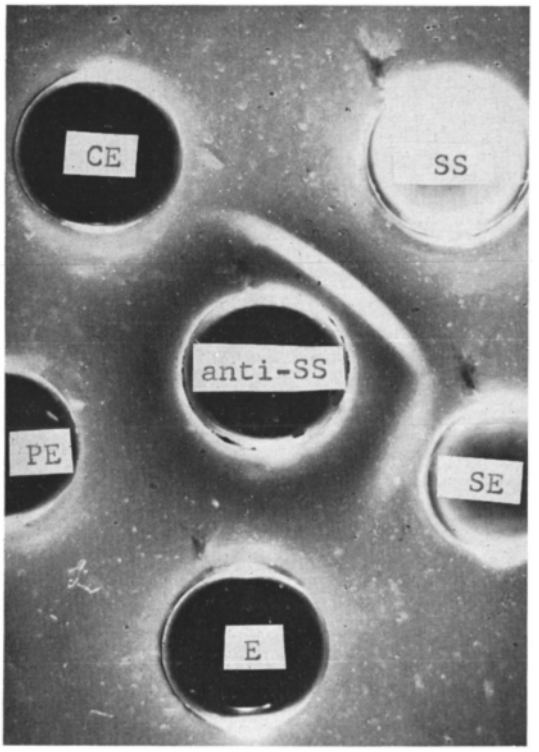

FIG. 8. Qualitative precipitin reaction using Ouchterlony's double diffusion method in agar. Anti-SS was reacted with SS, SE, E (egg albumin), PE (psychosine-egg albumin) and CE (deraminde-egg albumin) as antigens.

tra were taken with the Hitachi infrared spectrophotometer (EPI-2). The ultracentrifugal pattern was obtained with the Spinco Model E centrifuge. The specific rotation was measured by the polarimeter (EPU-2A) of Hitachi spectrophotometer.

RESULTS

Preparation of Conjugated Erythro-Dihydrosphingosine-Protein

Erythro-sphingosine. As described above, *erythro*-sphingosine was prepared from sphingomyelin. The time course of sphingosine release was estimated by ninhydrin reagent and it was found that the amide linkage of sphingomyelin was completely split in 2 hr by alkaline hydrolysis (N KOH in *n*-butanol). As shown in Figure 3, the sphingosine could be eluted with chloroform-methanol (8.5:1.5 and 8:2) solvent from the silicic acid column. The sphingosine showed only one spot, separated from that of dihydrosphingosine or psychosine by thin-layer chromatography (Fig. 4 and 5).

The infrared spectra of *erythro*-sphingosine and tribenzoyl-dihydrosphingosine are shown in Figure 6. The configuration of the sphingosine was demonstrated by catalytic hydrogenation, and benzoylation with benzoylchloride yielding a crystalline tribenzoyl-dihydrosphingosine which was identified by chemical analysis and infrared spectrum (Fig. 6). The analytical values for C,H and N, and the melting point of this tribenzoyl-dihydrosphingosine were identical with the results reported by Fujino and Carter (15). These findings demonstrated the *erythro*-configuration of the sphingosine obtained from sphingomyelin by alkaline hydrolysis.

N-p-nitrobenzoyl-sphingosine. As described above, this substance was synthesized from *erythro*-sphingosine and *p*-nitrobenzoylchloride by the Schotten-Baumann's method. This synthetic substance was identified as N-*p*-nitrobenzoyl-sphingosine by chemical analysis and infrared spectrum. As shown in Figure 7, the infrared spectrum of this substance showed the presence of amide linkage, but no ester linkage.

Analysis. $C_{25}H_{40}N_2O_5$ (448.6)

	C	H	N
Calculated	66.93	8.99	6.24
Found	67.43	9.30	5.80

mp = 48–50C $(\alpha)_{589}^{24^\circ} = +22.0$ (1% in chloroform)

N-p-Aminobenzoyl-Dihydrosphingosine. This substance, as already described above, was obtained by catalytic hydrogenation of N-*p*-

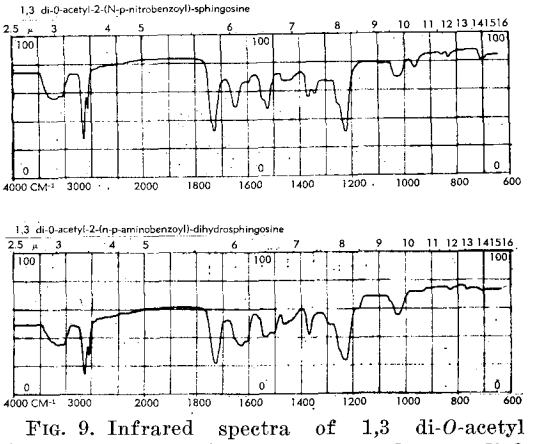

FIG. 9. Infrared spectra of 1,3 di-*O*-acetyl (N-*p*-nitro benzoyl)-sphingosine and 1,3 di-*O* acetyl-2-(N-*p*-nitro benzoyl)-sphingosine and 1,3 di-*O*-acetyl-2-(N-*p*-aminobenzoyl) - dihydrosphingosine. Prepared as KBr pellets.

nitrobenzoyl-sphingosine. This reduced material was identified as N-*p*-aminobenzoyl-dihydrosphingosine by chemical analysis and infrared spectrum. The *p*-nitro group absorption at 1600 cm^{-1} and 1350 cm^{-1}, and the trans double-bond absorption at 970 cm^{-1} in the infrared spectrum of N-*p*-nitrobenzoyl-sphingosine are absent in this spectrum (Fig. 7).

Analysis. $C_{25}H_{44}N_2O_3$ (420.6)

	C	H	N
Calculated	71.38	10.54	6.66
Found	70.60	9.83	5.90

mp = 120–125C $(\alpha)_{589}^{24°} = +23.0$ (1% in chloroform)

Synthetic Dihydrosphingosine-Protein. As already described above, the dihydrosphingosine-serum albumin (SS) and dihydrosphingosine-egg albumin (SE) were obtained through diazotization of the N-*p*-aminobenzoyl derivative. The UV and visible absorption spectra of SS were very similar to those of psychosine-serum albumin (PS), i.e. absorption maxima at 280 mμ, 375 mμ, and 430 mμ. The PS contained 6.5% galactose, that is, about 25–30 benzoyl-dihydropsychosine groups estimated from galactose content per one molecule or serum albumin (as molecular weight 70,000). A comparison of the spectra of PS and SS, showed that SS contained at least 20 benzoyl-dihydrosphingosine groups per one molecule of serum albumin. Similarily, SE contained also about 20 benzoyl-dihydrosphingosine groups per one molecule of egg albumin.

Preparation of Antibody Against Sphingosine-Protein

Complement Fixation Test. Antibody produced against SS did not fix complement with egg albumin, but fixed it with SE or SS as shown in Table I. This finding pointed out that antisera against SS contain at least two kinds of complement-fixing antibody, one each to the serum albumin and sphingosine moieties.

Qualitative Precipitin Reaction. The antisera prepared by immunization with SS were tested for the presence of precipitins against the homologous antigen (SS), and heterologous antigens of SE, E, PE and CE by the Ouchterlony's double diffusion method in agar. As shown in Figure 8, it is possible that anti-SS contains at least two kinds of antibody, namely, the antisphingosine and the antiserum albumin. The antiserum albumin exhibited a much higher potency than the antisphingosine. The antisphingosine did not cross-react with psychosine and ceramide-proteins. Although psychosine and ceramide are sphingosyl complexes, the sphingosine moiety of psychosine and ceramide-proteins differs from that of sphingosine-protein at the hydroxy groups at C_1 and C_3. The hydroxy groups of sphingosine of the latter are free. After acetylation of N-*p*-nitrobenzoyl-sphingosine and catalytic hydrogenation, the 1,3 di-*O*-acetyl-2-(N-*p*-aminobenzoyl)-dihydrosphingosine obtained (Fig. 9) was combined with a protein by the diazo-coupling reaction. This synthetic, acetylated sphingosine-protein did not react with anti-SS.

TABLE I

Two-dimensional complement fixation test with egg-albumin (E), sphingosine-egg albumin (SE) or sphingosine-serum albumin (SS) and rabbit antibody against SS. "0" representing no lysis, "4" representing complete lysis

Amount of E per test mixture	Antiserum dilution 1	1/2	1/10	1/20	Control with 5 C'H_{50}	3 C'H_{50}
μg 20	4	3	3	4	4	4
10	4	4	3	4	4	4
5	4	4	4	4	4	4
3	4	4	4	4	4	4
2	4	4	4	4	4	4
1	4	4	4	4	4	4
Control with 5 C'H_{50}	4	4	4	4	4	4
3 C'H_{50}	4	4	4	4	4	4
Amount of SE per test mixture						
μg 20	3	4	3	4	4	4
10	0	0	3	4	4	4
5	0	0	0	4	4	4
3	0	0	0	2	4	4
2	0	0	0	2	4	4
1	0	0	0	2	4	4
Control with 5 C'H_{50}	4	4	4	4	4	4
3 C'H_{50}	4	4	4	4	4	4
Amount of SS per test mixture						
μg 20	0	0	0	4	4	
10	0	0	0	4	4	
5	0	0	0	4	4	
3	0	0	0	4	4	
2	0	0	0	4	4	
1	0	0	0	4	4	
Control with 5 C'H_{50}	4	4	4	4	4	
3 C'H_{50}	4	4	4	4	4	

Preparation of Antibody Against Rabbit Spinal Cord. Each rabbit received a total of 0.5 ml of emulsion containing 200 mg spinal cord and 5 mg Mycobacterium. Two out of three rabbits developed complete paralysis or ataxia. The cross-reactivity of various artificial antigens with antisera of diseased rabbits was checked by the complement fixation test. As shown in Table II, it was found that psychosine-protein (PS), in particular, had a cross-reactivity with antisera against spinal cord.

Lactose-protein (LS), sphingosine-protein (SS) and N-acetylgalactosamine-protein (GalNS) had a very weak cross-reactivity. Glucose, galactose, mannose and N-acetylglucosamine-proteins had no cross-reactivity. On the other hand, the antisera of nondiseased rabbit had no cross-reactivity with any one of the artificial antigens by the complement fixation test. As already published by us, psychosine is antigenic when coupled with a protein. In the present experiment, the cross-reactivity of anti-PS with these artificial antigens was also checked by the complement fixation test. The result showed that anti-PS contained complement-fixing antibodies with serum albumin and psychosine, and also cross-reacted a little with lactose-protein (LE) and sphingosine-protein (SE). In view of these findings, one of the determinant groups of antigenicity of rabbit spinal cord may be related to glycolipids composed of psychosine-moiety.

Arthus Reaction. The rabbits immunized with rabbit spinal cord or psychosine-protein (PS) were intradermally injected with 0.1 ml of the saline solution of artificial antigens. The results of the skin reactions are shown in Table III. This demonstrates that circulating antibody capable of reacting with psychosine-protein is produced in the rabbits immunized with spinal cord or psychosine-protein.

Fluorescent Antibody Technique. When sections of mouse brain were stained with fluorescent antibody against psychosine or spinal cord, the fluorescence appeared consistently in the lumen of the cerebral blood vessel and in the plexus, but not in the myelin sheath of white matter, contrary to our expectation. In sections from kidney stained with the fluorescent antibodies, the tubules and the renal blood vessel show brilliant fluorescence, but the glomerules do not show (Fig. 10).

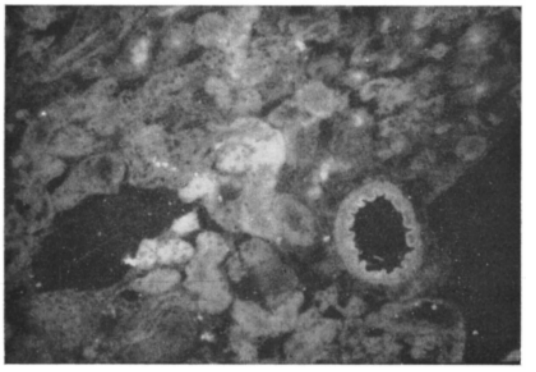

FIG. 10. Photograph of a kidney section stained with fluorescent antibody against psychosine.

DISCUSSIONS

As we have mentioned (16), we have the impression that such cell antigens as the ABO-blood group and Forssman's hapten are glycolipids, similar to the lipocarbohydrates which make up a structural part of the bacterial cell wall. It is generally supposed that antigenic determinants exist in the sugar moiety and that the fatty part have little or no direct relation to antibody. We found that psychosine is fully antigenic when coupled with a protein. In that case, it seemed that the terminal non-reducing galactose is the antigenic determinant. In the present experiments, following the previous procedure for the preparation of psychosine-specific antibody, it was found that the naturally occurring *erythro*-sphingosine also can be antigenic when coupled with a protein through diazotization of its N-*p*-aminobenzoyl derivative. It appears that the determining factor of *erythro*-sphingosine is due to the hydroxy groups at C_1 and C_3. As is well known, the limited solubility of lipids in aqueous medium is disadvantageous for the immunological study of lipids and the characteristics of the micelle of glycolipids in aqueous solution are critical in determining immunological reactivity. As already suggested (5), the water-soluble conjugated lipid-protein antigens may serve to elucidate the immunological reactivity of lipids in general.

So far, many workers (17–21) have challenged the observations that antigenic properties of nervous tissue have been detected and that circulating antibodies against nervous tissue have been formed in experimental allergic encephalomyelitis. While there are many opinions and ideas concerning these problems (22,23), there is a lack of decisive evidence.

We imagined that various artificial antigens of lipid-protein or sugar-protein would be useful and desirable for the investigation of these problems. The cross-reactivity of antibody against spinal cord with psychosine-protein (PS) was not strong enough to be assayed by the quantitative precipitin test, but was detected definitely by the complement fixation test. However, lactose-protein (LS), N-acetylgalactosamine-protein (GalNS) and sphingosine-protein (SS) had a very weak cross-reactivity with the antibody against spinal cord. It is interesting that the psychosine portion of cerebroside, which is constituted by galactose and sphingosine combined in glyco-

TABLE II

Two-Dimensional Complement Fixation Test with S(Serum-Albumin), LS(Lactose-Serum-Albumin), GalNS(N-acetylgalactosamine-Serum-Albumin), SS(Sphingosine-Serum-Albumin) and PS(Psychosine-Serum-Albumin), and Antispinal Cord

Amount of S per test mixture	Antiserum dilution						Control with	
	1/5	1/10	1/20	1/40	1/80	1/160	5 $C'H_{50}$	3 $C'H_{50}$
μg 20	4	4	4	4	4	4	4	4
10	4	4	4	4	4	4	4	4
5	4	4	4	4	4	4	4	4
1	4	4	4	4	4	4	4	4
0.1	4	4	4	4	4	4	4	4
Control with								
5 $C'H_{50}$	4	4	4	4	4	4	4	4
3 $C'H_{50}$	4	4	4	4	4	4	4	4
Amount of LS per test mixture								
μg 20	2	3	3	3	4	4	4	4
10	2	3	3	4	4	4	4	4
5	2	3	3	4	4	4	4	4
1	4	4	4	4	4	4	4	4
0.1	4	4	4	4	4	4	4	4
Control with								
5 $C'H_{50}$	4	4	4	4	4	4	4	4
3 $C'H_{50}$	4	4	4	4	4	4	4	4
Amount of GalNS per test mixture								
μg 20	3	3	3	4	4	4	4	4
10	3	3	4	4	4	4	4	4
5	4	4	4	4	4	4	4	4
1	4	4	4	4	4	4	4	4
0.1	4	4	4	4	4	4	4	4
Control with								
5 $C'H_{50}$	4	4	4	4	4	4	4	4
3 $C'H_{50}$	4	4	4	4	4	4	4	4
Amount of SS per test mixture								
μg 20	3	3	3	4	4	4	4	
10	3	3	3	4	4	4	4	
5	3	3	4	4	4	4	4	
1	4	4	4	4	4	4	4	
0.1	4	4	4	4	4	4	4	
Control with								
5 $C'H_{50}$	4	4	4	4	4	4	4	
Amount of PS per test mixture								
μg 20	0	0	0	2	4	4	4	4
10	0	0	0	3	4	4	4	4
5	0	0	0	3	4	4	4	4
1	2	2	3	4	4	4	4	4
0.1	3	4	4	4	4	4	4	4
Control with								4
5 $C'H_{50}$	4	4	4	4	4	4	4	4

TABLE III

Result of Arthus Reaction in Abdominal Skin or Rabbits Immunized with Rabbit Spinal Cord or PS[a]. After Injection of 0.1 ml of the saline solution containing 100 μg of various antigens, the skin reaction was recorded with "—" negative "± ~ +" edema and "++" erythema. PS:psychosine-serum albumin, PE:psychosine-egg albumin, LE:lactose-egg albumin, GNE:N-acetylglucosamine-egg albumin, GE:glucose-egg albumin.

After injection of antigens		Antigens					
		Control	PS	PE	LE	GNE	GE
Rabbit immunized with spinal cord	3 hr	—	+	+	—	—	—
	24 hr	—	+	++	—	—	—
Rabbit immunized with PS	3 hr	—	+	+	±	—	—
	24 hr	—	+	+	±	—	—

sidic linkage, is far more cross-reactive than sphingosine or galactose alone. In view of these findings, it seems that not only the sugar moiety, but also the fatty moiety contributes to the serological activity of glycolipids.

The ultracentrifugal pattern of psychosine-protein (PS) shows that it has a lipoprotein property in comparison with those of serum albumin (S) and lactose-protein (LS) (Fig. 11). The S_{20w} of PS, LS and S is 3.5, 4.5 and 4.1, respectively. Thus, the density of PS is much lower than that of S and LS.

The Arthus reaction in the abdominal region of rabbit serves as evidence for circulating antibody against spinal cord or PS. We attempted to clarify the localization of glycolipids in tissues by means of direct fluorescent antibody technique, but unfortunately failed in staining the myelin sheath of nervous tissue with fluorescent antibody against psychosine or spinal cord. Lack of specific staining may be explained if the fluorescent antibody did not combine with reactive sites of antigen in the myelin sheath because of steric hindrance or altered permeability. Further investigation of fixation or pretreatment of frozen sections for fluorescent staining are needed.

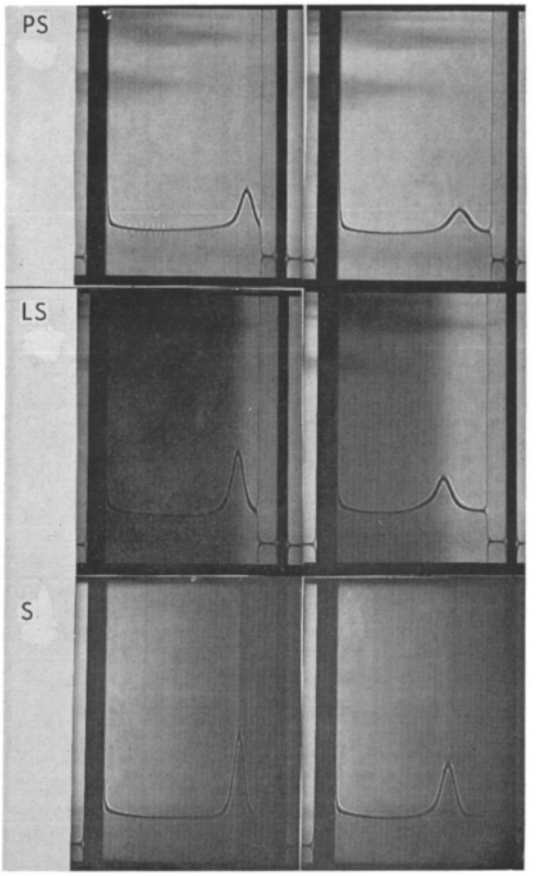

FIG. 11. Ultracentrifugal patterns of PS, LS and S on the left are the patterns at the start and on the right the patterns after 54 min of centrifugation at 59,780 rpm at 20.4C.

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[Received Oct. 18, 1965]

Naturally Occurring Epoxy Acids. IV. The Absolute Optical Configuration of Vernolic Acid

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ABSTRACT

Vernolic acid [(+)-*cis*-12,13-epoxyleic acid] was transformed by stereospecific reactions to a mixture of *threo*-9,10,12- and *threo*-9,10,13-trihydroxyoctadecanols. The four components of this mixture were separately isolated by chromatography on thin layers impregnated with glycol-complexing agents. The 9,10,12-trihydroxyoctadecanols so obtained were identical to the corresponding derivatives of D-(+)-ricinoleic acid, thereby proving the absolute optical configuration of the epoxy group of vernolic acid to be D. As a corollary to this the absolute configurations of some other oxygenated fatty acids have been deduced.

INTRODUCTION

EPOXY ACIDS HAVE TWO ASYMMETRIC carbon atoms at the oxirane ring and are thus capable of existing in enantiomorphic forms. Most of the epoxy fatty acids which have so far been shown to occur naturally as components of glyceride oils have been demonstrated, either directly or indirectly, to have optical activity (1-4). This is to be expected, as it seems to be a general rule that racemates are not produced in nature. More remarkable, however, is the fact that *cis*-12,13-epoxyoleic acid occurs naturally in both of its optically active forms (4,5). The (+)-acid (vernolic acid), which gives rise to predominantly (−)-*threo*-12,13-dihydroxyoleic acid by acetolysis, is present in the seed oils of a number of species of the families *Compositae, Euphorbiaceae,* and *Onagraceae* whereas the (−)-epoxy acid, which by similar chemical cleavage gives predominantly the (+)-dihydroxyoleic acid enantiomer, is a constituent of seed oils of several species of *Malvaceae.*

The absolute optical configuration of no natural epoxy acid has yet been ascertained. The elegant work of Serck-Hanssen (6,7) proving that ricinoleic acid, namely (+)-12-hydroxyoleic acid, has the D-configuration [i.e. is (*R*) according to the Cahn-Ingold-Prelog system (8)] provides the key for the determination of the absolute configuration of 12,13-epoxyoleic acid. If by stereospecific reactions the epoxy acid can be converted to 12-hydroxyoleic acid or a derivative of this acid then simple determination of the identity or enantiomorphism of the product with the corresponding derivative of ricinoleic acid will unequivocally ascertain the absolute configuration of the epoxy acid.

Some years ago, one of us described experiments which indicated fairly conclusively that vernolic acid has the D-configuration (9). The reactions involved and the specific rotations of the products obtained from epoxyoleate and from ricinoleate for comparison are very briefly summarised in Figure 1.

The epoxide cleavage reactions by hydrochlorination and by reduction with lithium aluminium hydride are known to be stereospecific in the manner indicated (10,11) so that the hydroxyl groups of their respective products, whether on the 12- or the 13-position, retain the absolute configuration of the parent epoxide group. Those products which could be compared to the reference derivatives from ricinoleic acid showed the same sign of rotation as the reference compounds even when, as in the sequence I ⟶ III ⟶ V ⟶ VII, the sign of rotation changed with each reaction step. This showed fairly conclusively that vernolic acid was of the same configuration as ricinoleic acid, namely D. The specific rotations of the products derived from vernolic acid, however, were always lower than the reference values indicating that some racemisation had occurred, probably during the $LiAlH_4$ reduction stages. This and the fact that, due to the very small amounts of the chlorohydrin isomers (II and III) we were able to isolate pure, it was not possible to prove unequivocally the structures of all the products are the reasons for this work not being published previously.

The recent discovery that the diastereoisomeric pairs of 9,10,12- and 9,12,13-trihydroxystearates could be separated by thin-layer chromatography (TLC) on adsorbents impregnated with glycol-complexing agents (12), suggested another approach to the determination of the absolute configuration of vernolic acid. This paper describes the reactions and

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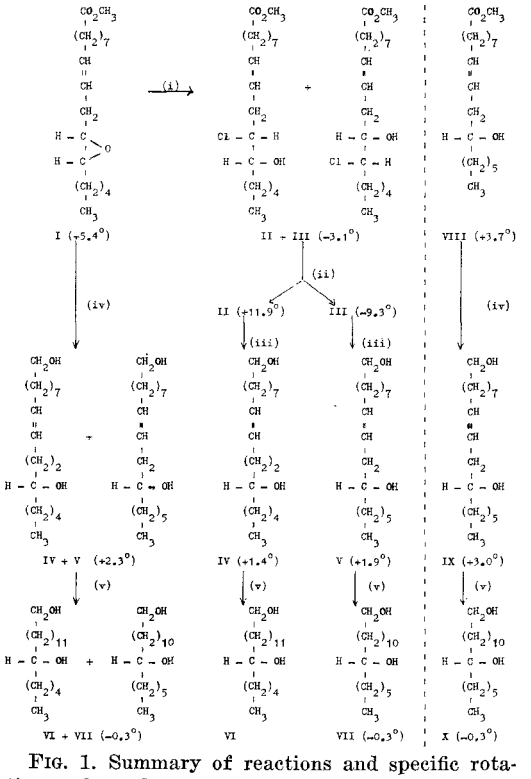

FIG. 1. Summary of reactions and specific rotations of products from earlier attempts (9) to determine the absolute optical configuration of methyl vernolate (I). All rotations are at the Na_D line, measured on ca. 10% solutions in chloroform. The reactions are: (i) reaction with anhydrous HCl in diethyl ether; (ii) separation of isomers by adsorption chromatography; (iii) reduction with $LiAlH_4/LiH$ mixture, by refluxing 2 days in tetrahydrofuran solution; (iv) reduction with $LiAlH_4$, at room temperature in ether solution; (v) hydrogenation over Adams catalyst in ethanol solution.

the chromatographic separations involved and demonstrates conclusively that vernolic acid has the D-configuration, thus verifying the earlier work summarised above.

EXPERIMENTAL AND RESULTS

Pure methyl *cis*-12,13-epoxyoleate was isolated from the mixed esters from *Vernonia anthelmintica* seed oil by adsorption column chromatography as described previously (13). The pure ester had an optical rotation, without solvent, of $\alpha_D^{25°} = +1.7°$ in a 1 dm tube and specific rotations of $[\alpha]_D^{25°} = +5.4°$ and $-0.3°$ as 10% solutions in chloroform and ethanol respectively. Methyl ricinoleate was isolated from castor oil mixed esters by a simple single withdrawal countercurrent distribution, in three separating funnels, between pre-equilibrated hexane and methanol-water (9:1). Both methyl vernolate and methyl ricinoleate so prepared were completely pure as judged by TLC and GLC.

Preparation of Polyhydroxy Derivatives

The reactions used to prepare the polyhydroxy derivatives from methyl vernolate and the reference derivatives from methyl ricinoleate are summarised in Figure 2. The stereospecificity of the reduction of epoxy compounds with $LiAlH_4$ is known (11) and leads to the isomers of the same configuration as the original epoxy group as shown.

trans-Hydroxylation of the double bond to give the *threo*-glycol derivatives was chosen because, by analogy with the corresponding 9,10,12-trihydroxystearic acids (14), the specific rotations of the *threo*-compounds were likely to be of much greater magnitude than those of the corresponding *erythro*-isomers. In addition, the individual diastereoisomers of each *threo* oxidation pair would be expected to have rotations of opposite signs whereas the *erythro*-compounds would be likely to have the same sign of rotation (14,15).

The reaction steps in the preparation of the derivatives from methyl epoxyoleate will be described in detail. The same reactions were

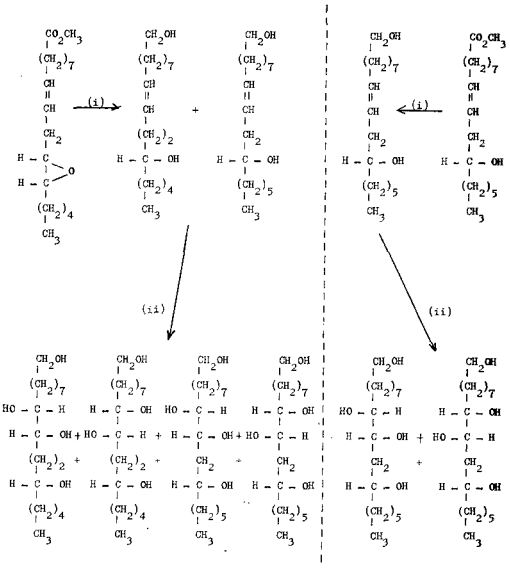

FIG. 2. Summary of reactions in preparation of trihydroxyoctadecanol derivatives from methyl vernolate and methyl ricinoleate. The reactions are: (i) reduction with $LiAlH_4$ in diethyl ether solution; (ii) hydroxylation with performic acid. Details are given in the text.

used to obtain the reference derivatives from methyl ricinoleate, with comparable yields.

$LiAlH_4$ Reduction

Methyl epoxyoleate (10 g) in anhydrous diethyl ether (50 ml) was added slowly to a solution of $LiAlH_4$ (2 g) in ether (100 ml), which had been refluxed for 1 hr to effect maximum solution of the hydride. The reaction mixture was boiled under reflux for 2.5 hr, stood overnight at room temperature, cooled in ice and the complex then decomposed by careful addition of excess 10% H_2SO_4. The product was extracted into the ether layer which was washed six times with distilled water and dried over anhydrous Na_2SO_4. The solvent was removed to yield 7.64 g (83% of theoretical yield) of product which migrated on TLC as a single zone with the same mobility as a sample of authentic ricinoleyl alcohol, showing that the reduction was complete. This product is a mixture of two positional isomers, as shown in Figure 2, but these are not separable by normal adsorption TLC.

Hydroxylation

Prior to hydroxylation of the double bond with performic acid, the reduction product was acetylated by refluxing with acetic anhydride. The diacetate thus obtained was mixed with formic acid (25 ml) and 4 ml of a 30% solution of hydrogen peroxide was added. The flask was tightly stoppered and shaken vigorously on a wrist-action shaker until the mixture was homogeneous (ca. 1 hr) and then for a further 3 hr. The formic acid was removed under reduced pressure and the residue was boiled under reflux for 1 hr with 100 ml of 3N sodium hydroxide in aqueous methanol. The hot solution was poured into stirred ice-water (1000 ml) and the precipitated solid was filtered off in a sintered glass funnel, thoroughly washed with distilled water till the washings were neutral and dried in a vacuum desiccator. The yield of mixed tetrahydroxyoctadecane isomers was 7.67 g, i.e. 75% overall yield from methyl epoxyoleate.

Isolation of Individual Isomers

From the above series of reactions, methyl ricinoleate gave a mixture of two *threo*-9,10,12-trihydroxyoctadecanols and methyl vernolate gave a mixture of four isomers, two *threo*-9,10,12-trihydroxyoctadecanols and two *threo*-9,10,13-trihydroxyoctadecanols, as illustrated in Figure 2.

The mixed product from ricinoleate was very clearly separated into its two components by TLC on silica gel G impregnated (10% w/w) with sodium arsenite, using methanol-ether (5:95) as developing solvent. An attempt to achieve this separation on a large scale by column chromatography on Davison 950 silica gel similarly impregnated and using chloroform as eluting solvent was abortive, probably due to overloading of the complexing capacity of the column. Because preparative TLC separations were so clearcut, even with large loads, this procedure was adopted for all separations and no further attempts were made to perfect the column chromatographic method.

In checking the fractions which were eluted from the trial column separation on nonimpregnated silica gel plates, it became evident that the products were being eluted as stable arsenite complexes. The free tetrahydroxy compounds, however, were readily regenerated by alkaline hydrolysis.

Preparative separation of the two *threo*-9,10,12-trihydroxyoctadecanols from ricinoleate was effected by TLC on arsenite impregnated silica gel layers, 1 mm thick on 20 × 20 cm plates, at a loading of about 150 mg of mixture per plate. After developing in diethyl ether-methanol (95:5) and spraying with dichlorofluorescein solution, two clearly separated bands could be seen under ultraviolet light. These were scraped off and the adsorbed complexes eluted with ether-methanol (1:1). The solvent was removed from each fraction and the residues were hydrolysed by refluxing for 30 min or by standing overnight with 10% KOH in methanol. The hydrolysates were diluted with water and the free hydroxy compounds were extracted into ether-methanol and washed with water-methanol (95:5) till neutral and recovered. Recrystallisation from acetone-water and from ether-methanol mixtures gave the two pure *threo*-9,10,12-trihydroxyoctadecanols.

From a series of ten such preparative plates, the yields of oily complexes were 0.93 g and 1.12 g for the upper and lower bands respectively and these in turn yielded 0.61 g and 0.58 g of the individual tetrahydroxy isomers. As with the *threo*-9,10,12-trihydroxystearates (12), the upper band from the arsenite plates corresponded to the higher melting compound (RAI) and the lower to the isomer of lower melting point (RAII). The melting points and specific rotations of these compounds are included in Table I and their migration behavior on arsenite and borate impregnated

plates is illustrated in Figure 3 (samples 1 and 2).

The product of reduction and hydroxylation of methyl vernolate was expected to be the four isomers illustrated in Figure 2 and TLC on arsenite impregnated silica gel did indeed show four component spots as illustrated in Figure 3 (a), sample 3. Of these, the components VAI and VAIV, which had respectively the greatest and the least mobility, were present in greater amounts than the intermediate components VAII and VAIII and, moreover, had the same mobilities as the reference derivatives RAI and RAII. The two components of least mobility, VAIII and VAIV, were barely separable in analytical amounts and were not separated on preparative chromatograms.

Preparative TLC of the product mixture from epoxyoleate on arsenite impregnated layers was carried out as described for the ricinoleate derivatives except that 20 × 40 cm plates were used and the amount of sample applied to each plate was somewhat reduced. Three distinct bands of material were evident after development of these plates, of which the most polar was a mixture of the two isomers VAIII and VAIV. An initial separation of about 1.5 g of the product mixture on fifteen 20 × 40 cm plates was followed by

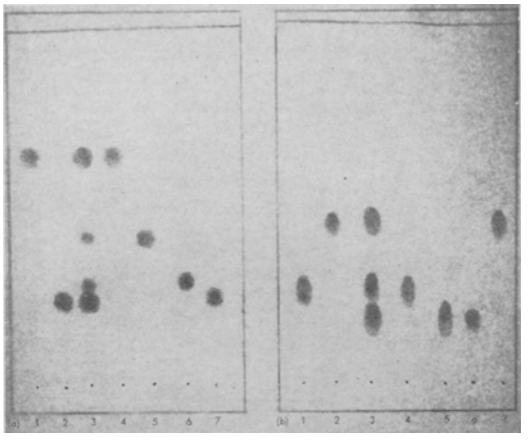

FIG. 3. Thin-layer chromatograms of trihydroxyoctadecanol derivatives on silica gel impregnated (a) with sodium arsenite and (b) with sodium borate. The chromatograms were developed in methanol-ether, (a) 5:95 and (b) 7.5:92.5. The spots were detected by spraying with 25% H_2SO_4 and charring and were reproduced by photocopying. Samples are: (1) = RA I; (2) = RA II; (3) = mixed product from methyl vernolate; (4) = VA I; (5) = VA II; (6) = VA III and (7) = VA IV; see text and Table I for details of this coding.

purification of the middle (VAII) and lowest (VAIII + VAIV) band components by rechromatography. After alkaline hydrolysis of the arsenite complexes so isolated, the products from the top and middle bands were recrystallised several times from ether-methanol to yield 375 mg of VAI and 64 mg of VAII, respectively. These migrated as single compounds on both arsenite and borate impregnated plates (Fig. 3, samples 4 and 5) and their melting points and specific rotations are given in Table I. The lowest band from the arsenite plates gave 800 mg of product after hydrolysis and was a mixture of VAIII and VAIV.

These two compounds were readily separated by chromatography on silica gel layers impregnated with sodium borate (10% w/w), as had been anticipated by analogy with the results obtained with the 9,10,12- and 9,12,13-trihydroxystearate isomers (12). Preparative TLC on borate impregnated plates (20 × 20 cm) was carried out in the same way as on arsenite impregnated plates, except that a more polar developing solvent (ether-methanol, 92.5:7.5) was used. Because of a tendency towards streaking on these plates even less sample could be applied than before and eighteen plates were required for isolation of the two components of this fraction. Each was then purified by rechromatography on additional plates. As with the arsenite plates, the compounds eluted from the borate plates proved to be intact complexes. Direct hydrolysis of these borate complexes seemed to be less effective than with arsenite complexes and the desired trihydroxyoctadecanols were finally recovered by shaking the borate complexes with a large excess of sodium arsenite solution, which resulted in an exchange of the complexing group, followed by hydrolysing the resulting arsenite complexes as before. After several recrystallisations of the trihydroxyoctadecanols regenerated in this way, the upper band from the borate plates yielded 286 mg of VAIV and the lower band gave 80 mg of VAIII. These compounds also migrated as single spots on both arsenite and borate impregnated layers (Fig. 3, samples 6 and 7) and their melting points and specific rotations are included in Table I.

It is clear from the migration of behaviour of the various purified products on both arsenite and borate impregnated plates and from the data in Table I that the products VAI and VAIV from vernolic acid are identical respectively to RAI and RAII from ricinoleate and are therefore the two *threo*-9,10,12-trihydroxy-

TABLE I

Melting Points and Specific Rotations

Compound	mp, C	$[\alpha]546.1$ mμ
RA I	101	−34.5°
RA II	77	+19.6°
VA I	101	−31.0°
VA II	91	+22.1°
VA III	102	−13.3°
VA IV	77	+20.0°

octadecanol isomers. This conclusion was further supported by mixed melting point determinations on VAI plus RAI and on VAIV plus RAII; no depression of melting point relative to the individual compounds was apparent. Components VAII and VAIII, therefore, are the two *threo*-9,10,13-trihydroxyoctadecanol isomers.

DISCUSSION

The stereochemical as well as chemical identity of the two tetrol derivatives (VAI and VAIV) from methyl epoxyoleate with the derivatives from methyl ricinoleate proves that the two parent compounds have the same configuration. This depends only on the assumption that the reductive cleavage of the epoxide group of methyl vernolate results in inversion of configuration at the point of nucleophilic attack and retention of configuration of the hydroxyl group so formed, as illustrated in Figures 1 and 2. However, the mechanism and stereochemistry of $LiAlH_4$ reductions of epoxide groups are now so well known (11,16) that this assumption is certainly valid. As ricinoleic acid is known to have the D-configuration (6,7), i.e. (*R*) in the Cahn-Ingold-Prelog system (8), then (+)-vernolic acid is proved to be D-*cis*-12,13-epoxy-*cis*-9-octadecenoic acid, i.e. (12*S*:13*R*)-*cis*-12,13,-epoxy-*cis*-9-octadecenoic acid. The (−)-epoxyoleic acid from seed oils of the *Malvaceae* (4) must therefore be of the L-configuration, i.e. (12*R*:13*S*).

The other compounds whose absolute optical configurations are immediately apparent are the *erythro*-12,13-dihydroxyoleates and -stearates derived from these epoxyoleate enantiomers. Bharucha and Gunstone (17) obtained *erythro*-12,13-dihydroxyoleic acid ($[\alpha]_D = -3.6°$, in ethanol) from vernolic acid in two steps which involved retention of configuration about one of the epoxide carbons and two inversions (i.e. retention) about the other, so that the product must be D-12,D-13-dihydroxyoleic acid; i.e. (12-*S*, 13-*R*). The *erythro*-12,13-dihydroxystearic acid ($[\alpha]_D = -1.7°$ in ethanol) derived from it (17) must, of course, have the same configuration and the enantiomeric compounds would be derived from the L-(−)-epoxyoleic acid.

Of the diastereoisomeric pairs of 9,10,12- and 9,12,13-trihydroxyoctadecanols prepared and separated in this study, it is not possible to state conclusively which isomer is which. A *cis*-1,3-diol grouping on positions 10 and 12 would be expected to engage in fairly strong intramolecular hydrogen bonding and thus to have a lower melting point than the isomer with a *trans* disposition of hydroxyls on positions 10 and 12. If this is the case then the compounds RAII and VAIV (mp 77C) are L-9,D-10,D-12-trihydroxyoctadecanol, i.e. (9-*R*, 10-*R*, 12-*R*) and the compounds RAI and VAI are D-9,L-10,D-12-trihydroxyoctadecanol, i.e. (9-*S*, 10-*S*, 12-*R*). The corresponding isomeric *threo*-9,10,12-trihydroxystearic acids derived directly from ricinoleic acid (14) would then also have these configurations for the lower and higher melting forms respectively but it should be emphasised that these particular configurational assignations are only tentative at present.

As a corollary to the findings described in this paper, the absolute optical configurations of some other oxygenated derivatives of epoxyoleic acid can be deduced with reasonable certainty. Thus Figure 3 (a), sample 3 shows that considerably more than 50% of the 12-hydroxy isomer was formed in the reductive cleavage of epoxyoleate, a fact borne out by the yields of the individual trihydroxyoctadecanol isomers isolated by preparative chromatography. This shows that nucleophilic attack is preferentially at the 13-position, presumably due to the influence of the 9,10-double bond, resulting in preferential cleavage of the ether bond to that carbon. In reactions with acidic reagents such as HCl or acetic acid, although the primary attack is by a proton to form the conjugate acid of the epoxide the actual cleavage of the epoxide results from nucleophilic attack on this conjugate acid (10,17). The influence of the 9,10-double bond in this case is likely to be in the same direction as in the $LiAlH_4$ reaction, again directing the nucleophilic attack predominantly to the 13-position. This will result in preferential cleavage of the ether bond to the 13-position so that the major product from such acidic reactions will have the epoxide oxygen retained as hydroxyl, in the same configuration, at the 12-position and the new substituent group inserted, with inversion, at the 13-position. Thus the major product from reaction of *cis*-12,13-epoxyoleate (I, Fig. 1) with anhydrous HCl should be the 12-hydroxy, 13-chloro-oleate isomer (III, Fig.

1). TLC of the chlorohydrins from epoxyoleate results in separation of the two isomers and demonstrates clearly that the isomer of lower mobility is present in greater amount. From the specific rotations of the mixture and of the individual pure compounds reported in Figure 1, it can readily be calculated that the more polar laevorotatory compound comprises 74% of the mixture. According to the ideas on reaction mechanisms expressed above, this compound should be the 12-hydroxy, 13-chlorooleate isomer and this structure was, in fact, fairly conclusively proved in the original work (9). Thus the chlorohydrin III from methyl vernolate ($[\alpha]_D = -8.3°$) is considered to be D-12-hydroxy, L-13-chloro-oleate, i.e. (12-*S*, 13-S), and the less polar chlorohydrin II ($[\alpha]_D = +11.9°$) is L-12-chloro, D-13-hydroxyoleate, i.e. (12-*R*, 13-*R*).

Acetolysis of methyl vernolate, which will have the same mechanism as reaction with HCl, is also partially specific since the *threo*-12,13-dihydroxyoleic acid product is optically active ($[\alpha]_D = -5.7°$ in EtOH). By fractional crystallisation, Hopkins and Chisholm (5) isolated one pure enantiomer ($[\alpha]_D = -19.0°$ in EtOH) from this mixture and from the product of acetolysis of the (−)-12,13-epoxyoleic acid from *Malope* oil, they isolated the other enantiomer ($[\alpha]_D = +18.9°$ in EtOH). From the specific rotations of the pure enantiomers and the mixed product, it is evident that the dihydroxyoleic acid product from acetolysis of methyl vernolate contains 65% of the (−)-isomer and 35% of the (+)-enantiomer. From the mechanistic considerations, the (−)-dihydroxyoleic acid from vernolic acid is considered to be D-12, L-13-dihydroxyoleic acid, i.e., (12-*S*, 13-*S*) and the dextrorotatory enantiomer to be, of course, L-12, D-13-dihydroxyoleic acid, i.e. (12-*R*, 13-*R*). The *threo*-12,13-dihydroxystearic acids produced from these compounds (5) and having rotations of −23.8° and +23.8° are similarly the D-12, L-13- and the L-12, D-13-dihydroxystearic acids, respectively.

Enzymatic hydration of vernolic acid in crushed, incubated *Vernonia* seed (18) gives the opposite enantiomer to that predominating in the chemical acetolysis product from the same acid, i.e., it gives the dextrorotarory L-12, D-13-dihydroxyoleic acid. Thus the enzymic attack must be at the 12-position resulting in inversion there and the oxygen of the D-13-hydroxyl groups is presumably the oxygen from the original epoxide group.

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[Received Sept. 9, 1965]

Metabolism of Brain Glycolipid Fatty Acids [1,2]

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ABSTRACT

The metabolism of the fatty acid moieties of brain cerebrosides, sulfatides, and gangliosides is reviewed and discussed. The methodology involved in the isolation of the fatty acids is described briefly. It seems clear now that most of these acids are made by chain elongation of intermediate length fatty acids by addition of acetate residues. The unsaturated acids are made by desaturation of the intermediate length acids (palmitic, heptadecanoic, stearic) followed by chain elongation. The hydroxy acids are made directly from the corresponding nonhydroxy acids, saturated, unsaturated, and odd-numbered. All the hydroxy acids undergo oxidative decarboxylation to yield fatty acids containing one less carbon atom. The odd-numbered acids are also made from propionate, which is elongated to intermediate length acids and then to longer acids. The major intermediate length "primer" acid seems to be palmitate, but there is evidence that the stearate used for cerebroside synthesis is also made *de novo* from acetate. The ganglioside fatty acids were found to turn over somewhat faster than the other fatty acids. Two metabolic pools for the cerebroside acids were found, one with a very high turnover rate, the other with a very low turnover rate.

INTRODUCTION

THE BRAIN GLYCOLIPIDS covered in this paper are the cerebrosides, sulfatides, and gangliosides. More specifically, these are ceramide galactoside, cerebroside sulfate (sulfuric acid ester of cerebrosides, attached to the 3-position of the galactose), and the ceramide polysaccharides containing neuraminic acid. These lipids occur as families, differing within each family as to the nature of the fatty acid in the ceramide residue. The cerebrosides and sulfatides contain NFA and HFA, saturated and unsaturated; the gangliosides, however, contain only NFA in which there are only traces of unsaturated acids. In the cerebrosides and sulfatides there are two clusters of FA: those around 18 carbons long and those around 24 carbons long. In the gangliosides there is only one cluster, centering around 18:0, with negligible amounts of 22:0 and 24:0.

Other points of contrast between gangliosides and the other two can be made: the former occurs primarily in brain gray matter, the latter are primarily in white. The former has glucose attached to the ceramide residue, the latter have galactose. The former has only traces of odd-numbered FA; the latter can contain considerable amounts of C_{23} and C_{25} FA. Further differences, particularly in the metabolism of the two groups of glycolipids, will be described below.

This paper describes mainly the studies carried out in this laboratory, generally with live rats. It discusses the special problems of isolation that were encountered, as well as the interpretation of the isotopic data that were obtained. It is hoped that the approaches used will be of value in other types of studies.

SPECIAL PROBLEMS OF ISOLATION

Technical problems made this study particularly difficult. These arose from several factors:

(a) Judging by experiments with adult animals the blood-brain barrier causes the brain to come out second-best when there is a competition for intraperitoneally administered isotopic precursors. The liver and other organs pick up most of the injected material, so that large amounts of radioactivity must be administered to each animal in order to get useful levels of incorporation into the brain. While intracranial injection yields much better utilization of labeled material, it does not seem to be reproducible or physiological enough to warrant use in turnover and other quantitative studies. Because of the blood-brain barrier and the relatively leisurely rate of metabolism in the brain, the glycolipid FA obtained from labeled acetate administration have low specific activities. This prevents use of the convenient GLC-ionization chamber combinations, which allow rapid determination of the radioactivity in each FA.

[1] Presented at the Prof. Ernst Klenk Symposium on Glycolipids and the Nervous System, AOCS meeting, Houston, April 1965.

[2] Abbreviations: RCA, relative carboxyl activity (activity in COOH group × 100/activity in total FA); FA, fatty acid, HFA, 2-hydroxy fatty acid; NFA, nonhydroxy fatty acid; 18:0, stearic acid; 18:1, oleic acid; 16:1[9], palmitoleic acid as is 16:1ω7. An h symbolizes a 2-hydroxy FA, thus h24:0 is cerebronic acid (2-hydroxylignoceric acid). A k indicates a 2-keto FA. C is chloroform; E, ether; H, hexane; M, methanol.

(b) If the individual FA in each glycolipid family is to be quantified and its relative radioactivity is to be measured, the glycolipid family must be isolated from the brain in nearly 100% yield. Methods which involve losses inevitably give unequal losses of the various individual members.

(c) Because of the high amounts of odd FA in many glycolipid samples, GLC separations of the highest quality are needed.

(d) Since GLC methods, on a preparative scale at least, do not give complete separation of saturated from unsaturated FA, and NFA from HFA, it is necessary to carry out preliminary class separations.

(e) The methyl esters of the 2-hydroxy FA do not, in our hands, undergo GLC separation without destruction. It is therefore necessary to protect the OH group prior to GLC. In experiments where the isolated HFA is to be degraded chemically, it is necessary to use an easily removed protecting group.

(f) Because of the great length of the glycolipid FA (up to C_{26}), particularly stringent requirements are placed on the techniques for GLC. "Bleed" materials from packings contaminate the GLC effluents.

This list of difficulties is offered to enlist not only the reader's sympathy but also his skepticism toward studies in which the importance of the factors was inadequately appreciated.

SPECIFIC METHODS OF ISOLATION

Ganglioside Fatty Acids

As in most of the recent methods for ganglioside isolation or analysis, we use a version of the later extraction-partitioning system of Folch, Lees, and Sloane Stanley (8,26,17). The upper layer of this solvent system contains nearly all the gangliosides, as well as an unknown lipid in which the FA are ester-linked. Isolation procedures which rely on dialysis as the primary purification step (following partition) cannot eliminate this impurity. The ester-linked FA account for nearly all the 18:1 in such extracts.

For simple analytical work, we remove most of the esterified FA by a back-extraction with "Folch lower phase" (8), but for isotopic work we saponify the ganglioside extract with mild aqueous alkali, extract the free FA, and then process the purified gangliosides. The FA are obtained by evaporating the solution to dryness, methanolyzing with HCl-M, and extracting with hexane. We have made the methanolysis step convenient and reliable by using a test tube with an O-ring closure (25).

Cerebrosides and Sulfatides

In our earlier work (36,18) we used Florisil to remove nonpolar lipids (mainly cholesterol), gangliosides, and nearly all the phosphatides from the total brain lipids. The potentialities of this interesting and economical adsorbent were thus brought to the attention of workers in the field of the complex lipids. Following the suggestion of Carroll (5) we have more recently been using Florisil deactivated by addition of water. We first dry the powder thoroughly by heating at 600C for 60 min, then add 8 ml water per 100 g powder. The separating power of such Florisil undergoes a rather sudden deterioration after 3 months, and we discard the outdated material. It is likely that cold storage of the wetted material would prolong its life. Perhaps the adsorbent, which is coprecipitated silica gel and magnesia, slowly reacts in the presence of water to form magnesium silicate.

Sulfatides are not readily separated from cerebrosides by Florisil, so we used synthetic ion exchange resins to isolate the sulfatides from the mixture (37). Impurities in the resins, as well as other exchangers we tried, kept us from preparing pure sulfatides by this approach. As Rouser and his co-workers have shown (39), it is possible to make ion exchange practical by purifying the ion exchanger and eluting reagent very extensively.

Our previous methods called for the use of large Florisil columns since much of the total brain lipids had to be retained by the adsorbent. We now carry out a preliminary cleavage of the ester-linked lipids, following removal of gangliosides by solvent partitioning (17). The lipids are stirred 1 hr at room temperature in 0.07 *N* NaOH in C-M 2:1, then partitioned with aqueous acetic acid to remove glycerophosphate esters. This treatment converts the ester-linked FA and part of the free FA (7) to methyl esters, which are then readily isolated by Florisil chromatography. The same column also yields brain cholesterol quantitatively. Cerebrosides and sulfatides are eluted with C-M 3:1. Because of the prior methanolysis step, the Florisil column has to adsorb just the cholesterol, cerebrosides, sulfatides, and lyso derivatives of the alkenyl and alkyl phosphatides. If the cholesterol is not wanted, it can be eluted with the methyl esters, thereby reducing the size of the column further.

This procedure is of additional interest as it yields the ester-linked FA in the form of

methyl esters, ready for GLC, by a very mild and rapid process. There is no contamination by acetals or aldehydes since the lysophosphatidal derivatives formed during transesterification are held back by the Florisil. Most of the free FA are also held by the Florisil under these conditions.

The availability of a superior quality of silica gel (Unisil, Clarkson Chemical Co.) has made it possible to get good separation of the two glycolipids obtained from Florisil columns (17). The mixture is applied to the Unisil column in C-M 98:2, and eluted further with this solvent to remove ceramide. Elution with C-M 94:6 gives the cerebrosides, and C-M 85:15 gives the sulfatides, slightly contaminated with cerebroside. This method is the simplest one available.

Isolation and Analysis of Cerebroside Fatty Acids

The methyl esters of the NFA and HFA can be separated readily with Florisil columns (18,20). The unsaturated and saturated esters in each group can be separated with Florisil by first forming the methoxy mercuriacetate derivative of the former esters. The mercury derivative is sufficiently polar that it sticks firmly to Florisil. The saturated esters are eluted with hexane or H-E, and the mercury derivative is eluted with chloroform-alcohol-HCl. The HCl regenerates the original unsaturated ester during elution. Now that good silica gel is available the above separations can be performed equally well with that adsorbent; however, Florisil is somewhat cheaper.

In the course of various problems we have also isolated the hydroxy acids as the copper chelate (20), and have separated the saturated from unsaturated FA esters with silver nitrate-silica gel columns (21). The OH group does not interfere in the latter method, but there is appreciable overlap in the elution of unsaturated NFA and saturated HFA.

The HFA methyl esters can under some conditions apparently be isolated by GLC without derivatizing the OH group (34). However, we found it necessary to form the methyl ethers (18) or acetate esters (11,20). The acetate esters are more convenient to make and are necessary when subsequent degradations are to be performed, but the methyl ethers separate more sharply and at lower temperatures in GLC.

Application of these techniques led initially to the discovery that the NFA and HFA in rat and human cerebrosides greatly resemble one another in chain length distribution, except for the relative absence of h18:0 (18,35). The saturated FA of both types differ in distribution from the unsaturated FA. On this basis we suggested that the NFA are made from the HFA or vice versa. A second major finding was the high concentrations of the C_{23} and C_{25} FA, the relative amounts increasing with age remarkably (19). As is now known, this is the result of conversion of the C_{24} and C_{26} acids to the odd-numbered FA by a 1-carbon degradation system.

CHAIN ELONGATION vs DE NOVO SYNTHESIS OF THE GLYCOLIPID FATTY ACIDS

Interpretation of Isotopic Data

It is now well established by in vitro work that there are two main pathways of biosynthesis of NFA. One is the de novo route, the other is chain elongation of intermediate length FA (13,15). The former system has been prepared in soluble form from various organisms and tissues, including brain (2). This system is able to synthesize 16:0 from acetyl CoA, via malonyl CoA, in one fell swoop without appreciable dilution by adjacent short-chain FA molecules. (The intermediate formation of malonate, incidentally, was first proposed to a scientific gathering at a Gordon Research Conference in 1958 by the senior author.) There is evidence that 14:0 is also formed by the soluble system, but it is not yet clear whether any 18:0 is formed in this way.

An enzyme system which elongates FA is found in mitochondria and acts by adding C_2 units (acetyl CoA or malonyl CoA) to medium length acids, such as 16:0 or 16:1. Microsomes are known to synthesize FA but the mechanism is not yet known. Since somewhat more formation of 18:0 than 16:0 is observed in microsomes (28), one might expect that both systems are present. However, on the basis of findings described below, we suggest that the microsomes make *both* acids by the de novo route.

A way of determining which route is operating in a live animal is to administer COOH-labeled acetate, isolate the FA of interest, and decarboxylate it. If the FA is made only by the de novo system, the radioactivity in each odd carbon atom should be the same, regardless of the time at which the animal is sacrificed. Thus the activity in the FA's carboxyl carbon should be 1/8 the total activity in the case of 16:0, 1/9 the total in

18:0, etc. On a percentage basis, the RCA for 16:0 should be 12.5%.

If a FA is made by chain elongation, the RCA can be greater than, equal to, or less than the de novo value, depending on when the animal is killed after the isotope has been administered. This can be seen by considering the relative activities in the acetate and palmitate pools. Shortly after injection of acetate-1-C^{14}, the brain acetate pool becomes highly radioactive. The 16:0 pool in brain is assumed to consist of free palmitic acid, previously formed by synthesis from cold acetate as well as breakdown of complex lipids. Each nonradioactive 16:0 molecule condenses with a labeled acetate residue, forming 18:0 in which all of the C^{14} is in the COOH group (RCA = 100%). As time passes, the 16:0 pool becomes highly radioactive and the acetate pool loses much of its activity due to the rapid metabolism characteristic of this intermediate. Thus 18:0 molecules made later will contain rather little activity in their COOH groups, the RCA thus falling below the de novo value.

Samples of 18:0 isolated at various time intervals will exhibit a wide range of RCA's, depending on the relative turnover rates of acetate, 16:0, and 18:0. At all times the isolated 18:0 will consist of a mixture of molecules formed at different times and with differing RCA's. If one were to carry out such a study with only a single time point, one might accidentally find an RCA characteristic of the de novo value.

As was indicated above, it is conceivable that the acetate pool might be used to form 16:0 de novo and then, without delay, used to elongate the 16:0 molecules. In this case it would appear that 18:0 was made de novo. However, it is known that free FA, including 16:0, occur in brain (7,40) and that many of the complex lipid molecules of brain undergo hydrolytic release of their FA moieties, presumably feeding FA into the free FA pool. Moreover, recent experiments with microsomal FA synthesis indicate that the newly synthesized FA are immediately incorporated into complex lipids, rather than released as free FA. One might therefore expect that the chain elongation enzymes would have available preformed, *non*radioactive 16:0. The above hypothesis is summarized in Figure 1.

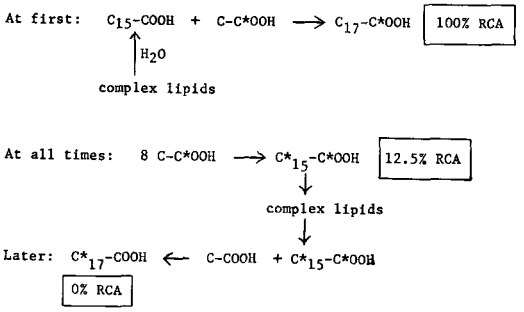


FIG. 1. Sequence of events in brain following intraperitoneal injection of acetate-1-C^{14}.

We have obtained the expected data in the case of 16:0 and 18:0 of the total brain lipids (11). Here, the RCA of 16:0 ranged between 12.2 and 12.9 during a 56 day time period, during which time the specific activity dropped 31-fold. (The theoretical value = 1/8 = 12.5%.) Meantime the 18:0 RCA dropped from 20.7 at 4 hr to 6.0 at 56 days (de novo value = 11.1%). Evidently all or most of the 16:0 and 18:0 fit the pattern predicted above.

Relative Carboxyl Activities of Ganglioside Stearate

Three groups of young rats were injected with acetate-1-C^{14}, then killed in subgroups of five at various time intervals (see Fig. 2). The three groups differed in their initial ages: 7, 13, and 22 days, chosen to represent the beginning of myelination, the time of most active myelination, and the point of slowing myelination. The brains of all five rats in each time point were pooled and the 18:0 was isolated from the gangliosides and decarboxylated (17). Figure 2 shows the RCA's obtained at each time point. It is evident that ganglioside 18:0 follows the predicted pattern of chain elongation from 16:0 and that there

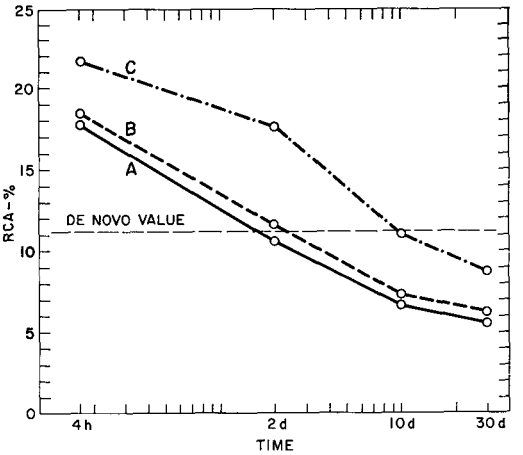


FIG. 2. Relative radioactivity in carboxyl carbon of ganglioside stearate as a function of age. *A*, rats injected at 7 days of age; *B*, rats injected at 13 days; *C*, rats injected at 22 days.

is no qualitative difference in mode of synthesis in the three age groups.

The older animals show higher RCA's at each time point, probably because the turnover rates of the FA are slower in the older animals.

Relative Carboxyl Activities of the Cerebroside Nonhydroxy Fatty Acids

Table I shows the results of two studies in which rats were given acetate-1-C^{14} and the cerebroside lignocerate (24:0) was isolated and decarboxylated. The de novo value for lignocerate RCA is 1/12 = 8.3%. It can be seen that high RCA's were found in the early time points, as with ganglioside and total brain 18:0, and the RCA's declined with time. However, only the youngest group of rats showed RCA's below the de novo value, evidently because of a considerable decrease in turnover rate with increasing age. It is evident that cerebroside 24:0 is made by chain elongation.

Fulco and Mead carried out a similar study (9), killing at only one time point, and found an RCA for 24:0 of 10.3%. Since this is moderately close to the de novo value of 8.3% they concluded that 24:0 is made directly from acetate, with little preliminary formation of shorter acids. In one of our experiments with rats of the same age (line 1, Table I), we found a similar value after two days (9.4%), but it is evident that this is only a coincidence of timing.

We also examined the 18:0 from cerebrosides in the above experiments and here (Table II) the RCA's come rather close to the de novo value (11.1%). Only one point (18.2%) indicates the existence of chain lengthening. There is thus good reason to believe that the 18:0 used for cerebroside synthesis is made by a hitherto uncharacterized synthetic system. Sphingosine (3), psychosine (6), and cerebroside (1) are made by microsomes, which are also known to utilize acetate to make labeled complex lipids (28). It is thus quite possible that these particles make 18:0 de novo and elongate it to form 20:0, 22:0, etc. Liver microsomes can convert malonyl-CoA to FA, mainly to 18:0 rather than 16:0 (28), and it would be of interest to isolate these particles after in vivo administration of labeled acetate to see if the microsomal lipids, freshly formed, contain uniformly labeled 18:0.

Direct Evidence for Chain Lengthening in Sphingolipids

Additional evidence for the chain elongation system was obtained by injecting emulsions of palmitate-1-C^{14} and stearate-1-C^{14} directly into the brains of 22-day-old rats (12). The major sphingolipids (cerebrosides + sulfatides + sphingomyelin) were isolated from the brains 24 hr later and their FA were decarboxylated. The 16:0 isolated after palmitate-C^{14} injection contained nearly all its radioactivity in the original position (96% RCA), showing that degradation to acetate and resynthesis of 16:0 (12.5% RCA) was a minor route, and that part of the injected FA was incorporated into the sphingolipids. Similarly, the 18:0 isolated after stearate-C^{14} injection was incorporated very directly, too (97% RCA).

The 18:0 isolated after *palmitate*-C^{14} injection was rather highly labeled and its RCA was 3%. Evidently the injected 16:0 was elongated with acetate that was relatively nonradioactive. The 16:0 isolated after *stearate*-C^{14} injection was not highly labeled, evidently because it was made purely by β-oxidation of 18:0 to acetate, followed by de novo formation. The RCA for this 16:0 was 15%, a little too high for the de novo value (12.5%); it is likely that a small amount of highly labeled 18:0 (97% RCA) contaminated the 16:0 during GLC.

Radioactivity was also found in 22:0 and 24:0 of the sphingolipids and here the RCA's were 3–5% with both groups of injected rats. This is direct evidence showing that both 16:0 and 18:0 can be elongated to 22:0 and 24:0, the elongation having taken place with acetate of relatively low specific activity.

TABLE I

Relative Carboxyl Activity in Biosynthesized Cerebroside Lignocerate following Injection of Acetate-1-C^{14}

Age of rats at time of injection	Time of sacrifice after injection						
	4 hr	10 hr	2 d	4 d	10 d	28–30 d	56 d
Days							
13	13.4	—	9.4	—	6.5	5.5	—
22	18.2	—	13.6	—	12.1	11.2	—
23	15.7	15.6	—	13.1	—	8.1	7.8

First 2 lines are part of one experiment (17); last line is part of (11). Data are in percents.

TABLE II

Relative Carboxyl Activity in Cerebroside Stearate following Intraperitoneal Injection of Acetate-1-C^{14}

Age of rats at time of injection	Time of sacrifice after injection						
	4 hr	10 hr	2 d	4 d	10 d	28–30 d	56 d
Days							
13	11.7	—	9.8	—	10.8	9.4	—
23	18.2	10.9	—	11.2	—	10.9	10.2

First line from (17), second line from (11). Data are in percents.

Formation of the Odd-Numbered Fatty Acids

The major odd-numbered NFA in the glycolipids are 23:0 and 25:1, with smaller amounts of 23:1 and 25:0. Small amounts of 15:0 and 17:0 occur in brain and elsewhere, and the results of in vitro work with those acids indicate that they are made by the same de novo system that makes 16:0 (4). The only difference is that the starting substrate is propionyl-CoA instead of acetyl-CoA. To see whether a similar system exists in brain, we injected propionate-1-C^{14} intraperitoneally into a group of rats, then isolated the saturated NFA from the cerebrosides (10).

The data showed that the odd-numbered acids exhibited the highest specific activities by far. Similar results were obtained when the *total* brain FA were isolated, in which case 15:0 and 17:0 had much more activity. In view of the previously described data for the even-numbered acids, it seems very likely that 15:0 and 17:0 are made from propionate + acetate (malonate) and that these are elongated with C_2 residues to form the longer odd-numbered FA so characteristic of the glycolipids.

Evidence for a second route of biosynthesis is presented later.

Formation of the Unsaturated Sphingolipid Acids

Surprisingly little is known from in vitro work about the mode of formation of the unsaturated acids. For many years the basic fact was known that 18:0 can be desaturated to form $18:1^9$ and it appeared likely that 16:0 is desaturated similarly, to form $16:1^9$ (palmitoleate). Presumably the same enzyme acts on both substrates, removing two hydrogen atoms in both cases at the 9,10 position (counting from the COOH end). In the case of the longer unsaturated NFA of the glycolipids, one would expect this enzyme to form $24:1^9$, $26:1^9$, etc., from the saturated analogs. However, the only isomers of known structure at the time we began this study were $24:1^{15}$ (nervonate), $22:1^{13}$, and $20:1^{11}$. From this observation it would appear unlikely that desaturation of long FA takes place.

Examination of the sphingolipid unsaturated FA from a different viewpoint—from the methyl or ω end—places the acids in a more coherent system. If we locate the double bonds by counting from the ω end, we find that they are all in the oleate family: 18:1ω9, 20:1ω9, 22:1ω9, and 24:1ω9. It is then reasonable to assume that the chain elongation system used by the brain to make the glycolipid acids acts similarly on oleate.

Fulco and Mead, in the same labeled acetate experiment mentioned above (9), isolated nervonate and decarboxylated it. Its RCA was 20.2%, much different from the 10.3% they found for the saturated analog. Since they had concluded that the latter is formed by de novo synthesis, they then concluded that 24:1 is formed by the alternative method, chain elongation. Noting the above-mentioned similarity in double bond position, they suggested oleate is the precursor of nervonate.

We sought to make a direct demonstration of the conversion by injecting oleate-1-C^{14} directly into rat brains, as in the labeled palmitate and stearate experiments. The results were somewhat different (22), for the long unsaturated FA showed very high RCA's: 74% for 24:1, 53% for 22:1, and 86% for 20:1. An additional surprise is that relatively little of the injected oleate was incorporated into the sphingolipids. Part of the explanation for the latter finding is that 18:1 is a very minor acid in this group of complex lipids, and it apparently turns over very slowly (if at all).

It would appear from the above results that very little of the injected oleate entered the cells making nervonate, etc., and much of the 18:1 was simply oxidized to acetate-1-C^{14}. The acetate did enter the synthesizing cells and was used to elongate the 18:1 already present in the cells. However, if labeled acetate were used to elongate only 18:1, the resultant 24:1 would have $\frac{1}{3}$ of its C^{14} in its COOH group (since three C_2 units are needed). Since the RCA for 24:1 was far above 33%, it must be made in part by elongation of 22:1 and 20:1. Evidently the sphingolipid-synthesizing cells contain pools of 22:1 and 20:1, as well as 18:1, all of which can be used for chain elongation. It is likely that similar pools of saturated FA are also present.

Evidence from Ozonolysis of the Sphingolipid Acids

Palmitoleate, 16:1ω7, exists in brain, albeit in much lower concentrations than oleate, and it too should be subject to chain elongation if the elongating enzymes are not too specific. 17:1 also exists and should give rise to the odd-numbered unsaturated FA, none of whose structures was known. In order to confirm these guesses, we processed pig brains to isolate the sphingolipids and their individual unsaturated FA. GLC yielded these acids as single peaks which showed the expected in-

frared absorption spectra for cis-FA. A micro-ozonolysis procedure was devised (21) which permitted quantitative determination of the fragments of each FA isomer, starting with 1.5–3 mg of sample. The results of analyzing the mono- and diunsaturated FA are shown in Table III, which gives the abundance of each isomer, classified according to its ω-family. Similar data are shown for the HFA from the same brains (23).

The number of isomers found within each chain length was unexpectedly large; most of these had previously not been reported in nature. The existence of even- and odd-numbered diunsaturated FA in the sphingolipids is also of interest.

The ω6 family occurs only in the dienes and is evidently due to chain elongation of linoleate. The ω7 family is found in all of the acids and presumably arises from palmitoleate. Elongation of 16:1ω7 should give rise to 18:1ω7 (cis-vaccenic acid) as well as the longer members of this family. Cis-vaccenic acid does indeed exist in brain, as we found in a later study (24) of the ester-linked FA of pig brain. While this acid had been known to occur in animals for some time, it was considered a rare acid. However, we found this to be the second most common monounsaturated FA, second only to oleate and much more abundant than palmitoleate. As was shown very recently in vitro (15), cis-vaccenate is formed by elongation of palmitoleate, confirming our suggestion that the latter is elongated to form a series of ω7 FA.

The odd-numbered acids of the ω7 family may be presumed to originate from the even-numbered members by 1-carbon degradation according to the hydroxylation-oxidation system described in a later section of this paper.

The occurrence of the ω8 family *only* in the odd-numbered FA must mean that these acids are formed by elongation of a primary acid, 17:1ω8. This acid is presumably formed by desaturation of 17:0 at the 9,10 position.

The oleate, or ω9 family of acids is like the ω7 family in that it is represented in all chain lengths and in the dienes. These acids are probably formed by elongation of oleate and the odd-numbered acids are formed by 1-carbon degradation of the longer acids. Thus all of the above unsaturated acids may be considered as arising initially from the desaturating enzyme which acts on the 9,10 position from the COOH end of 16:0, 17:0, and 18:0.

The ω10 family of Table III can probably be explained as arising by elongation of 16:1ω10. The odd-numbered acids presumably are formed from the longer acids (26:1ω10 and 24:1ω10) by the 1-carbon degradation system. The postulated "primer" acid, 16:1ω10, was found in the ester-linked acids (24) as was a long line of longer ω10 FA. Perhaps another desaturating enzyme exists in brain which is specific for the 6,7 position of FA (counting from the COOH end).

The above hypotheses are based on the assumption that the chain elongating enzymes can act on 16-,17-, and 18-carbon FA as their "primer." This is consistent with our previous proposal that 18:0 is made de novo in microsomes and lengthened to form the cerebroside acids and with the results cited above with intracranial injection of labeled 16:0 and 18:0. FA desaturation of 18:0 takes place in microsomes (29).

The role of palmitoleate in chain elongation was demonstrated by ozonolysis of the 24:1 isolated in the previously described experiment (22) in which oleate-1-C^{14} was injected into rats intracranially. The 24:1 yielded two dicarboxy acids, C_{15} and C_{17}, and two monocar-

TABLE III

Occurrence of the Unsaturated Fatty Acids of Pig Brain Sphingolipids

Fatty acid family[a]	Monounsaturated fatty acids					Diunsaturated acids		
	22:1	23:1	24:1	25:1	26:1	24:2	25:2	26:2
ω6 NFA	—	—	—	—	—	1.9	0.14	0.14
ω7 "	21	4.5	44	3.7	13	0.3	0.07	0.22
ω8 "	—	2.7	—	1.3	—	—	—	—
ω9 "	3.7	1.5	170	10	10	0.4	0.06	0.13
ω10 "	—	0.1	4.4	1.3	2.9	—	0.01	0.02
ω7 HFA	3.2	1.6	33	3.8	18			
ω8 "	—	0.7	—	0.7	—		not isolated	
ω9 "	0.5	0.4	44	2.8	7.3			
ω10 "	0.1	0.03	3.2	0.2	1.0			

[a] Position of double bond counting from the methyl end. In the case of the diunsaturated FA, it indicates the position of the double bond closer to the methyl end.

Data are in mg FA/100 g fresh brain.

boxy acids, 7:0 and 9:0. The distribution of activity in the original 24:1 isomers was as follows:

ω9 isomer (nervonate):

$$\overset{\text{18 cpm}}{\overbrace{}}\!\!-\!\!\overset{\text{3450 cpm}}{\overbrace{\phantom{CH=CH-(CH_2)_{13}-COOH}}}$$
$$CH_3-(CH_2)_5-CH_3-CH_2-CH=CH-(CH_2)_{13}-COOH$$

ω7 isomer:

$$\overset{\text{0 cpm}}{\overbrace{}}\!\!-\!\!\overset{\text{123 cpm}}{\overbrace{\phantom{CH_2-(CH_2)_{13}-COOH}}}$$
$$CH_3-(CH_2)_5-CH=CH-CH_2-CH_2-(CH_2)_{13}-COOH$$

These data are consistent with the idea that the injected oleate was primarily oxidized to acetate, which was then used to elongate nonradioactive palmitoleate and oleate.

FORMATION OF THE 2-HYDROXY FATTY ACIDS

Studies with Labeled Acetate

Fulco and Mead, in the same experiment mentioned (9), carried out the first examination of cerebronic acid (h24:0). They injected acetate-1-C^{14} intraperitoneally into 13-day-old rats and isolated brain h24:0 (as well as 24:0). Decarboxylation of the HFA yielded a RCA of 10.3%, identical to that of the 24:0. Indeed the C^{14} distribution in the first 3 carbon atoms of each acid showed very similar patterns. From these relations, and the finding that 24:0 had 16 times the specific activity of h24:0, they concluded that the HFA was probably made from the analogous NFA.

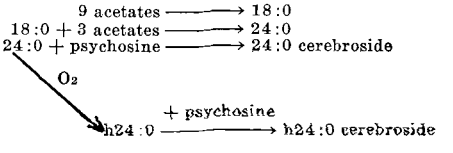
The force of the conclusion was weakened by the mode chosen for administering the isotopic acetate. The acetate was injected in 4 daily doses and the animals were killed 3.5 hr after the last dose. Reiner (38) has pointed out the difficulties that ensue from such a dosage plan, but the mathematical treatment of the problem was a formidable one. A simpler approach can be made by considering what would happen if one were to give an animal acetate-1-C^{14} for several months during its early life. It seems very likely that each FA molecule in the animal would become uniformly labeled (in the odd-numbered positions) and would reveal a RCA corresponding to the theoretical de novo value, even if the acid had been made by chain elongation. Lignocerate, for example, is made from 16:0 or 18:0 and acetate, all of which would have the same specific activity.

In the more typical 1-pulse type of isotope experiment, the molecules of 24:0 formed at first are made from highly labeled acetate and relatively unlabeled 16:0 or 18:0. A second pulse of labeled acetate would find itself adding on to 16:0 which is now more radioactive. Each successive pulse maintains the specific activity of the brain acetate, which is now adding on to highly and similarly labeled 16:0. The Fulco-Mead experiment was concluded shortly after the last injection, while the brain 16:0 was still at a high level of activity. Under this condition one would expect to find a RCA for each brain acid (or any other FA made from a slowly metabolizing FA) close to its de novo value. Indeed, Fulco and Mead confirmed this in a later analysis of the 18:0 from these rat brains (31). Here the RCA was 12.7% for cerebroside 18:0 and 11.7% for ester-linked 18:0 (de novo = 11.1%). They concluded from this that all brain 18:0, like 24:0, is made from acetate without dilution with inactive 16:0.

The liver 18:0 from the same rats showed a RCA of 30%, which indicates chain elongation must have taken place. Why the difference between brain and liver? FA metabolism is undoubtedly much faster in liver so that the 16:0 formed after the first 3 isotopic injections had already turned over and lost much of its radioactivity by the time the last pulse of acetate was administered. Thus this experiment with respect to liver was much like a typical 1-pulse experiment; during the 3.5 hr following the last injection the labeled acetate was adding on to relatively nonradioactive 16:0, producing 18:0 with a high RCA.

It should be evident from this discussion that the data resulting from a multipulse experiment can be misleading when the animals are sacrificed shortly after the last injection.

As part of our time study of the NFA of brain cerebrosides formed from labeled acetate (11), we also isolated the major saturated HFA. The RCA's of 24:0 and h24:0 were quite similar throughout the period examined, 4 hr to 56 days (see line 3 of Table I for the 24:0 data). This relation is consistent with the idea that 24:0 is the precursor of h24:0 (or vice versa). A probable sequence of events in this case is:



If the 2 cerebrosides, kerasin and cerebron, had somewhat different rates of synthesis and breakdown one would find the RCA's diverging with time. This follows from the fact that the RCA of a group of molecules is the average of the RCA's of the individual molecules. As new molecules of 24:0 and h24:0 are deposited in cerebroside form, they modify

the average RCA of the previously deposited molecules at similar rates.

Evidence from Intracranial Injection

As in the previously described experiments utilizing intracranial injection of labeled 16:0 and 18:0, we also injected lignocerate-1-C^{14} and cerebronate-1-C^{14} (12). In the case of the former compound, only the 24:0 and h24:0 of the cerebrosides were found to be highly labeled. (Unsaturated acids were not isolated). The RCA of the isolated h24:0 was 89%, which means that most of the cerebronate was made quite directly from the 24:0, although some must have been made from 24:0 formed endogenously from labeled acetate (itself derived by β-oxidation of some of the injected 24:0).

The reverse reaction apparently does not go, at least under these conditions. Almost none of the cerebronate radioactivity found its way into the NFA although incorporation of the cerebronate into the sphingolipids was observed.

Since the reaction probably is carried out by the microsomes, which are noted for their ability to hydroxylate substrates, it is likely that the reaction involves a direct hydroxylation in the 2-position. However, there is the possibility that the NFA is first dehydrogenated in the 2,3-position, then hydrated in the "unnatural" direction.

A preliminary note has appeared on an enzyme system in plants which converts 16:0 to h16:0 and then to 15:0 (14).

The Unsaturated Hydroxy Acids

Of two possible synthetic routes:

$$\text{unsaturated FA} \xrightarrow{+O_2} \text{unsaturated HFA}$$

$$\text{saturated HFA} \xrightarrow{-2H} \text{unsaturated HFA},$$

it is likely that the first is the actual one. The evidence presented earlier in this paper showed that it is likely that the unsaturated NFA are made by chain elongation of the medium length unsaturated NFA. It is these acids which are probably hydroxylated.

Further evidence comes from examination of the double bond positions in the HFA of brain (23). As described before, pig brains were processed to yield the individual unsaturated HFA (homogeneous with respect to chain length) and these were then examined by ozonolysis to determine the amounts of each unsaturated isomer. The even- and odd-numbered HFA between h22:1 and h26:1 were studied (Table III). Each acid contained a member of the ω7 family and the ω9 family, presumably derived by elongation of palmitoleate and oleate followed by hydroxylation.

Also present, but only in the odd-numbered HFA, were members of the ω8 family. As explained before, these are probably formed by elongation of 17:1ω8.

Trace amounts of the ω10 family were also found, as in the unsaturated NFA. Again the evidence points to simple hydroxylation of the analogous unsaturated NFA.

There is a marked difference between HFA and NFA in the relative abundances of the ω7 and ω9 families. The total amounts of the two families in the HFA are 60 and 55 mg/100 g brain, respectively; in the NFA they are 86 and 195 mg/100 g brain. Perhaps this reflects a tendency of the hydroxylating enzyme to prefer FA having a longer saturated chain next to the COOH group. This preference is shown also in a comparison of the relative abundances of the NFA and HFA of all the sphingolipid acids of the same pig brains (Table IV). Here we see that the relative amount of HFA rises with increasing length of the CH_2 chain next to the COOH group (26:0 is a minor exception).

THE 1-CARBON DEGRADATION SYSTEM

Evidence from Degradation Experiments

In one of the turnover experiments described above (11), in which weanling rats were injected with acetate-1-C^{14}, the cerebroside h23:0 acid was isolated and decarboxylated. As predicted from the elongation theory, the RCA was high in the early time points and low in the late time points. Indeed, the RCA went to the lowest observed value in this study, 2.9%. From this it would appear that an odd-numbered FA, probably 17:0, is formed first

TABLE IV

Relative Abundances of Hydroxy and Nonhydroxy Acids in Pig Brain Sphingolipids

Acid type	Number of CH_2 groups attached to COOH group[a]	Ratio of HFA/NFA
22 Monoenes	14	0.15
23 "	15	0.31
24 "	14	0.35
25 "	17	0.88
26 "	18	0.97
22 Saturateds	21	1.6
23 "	22	2.1
24 "	23	2.1
25 "	24	2.3
26 "	25	1.5

[a] Calculated for the major isomer in the case of the monoenes (in the case of 25:1 the major NFA isomer is ω9 and the major HFA isomer is ω7). Data are derived from Table III.

TABLE V

Distribution of Radioactivity in the Individual Carbon Atoms of Cerebroside Acids after Injection of Acetate-1-C^{14}

Age of rat at the time of		Fatty acid	Carbon atom number[a]				
Injection	Sacrifice	degraded	20	21	22	23	24
23 d	51 d	h23:0	—	—	8.5	4.3	—
23 d	79 d	h22:0	—	1.4	9.1	—	—
		h23:0	—	—	8.3	2.9	—
		h24:0	—	—	—	0.8	8.4
15 d	168 d	h23:0	—	2.8	5.8	0.3	—
		h24:0	—	1.0	5.3	0.6	9.3
		23:0	6.4	1.9	4.9	0.8	—

[a] Counting from the methyl end of the FA.
Data are in percent of activity in the carbon atom compared with the entire molecule. The lower three lines are from Mead and Levis (32).

and is then elongated with 3 acetate residues to form 23:0, which is then hydroxylated. The rapid drop-off in RCA suggests that this acid has a high turnover rate. However, the total radioactivity per brain in cerebroside h23:0 showed the most prolonged rise with time of all the cerebroside acids, which suggests that this acid undergoes little or no breakdown. Moreover, the deposition in brain of h23:0 is the most prolonged and striking, which could mean that this acid does not undergo metabolic attack.

The conflicting pieces of evidence were clarified by degrading the h23:0 further to learn the relative activity of carbon 2. The data are shown in Table V and include similar degradation data for h22:0 and h24:0. By looking at lines 1 and 3, we see that the second carbon atom of h23:0 (analyzed at 2 different time points) has an unexpectedly high amount of C^{14}. If h23:0 were made only by elongation with acetate-1-C^{14}, the α-carbon atom should contain virtually no radioactivity. Indeed, analysis of the *even*-numbered HFA (lines 2 and 4) does show there is little C^{14} in the α-atoms. (There should be even less, according to the elongation theory.) The most plausible explanation of the unexpected finding is that part of the h23:0 is made by a 1-carbon degradation of h24:0. The β-carbon of h24:0, which should have a C^{14} content similar to that of the COOH carbon, becomes the α-carbon of h23:0 and the α-carbon of h24:0 becomes the COOH of h23:0.

The relative activities in the individual atoms are shown in Table V in columns which align the data for identical atoms.

The 1-carbon degradation scheme is shown in Figure 3.

It is very likely that part of the h23:0 is further degraded to h22:0. This explains the small but appreciable activity in the α-carbon of the latter acid. (An alternative explanation, which must account for part of the activity in the α-atoms of all FA, is that part of the injected acetate is metabolized to labeled CO_2, and part of this finds its way to form acetate labeled in the methyl position. Such acetate, during chain elongation, would yield FA containing some C^{14} in the even-numbered carbon atoms.)

Mead and Levis (32) carried out a similar experiment which is also summarized in Table

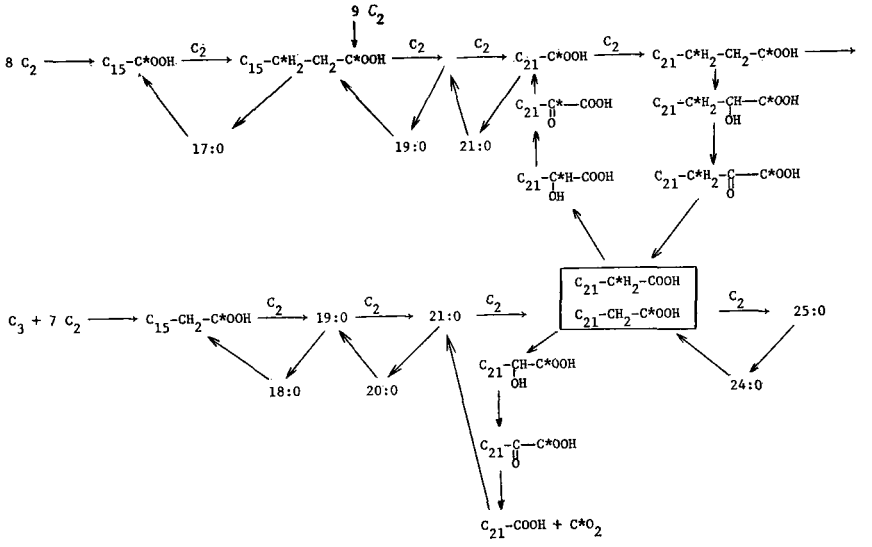


FIG. 3. Formation of the odd-numbered fatty acids by two biosynthetic routes.

V (last 3 lines). Substantially the same results were found. Incidentally, their RCA for h23:0 was even lower than ours, evidently because the rats were kept for a much longer period before sacrificing.

Evidence from in Vitro Work

Following this decarboxylation study, Levis and Mead succeeded in demonstrating the 1-carbon degradation reaction in vitro (27). They showed that h18:0 and h23:0 are degraded by rat brain microsomes to form CO_2 and the acid with one carbon atom less. ATP and NAD are necessary for the system as well as unknown material found in the cell supernatant. They found that k18:0 (2-ketostearate) was also attacked by the enzyme system, somewhat more rapidly than the h18:0. Presumably the hydroxy acid is decarboxylated via the keto acid as intermediate.

At the kind invitation of Dr. Mead, the senior author and Dr. Amiya Hajra spent a month at his laboratory to become familiar with the problem. We have since been studying the enzymatic attack of the brain enzymes on k18:0. Drs. S. S. Parmar and W. E. Davies have also worked in this laboratory on the problem, using an acetone powder of pig brain as the enzyme source and following the reaction by measuring the $C^{14}O_2$ produced from the keto acid.

It appears that the same cofactors are needed for k18:0 and h18:0 oxidation: ATP, NAD, and cell supernatant. Part of the activity in the cell supernatant is removed by Dowex 50-H^+; this seems to be Mg^{++}. Another part is removed by Dowex 1-OAc^-; this can be recovered from the resin by elution with dilute acid. The acidic cofactor is stable to heat in acid but not in alkali and shows the properties of ascorbic acid. Synthetic ascorbic acid does replace the acidic cofactor but is inhibitory at higher concentrations. The optimal concentration in our system is 5×10^{-5} *M*, which is unusually low for ascorbic-catalyzed enzyme reactions.

The complete enzyme system, containing ATP, NAD, ascorbate, and Mg^{++}, is seriously inhibited by an iron chelator (*o*-phenanthroline) or by the more general chelator, EDTA. The EDTA effect is not reversed by washing the particulate enzyme with water unless ferrous chloride is added, so it appears that bound Fe^{++} is also needed for activity.

Incubation under nitrogen drastically lowers activity. Further evidence that the reaction is oxidative was obtained by isolating the non-radioactive product, 17:0 acid, by means of TLC and GLC. The enzyme preparation itself contains some free FA (even-numbered), so no carrier was needed. We did add 16:0 aldehyde before isolating the lipoidal products, but reisolation of the aldehyde showed only one peak (16:0) on GLC.

TURNOVER OF THE GLYCOLIPID FATTY ACIDS

Turnover data normally consist of measurements of the activity of compounds which are isolated from animals previously injected with a single dose of a radioactive precursor. The animals are sacrificed at various times after the injection and the activities are plotted against time. In order to minimize individual variation among animals and obtain smooth curves, one must sacrifice groups of animals at each time point. Thus such experiments tend to involve a great deal of effort and radioactive material. They are carried out because they yield information that cannot be obtained in other ways.

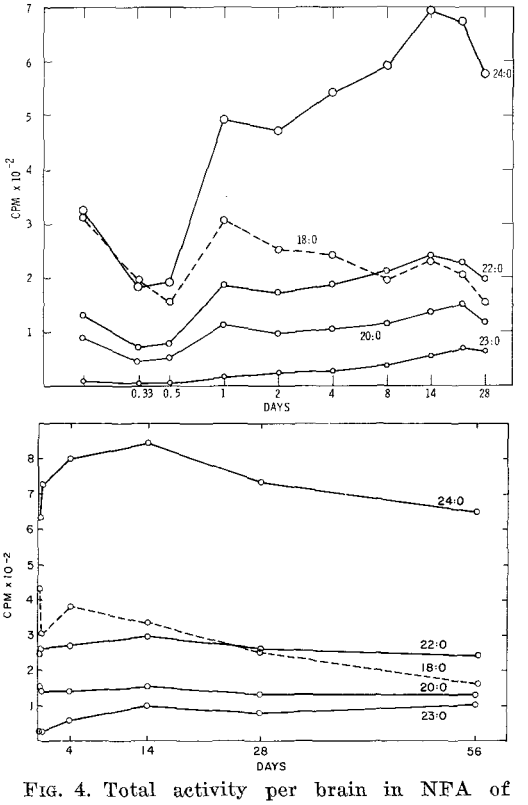


FIG. 4. Total activity per brain in NFA of cerebrosides. Upper figure, rats injected with tritium-labeled acetate. Lower figure, rats injected with COOH-labeled acetate. (From ref. 11, reprinted by permission of the publisher.)

The Cerebroside Fatty Acids

Figure 4 shows the results of administering a single dose of acetate-2-H^3 (upper curves) *or acetate-1-*C^{14} *(lower curves) to male weanling* rats, then isolating the cerebroside NFA (11). Let us examine the curves singly, from the top down.

The curve for 24:0 shows a maximal value was reached soon after the injection, at approximately 4 hr. (The zero point is not shown because the data are plotted on semilog paper). The activity then declined rapidly, showing rapid metabolism of the cerebroside-bound 24:0. Whether the metabolism involved hydrolytic breakdown or, perhaps, sulfation to form sulfatide, we cannot tell from these data. A second rise in activity appears, much more gradual, reaching a peak at roughly 14 days. Again the activity drops off, also much more gradually than the first time. The presence of two separate turnover patterns for one substance must mean that we are observing two pools of 24:0 cerebroside (kerasin). The first peak shows there is a pool of kerasin in brain which is formed and destroyed rapidly, with a half-life of roughly one third of a day. The second peak shows there is a pool with a much more leisurely breakdown rate. Since we did not prolong the experiment long enough we must consider the possibility of a third pool which might not, perhaps, undergo any breakdown at all.

The first pool of 24:0 is made from 16:0 and/or 18:0 by elongation with highly radioactive acetate. Since the tritium-labeled acetate is known to undergo very rapid oxidation to T_2O and CO_2, the slowly metabolizing 24:0 must be made from some other radioactive precursor. As our decarboxylation and other experiments showed, this radioactive precursor must be labeled 16:0 or 18:0, formed in the initial hours from labeled acetate. The labeled medium-length FA are in large part incorporated into the glycerophosphatides, which show a more rapid metabolism than the cerebrosides (11). As the phosphatides undergo breakdown, the liberated FA are in part utilized for chain elongation to form 24:0. Thus the precursor FA pool loses activity only quite slowly and continues to add tritium over many weeks to the even more slowly metabolizing cerebroside pool.

It is tempting to assign the slowly metabolizing kerasin to myelin and the rapidly metabolizing kerasin to the other structural elements of the brain. Alternatively, the rapidly metabolizing kerasin could be in the gray matter and the other pool in the white. Several workers, including our own group, have shown that cerebroside is not localized only in myelin.

Let us now examine the next curve, for cerebroside 18:0. This FA also shows 2 components, but the second one reaches its maximum fairly soon too. It then shows a gradual decline in activity for the next 27 days or more. The difference between kerasin and 18:0 cerebroside is quite striking with respect to the more slowly metabolizing cerebroside. Perhaps the difference lies in a different subcellular distribution of the two cerebrosides, or in readier attack by enzymes on the less lipoidal 18:0 cerebroside (33).

The next 2 curves, for 22:0 and 20:0, resemble the 24:0 curve somewhat. The 23:0 curve is atypical, showing a very prolonged rise. The slight dip at the 28-day point may be due to experimental variation. This long period of accumulating isotope indicates that a radioactive precursor is slowly being made available for 23:0 formation. We now know from the experiments described earlier in this paper that *two* long-lived precursors are involved: labeled 17:0, formed early from propionate and labeled acetate, and labeled h24:0. Both FA are made available by constant release from glycerophosphatides and cerebrosides.

The lower set of curves, obtained in the C^{14} experiment, illustrates an important point of general validity. Note that the very early rise and fall in activity seen in the upper curves is not visible here, except for the cerebroside 18:0. If we had not run the same experiment with tritium-labeled acetate, we would have missed finding the rapidly metabolizing cerebrosides. The explanation for the difference lies in the nature of FA degradation. When tritium-labeled 16:0 or 18:0 is degraded by β-oxidation, most of the tritium is lost as water (and greatly diluted by the body water). The acetate residues formed in β-degradation are thus only slightly radioactive so that reutilization of these residues will give rise to rather slightly labeled FA. On the other hand, when C^{14}-labeled 16:0 or 18:0 is oxidized, the resultant acetate residues still have all their radioactivity. Of course much of this endogenous acetate is metabolized via the Krebs cycle, but still part is reutilized for FA synthesis. The net effect is that the radioactivity in the acetate-C^{14} pool dies away somewhat more slowly than that in the acetate-H^3 pool. This acts to cover up rapid changes in FA pools in the first day after injection. It is very likely

that other studies of FA metabolism in similar experiments have missed similar disclosures of rapid turnover fractions because of the use of C^{14}.

It should be noted also that many turnover studies in brain have neglected the early time points altogether and could not have picked up such fractions anyway.

Returning to the lower part of Figure 4 we see that the curves are similar to the upper part with respect to the slow turnover fraction. This experiment covered a somewhat longer time period (and is plotted arithmetically), so the long period of decline in activity is better delineated. The slowness in decline of 24:0 activity hints at the presence of a nonmetabolizing fraction. Again we see that 18:0 cerebroside occupies a distinctly different position in the metabolic scheme, as does the 23:0 cerebroside.

Our analytical data from GLC also show the atypical nature of 23:0 and h23:0 cerebrosides, both of which accumulate markedly with age. Evidently the 1-carbon degradation system becomes a prominent system in older animals, in which the synthetic enzymes seem to slow down.

The turnover curves for the HFA of cerebrosides (not shown here) are qualitatively similar to those of Figure 4 except that their second activity peak (with acetate-H^3) comes somewhat later. This is evidently due to their being formed from the corresponding NFA.

In a later study with tritium-labeled acetate we confirmed the existence of the rapidly metabolizing fraction and found the first activity maximum to be closer to 2 hr than 4. This fraction exists also in somewhat older rats. Incidentally, the sphingosine in cerebroside was also found to undergo turnover, so it is apparent that cerebroside molecules undergo complete breakdown or are used in toto in the course of their metabolism.

We also made a turnover study with acetate-1-C^{14} using rats of three different ages, as described in Figure 2. The rats were killed 4 hr, 2 days, 10 days, and 30 days after injection and the activity incorporated into the cerebroside acids was measured (17). The highest activity was found in rats injected at 13 days of age, when myelination is fastest. Some qualitative differences between the 3 age groups were seen. A striking difference was noted in the 18:0 from the youngest rat group which did not show the rapid decline seen in the older rat groups. Moreover, the activity found in 18:0 of the youngest rats was higher than that found in 24:0 (in contrast to the reverse situation in the older rats). Evidently very young rats make more 18:0 cerebroside than 24:0 cerebroside. This finding is in agreement with that of O'Brien, who found 18:0 cerebroside to be somewhat more plentiful in early childhood (33). The same is true for the FA of human brain sphingomyelin (41).

Another interesting observation was that the data from the rats injected at 7 days revealed the existence of the rapidly metabolizing cerebroside fraction even though the isotope used here was C^{14}. This observation is consistent with the suggestion that the slowly metabolizing fraction is myelin-bound. Myelin formation in rat brain appears to be insignificant until about 9 days of age (16), so during the 2-day period following the C^{14} injection there must have been incorporation only into the labile cerebrosides and none into the myelin cerebrosides.

The Sulfatide Fatty Acids

The 18:0 and 24:0 of the sulfatide FA were isolated in the experiment described just above. As with the cerebrosides, the 24:0 activity curve showed a steady rise with time. Surprisingly, the 18:0 curve also showed a similar steady rise; indeed, in the 7-day and 13-day rat groups the 18:0 rose even more rapidly than the 24:0. In the cerebrosides, the 18:0 activity declined (except in the early period) so the difference is quite marked. The difference may indicate a relatively rapid conversion of 18:0 cerebroside to 18:0 sulfatide. That such a conversion does occur was suggested by our early work with labeled galactose (37) and a recent report based on in vitro work (30). Presumably the conversion is by direct sulfation with phosphoadenosyl phosphosulfate.

The Ganglioside Fatty Acids

Virtually all the ganglioside FA are accounted for as 16:0, 18:0, and 20:0. These were examined as part of the 3-age turnover study described above (17). The curves for the specific activity of 16:0 showed a very rapid rise followed by a fairly rapid fall. The rate of fall dropped off with time, particularly with increasing age of the rat group. Comparison with the 16:0 of glycerophosphatides showed quite similar drop-off rates and specific activities, so this sphingolipid is evidently much more active metabolically than most of the other glycolipids. A similar conclusion was drawn from our earlier study with galactose-C^{14} (37).

The 18:0 of ganglioside yielded curves similar to the 18:0 of glycerophosphatides. In both groups of acids (with only one exception) the curves were surprisingly like the theoretical precursor-product curves predicted by Zilversmit, Entenman, and Fishler (42). The 18:0 curves rose more slowly than the 16:0 curves, then the latter intersected the maximum of the 18:0 curves, and then the two curves descended together (with the 18:0 now more radioactive). The curves are thus quite consistent with the elongation theory and evidence. The curves for 20:0 were similar to those for 18:0.

Although ganglioside deposition slows down somewhat after the rat reaches 21 days of age, the turnover curves show that ganglioside FA is being metabolized even at 52 days. (However, the 20:0 ganglioside appears to be inert after 32 days).

General Conclusions from Turnover Studies

The common belief that the brain FA are inert arose from two observations: (1) Labeled precursors injected systemically into animals gave rise to low activities in the brain lipids; (2) Long-term turnover studies of brain lipids have shown remarkably long persistence of radioactivity. The first type of observation was made without consideration of the blood-brain barrier, which calls for the use of large doses of radioactivity in order to get measurable activities into the brain. The barrier, however, has little to do with the *rate* of brain metabolism; it simply reduces the choice of precursors which the brain can draw upon. The researchers who made these experiments did not appreciate the need to consider time as a variable in discussing reaction rates.

The second type of observation was made without examining the early time points so that the rapidly metabolizing fractions were missed. Unquestionably they point to the existence of a fraction in brain which metabolizes very slowly and perhaps not at all. This might well be myelin or some lipid structure concerned with the preservation of memory (37). Our shorter term experiments supplement the long term experiments and show that a portion of the brain lipid undergoes breakdown even during the period of rapid deposition. Indeed, the breakdown rates seem to be highest during the periods of most rapid synthesis or deposition. Lipid breakdown continues well after the period of most rapid myelination. At the moment there is no way to measure the relative amounts of the rapidly and slowly metabolizing fractions.

Our turnover and decarboxylation studies indicate the presence of several different metabolic pools and future work must examine the various subcellular regions separately.

FINAL COMMENTS

This paper on glycolipid fatty acids was based primarily on the work of this laboratory and covers work by Drs. Amiya K. Hajra, W. Ewart Davies, Krystyna C. Kopaczyk, and S. S. Parmar. We are indebted to Dr. Bernard Agranoff, Coordinator of Biological Sciences in this Institute, for many valuable insights into our data. For reasons of space we have neglected to mention relevant work from other laboratories. Our critical comments are offered only to clarify certain approaches to the study of brain metabolism and will, we hope, be taken good-naturedly.

ACKNOWLEDGMENT

Supported in part by grant No. NB03192 from the Institute of Neurological Diseases and Blindness, USPHS.

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[Received July 7, 1965]

Gas-Liquid Chromatographic Analysis of Long Chain Isomeric Glyceryl Monoethers[1]

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ABSTRACT

A quantitative method is described for the gas chromatographic analysis of glyceryl ethers using their trifluoroacetate (TFA) and trimethylsilyl (TMS) ether derivatives. Both derivatives are prepared at room temperature by reactions that proceed virtually to completion in less than 15 min, eliminating time-consuming derivative preparations and laborious clean-up steps of unreacted materials required by other methods. Gas-liquid chromatography (GLC) resolves the 1- and 2-isomers of the glyceryl ether TFA derivatives, which have not been separated previously. Purified synthetic 1- and 2-glyceryl ethers, including saturated and mono- and di-unsaturated, were used to evaluate several polar and nonpolar liquid phases for the analysis of the TFA and TMS derivatives. Analyses can be made on some liquid phases normally used for methyl esters, while others are unsatisfactory. A mixture of isomeric C_{18} saturated and mono- and di-unsaturated TFA derivatives was partially resolved; however, a complete analysis of this mixture can be made by preliminary separation of the unsaturates on silver-ion-impregnated thin-layer plates or by GLC analysis alone with three different phases.

INTRODUCTION

GAS-LIQUID CHROMATOGRAPHY (GLC) has been the most successful method for the analysis of glyceryl ethers. It has developed slowly, however, because suitable derivatives for separating 1- and 2-glyceryl ether isomers were not available. Several derivatives of the intact glyceryl ether molecule have been used for GLC analysis. Hanahan et al. (1) reported the GLC analysis of glyceryl ethers as their isopropylidene derivatives, but the method does not yield derivatives of the 2-isomer and the reaction involving the 1-isomer is incomplete, requiring a clean-up step to remove unreacted glyceryl ethers. Hallgren and Larsson (2) prepared the dimethoxy derivatives of the glyceryl ethers, which were then analyzed by GLC. Like the isopropylidene derivatives, the dimethoxy derivatives required a clean-up step to remove unreacted and partially unreacted material before GLC analysis. Dimethoxy derivatives of the 2-isomers can be prepared by this method; however, their resolution from the dimethoxy derivatives of the 1-isomer by GLC has not been reported. Blomstrand and Gürtler (3) analyzed glyceryl ethers by GLC as their acetylated derivatives. However, the acetylated derivatives have long retention times, and Hanahan et al. (1) have reported that resolution of the saturated derivatives from the corresponding unsaturated derivatives is not satisfactory. Indirect GLC analysis of the aldehydes resulting from periodic acid oxidation of the glyceryl ethers have been reported by Malins (4). This method, like the others, does not analyze for the 2-isomer. Alkyl iodides and hydrocarbons derived from glyceryl ethers have been analyzed by GLC by Guyer et al. (5). Their method required long preparation time, and the hydrocarbons were prepared from the alkyl iodides. Unfortunately, the latter derivative cannot be prepared quantitatively from unsaturated glyceryl ethers according to Hanahan (6).

A nonspecific colorimetric method by Karnovsky et al. (7,8) is commonly used for the analysis of glyceryl ethers. This involves measurement of the formaldehyde formed from the 1-isomer after periodate oxidation; the 2-isomer does not react and the results can be affected by other glycol type compounds.

In view of the limitations of existing analytical methods for analysis of the 1-isomer of glyceryl ethers and the lack of a method for analysis of the 2-isomer, we investigated the use of trifluoroacetate (TFA) and trimethylsilyl ether (TMS) derivatives for the analysis of 1- and 2-glyceryl ethers by GLC.

EXPERIMENTAL

Nomenclature

Only a few glyceryl ethers have been given common names: batyl, chimyl, and selachyl

[1] Presented at the AOCS meeting, Cincinnati, October, 1965.

[2] USAEC Postdoctoral Fellow under appointment from ORINS.

[3] Under contract with US Atomic Energy Commission.

alcohols. Rather than assign names to the unnamed ones or use the long International Union of Pure and Applied Chemistry (IUPAC) nomenclature, a shorthand system is suggested similar to that introduced by Farquhar et al. (9) for fatty acids. The hydrocarbon chain of the glyceryl ether is represented by a number equal to the number of carbon atoms it contains; this integer is followed by a second, separated by a colon, that represents the number of π bonds in the chain. The location and configuration of the double bond(s) can be represented by a number and a small letter (c = *cis* and t = *trans*), respectively, in parentheses if they are known. The isomeric point of bonding the hydrocarbon chain to the glycerol is designated by a number separated by a dash. The specific enantiomeric forms, or a racemic mixture of the 1-isomers, can be represented by D or L and RM, respectively, in front of the abbreviated form. An example will serve to illustrate the system. One-selachyl alcohol (classical name) of *cis*-9-1, 2-propanediol-3-octadecenyl ether (IUPAC name) can be represented simply as 18:1 (9c)-1. This abbreviation is a convenient time and space-saving system that can be applied to monoglycerides as well as glyceryl ethers.

The 1-isomers used in this study were racemic mixtures. The mono- and di-unsaturated glyceryl ethers were RM 18:1 (9c)-1, 18:1 (9c)-2 and RM 18:2 (9c,12c)-1, 18:2 (9c,12c)-2, respectively. The enantiomeric form, location and configuration of the double bond given here will apply in the remainder of this report unless otherwise stated and will, therefore, be omitted from the shorthand nomenclature system.

Materials

The 1- and 2-isomers of the saturated and mono-unsaturated glyceryl ethers used in this study were more than 97% pure and the di-unsaturated ethers were more than 95% pure. The purity was determined by infrared and nuclear magnetic resonance spectroscopy, melting-point determinations, and TLC analysis; these purity figures were confirmed by GLC data obtained by the methods reported here. Synthesis, purification, and chemical and physical properties of the glyceryl ethers used here will appear in a separate publication. Other materials were obtained as follows: hexamethyldisilazane from Peninsular Chemical Research, Gainesville, Fla; trimethylchlorosilane from K and K Laboratories, Plainview, N. Y.; trifluoracetic anhydride from Eastman Organic Chemicals, Rochester 3, N.Y. Solvents and other reagents were reagent grade and used without further purification.

Preparation of Glyceryl Ether Derivatives

The TMS derivatives of the glyceryl ethers were prepared according to the procedure described by Wood et al. (10). The TFA derivatives were prepared by placing 1 mg or more of the glyceryl ether sample in a 5-ml glass-stoppered conical centrifuge tube, followed by the addition of 1 ml of trifluoroacetic anhydride. The glyceryl ethers were mixed with the trifluoroacetic anhydride and allowed to stand at room temperature for 15 min, at which time the trifluoroacetic anhydride and the trifluoroacetic acid formed during the reaction were evaporated under a stream of dry nitrogen. Samples were then diluted to desired concentration with trifluoroacetic anhydride for GLC analysis.

Gas Chromatography

An Aerograph Model 600D Hy-Fi Gas Chromatograph, (Wilkens Instruments and Research Inc., Walnut Creek, Calif.) equipped with a hydrogen flame ionization detector, was used in this study. The oven temperature of the chromatograph was controlled (±0.1C) with a Wilken's Model 328 isothermal temperature controller. The signal from the gas chromatograph was recorded by a 1.0 mv Brown recorder (Minneapolis-Honeywell Reg. Co., Philadelphia, Pa).

The liquid phases and conditions used for the GLC analysis of the glyceryl ethers as their TFA and TMS derivatives are reported in Table I.

The columns with the exit end plugged with glass wool were attached to a water aspirator vacuum line. The packing material was then poured into a funnel at the inlet end of the column and the column was allowed to fill. Finally, the inlet end was plugged with glass wool and the column was coiled around an appropriate size reagent bottle. The columns were then connected to the chromatograph and the flow rate was measured at 20 psi; columns with a flow rate less than 100 ml/min at this pressure were rejected. The temperature of the columns was raised to approximately 200C and a test mixture of methyl esters was injected to test the resolving power of the columns. Polar columns that failed to give base-line separation between methyl stearate and methyl oleate were rejected. Acceptable columns were then conditioned (see Table I) for 24 hr under a flow rate of 80–100 ml/min of He gas.

TABLE I

Conditions Used to Evaluate Liquid Phases for the Analysis of TFA and TMS Glyceryl Ether Derivatives

Liquid phase	% Liquid phase	Support	Column dimensions [a]	Thermal cond [b] Temp. (°C)	Column Temp. (°C) for TFA analysis	Column Temp. (°C) for TMS analysis
Methyl silicone polymer SE-30[c]	5	Chromosorb W 60–80 mesh[d]	5′ × ⅛″	300	210	230
Cyanoethylmethyl silicone polymer XE-60[e]	3	Gas-Chrom Q 80–100 mesh	5′ × ⅛″	250	200	200
Apiezon L[f]	5	Chromosorb W 60–80 mesh[g]	5′ × ⅛″	280	220	
Apiezon L[f]	5	Chromosorb W 60–80 mesh[g]	6.75′ × ⅛″	280		250
Ethylene glycol succinate cyanoethyl silicone polymer ECNSS-S[e]	10	Gas-Chrom P 100–120 mesh	5′ × ⅛″	220	170	190
Ethylene glycol succinate methyl silicone polymer EGSS-X[e]	10.5	Gas-Chrom P 100–120 mesh	5′ × ⅛″	220	170	170
Ethylene glycol succinate polyester EGS[e]	16	Chromosorb W 100–140 mesh	3′ to 6′ × ⅛″	220	200	215
Ethylene glycol adipate polyester EGA[e]	11	Chromosorb W 100–140 mesh[d]	5′ × ⅛″	220	200	210
Diethylene glycol succinate polyester DEGS[f]	15	Chromosorb W 60–80 mesh[g]	5′ × ⅛″	220	200	210

[a] All columns were stainless steel except the EGS and EGSS-X columns which were both aluminum and stainless steel.
[b] Detector end of columns disconnected during column conditioning.
[c] Pretested packed columns obtained from Wilkens Instruments and Research, Inc., Walnut Creek, California.
[d] Acid washed support.
[e] Obtained from Applied Science Laboratories, State College, Pa., coated on support.
[f] Stationary support was coated with liquid phase by the flash evaporation technique.
[g] Hexamethyldisilazane treated support obtained from Applied Science Laboratories.

Since the flame ionization detector was located directly at the exit end of the column in the forced-air oven, its operational temperature was equal to that of the column. Air and hydrogen at 300 and at 25 ml/min, respectively, were used to maintain the flame. An Oscar Model 55 aquarium pump (The Oscar Company, Berkeley, Calif.) was used to supply the oxygen, while the hydrogen was supplied from a compression cylinder. The flash evaporator was always operated between 240 and 250C. Column pressures ranging from 4–18 psi were used; carrier gas flow rate was not measured due to the difficulty encountered in measuring flow rates from the flame detector at operating temperatures.

Thin-Layer Chromatography

Silver ion impregnated plates were prepared by mixing aqueous silver nitrate (12.5%) solution with silica gel G (2:1 wt/wt). Uniform 0.25-mm layers were spread on 2- × 20- and 20- × 20-cm glass plates with a Colab applicator modified by us (11) (Colab Laboratories, Inc., Chicago Heights, Ill.). After the chromatoplates had air dried for 30 min, they were activated in an oven for 30 min at 110C. Development of the chromatoplates was carried out in a chamber saturated with chloroform-ethanol, 90:10 (v/v). The separations were visualized either by spraying with 0.2% 2′, 7′-dichlorofluorescein in ethanol and viewing under UV light, or by charring according to the procedure of Privett and Blank (12) or Barrett et al. (13). The charring procedure was used when the results were to be documented by photography. After UV visulization the desired regions were scraped from the plate and the glyceryl ethers eluted with diethyl ether.

RESULTS AND DISCUSSION

TFA Derivatives

A number of investigators have used the N-trifluoroacetyl derivatives for the GLC analysis of amino acids (14–19) and long-chain fatty amines (20). The more severe conditions used in previous studies for the preparation of the

N-trifluoroacetyl derivatives were unnecessary and are unadvisable for the preparation of the O-trifluoroacetyl (TFA) derivatives of long-chain glyceryl ethers. The O-trifluoroacetyl derivatives are more easily prepared than the N-trifluoroacetyl and as a result are more labile, which will become apparent later. The TFA derivatives of the glyceryl ethers were prepared at room temperature. Formation of the TFA derivatives proceeded to completion within 5 min, although 15 min was always allowed to assure a complete reaction. The reaction mixture can be used directly for GLC analysis, but the trifluoroacetic acid formed in the reaction caused tailing of the solvent peak. This undesirable tailing of the solvent peak was partially eliminated by evaporation of the reaction mixture to dryness under a stream of dry nitrogen and rediluting with trifluoroacetic anhydride. Samples that contained only saturated and mono-unsaturated glyceryl ethers were stored in a refrigerator at 0C until analyzed by GLC. Samples were stored several weeks under these conditions without detectable changes.

In an attempt to eliminate the tailing of the solvent peak, the reaction mixture was evaporated to dryness and the TFA derivatives were rediluted in such solvents at CCl_4, $CHCl_3$, CH_2Cl_2, CS_2, benzene, diethyl ether, and hexane. Surprisingly, the TFA derivatives could not be chromatographed in these solvents since hydrolysis was virtually complete within 20 to 30 min. The lability of the O-trifluoroacetyl derivatives had also been observed by Lamkin and Gehrke (17) for the hydroxy amino acids.

The temperature of the reaction was not critical unless the sample being acetylated contained dienes. When the TFA derivatives of 18:2 glyceryl ethers were prepared, a yellow color developed, which turned brownish-black upon standing long periods. This color change can be accelerated to a few minutes by increasing the temperature of the reaction mixture. On GLC analysis the quantity of the 18:2 TFA derivatives decreased as the color darkened. In spite of this apparent degradation of the 18:2 TFA derivatives, nearly quantitative results were obtained when GLC analysis was carried out *immediately* after the derivatives were prepared. The saturated and mono-unsaturated glyceryl ether reaction mixture produced no such color change, nor could the GLC analysis reveal any decrease in the TFA derivatives even when the reaction mixture was heated to 70C. These results suggest that the attack of the trifluoroacetic anhydride

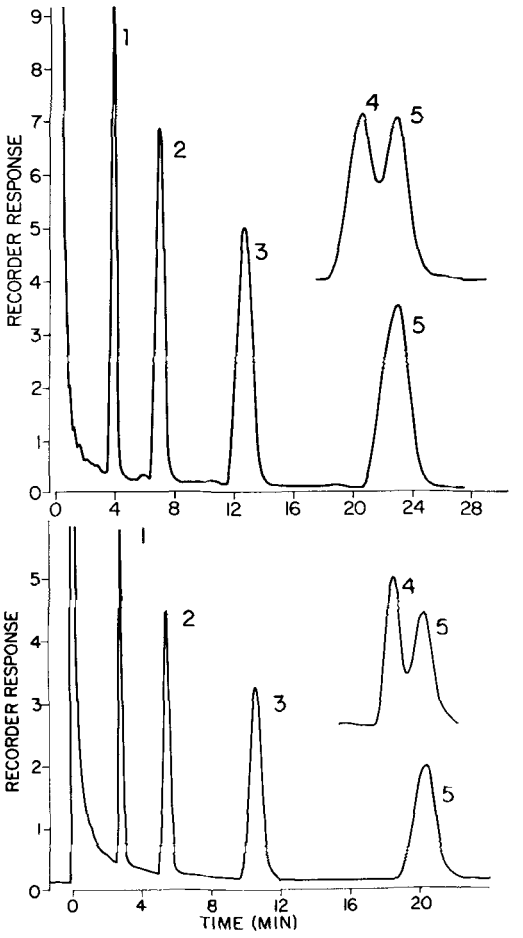

FIG. 1. Chromatograms of glyceryl ether TMS (top) and TFA (bottom) derivatives obtained on a 5 ft × ⅛ in. stainless steel column packed with 5% SE-30 operating at 230C and 210C, respectively. Each peak consists of a mixture of the 1- and 2-isomers and the numbered peaks are: 1) 12:0; 2) 14:0; 3) 16:0; 4) 18:1; and 5) 18:0.

or acid occurs at the methylene carbon between the two double bonds of the 18:2 glyceryl ether.

TMS Derivatives

The reaction involving the formation of the TMS derivatives of the glyceryl ethers proceeded with equal rapidity as that previously noted (10,21) for monoglycerides and hydroxy acids.

Gas Chromatography

Satisfactory analyses of TFA and TMS derivatives of glyceryl ethers depend primarily on the gas chromatographic column, the most important and unpredictable component of the system. Short columns that give high resolution with short retention times at low column

pressure are ideally suited for such analyses. Such columns have previously been used for the analysis of TMS derivatives of monoglycerides and hydroxy acids (10,21).

Columns used for the analyses of the TFA derivatives were conditioned with trifluoroacetic anhydride after the 24-hr temperature-conditioning process by injecting several 1.0-μl injections of trifluoroacetic anhydride. The first injection usually had a broad solvent peak (trifluoroacetic anhydride and trifluoroacetic acid) that tailed severely. With each successive injection, the solvent peaks became narrower and the tailing was reduced to a point where additional injections had no effect. After this trifluoroacetic anhydride conditioning process, the first 4–6 sample runs were discarded before quantitative data were collected. The first two sample runs obtained after the column had been allowed to cool or sit idle for more than 4 hr were discarded as extra precaution against any new active sites being formed during that time which might affect the results. The trifluoroacetic anhydride conditioning process presumably deactivates sites on the column, stationary support or liquid phase by reacting with certain compounds on the column. Evidence in support of this was obtained when a peak appeared after an injection of trifluoroacetic anhydride into a column in which a sample of free 18:0 had previously been injected. After three or four injections of trifluoroacetic anhydride the peak would disappear; however, its reappearance could be brought about simply by injecting the free 18:0 glyceryl ether followed by the trifluoroacetic anhydride. Trifluoroacetic anhydride appears to be a useful reagent for the cleaning of the chromatographic system, reduction of active sites on new columns and restoration of old columns by removal of adsorbed polar materials.

Several liquid phases were evaluated for the GLC analysis of glyceryl ethers as their TFA and TMS derivatives. A homologous series of synthetic glyceryl ethers ranging from 12:0 to 18:0 was used to prepare the saturated standard mixtures of the racemic 1-isomers, 2-isomers, and a mixture of the 1- and 2-isomers. Each of the isomeric mono- and diunsaturated glyceryl ethers was analyzed individually with the corresponding 14:0 isomer in addition to mixtures. This facilitated the quantitative evaluation where resolution of the saturated from the unsaturated glyceryl ethers was unsatisfactory in mixtures.

Nonpolar Liquid Phases

Two typical chromatograms obtained for the standard mixture of the saturated 1- and 2-glyceryl ether TMS and TFA derivatives on SE-30 are shown in Figure 1, top and bottom, respectively. The partial resolution of 18:1 (Peak 4) and 18:0 (Peak 5) of the 1- and 2-isomers for each of the derivatives is also shown. Resolution through 18:0 was achieved in less than 30 min with symmetrical peaks for each of the derivatives. The 1- and 2-isomers were not separated in either the TMS or TFA derivatives. The partial resolution of the 18:1 and 18:0 glyceryl ether derivatives was surprising since saturated and mono- and diunsaturated fatty acid methyl esters of the same chain length were not resolved at this temperature. At lower temperatures dienes were resolved from the single peak containing monoenes and saturated methyl esters. The TFA and TMS ether derivatives of the glyceryl ethers gave the opposite effect. The 18:2 and 18:1 were eluted as a single peak and only partially resolved from the 18:0, with the TFA derivatives giving the best resolution.

Two different columns were used for the analyses because SE-30 columns previously used for the analysis of the TFA derivatives decomposed the TMS derivatives. This was presumably caused by trifluoroacetic acid being bound or adsorbed to the column, causing acidic conditions. The TMS derivatives have previously been shown to decompose under acidic conditions (10). The acidic condition of the SE-30 column was not permanent; after 2 or 3 days at operating temperatures, they could be used for the analysis of TMS derivatives. All of the columns used showed varying degrees of decomposition of the TMS derivatives if used immediately after the columns had been used for the analysis of TFA derivatives. The rate at which the columns used for the analysis of TFA derivatives returned to a usable condition for the analysis of TMS derivatives ranged from a few hours with the polyester and organosilicone polyesters to 1 to 2 days with the SE-30 Apiezon L columns. Polar columns used for the analysis of both derivatives were allowed to stand overnight at operating temperature (if they had previously been used for the analysis of TFA derivatives) before being used for the analysis of TMS derivatives.

The resolution of a synthetic mixture of saturated 1- and 2-glyceryl ether TMS and TFA derivatives obtained on two Apiezon L columns is shown in Figure 2, top and bottom,

respectively. A tracing showing the separation of an isomeric mixture of the 18:1 and 18:0 TFA derivatives also appears at the bottom of Figure 2. The separation of the glyceryl ethers through 18:0 was achieved with good peak symmetry for both derivatives in less than 20 min. The isomeric glyceryl ethers were not resolved in either case. Resolution of the 18:1 from the 18:0 was virtually complete with the TFA derivatives (Fig. 2), while that obtained with the TMS derivatives was very poor. The TFA of the 18:2 was shouldered on the 18:1 glyceryl ether peak and no separation was observed for the TMS derivatives.

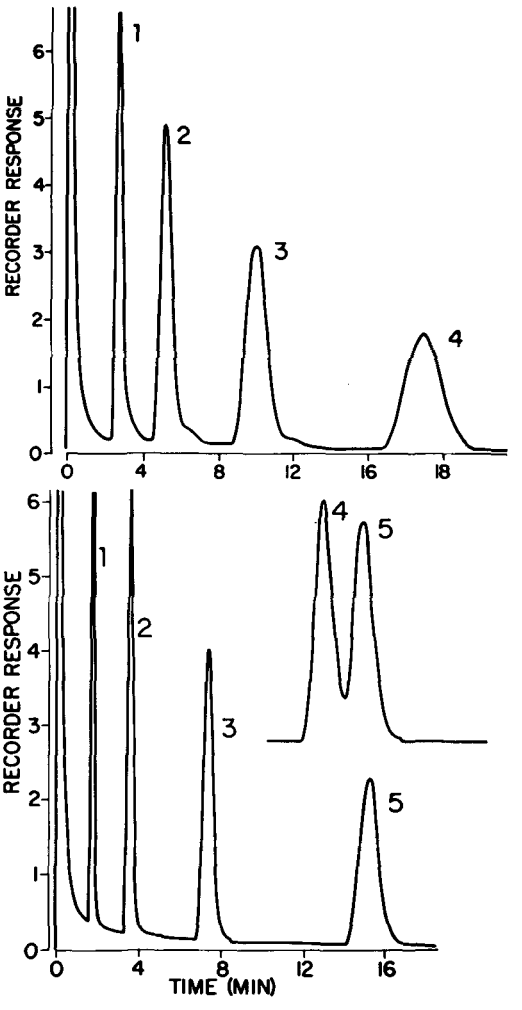

FIG. 2. GLC analysis of an isomeric mixture of purified saturated glyceryl ether TMS derivatives (top) and TFA derivatives (bottom). The analyses were carried out on a 5 ft and a 6.75 ft × ⅛ in. stainless steel column packed with 5% Apiezon L on 60–80 mesh Chromosorb W operating at 250C and 220C, respectively. The major peaks are: 1) 12:0; 2) 14:0; 3) 16:0; 4) 18:0 (top) 18:1 (bottom); and 5) 18:0. Only slight resolution of 18:1-1 and 18:1-2 from 18:0-1 and 18:0-2 was obtained for the TMS derivatives.

Semipolar Liquid Phase

The GLC analysis of a synthetic mixture of saturated 1- and 2-glyceryl ether TMS and TFA derivatives obtained on an XE-60 column is shown in Figure 3, top and bottom, respectively. Resolution of the 1- and 2-isomers of the glyceryl ether TFA derivatives from 12:0 to 18:0 was obtained in approximately 20 min with good peak symmetry. The 2-isomer eluted after the 1-isomer, which is the opposite of that obtained by Wood et al. (10) for the TMS derivatives of the isomeric monoglycerides. This pattern of elution was expected and was actually predicted from their structural formulas based on the relationships between the ether and ester groups of the isomeric forms. It was also predicted that the TMS derivatives of the isomeric glyceryl ethers could not be separated, or only with difficulty because of the similarity of the two types of ether bonds involved. The lack of resolution of the isomeric glyceryl ether TMS derivatives (Fig. 3, top) on the same column where the isomeric glyceryl ether TFA derivatives were easily resolved supported the hypothesis. It was further substantiated by the fact that in all the liquid phases tested not one gave resolution of the isomeric glyceryl ether TMS derivatives. Neither the TFA nor TMS derivatives of the saturated and unsaturated glyceryl ethers were resolved on the XE-60. The acidic condition of the column caused by the TFA derivatives was very slight with the XE-60 column and it usually disappeared within one or two hours; however, the usual overnight period was always allowed before the TMS derivatives were chromatographed.

Polar Liquid Phases

Several polar polyester and organosilicone polyester liquid phases were tested before a satisfactory one was found. Resolution of the 1- and 2-isomers of a synthetic mixture of saturated glyceryl ether TFA derivatives similar to that shown in Figure 3 (bottom) was obtained using EGSS-X as the liquid phase. The 1- and 2-isomer of 14:0, 16:0, and 18:0 were resolved within 20 min practically to base line at an isothermal column temperature of only 170C. The isomers of 12:0 were also completely resolved at lower temperatures. The solvent was separated into two peaks; the first

was found to be trifluoroacetic anhydride and the second trifluoroacetic acid. The group of partially resolved peaks shown in Figure 4 consisted of the 1- and 2-isomers of a mixture of 18:0, 18:1, and 18:2 glyceryl ether TFA derivatives. These chromatograms were obtained in approximately 25 min on EGSS-X at an isothermal temperature of 170C. The identity of the peaks are: 1) 14:0-1 and 14:0-2 used as a marker; 2) an unknown artifact; 3) 18:0-1; 4) 18:1-1; 5) 18:0-2; 6) 18:1-2; 7) 18:2-1; and 8) 18:2-2. At higher temperatures accompanied by lower flow rates the resolution of 18:0, 18:1, and 18:2 was improved so that a quantitative analysis could be made from a mixture of either the 1- or 2-isomers, but not from a mixture of both. When a mixture of both isomers were chromatographed under these conditions, peaks 4 and 5 eluted as one peak (4 + 5) similar to that of peak 6 + 7. The higher temperatures and lower flow rates allow greater resolution between peaks 3 and 4 + 5 and between 4 + 5 and 6 + 7, which can be used to an advantage to calculate percentage composition (see section on mixture analysis). The EGSS-X organosilicone polyester is remarkable in that 18:0, 18:1, and 18:2 glyceryl ether TFA derivatives with molecular weights of 536, 534, and 532, respectively, can be resolved at low temperatures (165–180C) in less than 20 min. These EGSS-X columns can be used to analyze glyceryl ethers of much longer chain length since column temperatures up to 220C have been used without serious bleed problems.

The chromatogram tracing shown in Figure 4 was obtained using a 5-ft aluminum column; however, similar separations were obtained using stainless steel columns, but the conditioning time required for the aluminum columns was considerably less than that required for the stainless steel. Good results can be obtained for the glyceryl ether TFA derivatives on newly packed EGSS-X aluminum columns with only trifluoroacetic anhydride conditioning (2 or 3 1.0 μl injections) at operating temperature. This was also observed with aluminum columns packed with EGS. Since the columns were packed from the same lot of packing material and used in the same instrument, apparently the composition of the metal tubing is responsible for the observed difference. Possibly the aluminum columns form a protective oxide layer more quickly or, equally possible, the stainless steel may require longer time to form a deactivated surface. Peak number two, Figure 4, earlier referred to as an unknown artifact, appears in the region of 16:0-1 of the chromatogram when aluminum columns are used for the analysis of TFA derivatives. The peak appears after approximately 2 days of column use and increases slowly in size. The peak once present will appear again and again when trifluoroacetic anhydride is injected alone. This problem can quickly be eliminated by simply replacing the glass wool plug and a small amount (0.5 cm) of the packing material at the inlet end of the column with new material.

The EGSS-X was found to be unsatisfactory for the analysis of glyceryl ether TMS derivatives. Retention times were similar to those obtained for TFA derivatives, but 18:0 was not resolved from 18:1 and isomeric glyceryl ethers were not separated; component peaks were always skewed at the leading edge.

Other polar liquid phases were tested and EGS was found to be second best to EGSS-X. The glyceryl ether TFA derivatives were eluted quickly from the column and separation of 18:0 and 18:1 was comparable to that obtained on EGSS-X. In spite of these good qualities, the liquid phase always produced asymmetrical peaks with tailing.

The TMS derivatives of the glyceryl ethers were separated only by chain length on EGS and, as with the TFA derivatives, peaks were asymmetrical with tailing.

The separation of the glyceryl ether TFA and TMS derivatives on the EGA liquid phase were approximately the same as those obtained with the EGS, and the lack of peak symmetry persisted with all derivatives.

Glyceryl ether TFA derivatives could not be chromatographed on DEGS and ECNSS-S liquid phases. After their injection, a large distorted peak was eluted over several minutes. Blau and Darbre (15) have also reported that TFA derivatives of hydroxy amino acids could not be chromatographed on DEGS.

The glyceryl ether TMS derivatives were resolved according to chain length only with the ECNSS-S liquid phase, and the leading edge of the peaks was always skewed. The leading edge of peaks was skewed on the EGSS-X and ECNSS-S organosilicone polyesters, whereas tailing of peaks was observed on the EGS and EGA polyesters.

Analysis of the glyceryl ether TMS derivatives on the DEGS liquid phase gave results similar to those obtained on EGS and EGA phases.

Quantitation

Table II shows a comparison of known percentage composition of synthetic mixtures of

1- and 2-glyceryl ethers with that obtained by GLC analysis of their TFA and TMS derivatives on several liquid phases. The determined values for each of the derivatives, in general, agreed well with the known values, with the exception of 18:2 TFA derivatives. However, with the EGSS-X liquid phase, 18:2 values were in close agreement with the known values when the samples were analyzed immediately after derivative preparation. The small observed differences can be accounted for by the lack of absolute purity of each component in the standard mixture, errors in measuring peak area and errors in the weighing of the standards. The determined values for 12:0 and 18:0 were approximately 3% higher and 3% lower, respectively, than the known weight percentage. In general, experimental values obtained for the TFA derivatives agreed more closely to the known *molar percent* than with the known *weight percent.* Excellent agreement between determined values and known molar percent was obtained with the EGSS-X liquid phase. Differences between the response obtained for each of the two isomeric forms of either derivative were not observed.

From the data shown in Table II, it follows that glyceryl ethers can quickly and quantitatively be determined by GLC as their TFA and TMS derivatives. The authors are of the opinion that the design and type of construction of a gas chromatograph can affect the quantitative results obtained as well as type of liquid phase. With this in mind, the operator should check the performance of the column and instrument with a standard mixture of known composition before analyzing samples of unknown composition.

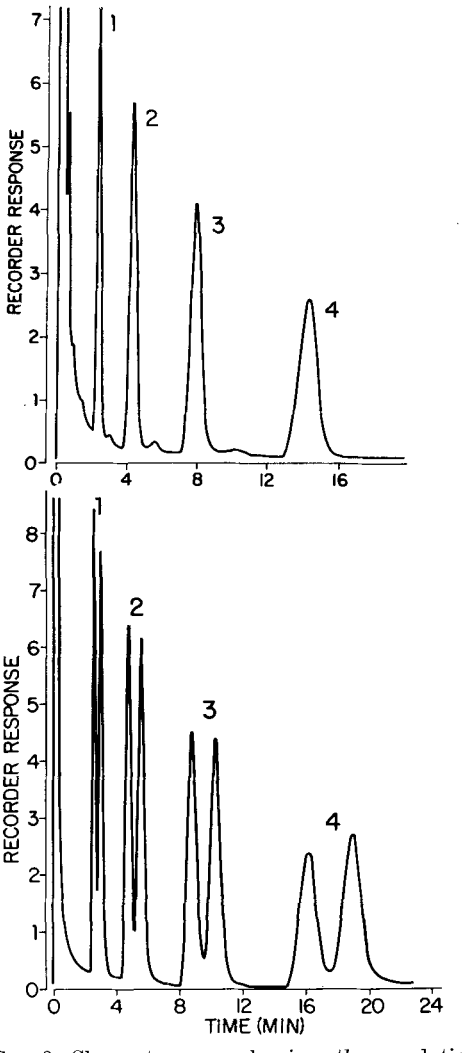

FIG. 3. Chromatograms showing the resolution of purified synthetic 1- and 2-glyceryl ether TMS derivatives (top) and TFA derivatives (bottom). Resolutions were obtained with a 5 ft × ⅛ in. stainless steel column packed with 3% XE-60 on 80–100 Gas-Chrom Q operating at an isothermal temperature of 200C. The numbered peaks are: 1) 12:0; 2) 14:0; 3) 16:0; and 4) 18:0. The 2-isomers were eluted after the 1-isomers for the TFA derivatives.

Mixture Analysis

The data presented to this point have dealt mainly with the evaluation of the TFA and TMS derivatives for the GLC analysis of glyceryl ethers. Synthetic mixtures, unlike those that occur naturally, of a particular composition were used to illustrate specific points. A more difficult problem encountered in the GLC analysis of a mixture of naturally occurring glyceryl ethers is illustrated by the lack of sufficient resolution for quantitative analysis of the C-18 synthetic mixture shown in Figure 4. Although the concentration of the 2-isomer occurring in natural sources may be much lower or even absent, the establishment of this fact is equally important and difficult. One approach to the problem that has been used and two alternate ones will be discussed separately.

Malins and co-workers (4,22,23) reported the separation of diacylglyceryl ethers and free glyceryl ethers from other lipid classes by TLC. The isolation of the glyceryl ether diesters is useful only for the analysis of these units themselves while the isolation of the free

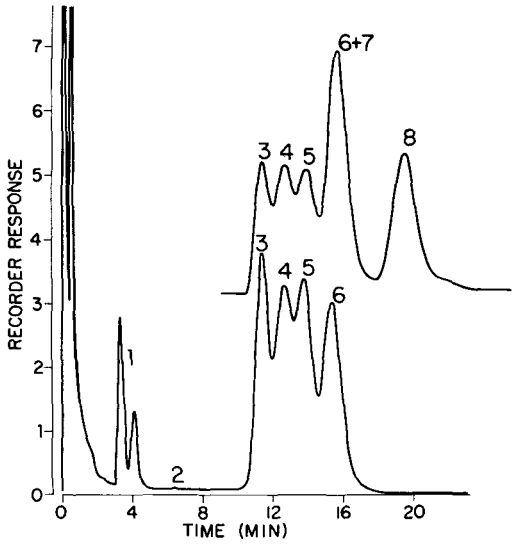

FIG. 4. GLC chromatograms of a mixture of saturated, mono- and di-unsaturated C_{18} isomeric glyceryl ether TFA derivatives. Analyses were obtained with a 5 ft × ⅛ in. aluminum column packed with 10.5% EGSS-X on 100–120 mesh Gas-Chrom P at 170C. The numbered peaks are: 1) 14:0-1 and 14:0-2 (used as a marker); 2) an artifact peak described in text; 3) 18:0-1; 4) 18:1-1; 5) 18:0-2; 6) 18:1-2; 7) 18:2-1; and 8) 18:2-2.

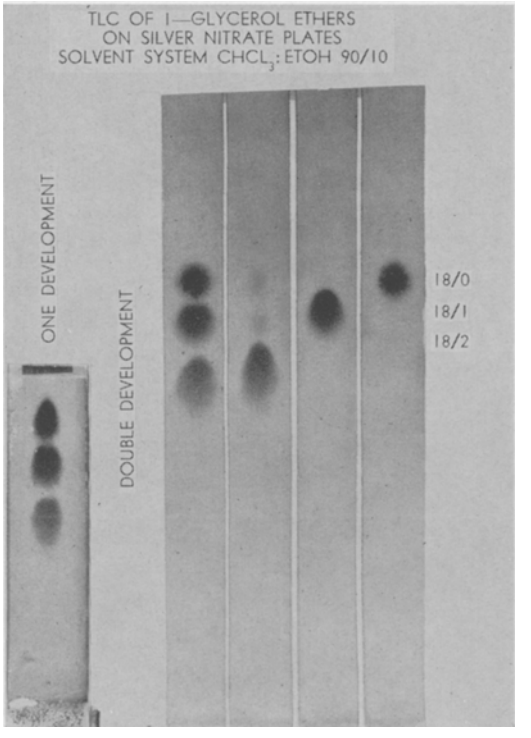

FIG. 5. Separation of a mixture of C_{18} free glyceryl ethers according to degree of unsaturation on silver ion impregnated silica gel G plates. The chromatoplates were developed in a solvent system of chloroform-ethanol 90:10 (v/v) using either one or two developments.

glyceryl ethers from other unsaponifiable material has proved to be most useful. Further separations are possible by silver ion impregnated TLC, routinely used for the separation of similar compounds according to degree of unsaturation. Separations become increasingly difficult as polarity of the compounds increases, and resolution of the glyceryl ethers by silver ion TLC has not previously been reported. A typical chromatogram depicting the resolution of 18:0-1, 18:1-1, and 18:2-1 glyceryl ethers after one or two solvent developments on silver ion plates is shown in Figure 5. Resolution of 1-glyceryl ethers is shown here, but the 2-isomer or a mixture of the two are resolved equally well. Each component represents approximately 40 μg, and usually sufficient material for quantitative GLC analysis of each band can be obtained from one 20- × 20-cm plate. The preliminary use of silver ion TLC circumvents the problem of overlapping GLC peaks of isomeric glyceryl ethers having equal chain length.

An alternate approach to the problem involves preliminary separation of the 1- and 2-isomers by preparative GLC followed by quantitative analysis of each isomer. The 1- and 2-isomers of each chain length can be separated on a preparative XE-60 column and collected by a fraction collector described by Wood and Reiser (24). Each fraction can then be quantitatively analyzed on an EGSS-X analytical column. Even though odd-numbered carbon chain lengths and branched chain glyceryl ethers, when present, can still present a problem, this approach has the advantage that a component can be collected and its identity established by other methods.

A second alternate approach to the problem of peak overlapping makes use of the information obtained from three different liquid phases. The scheme that follows will show how a mixture of C-18 glyceryl ether TFA derivatives, such as that shown in Figure 4, can be analyzed without preliminary fractionation. First the percentage of each isomer can be obtained from the XE-60 column (Fig. 3, bottom, Peak 4). A second piece of needed information, the percentage saturation and unsaturation, can be obtained from the Apiezon

TABLE II

Comparison of Percentage Composition of Synthetic Mixtures of Glyceryl Ethers by GLC as Their TFA and TMS Derivatives on Various Liquid Phases[a]

Column	Deriv.	12:0	14:0	16:0	18:0	18:1[b]	18:2[b]
		1-Isomers					
XE-60	TFA	20.5	22.9	28.0	28.6	63.9	51.5
	TMS	15.5	20.5	29.0	35.1	65.7	55.9
SE-30	TFA	16.7	21.1	28.4	33.8	61.0	53.2
	TMS	16.3	24.1	28.4	31.3	65.7	60.0
Apiezon L	TFA	16.3	21.2	29.6	32.8	65.9	.....
	TMS	18.6	22.2	27.8	31.4	66.2	55.1
EGSS-X	TFA	16.3	23.2	28.8	31.6	62.9	61.9[c]
Known wt. %		14.3	21.4	28.6	35.7	66.7	60.3
Known molar %		17.0	23.0	27.9	32.1	62.8	59.7
		2-Isomers					
XE-60	TFA	18.2	23.5	27.8	30.4	62.9	53.5
	TMS	13.8	22.7	29.9	33.6	66.7	60.1
SE-30	TFA	16.4	23.2	27.2	33.2	64.0	48.6
	TMS	19.2	22.0	27.6	31.2	62.3	60.4
Apiezon L	TFA	17.8	21.9	27.6	32.7	65.6	.....
	TMS	15.1	24.5	28.4	32.1	61.3	56.9
EGSS-X	TFA	17.4	23.4	27.8	31.5	63.6	63.9[c]
Known wt. %		14.3	21.4	28.6	35.7	66.7	66.7
Known molar %		17.0	23.0	27.9	32.1	62.8	62.8

[a] Each figure represents the mean of three determinations.
[b] Determined individually from a mixture with the corresponding 14:0 isomer.
[c] Determinations made from a newly prepared sample, whereas other 18:2 determinations were made from standard mixtures several hours old.

L column (Fig. 2, bottom, Peaks 4 and 5). Using the foregoing information, together with that obtained from an EGSS-X column (Fig. 4) operating at conditions described earlier in which peaks 4 and 5 elute together, the percentage of each component can be calculated. The percentage of 18:0-1 can be obtained directly from the area of peak 3 on the EGSS-X column. The difference between 18:0-1 and the percentage saturation obtained on the Apiezon L column gives the percentage 18:0-2 in peak 4 + 5 of the EGSS-X chromatogram and the percentage 18:1-1 is calculated by difference. The sum of the percentage 18:0-1 and 18:1-1 from the total percentage of the 1-isomer obtained from the XE-60 column gives the percentage 18:2-1 in peak 6 + 7 and the percentage 18:1-2 is calculated by difference. The percentage 18:2-2 can be calculated directly from the area of peak 8 (Fig. 4) of the EGSS-X chromatogram. The simplicity of this approach dissipates when mixtures of naturally occurring glyceryl ethers possessing branched and odd numbered carbon atoms in their hydrocarbon chain are analyzed.

Each of the approaches described here has merits that can be used advantageously in certain applications to the solution of the problem of peak overlapping in the GLC analysis of isomeric mixtures of glyceryl ethers having equal chain length. The methods described here for the quantitative GLC analysis of TFA and TMS derivatives of isomeric glyceryl ethers are now being applied to the analysis of glyceryl ethers from many natural sources and will appear at a later date.

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[Received Oct. 15, 1965]

Isolation of Methyl *cis*, *cis*-5,13-Docosadienoate from *Limnanthes douglasii* Oil

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ABSTRACT

Methyl *cis,cis*-5,13-docosadienoate has been isolated from the mixed methyl esters of the fatty acid moiety of *Limnanthes douglasii* oil by a combination of low temperature fractional crystallization and fractionation of mercuric acetate adducts. The methyl ester and its free acid have been characterized.

INTRODUCTION

THE RECENT PUBLICATION (1) of the synthesis of *cis,cis*-5,13-docosadienoic acid prompts us to publish our work on the isolation in pure form of the methyl ester of this acid from the mixed methyl esters derived from *Limnanthes douglasii* oil, and characterization of the methyl ester and its free acid. Bagby et al. (2) reported isolation of the acid in impure form and proof of structure by degradation of the tetrahydroxy compound derived from the acid by epoxidation followed by hydrolysis. No method for isolating moderate quantities of either *cis,cis*-5,13-docosadienoic acid or its methyl ester from *Limnanthes douglasii* oil has been reported. Since linoleic acid has been successfully purified either by low temperature fractional crystallization (3) or by urea complex fractionation (4,5), the possibility of using these techniques for purifying docosadienoic acid or its methyl ester was explored. Preliminary experiments indicated that while either technique was useful for concentrating tne diene, neither appeared very promising as a means of purifying it in reasonable yield. The separation of methyl esters having different degrees of unsaturation by chromatography on a silver nitrate impregnated column has been reported by De Vries (6). While we were able to prepare methyl docosadienoate of approximately 99% purity, by gas-liquid chromatographic (GLC) analysis, by this method, the large quantities of the highly inflammable solvents, diethyl ether and petroleum ether, which would be required to prepare moderate quantities of material by this method made it unattractive. Stearns et al. (7) prepared 95% methyl linoleate by partitioning the mercuric acetate adducts of safflower methyl esters between methanol and petroleum ether. Our preliminary experiments indicated that the methyl docosadienoate could be more effectively purified by extraction of impurities from the methanol-free adduct with petroleum ether and that this technique could be combined with low temperature crystallization of the methyl esters to give a reasonably good yield of essentially pure methyl *cis,cis*-5,13-docosadienoate.

EXPERIMENTAL PROCEDURE AND DATA

Materials

Limnanthes douglasii oil was obtained by hexane extraction of the ground seed. The oil was converted to the methyl esters by alcoholysis with methanol employing sodium methoxide as catalyst. The composition of the methyl esters, as determined by GLC, was: $C_{16:0}$, 0.4; $C_{16:1}$, 0.2; $C_{18:0}$, 0.2; $C_{18:1}$, 1.9; $C_{18:2}$, 0.8; $C_{20:0}$ 0.6; $C_{20:1}$, 63; $C_{20:2}$, 0.6; $C_{20\text{-unknown}}$, 0.2; $C_{22:1}$, 20; $C_{22:2}$, 11; $C_{22\text{-unknown}}$, 0.4; $C_{22\text{-unknown}}$, 0.7%.

Unsaturated methyl esters and cyclic olefins used to prepare aldehyde standards were obtained from Hormel Institute and K& K Laboratories, Inc., respectively. ECNSS-S organosilicone polymer was obtained from Applied Science Laboratories, stabilized DEGS from Analabs and other GLC column packing materials from Wilkins Instrument Co.

Analytical Methods

GLC analyses were made on an Aerograph A-700 gas chromatograph equipped with a thermal conductivity detector, and employing ¼ in. O.D. aluminum columns. In all cases helium was used as flow gas and 80–100 mesh regular chromosorb W as stationary phase. All quantitative GLC analyses of methyl esters were carried out on a 10 ft stabilized-DEGS (20%) column operated at 245C and at a flow rate of 50 or 75 ml/min. Peak areas were determined by triangulation and corrected by multiplication by the square root of the molecular weight of the compound (8). Other columns and conditions were used for other special applications and these are described at the appropriate place in the paper.

[1] So. Util. Res. Dev. Div., ARS, USDA.

Iodine values were determined by the AOCS Official Method (9) and hydrogen-iodine values by the method of Pack and Planck (10). Elemental analyses were made by Midwest Microlabs, Inc.

Purification of Methyl cis,cis-5,13-Docosadienoate

Mixed methyl esters derived from *Limnanthes douglasii* oil, 1000 g, was dissolved in 9 liters of acetone and crystallized in a step-wise manner, first at −56C and then at −75C. The −75C precipitate fraction, 107.5 g, contained 70% C-22 diene by GLC.

A mixture of 50.0 g of the 70% methyl docosadienoate, 97.0 g of mercuric acetate and 100 ml of methanol was refluxed for 15 min, then cooled to room temperature and mixed with 500 ml of water. After the layers separated, the aqueous methanol was decanted, and then the residue was washed two additional times with water. The wet residue was transferred to a liquid-liquid extractor and extracted with boiling petroleum ether (bp, 30–60C). The progress of the extraction was followed by GLC analysis of esters regenerated by treatment with HCl from aliquots taken from the residue at intervals. Extraction was stopped after 5½ hr at which time the methyl esters regenerated from an aliquot of the residue contained 95% of methyl docosadienoate and no further purification appeared to be being effected. Adduct which oiled out of the petroleum ether extract collected during the last 2 hr of extraction had the same methyl docosadienoate content as the residue and was combined with it. Adduct recovered from the supernatant liquid from the last 2 hr of extraction was combined with adduct which oiled out of the remainder of the extract at −10C and reextracted with boiling petroleum ether. The two residues were combined, dissolved in ether, and first washed twice with concentrated HCl, then 3 times with water. The amber-colored residue, 18.5 g, remaining after drying over sodium sulfate and removal of the ether was distilled rapidly (mostly at 153–156C/50 μ) through a 3 in. Vigreaux column to yield 16.2 g of colorless liquid having the same methyl docosadienoate content, 95%, as the undistilled material.

Two recrystallizations from 10 ml of petroleum ether (bp 30–60C)/1 g of ester at −75C yielded 12.43 g of ester, which had mp −23.5 to −21.5C (uncorr) and $n_D^{25.0}$, 1.4617. Analysis on both the stabilized DEGS column and a 20% Apiezon L column, 4 ft in length, operated at 295C and a flow rate of 100 ml/min, indicated that the sample was pure methyl docosadienoate.

Anal. Calc. for $C_{23}H_{42}O_2$: C, 78.79; H, 12.07; O, 9.12; I.V. and hydrogen - I.V., 144.8. Found: C, 78.58; H, 12.10; O, 9.51; I.V., 145.8; hydrogen - I.V., 143.0.

No evidence of the presence of *trans* bonds was discernible when the sample was examined as a liquid film between salt plates on an "Infracord" infrared spectrophotometer.

Methyl docosanoate recovered from the hydrogen-I.V. determinations had the following constants: mp 51.6–52.7C (corr) (lit. (11) 53.3); $n_D^{59.2}$, 1.4357 (lit. (12) 1.4344).

Anal. Calc. for $C_{23}H_{46}O_2$: C, 77.90; H, 13.08; O, 9.02. Found: C, 77.90; H, 13.00; O, 9.02.

Location of Ethylenic Bonds in Methyl Docosadienoate

Methyl docosadienoate was converted to aldehyde fragments by reductive ozonolysis employing the method of Privett and Nickell (13). The aldehydes were analyzed by GLC employing columns of three different polarities: 10% EGNSS-S, 5 ft in length, programmed at 100–200C, gas flow, 75 ml/min; 15% QF-1, 4 ft in length, programmed at 80–200C, gas flow, 25 ml/min; and 20% Apiezon L, 4 ft in length, programmed at 100–225C, gas flow, 50 ml/min. In order to test the suitability of the GLC method for identification of aldehydes of the type expected from the methyl docosadienoate, a standard mixture consisting of pentanal, hexanal, octanal, nonanal, decanal, dodecanal, heptanedial, octanedial, methyl formylbutanoate, methyl formylpentanoate, and methyl formyloctanoate was prepared by addition of pentanal, octanal, and decanal to the products of reductive ozonolysis of methyl arachidonate, methyl oleate, methyl petroselinate, cycloheptene and cyclooctene. All components of the mixture were resolved except dodecanal and methyl formylbutanoate on the EGNSS-S column, nonanal and methyl aldehydopentanoate, and heptanedial and methyl formylpentanoate on the QF-1 column, and nonanal and heptanedial on the Apiezon L column; and the order of elution for the various aldehydes was different on each column. The aldehydes derived from methyl docosadienoate were shown to be methyl formylbutanoate, octanedial and nonanal by chromatographing on all three columns, both alone and mixed with known samples of these three aldehydes. Thus the ester is methyl 5,13-docosadienoate.

5,13-Docosadienoic Acid

A mixture of 0.18 g sodium hydroxide, 0.25 ml water, 6 ml ethanol and 1.00 g of methyl 5,13-docosadienoate was refluxed for 1 hr, cooled, diluted with 100 ml of water and extracted 3 times with 50-ml portions of diethyl ether. The soap solution was then acidified with 6 ml of 0.6 *N* HCl and extracted 3 times with 50-ml portions of petroleum ether. The petroleum ether solution was washed with water and dried over sodium sulfate, after which solvent was removed by stripping under vacuum with nitrogen gas. The acid, 0.76 g, had the following constants: mp −0.5 to 1.5C (uncorr); $n_D^{25.0}$, 1.4684 (lit. (1) mp −4C; n_D^{22}, 1.4697).

Anal. Calc. for $C_{22}H_{40}O_2$: C, 78.51; H, 11.98; O, 9.51. Found: C, 78.55; H, 12.17; O, 9.43.

DISCUSSION

Although attempts to purify methyl *cis,cis*-5,13-docosadienoate or its derived acid by several methods which have been used successfully with another *cis,cis*-diene, linoleic acid, were unsuccessful, methyl *cis,cis*-5,13-docosadienoate was isolated from the mixed methyl esters derived from *Limnanthes douglasii* oil in a yield of 24% of that present in the original esters by a combination of low temperature crystallization and mercuric acetate adduct fractionation techniques. The various analyses performed on the methyl ester indicate that it is essentially pure *cis,cis*-5,13-docosadienoate. The compound possesses some unusual infrared and nuclear magnetic resonance (NMR) characteristics which are being explored further and will be the subject of a future publication.

ACKNOWLEDGMENT

Limnanthes douglasii oil extraction by A. J. Crovetto.

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[Received July 30, 1965]

The Effect of Dietary Fat on Fatty Acid Synthesis in Cell-Free Preparations of Lactating Mammary Gland[1]

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ABSTRACT

Cell-free preparations of lactating mammary gland of rats maintained during lactation on a fat-free diet incorporated C^{14}-acetate into fatty acids to a greater degree than preparations made from rats fed a similar diet containing 20% fat. The type of fat used did not affect the degree of inhibition of synthesis. C^{14}-acetate was incorporated mainly into dodecanoic and tetradecanoic acids although labeling was observed in fatty acids from 8–18 carbons. The pattern of labeling was not significantly different in the various groups except for slightly decreased amounts of C^{14} in the shorter chain fatty acids of preparations made from glands of rats on the fat-free or coconut oil-containing diet. The fatty acids characteristic of the fed fat became prominent components of the microsomes and mitochondria as well as of the fat floating on the centrifuged homogenates (presumably milk fat).

INTRODUCTION

THE LACATATING MAMMARY GLAND is very active in the synthesis of fatty acids and offers a unique system for the study of the regulation of fat synthesis. Responses of liver and adipose tissue to inclusion of fat in the diet have been well established. Thus, Masoro et al (1) found that liver slices prepared from rats fed a high-fat diet for as short a period as three days had little ability to synthesize C^{14}-fatty acids from C^{14}-glucose. Similar findings were reported for adipose tissue by Hausberger and Milstein (2). The effect of dietary fat on fat synthesis in mammary gland has been studied much less extensively, and most of the studies available are on cows (3). It was of interest, therefore, to determine the nature of the influence of dietary fat on fatty acid synthesis in lactating mammary gland of rats. In addition, a study was made of the effect of the dietary fat on the fatty acid composition of mammary gland microsomes and mitochondria and of the fat floating on the centrifuged homogenates.

[1] Presented at the AOCS Meeting, Chicago, October 1964.

EXPERIMENTAL

Animal Studies

On the day of parturition Sprague-Dawley rats were placed on one of the following purified diets: basic, fat-free (FF); basic, plus 20% by weight of a commercial corn oil (CNO); basic, plus 20% by weight of a commercial coconut oil (CO); basic, plus 20% by weight of a commercial vegetable shortening (VS) (Crisco containing added polyunsaturates. Procter & Gamble Co., Cincinnati, Ohio). The basic diet contained sucrose, casein, salts, and adequate vitamin supplements, and its exact composition has been described (4). The major fatty acid composition of each of these diets is shown in Table I. Only rats suckling six or more pups were used and the number of pups of experimental and control rats was kept identical. The animals were killed at 20–25 days postpartum. Mammary glands were chilled in ice immediately after removal, and all subsequent operations were carried out at 4C. After washing once with homogenizing medium and mincing finely with scissors, the glands were homogenized in 1½ volumes of 0.25 M sucrose containing 0.03 M nicotinamide. Homogenates were centrifuged for 10 minutes at 600 × g, at 10,500 × g for 20 min, and finally at 105,000 × g for 60 min. For most of the experiments the amounts of cofactors used were those found by Dils and Popják (5) to be optimal for fatty acid synthesis from acetate. These were as follows: L(+) cysteine (10 mM), ATP (20 mM), CoA (0.13 mM), DPNH (0.33 mM), TPN (0.66 mM), glucose-6-phosphate (20 mM), $KHCO_3$ (50 mM), $MnCl_2$ (3.3 mM), sodium malonate (50 mM), C^{14}-acetate (10 mM), potassium phosphate buffer, pH 7.4 (130 mM), final volume 1.5 ml. Incubations were done at 37C under nitrogen for time periods up to 2 hr.

Analyses

Fatty acids were isolated by extraction with petroleum ether (bp 40–60C) after hydrolysis with potassium hydroxide. C^{14}-activity was determined by liquid scintillation counting of an aliquot of the extracts at an efficiency of 78%. C^{14}-activity of individual fatty acids was determined by collecting the effluent from gas-liquid chromatographic columns in anthra-

TABLE I

Major Fatty Acid Composition of Diets

Fatty acid	% of Total fatty acids			
	Diet FF[a]	Diet CNO	Diet VS	Diet CO
6:0	2.9	—	trace[b]	—
8:0	1.0	—	trace	9.3
10:0	2.6	—	trace	7.1
12:0	2.2	trace	trace	53.7
14:0	9.5	trace	trace	19.4
16:0	28.5	10.1	8.6	4.9
16:1	2.2	trace	trace	trace
18:0	16.0	1.1	3.9	0.9
18:1	29.5	26.0	54.6	3.6
18:2	2.7	61.9[c]	30.1	1.3
18:3	1.4	0.7	1.9	trace

[a] FF—fat-free; CNO—corn oil; VS—vegetable shortening; CO—coconut oil.
[b] Trace = less than 0.2%.
[c] Unusually high value for linoleic acid was verified by reanalysis.

TABLE II

Effect of Dietary Fat on Incorporation of C^{14}-Acetate into Fatty Acids by Cell-Free Preparations of Mammary Gland

Diet fed	C^{14} Incorporated in fatty acids (μ moles per mg protein)	
	105,000 × g Supernatant	10,500 × g Supernatant
Fat-free	0.087	0.026
Vegetable shortening	0.033	0.010
Fat-free	0.113	0.058
Corn oil	0.045	0.015
Fat-free	0.106	0.037
Coconut oil	0.045	0.015

Components of the incubation medium are given in the experimental section. Incubation time was 2 hr; 5 μc substrate ($CH_3C^{14}OONa$) were used. In each experiment preparations made from animals fed the fat-free or fat-containing diet were incubated with the same medium.

cene cartridges and subsequent determination in the liquid scintillation spectrometer. An 8 ft column (I. D., 4 mm) of diethylene glycol succinate polyester (12%) on 110/120 mesh Chromosorb P maintained at 195C was used for the separation. The effluent from the column was split, approximately 80% being collected and 20% furnished to the argon detector. In preparing methyl esters for gas-liquid chromatography, fatty acids of short chain length were lost to varying degrees. Thus, about 25% of octanoic acid and about 20% of decanoic acids were lost as shown by use of C^{14}-labeled standards. Recovery of dodecanoic and tetradecanoic acids was quantitative. Corrections have not been made for these losses in the tables. Known samples of methyl laurate-1-C^{14}, methyl myristate-1-C^{14}, and methyl stearate-1-C^{14} were used for determining the recovery of C^{14}-compounds in the anthracene cartridges. Values found were 60% for methyl laurate, 70% for methyl myristate, and 80% for methyl stearate. These values were used for correction of the data obtained by use of anthracene cartridges. Standards of methyl esters of fatty acids obtained from the National Heart Institute and Applied Science Corporation were used for checking the quantitative response of the Barber Colman argon detector.

RESULTS

The data in Table II show that addition of any of the three types of fats to the basic diet decreased fatty acid synthesis in cell-free preparations made from mammary gland of lactating rats to about one-third the amount obtained in preparations made from animals on the fat-free diet. Results of tests using any one diet were similar; therefore, only a typical experiment for each diet is shown in the table. The number of experiments in each case was: VS, 6; CNO, 4; CO, 4. In each experiment a preparation from a lactating rat maintained on the fat-free diet was included. Varying the type of fat did not alter the amount of decrease in the incorporation of C^{14}-acetate into fatty acids in a consistent manner.

A preparation containing the 105,000 × g soluble supernatant plus the microsomes sedimented at this centrifugation (this is equivalent to the supernatant obtained after centrifuging the homogenate at 10,500 × g for 20 min) from each of these experiments was also incubated with C^{14}-acetate. The results were similar to those obtained with the soluble supernatants alone except for a smaller percentage incorporation. The preparations from rats on the VS diet gave consistently the lowest C^{14}-incorporation.

In an attempt to stimulate synthesis of fatty acids by the fraction containing microsomes, as reported by Howard and Lowenstein (6), α-glycerophosphate was added to the incubation flasks in some experiments. In a few of these experiments a stimulatory effect was noted but the increase in synthesis was small. The effect was observed only in about one half of the experiments. The dietary fat was not a factor in the response.

The effect of the dietary fat on the composition of the fat floating on the centrifuged homogenates (presumably milk fat; see reference 5) was studied. The major fatty acid composition of these samples is summarized in Table III. The types of fatty acids fed determined the major fatty acids found in the milk fat obtained from the gland. Dietary coconut oil resulted in large concentrations of dodecanoic and tetradecanoic acids, dietary corn oil in octadecadienoic acid, and dietary

TABLE III

Effect of Dietary Fat on Fatty Acid Composition of the Fat Floating on the Centrifuged Homogenates ("milk" fat)

Fatty acid	% of Total fatty acid			
	FF Diet	VS Diet	CO Diet	CNO Diet
8:0	7.7 ± 2.6[a]	3.9 ± 1.5	4.4 ± 1.6	6.2 ± 2.0
10:0	19.2 ± 3.7	4.1 ± 1.2	9.5 ± 2.7	7.0 ± 2.6
12:0	11.9 ± 1.0	1.1 ± 0.4	28.9 ± 2.8	2.7 ± 1.3
14:0	8.2 ± 1.1	1.3 ± 0.3	14.2 ± 1.9	1.1 ± 0.4
16:0	20.9 ± 1.6	18.2 ± 2.4	19.2 ± 2.4	14.4 ± 1.0
18:0	2.8 ± 0.4	4.8 ± 0.5	2.7 ± 0.5	2.2 ± 0.4
18:1	21.4 ± 3.1	44.0 ± 2.0	17.2 ± 4.6	22.9 ± 3.0
18:2	3.8 ± 0.6	20.5 ± 2.1	2.1 ± 0.5	40.4 ± 2.1

[a] Average ± standard error of the mean.

TABLE IV

Incorporation of C^{14} from Acetate into Individual Fatty Acids by Soluble Supernatant Fractions of Mammary Gland

Fatty acid	% of Total C^{14}			
	FF Diet	VS Diet	CO Diet	CNO Diet
8:0	2.0 ± 0.9	6.0 ± 3.2	nil	3.7 ± 0.1
10:0	10.8 ± 2.0	20.1 ± 2.8	7.1 ± 2.0	12.3 ± 5.8
12:0	17.8 ± 1.7	32.6 ± 5.3	13.2 ± 4.0	21.3 ± 2.9
14:0	41.2 ± 1.3	35.4 ± 3.5	43.7 ± 3.8	33.2 ± 4.1
16:0	24.4 ± 4.6	12.9 ± 1.9	30.2 ± 8.3	20.1 ± 8.8
18:0	1.1 ± 0.4	nil	nil	nil

For P values see text.

vegetable shortening in octadecenoic acid. The fat-free diet resulted in relatively large amounts of fatty acids of chain lengths shorter than hexadecanoic. Very small concentrations of octadecadienoic acid were observed in fat obtained from preparations made from animals fed the fat-free or coconut oil diets.

Incorporation of C^{14} from acetate into fatty acids of chain lengths from 8–18 carbons was observed, but the amount of label found in 18 carbon fatty acids and in the monoenes was very small. Predominant labeling was observed in dodecanoic and in tetradecanoic with significant quantities also seen in decanoic and hexadecanoic acids. Less C^{14} was found in the shorter chain fatty acids isolated from preparations of rats on the FF diet than those on the VS diet (P = .005). Similar findings were obtained in the CO-fed animals when compared to the animals on the VS diet (P = .015). The largest amount of C^{14}-incorporation into dodecanoic acid occurred in animals on VS diet. Incorporation into tetradecanoic acid was high in all groups and highest in the FF and CO groups (P = .05 between animals on FF and VS diets; P = .15 between CO and VS). C^{14} in hexadecanoic acid was greater in preparations made from rats on FF and CO diets than from those on VS diet (P = .04 between FF and VS diets; P = .05 between CO and VS diets). C^{14} distribution in fatty acids synthesized by preparations of soluble supernatant plus microsomes was in general similar to that for soluble supernatant alone. Preparations made from rats on CO or CNO diets apparently synthesized fatty acids of shorter chain length to a greater extent than did soluble supernatant alone. However, these differences did not test statistically significant due to wide variation in the results.

The effect of the various diets on the fatty acid composition of the mammary gland tissue was studied by determination of the fatty acid composition of washed microsomes and mitochondria from these preparations. Results are summarized in Table V. Significant differences in fatty acids of microsomes of different groups were found. Dodecanoic acid was highest in the CO group (P = .02 between the FF and CO groups); tetradecanoic acid concentration of CO groups was high (but not significantly higher than FF group); octadecadienoic acid content was high in the glands of VS and CNO

TABLE V

Effect of Dietary Fat on Fatty Acid Composition of Microsomes and Mitochondria of Mammary Gland

Fatty acid	% of Total fatty acids							
	FF Diet		VS Diet		CO Diet		CNO Diet	
	Microsomes	Mitochondria	Microsomes	Mitochondria	Microsomes	Mitochondria	Microsomes	Mitochondria
8:0	trace	trace	trace	trace	trace	trace	trace	trace
10:0	4.4 ± 1.2	10.3 ± 3.7	1.0 ± 0.1	3.0 ± 0.9	2.8 ± 1.1	8.0 ± 2.5	1.5 ± 1.0	1.5 ± 0.9
12:0	4.8 ± 0.7[a]	7.2 ± 2.2	0.9 ± 0.1	1.9 ± 0.8	9.8 ± 2.7	28.7 ± 1.3	1.0 ± 0.3	1.3 ± 0.6
14:0	6.0 ± 0.9	6.1 ± 0.9	1.1 ± 0.1	2.0 ± 1.2	8.7 ± 1.3	11.5 ± 1.6	1.2 ± 0.2	1.9 ± 0.3
16:0	23.7 ± 1.3	20.3 ± 1.8	18.6 ± 1.3	16.7 ± 3.4	20.1 ± 1.4	12.9 ± 0.9	14.5 ± 1.2	14.0 ± 1.1
16:1	3.3 ± 0.1	2.7 ± 0.3	2.0 ± 0.3	1.2 ± 0.2	2.0 ± 0.3	1.7 ± 0.4	0.8 ± 0.2	0.4 ± 0.2
18:0	14.9 ± 1.7	11.8 ± 1.8	17.1 ± 2.0	15.4 ± 2.1	14.5 ± 2.5	7.2 ± 1.4	20.0 ± 0.6	20.3 ± 1.0
18:1	21.8 ± 3.8	16.9 ± 2.4	25.9 ± 1.7	25.1 ± 3.3	18.9 ± 1.8	8.0 ± 1.4	17.8 ± 0.9	15.6 ± 0.9
18:2	8.3 ± 1.8	6.2 ± 0.6	23.7 ± 2.2	23.2 ± 1.9	11.2 ± 0.9	10.0 ± 0.8	38.9 ± 0.9	42.1 ± 1.1
20:3	1.6 ± 0.1	—	trace	—	0.8 ± 0.2	—	trace	—
20:4	9.4 ± 1.3[b]	13.2 ± 2.2	8.3 ± 1.3	9.1 ± 1.3	9.5 ± 0.7[c]	9.5 ± 0.8	4.8 ± 0.9	7.7 ± 1.2

[a] P value for dodecanoic acid between FF and CO = .02.
[b] P value for eicosatetraenoic acid between FF and CNO = .03.
[c] P value for eicosatetraenoic acid between CO and CNO = .01.

animals. Of great interest was the observation that the concentration of eicosatetraenoic acid was no less in FF or CO animals than in the CNO or VS rats. Actually, the eicosatetraenoate concentration of the CNO group was lower than that of the CO animals ($P = .01$) and of the FF group ($P = .03$). Traces of a fatty acid with a retention time corresponding to an eicosatrienoic acid was observed in microsomes of the animals on the FF or CO diets.

The changes seen in the fatty acids of the mitochondria were similar in nature to those of the microsomes. Shorter chain fatty acids were increased in concentration in samples from animals on FF and CO diets more than in those from animals on VS or CNO diets. The concentration of dodecanoic acid was highest in the CO preparations while the concentration of octadecenoic acid was highest in the group fed the VS diet. In glands of animals fed CNO or VS diets the concentration of octadecadienoic acid was the highest. The greatest amount of eicosatetraenoic acid was in the animals fed the FF diet, although differences in the various groups were not statistically significant.

DISCUSSION

These experiments demonstrated that the mammary gland responds to dietary fat in the same manner as liver and adipose tissue, i.e., fatty acid synthesis was decreased by addition of fat at a level of 20% by weight to an otherwise fat-free diet. Supernatants obtained after centrifugation at 105,000 × g and supernatants containing microsomes responded similarly.

The type of fat in the diet had no significant effect on the extent of inhibition of synthesis. Because different fatty acids were furnished by the various dietary fats used, it had been hypothesized that inhibition of synthesis by these fats might be sufficiently different to change the C^{14} distribution in the synthesized fatty acids. However, the only difference obtained was a greater C^{14} incorporation in the shorter chain fatty acids of animals fed the VS diet compared to rats on the FF diet. Thus, synthesis was apparently inhibited in the same manner in all preparations. If synthesis was inhibited by the accumulation of long chain fatty acyl CoA compounds (7), the difference in chain lengths of fatty acids fed was not very important. The C^{14} incorporation into the fatty acids was no different when microsomes were left in the preparation than when they were omitted. These results obtained in mammary gland of the rat are different than those obtain in preparations of liver (8) or preparations of rabbit mammary gland (9). In those instances the preparations containing microsomes resulted in more C^{14} incorporation in fatty acids of longer chain lengths than in experiments with supernatants alone.

The different diets did, however, affect the fatty acid composition of the milk. In each case the fatty acids characteristic of the fed fat became prominent components of the fat skimmed off the top of the centrifuge tube after centrifugation. Such fat obtained from preparations of rats fed the fat-free diet contained larger amounts of shorter chain acids than did fat obtained from preparations of rats fed diets containing fats. These results, in part, are similar to those reported by Insull et al (10) in their studies on human milk. These investigators reported that during energy equilibrium milk fat closely resembled dietary fat. When excess (1000 calories above maintenance requirements) nonfat calories were fed, the milk showed an increase in tetradecanoic and dodecanoic acids, presumably from mammary gland synthesis. Beare (11) found that rats given maize oil transferred linoleic acid to milk, while in a low fat diet the milk fat produced was characterized by an increased proportion of shortchain saturated fatty acids.

The diet was also effective in changing the fatty acid composition of the subcellular particles of mammary gland. The fatty acid predominant in the dietary fat increased in concentration in the microsomes and mitochondria. Omission of essential fatty acids in the diet (FF and CO diets) for a period of 20–25 days led to traces of an acid of retention time indicative of eicosatrienoic acid (presumably the 5, 8, 11 isomer which accumulates in fat deficiency) (12). An interesting finding was the lack of decrease of eicosatetraenoic (presumably arachidonic) acid in the microsomes and mitochondria in spite of a low concentration of octadecadienoic acid. The subcellular structures containing this metabolite must thus not be subject to very rapid turnover.

ACKNOWLEDGMENTS

Technical assistance in early part of this work by Mark McCalla.

Supported by grant No. AM 06483 from the National Institutes of Health.

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[Received Dec. 28, 1964]

Synthesis of *trans*-3-Hexadecenoic Acid and of *trans*-3-Hexadecenoic-1-C^{14} Acid

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ABSTRACT

The *trans*-3-hexadecenoic acid has been synthesized. Physical properties and chemical degradation prove its identity with the acid earlier isolated from several plant lipids. In the sequence of the synthesis, the introduction of a terminal triple bond into commercially available 1-tetradecene was performed by bromination and debromination with KOH and $NaNH_2$. Chain elongation by a Grignard reaction with CO_2 gave a carboxylic acid with a triple bond in the 2-position. Reduction with $LiAlH_4$ yielded the corresponding alcohol, and reduction of the triple to the *trans* double bond was accomplished with Na in ethanol. Bromination of the alcohol with PBr_3 and conversion of the bromide to the nitrile with KCN or $KC^{14}N$ elongated the carbon chain to the desired length. Methanolysis with HCl in methanol and saponification with KOH formed the acid with acceptable yields, and in the case of the C^{14}-labeled carboxyl group, with high specific activity.

INTRODUCTION

THE TRANS-3-HEXADECENOIC acid was first detected, isolated in pure form and identified as such from the lipids of the fresh water algae, *Scenedesmus obliquus*, by Klenk and Knipprath (1), and a possible significance as an intermediate product in the biogenesis of fatty acids was suggested. Since then the acid has been shown to be present also in the lipids of higher plants by Debuch (2) and by Weenink and Shorland (3). The last-mentioned authors found it to be exclusively a part of the phosphatidyl glycerol fraction. This occurrence, coupled with its unique structure may indicate that it participates in the photosynthesis reactions of the plant. The unusual position of the *trans* double bond close to the carboxyl group raises the question of the metabolic significance of the acid. In the cases examined, however, the acid was always a minor constituent of the total fatty acid mixture. Davidoff and Korn (4) found that the CoA-derivatives of this acid and the C_{14} and C_{18} homologues were synthesized from C_{14}, C_{16}, and C_{18} fatty acyl-CoA by subcellular fractions from homogenates of the slime mold, *Dictyostelium discoideum*, together with the CoA-compounds of *trans*-2-, and *cis*-3-unsaturated fatty acids and D(−)-β-hydroxy acid. The authors demonstrated that direct isomerization of the unsaturated fatty acids occurs. In a recent work the same authors (5) used enzymes from guinea pig liver mitochondria and found the formation of the CoA-derivatives of the same unsaturated fatty acids and of l-β-hydroxy palmityl-CoA from palmityl-CoA and the interconversion of the substrates. Under anaerobic conditions with an electron donor the unsaturated components were reduced to palmityl-CoA.

The synthesis described here gives the final proof for the structure of the acid. In the preparations, the common methods for the introduction of a C^{14}-labeled carboxyl group either could not be applied at all or gave unsatisfactory yields, mainly because of the position of the double bond. For example a reaction of the Grignard compound of the intermediary 1-bromo-*trans*-2-pentadecene with CO_2 resulted only in a very small amount of the desired carbon chain elongation, even at lower temperatures, the main product possibly being a branched chain compound. Also the reactions with $Cu(CN)_2$ at 165C and with KCN in alcohol failed. The only method found to give good yields of the desired product without significant side reactions, was that described by Sugimoto et al. (6), which is the basis of this communication. This method employs relatively mild conditions, and the progress of the reaction can be followed by gas chromatographic analyses.

EXPERIMENTAL

The reaction sequence described here is shown in Figure 1. Melting points were taken on a Fisher melting point block and are corrected. The gas chromatographic analyses were performed on a Barber-Colman Model 10 apparatus with a 3 ft × 0.25 in. column of ethylene glycol succinate, 13% on siliconized fire brick, 100 to 115 mesh. Gas chromatographic analysis of the C^{14}-labeled acid was performed on a Loenco Model 70 Hi-Flex apparatus with a

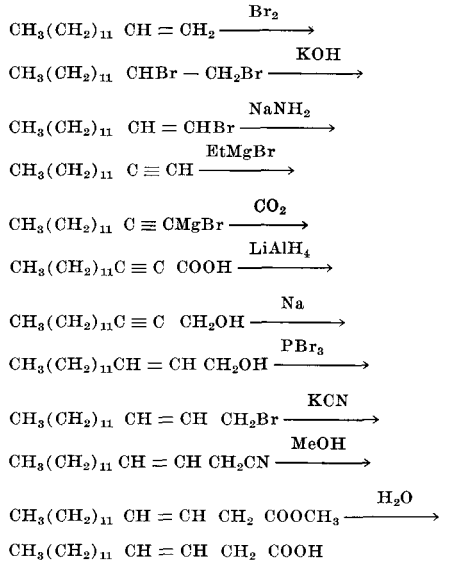

FIG. 1. Flow diagram of the reaction sequence.

5 ft × ¼ in. column of SE, 10% on Chromosorb W.

1,2-Dibromotetradecane was obtained as described by Kraft (7) by bromination of commercially available 1-tetradecene. 1-Tetradecene (95 g, 0.48 mole) in 500 ml CCl_4 was reacted by the dropwise addition of 79 g (0.49 mole) Br_2. Removal of the solvent by distillation and fractionation of the residual oil gave 154.6 g (0.43 mole) (90% yield) 1,2-dibromotetradecane, bp 140–56C, 0.22 mm, as a slightly yellow clear liquid.

1-Bromo-1-tetradecene was synthesized according to the method of Kraft and Reuter (8). Dehydrobromination of 154.6 g (0.43 mole) 1,2-dibromotetradecane was accomplished by heating in a solution of 75 g KOH in 700 ml ethanol at 40–45C for several hours. After completion of the reaction, as indicated by the disappearance of the rather insoluble dibromide, the solution was poured into water, extracted with ether, and dried over $MgSO_4$. Distillation gave 109 g (0.40 mole) (91% yield), bp 118–120C, 0.2 mm.

1-Tetradecyne was prepared according to the method of Lespieau and Bourguel (9). Sodium amide prepared from 36 g (1.57 g at.) Na in 600 ml liquid NH_3, was suspended in 300 ml mineral oil (bp > 250C). 1-Bromotetradecene (95 g, 0.35 mole) was added and the mixture heated for 2 hr at 165C. The excess $NaNH_2$ was decomposed by the addition of dilute HCl. The organic phase was extracted in ether, washed, and dried over $MgSO_4$. Distillation at 127C, 15 mm gave 67 g product contaminated by mineral oil. In order to purify the acetylene compound, it was dissolved in 160 ml ethanol and reacted by the dropwise addition of a solution of silver nitrate in ethanol/water (9:1 v/v). When the precipitate had settled and further addition of the reagent left the liquid phase clear the white microcrystalline powder was isolated by filtration on a Buchner funnel and washed with ethanol, then with ether. Decomposition of the precipitate in order to recover the acetylene compound was carried out by shaking it with dilute HCl at room temperature. The organic layer was separated, and after washing and drying over $MgSO_4$, the 1-tetradecyne weighed 60.5 g (0.31 mole) (90% yield).

2-Pentadecynoic acid. To an ethyl Grignard reagent prepared in the usual manner from 47.9 g (0.44 mole) bromoethane and 10.1 g (0.42 g at.) Mg in 60 ml ether, was added dropwise 60 ml (0.31 mole) 1-tetradecyne [Osbond et al. (10)]. When the evolution of ethane ceased, CO_2 was bubbled through the solution causing a slight increase in temperature and viscosity. The product was poured on ice, acidified by aqueous HCl, extracted by ether, washed, and dried over $MgSO_4$. The crude product was purified by chromatography on silicic acid/Celite (10:1 w/w) in a 8 × 15 cm column. The procedure was carried out with batches of 15 g each, and the elution from the column was performed with n-pentane (200 ml) and by n-pentane containing 10% ether, which eluted the acid and was run through the column until no more acid was eluted. The acid-containing fractions were combined and the solvent removed. Further purification by crystallization from acetone at −20C gave 67 g (0.28 mole) (91% yield) 2-pentadecynoic acid, mp 42.5–43C.

2-Pentadecyn-1-ol was prepared from the corresponding acid by a modification of the $LiAlH_4$ reduction procedure described by Crombie (11). To 15 g (0.063 mole) 2-pentadecynoic acid in 80 ml ether was added dropwise with stirring 2.6 g (0.069 mole) $LiAlH_4$ in 50 ml ether. Acidification with aqueous HCl, washing, drying ($MgSO_4$), and solvent removal gave the crude alcohol. Crystallization from 40–60C petroleum ether gave 12.3 g (0.055 mole) (87% yield) 2-pentadecyn-1-ol, mp 35–36.5C.

Trans-2-Pentadecen-1-ol. The reduction of a triple to a *trans* double bond requires a very large excess of Na in ethanol [Jenny and Grob (12)]. 2-Pentadecyn-1-ol (6 g, 0.027 mole) in 1.8 l 95% ethanol was reduced by the slow addition of 210 g Na. The viscous solution was

refluxed in an oil bath until the Na disappeared. The mixture was poured on ice, diluted with water and extracted with ether, the extract being washed until neutral. After drying ($MgSO_4$) and solvent removal, the product was crystallized from petroleum ether to yield 5.8 g (0.026 mole) (96% yield) *trans*-pentadecen-1-ol, mp 47–8C.

1-Bromo-trans-2-pentadecene. Following the method of Newman and Wotiz (13) 5.8 g (0.026 mole) *trans*-2-pentadecen-1-ol in ether was reacted with 2.7 g (0.01 mole) PBr_3 in the presence of a catalytic portion of pyridine. The mixture was not refluxed as recommended by the authors. After adding ice, washing, and drying (Na_2SO_4), the crude bromide weighed 5.3 g.

1-Cyano-trans-2-pentadecene. The method of Sugimoto et al. (6) proved to be an efficient and easy way to replace the halogen of the previous compound by a nitrile group. The progress of the reaction was followed by gas chromatographic analysis. A solution containing 1 g (0.015 mole) KCN in 6 ml H_2O and 5 ml of 60% aqueous phenyl trimethyl ammonium chloride was stirred rapidly. 1-Bromo-*trans*-2-pentadecene (2.6 g, 0.009 mole) was added dropwise at room temperature followed by refluxing for 90 min. The mixture was extracted with ether, washed and dried (Na_2SO_4). Removal of the solvent gave 1.8 g (0.0076 mole) (86% yield) crude product.

trans-3-Hexadecenoic acid. The steps involved were the methanolysis of the nitrile to the methyl ester and the ester's saponification. The crude nitrile (1.8 g, 0.0076 mole) was dissolved in 22 ml of methanol/H_2O (10:1 v/v), and HCl gas was passed through the solution. The solution began to boil and when the reaction temperature decreased again, the mixture was heated on the steam bath for 90 min with HCl gas passing through at a low rate. The completeness of the reaction was checked by gas chromatographic analysis.

The methanol solution was evaporated, and an ether solution of the residue, washed free of acid and dried over $MgSO_4$, was evaporated to give 1.5 g (0.0056 mole) (71% yield) methyl ester. The ester was saponified by refluxing in 50 ml 0.5 N KOH in 95% methanol. After 0.3 g of unsaponified material was removed by extraction with pentane the solution was acidified by dilute HCl and the crude product extracted with ether. Purification was accomplished by chromatography on a column of silicic acid/Celite (10:1 w/w) and elution with 10% ether in n-pentane, followed by crystallization from 10 ml pentane at −20C. The crystallized *trans*-3-hexadecenoic acid, at least 99.9% pure by gas chromatography (methyl ester) weighed 0.82 g (0.0032 mole) (55% yield) and melted at 54–5C.

The acid obtained from algae (1) melted at 53.5–54C. Further identification was obtained by hydrogenation to palmitic acid, by ozonization and reductive cleavage to tridecanal from the methyl end of the fatty acid chain, and by the strong bond at 10.3 μ of the infrared spectrum indicative of a *trans* olefin, see Figure 2. The pentane mother liquor yielded 0.13 g which was about 50% pure.

trans-3-Hexadecenoic-1-C^{14} acid. The reaction of Sugimoto et al. (6) was repeated with $KC^{14}N$. 1-Bromo-*trans*-2-pentadecene (2 g, 0.007 mole) was reacted with 0.14 g (0.002) KCN as described above, and the start of the reaction was noted by the presence of nitrile in the gas chromatogram. $KC^{14}N$ (4.5 mg) (18.3 mc/m*M*) was added, and then after 30 min 0.200 g (0.003 mole) KCN. After another 90 min the reaction was complete, and the free acid was obtained as described above. There was obtained 0.42 g pure acid, mp 54C, 0.13 g impure acid, and 0.17 g acid isolated from the

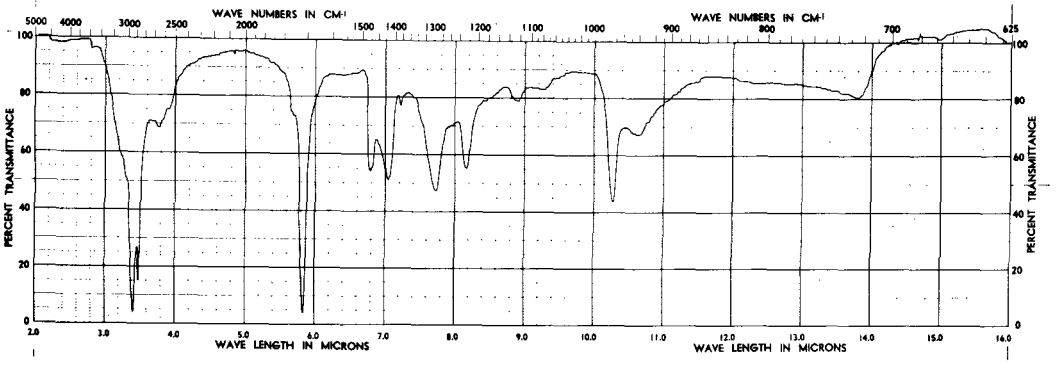

FIG. 2. IR-spectrum of the *trans*-3-hexadecenoic acid.

mother liquor. The analysis by gas liquid chromatography of the methyl ester indicated a purity of the 0.42 g fraction of at least 99%. Chromatography with the Loenco radio-gas chromatograph showed that the radioactivity of the sample (0.122 mc/mM) was exclusively in the methyl ester peak.

The $KC^{14}N$ run was made with less than a stoichiometric amount of cyanide to insure an extensive uptake of the radioactive material. In this case, the yield of pure acid from the bromide precursors was 24% as compared to the 36% obtained in the unlabeled run.

ACKNOWLEDGMENTS

Supported in part by lipid training grant USPHS TI HE-5306 and in part by a grant from the Nutrition Foundation.

IR spectrum determined by George Alexander.

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[Received Aug. 2, 1965]

COMMUNICATIONS

Quantitative Analysis of Phospholipids by Thin-Layer Chromatography and Phosphorus Analysis of Spots

PROCEDURES FOR ANALYSIS of phospholipid composition by thin-layer chromatography (TLC) and phosphorus analysis have been reported from a number of laboratories. These procedures usually depend upon one-dimensional TLC and elution of spots before analysis. The method reported here has the advantage of improved separations by two-dimensional TLC, direct aspiration of spots by suction, and phosphorus analysis without prior elution.

Our procedure depends upon two-dimensional TLC with the solvent pairs 1) chloroform/methanol/water 65/25/4 and n-butanol/acetic acid/water 60/20/20; and 2) chloroform/methanol/28% aqueous ammonia 65/35/5 followed by chloroform/acetone/methanol/acetic acid/water 5/2/1/1/0.5. The adsorbent composed of silica gel plain/magnesium silicate 9/1 (1) after spreading with a conventional Desaga spreader (0.25 mm layer) is heat activated for 20 min at 120C, cooled for 30 min, spotted, and chromatograms developed in chambers lined with solvent-saturated paper (2). Spots are detected by spraying with a 0.6% solution of potassium dichromate in 55% (by wt) sulfuric acid followed by heating for 30 min at 180C in a forced draft oven or by exposure to iodine vapors. After development, spots are circled and lettered for identification and several blank areas corresponding in size to the sample spots are marked off. A typical chromatogram of each series is photographed (Polaroid camera) and the spots recovered by aspiration.

Aspiration of the spots directly into 30 ml Kjeldahl digestion flasks is accomplished by fitting a rubber stopper with two plastic tubes removed from plastic wash bottles. One tube with a pointed end serves as the intake and the other tube for attachment to a water pump for suction. Adsorbent is prevented from passing out of the digestion flask during aspiration by adding 0.9 ml of 72% perchloric acid (used subsequently for digestion) to the flask to act as a liquid trap by moistening the lower bulb portion of the flask and by insertion of a 1 cm square of "Kimwipe" or similar light weight paper into the end of the suction tube to serve as a filter. After aspiration, the plastic tubes are tapped to remove any dry powder and the paper filter pushed with a wire plunger into the flask.

Digestion of the flask contents is carried out on an electrically heated Kjeldahl rack with water-pump suction to remove any escaping fumes. The heaters are adjusted to give gentle refluxing so that digestion is complete in about 20 min.

After digestion, the sides of the flask are rinsed with 5 ml of distilled water, 1 ml of 2.5% ammonium molybdate solution is added, the flask swirled for mixing, 1 ml of 10% ascorbic acid solution is added, and finally 2 ml of distilled water are added. The solution is transferred to a centrifuge tube, heated in a boiling water bath for 5 min, cooled, and suspended adsorbent removed by centrifugation for 5–10 min. Samples and blanks are transferred to cuvettes and the optical density determined at 820 mμ after zero adjustment with water. Sensitivity can be increased by using a 10 ml digestion flask and one half of the specified amounts of reagents. Glassware should be acid cleaned.

Corrected optical densities are determined by subtraction of the reading obtained from a blank area corresponding in size to that of the sample. The values are then converted to μg of phosphorus using a factor derived from a standard curve prepared using Na_2HPO_4. The factor in our laboratories is 11.0 for standard amounts and twice that for half amounts of reagents. Molar ratios of phospholipids are obtained by expression of results as percent of the total phosphorus in the sample. Determination of the total phosphorus is conveniently accomplished by spotting 50–100 μg of total sample in a blank area (upper right corner) after development with both solvents. The total sample is then charred, etc., in the same manner as the samples. For expression of results as percent of the total lipid, phosphorus values for brain lipids are multiplied by the following factors: phosphatidyl inositol, 31.4; phosphatidyl serine, 26.2; lecithin and phosphatidyl ethanolamine, 25.4; phosphatidic acid, 25.0; sphingomyelin, 24.8; and cardiolipin, 24.4

Animal tissue lipid extracts are spotted at levels of 200–1000 μg for determinations and at least four chromatograms are developed with each of the two-dimensional systems. Average values for the major lipid classes (lecithin, sphingomyelin, phosphatidyl ethanolamine and phosphatidyl serine) are thus obtained from eight determinations. Usually spots from two chromatograms are pooled for minor components.

The values obtained from a normal adult human brain by the present procedure and the

TABLE I
Human Brain Phospholipid Composition

		% Total lipid by TLC		
	μg P	System 1	System 2	DEAE-TLC
Phosphatidyl ethanolamine	2.40	15.2	15.1	15.1
Lecithin	2.05	12.9	13.6	12.0
Sphingomyelin	1.05	6.5	6.4	6.0
Phosphatidyl serine	1.00	6.5	7.1	6.1
Phosphatidyl inositol	0.12	0.9	0.9	1.0
Origin	0.07			
	6.69			

Total sample = 6.91 μg P
Recovery = 96.9%

DEAE-TLC procedure (1) are shown in Table I. Good agreement between the two methods is seen. Recovery of phosphorus is frequently low because minor components are not determined. Low recoveries may be obtained when lipid extracts are not handled with great care and artifacts and fragments are produced that are not visualized on chromatograms.

The present procedure was compared to a similar procedure using hydrolysis of aspirated spots with 1 N methanolic HCl and filtration to remove adsorbent (3). For beef heart mitochondrial lipids the percent of the total phosphorus was: diphosphatidyl glycerol, 18.6; phosphatidyl ethanolamine, 37.2; and phosphatidyl choline, 40.1 with methanolic HCl. The corresponding values (19.1, 37.3, 39.3) obtained by the more rapid charring procedure were in close agreement.

The phosphorus analysis procedure must be used cautiously with lipid extracts from organs that have not been investigated by other procedures since some spots may represent more than one lipid class and new lipids may be encountered. When values in the two chromatographic systems are not in close agreement, spot overlap in one system is indicated and may be related to an unknown component. Large errors may be introduced if lipid samples are not handled (prior to spotting) with care to prevent oxidation and/or hydrolysis producing artifacts that may migrate with native lipids.

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ACKNOWLEDGMENTS

Supported by USPHS Grants NB-01847-07, CA-03134-09, and GM 12831-02; and Contract DA 18-035-AMC-335 (A) from US Army Edgewood Arsenal, Maryland; and an established investigatorship to one of us (S.F.) from the American Heart Association.

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[Received Oct. 18, 1965]

Occurrence of Short Chain Triglycerides in Human Lens

THE HUMAN LENS contains approximately 175 μg of triglycerides. We recently isolated 4–5 mg of this lipid in crude form in the course of our studies of the lenticular glycolipids (1,2). These crude triglycerides were purified by preparative thin-layer chromatography followed by passage through a 2.5 × 20 cm column of DEAE (diethylaminoethyl cellulose). The resulting preparation was homogeneous by thin-layer chromatography on silica gel G with a solvent system of chloroform/benzene 1:1, v/v.

The purified triglycerides were subsequently analyzed by gas-liquid chromatography (3). The results obtained are shown in Figure 1. Note the peaks eluting shortly after the solvent front indicating triglycerides of short chain

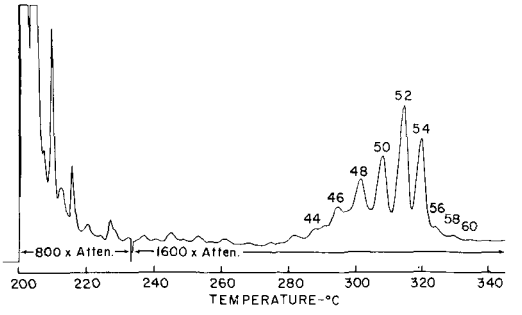

FIG. 1. Chromatogram of human lenticular triglycerides. Operating conditions: 3% JXR on 100/120 Gas Chrom Q in 2 ft × ⅛ in. stainless steel columns; temperature programmed from 200–330C at a rate of 4C/min, helium carrier gas at 100 ml/min, hydrogen flame ionization detector.

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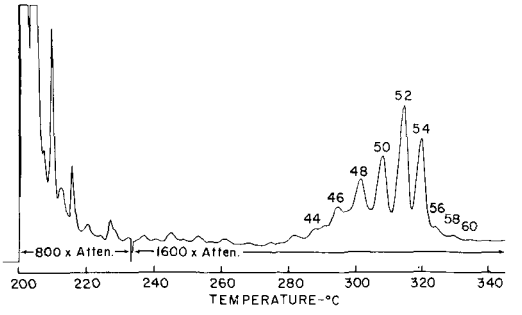

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length. Such an interpretation is consistent with our previously reported observation of appreciable quantities of fatty acids with chain lengths of less than 12 carbon atoms (4). Similar results are obtained with the triglycerides of cattle lenses. A detailed study of the lenticular triglycerides will be reported later.

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ACKNOWLEDGMENTS

Triglyceride analyses by C. Litchfield and R. Harlow. Supported in part by USPHS Grant NB-04277-04 from the National Institute of Neurological Diseases and Blindness.

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[Received Nov. 17, 1965]

The Incorporation in toto of Octadecanoic Acid (Formed from Ingested Octadecane-1-C^{14}) into the Lipids of the Sebaceous Glands of the Rat

WHEN RATS WERE FED traces of octadecane-1-C^{14}, radioactive lipid was excreted onto the skin surface of the back through the sebaceous glands (1). Since the activity was found in fractions other than the hydrocarbons, the octadecane must have been oxidized, then incorporated in some fashion into the lipids. Three alternate modes of incorporation are suggested: 1) complete breakdown of the hydrocarbon chain into 2 carbon fragments from which the fatty chains of sebum components are synthesized, 2) partial breakdown of the hydrocarbon chain (very likely by splitting off one or more 2 carbon fragments) then incorporation of the larger and possibly the smaller fragments into the fatty chains of sebum components, or 3) no breakdown of the hydrocarbon chain but oxidation of one of its terminal carbon atoms to an alcohol or an acid, then incorporation of either alcohol and/or acid directly of after chain elongation into the esters of sebum. Of course, all three or any combination of these alternatives could operate simultaneously. The formation of the oxidation products could occur anywhere in the rat body and be transported to the skin via the blood, but final synthesis of sebum components undoubtedly occurs in the sebaceous gland.

To decide between these alternatives, a portion of the radioactive surface lipid, obtained as already described (1), was saponified with 10% KOH in 90% ethanol, the fatty acids

TABLE I

Specific Activities of Saturated Fatty Acids of Rat Surface Lipid Obtained After Ingestion of Octadecane-1-C^{14}

Fatty acid as methyl ester[a]	Counts per min[b]	Counts per min above backgrd[c]	Efficiency %	Disintegrations/min above bkgrd. (DPM)	Weight mg	Specific activity DPM/mg
iso-C_{14}	22	0	....	....	0.15	....
n-C_{14}	23	0	....	....	0.11	....
anteiso-C_{15}	21	0	....	....	0.22	....
n-C_{15}	19	0	....	....	0.06	....
iso-C_{16}	34	14	70	20	0.90	22
n-C_{16}	38	18	70	26	1.36	19
anteiso-C_{17}	27	7	71	10	0.38	27
n-C_{17}	22	0	....	....	0.09	....
iso-C_{18}	22	0	....	....	0.21	....
n-C_{18}	51	31	71	43	0.23	186
anteiso-C_{19}	23	0	....	....	0.13	....
n-C_{19}	..	....	....	....	trace	....
iso-C_{20}	30	10	70	14	0.25	56
n-C_{20}	29	9	74	12	0.13	90
anteiso-C_{21}	25	0	....	....	0.20	....

[a] Assignments of structure of the fatty acid methyl esters is by retention data as in ref 2.
[b] Maximum error estimated at ±2 counts/min.
[c] Background = 20 counts/min.

length. Such an interpretation is consistent with our previously reported observation of appreciable quantities of fatty acids with chain lengths of less than 12 carbon atoms (4). Similar results are obtained with the triglycerides of cattle lenses. A detailed study of the lenticular triglycerides will be reported later.

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ACKNOWLEDGMENTS

Triglyceride analyses by C. Litchfield and R. Harlow. Supported in part by USPHS Grant NB-04277-04 from the National Institute of Neurological Diseases and Blindness.

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n-C_{14}	23	0	....	....	0.11	....
anteiso-C_{15}	21	0	....	....	0.22	....
n-C_{15}	19	0	....	....	0.06	....
iso-C_{16}	34	14	70	20	0.90	22
n-C_{16}	38	18	70	26	1.36	19
anteiso-C_{17}	27	7	71	10	0.38	27
n-C_{17}	22	0	....	....	0.09	....
iso-C_{18}	22	0	....	....	0.21	....
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[a] Assignments of structure of the fatty acid methyl esters is by retention data as in ref 2.
[b] Maximum error estimated at ±2 counts/min.
[c] Background = 20 counts/min.

recovered in the usual manner, then methylated with BF_3 and methanol. The methyl esters were separated on a column of silver nitrate impregnated silicic acid into saturated and unsaturated methyl esters by the method of DeVries according to details described in (2). The saturated esters were separated into their molecular species by preparative gas chromatography on silicone rubber SE-30 (2). The amounts of methyl ester recovered was estimated from the areas of the gas chromatographic peaks and checked by direct weighing on a Cahn microbalance. Each ester was then counted on a Nuclear Chicago scintillation counter by the channels ratio technique.

Although alternate explanations of the data (Table I) are possible (e.g., assumptions that fatty acids are not only produced by sebaceous glands but are also degraded by them, and that these processes can proceed at different rates), the simplest explanation is that *A*, a significant amount of octadecane is oxidized to n-C_{18} fatty acid which is then incorporated in toto into the sebaceous gland lipids, i.e., mode 3 above since maximum activity appears in the n-C_{18} fatty acid; *B*, fatty chains are being extended, since appreciable activity appears in n-C_{20} fatty acid; and *C*, mode 1 and possibly mode 2 are also operating to some degree, since activity is found in fatty acids with chain lengths below n-C_{18}.

If fatty acids can be incorporated in toto into the lipids of sebum, one might then expect the fatty acids being transported by the blood stream (i.e., the mobilizable fatty acids of adipose tissue or of dietary origin) to influence directly the composition of the fatty acids of sebum.

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ACKNOWLEDGMENT

Work initiated at the University of Oregon Medical School and supported by USPHS grants No. AM-05120, AM-10010 and 5TI-AM-5300 and US Army Medical Research and Development Contract DA-49-139-MD-2184.

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[Received Nov. 12, 1965]

Phospholipids of Human Serum[1]

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ABSTRACT

Phospholipids extracted from normal human serum were fractionated into lecithin, lysolecithin, sphingomyelin, phosphatidyl ethanolamine, lysophosphatidyl ethanolamine, phosphatidyl serine, and phosphatidyl inositol. Identification of each was established by thin-layer chromatography and infrared spectrophotometry. The content of plasmalogen was determined in both lecithin and phosphatidyl ethanolamine fractions. The composition of fatty acids and fatty aldehydes in isolated phospholipids is presented. The degree of unsaturation as reflected in the average content of double bonds per molecule of the fatty acids in phospholipids was: lecithin 1.2, choline plasmalogen 2.1, lysolecithin 0.6, sphingomyelin 0.2, phosphatidyl ethanolamine 2.8, lysophosphatidyl ethanolamine 1.0, phosphatidyl serine 1.0, and phosphatidyl inositol 1.8. Both choline and ethanolamine plasmalogen aldehydes were predominantly saturated. Molecular weight of each phospholipid was calculated from determined fatty acid and fatty aldehyde compositions; the phosphorus factor for each phospholipid was computed. On a weight percent basis, lecithin, sphingomyelin, and lysolecithin accounted for 95% of the total phospholipids. The ethanolamine-containing phospholipids accounted for 2.5%, and the remainder was divided among phosphatidyl inositol, choline plasmalogen and phosphatidyl serine.

INTRODUCTION

THE COMPOSITION of the phospholipids of human serum has been studied in recent years by several investigators using the techniques of column (16,24,26,28,30,37), thin-layer (6,14,32), and paper chromatography (2,6,26), or successive hydrolysis (3). Only a few reports have appeared on the fatty acid composition of the individual phospholipid classes of this tissue (16,24,37). In some of these studies, the phospholipid classes analyzed, particularly those occurring in small amounts, were not completely resolved (6,14,24,28,32). This has led not only to discrepancies in the reports on the fatty acid composition of the various phospholipids (24,37), but also to disagreement as to the levels of minor components such as phosphatidyl ethanolamine or phosphoinositide present in serum. Also, as far as we are aware, the fatty aldehydes present in the serum plasmalogens have not been determined. Therefore, it appeared worthwhile to carry out further studies of the levels of phospholipid present in human serum and of the fatty acid and fatty aldehyde composition of each of the individual classes of phosphatides.

The chromatographic separation of the various types of phospholipid of human serum is complicated by the fact that the three choline-containing lipids, lecithin, lysolecithin, and sphingomyelin, constitute at least 90% of the phospholipids. Therefore in the present study, extracts were prepared from large amounts of serum in an attempt to obtain sufficient lipid for adequate characterization of the phospholipids occurring in small amounts. Furthermore, the extract was chromatographed on silicic acid columns and the various fractions thus obtained were refractionated until preparations were isolated which were homogeneous enough on the basis of thin-layer chromatography (TLC) and infrared spectrophotometry so that the fatty acids and fatty aldehyde composition could be determined by gas-liquid chromatography (GLC).

EXPERIMENTAL

Materials

Four liters of blood were collected by venipuncture into dry sterile collection bottles from 8 fasting male medical students ranging in age from 22 to 24 years. After the blood had clotted for approximately 6 hr most of the clear serum was removed by siphon. A small additional portion was obtained by centrifugation. The combined yield was 1760 ml of clear, straw-colored serum with no evidence of hemolysis.

Silicic acid, 100 mesh, analytical reagent from Mallinckrodt Chemical works, was suspended several times in distilled water to remove fine particles by decanting, washed with methanol and reactivated at 120C. Aluminum oxide, suitable for chromatographic adsorption,

[1] Presented in part at the AOCS Meeting, Houston, April, 1965.

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was obtained from Merck and Company, and spectrograde solvents from Fisher Scientific Company. All other solvents used were freshly redistilled.

Crotalus adamanteus venom, used as a source of phospholipase A, was obtained from the Ross Allen Reptile Institute, Silver Springs, Florida.

Source of Reference Compounds

Phosphatidyl inositol was obtained from the Sigma Chemical Company. All other reference compounds were isolated from serum or egg yolk in this laboratory. Serum and egg lecithin were isolated and purified by silicic acid chromatography. Serum lysolecithin was prepared by enzymatic hydrolysis from lecithin (38). Phosphatidyl ethanolamine and phosphatidyl serine from agg were separated on a hydrated silicic acid silicate column (34). Lysophosphatidyl ethanolamine was prepared by enzymatic hydrolysis of phosphatidyl ethanolamine (21). Serum sphingomyelin, after elution from a silicic acid column, was purified by selective alkaline hydrolysis to remove contaminating phosphoglycerides followed by acid treatment to destroy plasmologens (35).

All prepared reference compounds were homogeneous on TLC and exhibited the characteristic bands in the infrared of the given reference compound but not those of other phospholipids (8,20,24,33,36).

The following methyl esters used as reference compounds in GLC were obtained from Applied Science Laboratories: Methyl caprate, undecanoate, laurate, tridecanoate, myristate, myristoleate, pentadecanoate, palmitate, palmitoleate, heptadecanoate, stearate, oleate, linoleate, nonadecanoate, linolenate, arachidate, heneicosanate, arachidonate, behenate, erucate, tricosanoate, lignocerate, and methyl nervonate. The dimethyl acetals of n-undecylaldehyde, myristaldehyde, palmitaldehyde, and stearylaldehyde were kindly donated by Dr. Kazbo Arai of the Medical School of the University of Texas.

Lipid Extraction

In order to minimize the possibility of oxidation, lipids were left at all times in an atmosphere of nitrogen.

Each 220 ml portion of serum was added gradually to 264 ml of methanol and thoroughly mixed in a glass stoppered Erlemeyer flask. After the addition of 264 ml of chloroform, the flask was placed in a 55C water bath for 15 min and occasionally swirled. The extract was filtered on a Buchner funnel, and the residue was extracted twice with 132 ml portions of chloroform in a Waring blendor at low speed for 2 min. The extract was left overnight in order for the phases to separate (7). The lower layer contained the lipid extract. Negligible amounts if any of phospholipid were present in the discarded aqueous layer since phospholipids were not detected after TLC of aliquots of this phase which had been concentrated 500-fold.

Chromatographic Separations

Silicic acid columns were prepared in chloroform with a minimum of 50 mg of silicic acid to 0.04 mg of phospholipid phosphorus. The lipid extract from 1760 ml of serum was added in chloroform to a 4 × 32 cm column containing 200 g of silicic acid. Neutral lipids were eluted with 2 liters of chloroform and pigments with 365 ml of acetone. Only minute traces of phosphorus were detected in pigmented effluent indicating that little if any oxidation of phospholipids had occurred up to this point (25). The scheme for the elution of the phospholipids is presented in Figure 1. All phospholipids shown in this figure were identified by appropriate spot tests after TLC and by cochromatography with reference compounds. The elution pattern from the silicic acid column was that which might be expected on the basis of previous work with the exception that phosphatidyl ethanolamine was eluted as two separate peaks.

Fractions 20 to 22 contained a small proportion of the phosphatidyl inositol and the middle fractions No. 36 to 54 contained most of the lecithin; an amount which represented 55% of the total lipid phosphorus. The other fractions contained two or more phospholipids and were combined into 4 major fractions designated A, B, C, and D in Figure 1. Each major fraction was subjected to further column chromatography.

Hanahan's (15) aluminum oxide column with some modification of the solvent systems was used to separate the mixtures in major fractions A and B. The components present in the major fractions are illustrated in Figure 1. For fraction A phosphatidyl ethanolamine was eluted with ethanol-chloroform-water 5/2/0.75 (v/v/v) followed with ethanol-chloroform-water 5/2/2 (v/v/v) to elute phosphatidyl inositol. These components did not overlap in any of the fractions and no lysophosphatidyl ethanolamine was detected on the thin-layer

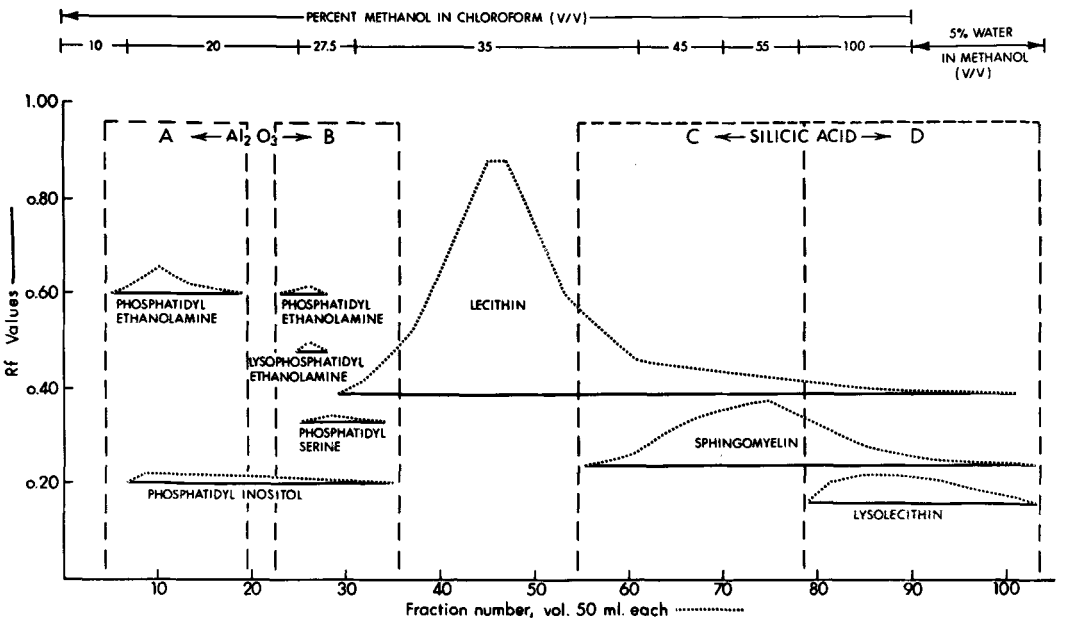

FIG. 1. Silicic acid column chromatography. The base of each eluted peak corresponds to the R_f values found on thin-layer chromatoplates. Eluted peaks were drawn on the basis of relative intensity of spots developed with the molybdenum blue spray (5). The regions indicated A, B, C, and D, where more than one component was eluted, were separated on additional columns. For details see text.

plates. Major fraction B was put on another aluminum oxide column. Lecithin was eluted with methanol-chloroform 1/1 (v/v) phosphatidyl ethanolamine plus lysophosphatidyl ethanolamine and a separate fraction containing phosphatidyl serine with ethanol-chloroform-water 5/2/0.75 (v/v/v) and phosphatidyl inositol with ethanol-chloroform-water 5/2/3 (v/v/v). No other phospholipids were detected on TLC of the eluate. The mixture of phosphatidyl ethanolamine and its lyso compound were clearly separated on a silicic acid column with 18% methanol in chloroform (v/v) to remove the phosphatidyl ethanolamine followed by methanol to bring down lysophosphatidyl ethanolamine.

The separation of the components in major fraction C (fractions 55–78) Figure 1 and major fraction D (fractions 79–104) was achieved on a silicic acid column with the solvent systems described by Phillips (27). Each major fraction was added to a separate column. Twenty per cent methanol in chloroform containing 1.35% water (v/v/v) eluted lecithin as a sharp peak followed by sphingomyelin as a flat peak. Lysolecithin was eluted with methanol containing 4% water (v/v). Some overlapping occurred between sphingomyelin and lecithin but complete separation was achieved by chromatography once more on silicic acid with Phillips solvents (27).

Thin-Layer Chromatography

An aliquot of each fraction eluted from the columns was concentrated in vacuo at least tenfold and spotted on thin-layer chromatoplates coated with silica gel G. TLC was conducted according to Stahl, with solvent system according to Wagner (40). Spots were visualized with the molybdenum blue test for phosphorus containing compounds (5), the ninhydrin reaction for aminophosphatides (22), the Dragendorf reagent for choline containing phospholipids (22), and the 2,4-dinitrophenylhydrazine test for plasmalogens (22). The samples were cochromatographed with the reference compounds to provide further proof of their identity. The relative order of movement of phospholipids in this system is given in Figure 1.

Infrared Spectrophotometry

Infrared spectrophotometry of isolated phospholipids was conducted in chloroform solutions with a Beckman IR8 double-beam recording spectrophotometer, equipped with filter-grating system, thermocouple detector

with potassium bromide optics, and a set of matched sodium chloride cells.

Phosphorus Determination

Phosphorus was determined on appropriate aliquots of the combined fractions by the method of Beveridge and Johnson (1).

Hydrolysis of Plasmalogens

Aliquots of the lecithin and phosphatidyl ethanolamine isolated by means of the column chromatographic procedures previously described were each incubated at 38C in 90% acetic acid in order to liberate plasmalogen aldehydes. This procedure was adapted from Gray (11). After neutralization of the hydrolysis mixture and extraction with chloroform-methanol 2/1 (v/v) in order to separate the glycerophosphatides and aldehydes from the water soluble products, the glycerophosphatides and aldehydes were separated on silicic acid columns. In the case of lecithin, aldehydes were eluted with 5% methanol and lecithin and lysolecithin were brought down in sequence with 20% methanol in chloroform containing 1.35% water and methanol containing 5% water, respectively.

The aldehydes of ethanolamine plasmalogens also were eluted with 5% methanol in chloroform (v/v) followed with 18% methanol in chloroform (v/v) to elute phosphatidyl ethanolamine and methanol to elute lysophosphatidyl ethanolamine.

Phosphorus was determined on both pairs of incubation products; that is, lecithin-lysolecithin and phosphatidyl ethanolamine-lysophosphatidyl ethanolamine. The ratios of the phosphorus contents in each pair served for calculation of the plasmalogen content in the lecithin and phosphatidyl ethanolamine fractions.

Enzymatic Hydrolysis of Lecithin

Enzymatic hydrolysis of lecithin with snake venom (Phospholipase A) in diethyl ether solution was achieved by the procedure of Tattrie (38). Enzymatic hydrolysis products were separated on silicic acid column. Fatty acids were eluted with chloroform and lysolecithin with 4% water in methyl alcohol. No unreacted lecithin was detected.

Preparation of Methyl Esters

The methyl esters of phosphoglyceride fatty acids were prepared by Hornstein procedure (18,19); saponification of phosphoglycerides with methanolic potassium hydroxide and liberation of fatty acids with hydrochloric acid, followed by synthesis of the methyl esters on basic ion exchange resins with anhydrous methanolic hydrochloric acid.

Since sphingomyelin is known to be resistant to alkaline hydrolysis (35), this phospholipid was hydrolyzed and esterified in one step with 5% anhydrous methanolic hydrochloric acid as previously described.

Preparation of Dimethylacetals

The aldehydes were converted to their dimethylacetal derivatives by the Gray method (12).

Gas-Liquid Chromatography

Gas-liquid partition chromatography was carried out on F & M Scientific Corporation Model 400, equipped with hydrogen flame ionization detector and disc chart integrator. The chromatographic column was packed with chromasorb W, impregnated with 17% ethylene glycol adipate. The identities of most fatty acids were established by direct comparison of retention times of methyl esters with those of reference compounds (see "Materials"). Where reference methyl esters were not available, the unknowns were identified from relative retention data. A similar approach was used for identification of dimethylacetals. The data were calculated as mole percent from the relationship between the peak areas and the corresponding molecular weights of the fatty acid, since it has been verified in many laboratories that peak areas obtained with the hydrogen flame ionization detector give a direct weight percent measurement for the component fatty acids (17).

RESULTS AND DISCUSSION

All phospholipids isolated, other than sphingomyelin and inositol phospholipid, gave only one spot on TLC. In some fractions the sphingomyelin spot showed a tendency to separate into two spots, each of which gave the tests characteristic of this phospholipid. Wood and Holton (41) have also reported this phenomenon and attribute it to the separation of sphingolipids differing in fatty acid composition. Phosphatidyl inositol showed some tailing which in some fractions appeared as one or two additional faint spots. None of these components of the phosphatidyl inositol gave ninhydrin or choline tests. The presence of more than one phosphoinositide in this

fraction thus cannot be eliminated. In this connection, Renkonen (30) has observed the presence of lysophosphatidyl inositol in human serum. All isolated phospholipids, other than additional faint spots of phosphatidyl inositol, cochromatographed with pure reference compounds.

The infrared spectra of the isolated phospholipids were taken as additional criteria of their purity. The following bands were used for evaluation of purity: 5.8 μ, the ester carbonyl band of the glycerophosphatides; 6.1 μ, the amide I band of sphingolipids; and 10.3 μ, the characteristic band of choline containing phospholipids. The 9.8 μ band, believed to be characteristic for cephalins (36), is poorly if at all developed in naturally occurring cephalins as compared with that of the synthetic product and appears rather as an inflection.

Both lecithin and lysolecithin isolated from serum showed the 5.8 μ and the 10.3 μ bands, and the absence of the 6.1 μ amide I band of sphingomyelin. In the sphingomyelin purified in this study, the 5.8 μ band was absent and the 6.1 μ and 10.3 μ bands were developed. In all of the cephalins there was no 10.3 μ band characteristic of choline phospholipids. The ester carbonyl band was developed, and there was some absorption in the 6.1 μ region. The absorption in the latter region was stronger in phosphatidyl serine than in phosphatidyl ethanolamine, a phenomenon usually exhibited by cephalins (33). In phosphatidyl inositol, both the 6.1 μ and 10.3 μ bands were absent but the ester carbonyl band at 5.8 μ was strongly developed.

The fatty acid composition of the serum phospholipids of fasting normal young men is given in Table I. The serum cholesterol levels of the eight donors varied from 177 to 231 and triglyceride contents from 60 to 186 mg%. These levels of triglyceride and cholesterol are within the range of normal in men of this age.

As can be seen in Table I, in lecithin free of plasmalogen about 85% of the fatty acids were composed of palmitic, stearic, oleic, linoleic, and arachidonic acids. The contribu-

TABLE I

Fatty Acid Composition of Human Serum Phospholipids (Mole %)

Fatty acid	L			CPL	LL	SPH	PE	LPE	PS	PI
	Total	α[a]	β[a]							
10:0	0.6	0.3	0.3	1.1	1.5	0.8	0.5	4.2	4.9	0.3
11:0	....	....	....	....	0.2	....	0.6	1.4	1.6	0.2
12:0	0.3	0.2	0.1	1.3	0.8	0.2	0.8	6.1	3.2	2.5
13:0	....	....	....	0.4	0.9	....	....	0.7	1.0	0.4
ISO 14:0	....	....	....	....	0.1	....	0.2	0.1	0.1	. .
14:0	0.3	0.2	0.1	1.0	1.0	1.5	0.5	2.3	2.1	0.8
14:1	....	....	....	....	....	0.1	....	0.1	....	....
15:0	0.4	0.3	0.1	0.7	0.7	0.3	0.3	1.6	1.8	0.6
ISO 16:0	0.1	0.1	....	0.1	0.1	0.2	0.8	0.7	....	0.1
16:0	27.8	25.8	2.0	13.6	44.0	42.5	7.6	18.3	21.3	5.3
16:1	1.7	1.0	0.7	1.9	2.4	0.2	0.5	2.0	0.7	1.1
17:0	0.3	0.2	0.1	0.3	0.4	1.0	0.3	0.8	0.6	0.7
ISO 18:0	0.1	0.1	....	0.1	0.1	....	1.2	1.2	0.3	0.3
18:0	12.5	11.9	0.6	5.7	14.2	10.9	10.3	18.6	18.9	31.5
18:1	13.0	3.2	9.8	18.4	10.3	1.9	7.6	14.6	10.2	11.6
18:2	24.5	0.4	24.1	20.0	12.3	0.8	11.6	12.8	7.4	6.3
18:3	....	. .	. .	....	....	....	0.4	0.3	0.5	0.6
20:0	1.7	0.7	1.0	....	0.2	4.6	0.9	1.0	3.7	0.1
20:2	....	....	....	0.4	0.2	0.6	0.3	0.1	0.8	0.4
20:3	2.1	....	2.1	4.3	1.1	0.5	2.3	1.2	3.7	2.6
20:4	6.9	....	6.9	23.8	1.4	....	29.3	9.4	9.5	24.2
22:0		. .		....	....	11.2	....	....	0.1	. .
22:1	3.8	3.6	0.2	4.1	2.6	0.4	2.5	0.6	4.9	1.0
23:0	....	....	....		....	5.2	....	....	....	....
22:4	....	....	....	3.7	....	0.8	....	....	....	1.0
24:0	....	....	....	2.6	0.1	7.1	1.0	0.2	0.2	0.4
24:1	1.7	0.8	0.9	0.5	3.1	8.3	0.4	....	....	....
22:5	1.2	0.1	1.1	0.5	2.3	0.5	4.1	0.3	1.9	4.3
22:6	1.0	0.1	0.9	5.5	....	0.4	16.0	1.4	0.6	3.7
Saturated	44.1	39.8	4.3	26.9	64.3	85.5	25.0	57.2	59.8	43.2
Unsaturated	55.9	9.2	46.7	73.1	35.7	14.5	75.0	42.8	40.2	56.8
Double bonds per molecule	1.2	....	....	2.1	0.6	0.2	2.8	1.0	1.0	1.8

L, lecithin free of plasmalogen; CPL, choline plasmalogen; LL, lysolecithin; SPH, sphingomyelin; PE, phosphatidyl ethanolamine together with ethanolamine plasmalogen fatty acids; LPE, lysophosphatidyl ethanolamine; PS, phosphatidyl serine; PI, phosphatidyl inositol.

[a] Lecithin was hydrolyzed with phospholipase A as described in text. The contents of fatty acids in the total unhydrolyzed lecithin were distributed between α and β positions from the ratios of fatty acids found upon hydrolysis.

tion of fatty acids with shorter chain length than 16 carbons was negligible. The chief saturated acid, palmitic acid, and the chief unsaturated acid, linoleic acid, were not far from a 1:1 ratio and contributed over one-half the amount of total fatty acid present. About 56 mole % of the lecithin fatty acids were unsaturated with an average of 1.2 double bonds per molecule.

The chief saturated acids in lecithin, stearic and palmitic acids, were present almost exclusively in the α-position and di- and polyunsaturated fatty acids in the β-position of lecithin. Seventy-five percent of the oleic acid which was the major monounsaturated acid was present in the β-position.

The fatty acids of the choline plasmalogen were almost twice as unsaturated as the ester form of lecithin. The chief unsaturated acids were arachidonic and linoleic acids. Half of the total double bonds present were contributed by the 24 mole % of arachidonic acid in this plasmalogen. The two main saturated fatty acids, palmitic and stearic acids, were found in about one-half the amount found in the ester form of lecithin.

Lysolecithin fatty acids were two times as saturated as lecithin fatty acids. Palmitic acid made up 44 mole % of the saturated acids. The content in this phospholipid of polyenoic acids was negligible.

Sphingomyelin was the most saturated phospholipid obtained since 85 mole % of the fatty acids were saturated acids. Palmitic acid represented half of these saturated acids. The only unsaturated fatty acid present in more than trace amounts was nervonic with 8 mole %. Behenic, lignoceric and nervonic acids occurred as major fatty acids only in sphingomyelin.

The fatty acids of phosphatidyl ethanolamine are presented together with those of the ethanolamine plasmalogen. This fraction showed the highest degree of unsaturation as reflected in the average amount of 2.8 double bonds per molecule and the 75 mole % of total fatty acids as unsaturated fatty acids. Only 15% of the unsaturated acids were monoenoic acids. Arachidonic and docosahexaenoic each contributed 42 and 34%, respectively, of the total double bonds present. Two saturated acids, stearic and palmitic acid, made up 72% of the saturated acid fraction, and 12% of this fraction was scattered among six fatty acids with carbon chains shorter than 16 carbon atoms.

Lysophosphatidyl ethanolamine and phosphatidyl serine, in general, had similar fatty acid composition. Both of them contained a greater proportion of fatty acids shorter than 16 carbons than did other phospholipids. The degree of unsaturation as measured by the number of double bonds per molecule was similar to that noted in lecithin but about one third that found in phosphatidyl ethanolamine. The main saturated fatty acids were palmitic and stearic acids. It is evident that a marked decrease in unsaturation occurs in each of the pairs, lecithin-lysolecithin, and phosphatidyl ethanolamine-lysophosphatidyl ethanolamine.

Phosphatidyl inositol had a high degree of unsaturation with an average of 1.8 double bonds per molecule. Over half of the double bonds were contributed by arachidonic acid. Seventy-three percent of the total saturated fatty acids were present in 31 mole % of stearic acid, the highest mole % content for this acid among the listed phospholipids. Metabolic studies have shown phosphatidyl inositol to have high specificity for this saturated fatty acid (4).

It is of interest to compare the fatty acid composition of the phospholipid classes isolated in this study with those published by other workers. As far as we are aware, such data have been reported for serum lecithin (16,24,31), phosphatidyl ethanolamine (24), sphingomyelin (16,24,37), and lysolecithin (24).

The fatty acid composition of lecithin in the present study is, in general, similar to that which has been reported by other workers (16,24). Also, the distribution of main fatty acids between the α and β positions is in agreement with previous work of Hanahan et al. (16) and Renkonen (31).

On the other hand, the fatty acids found in serum phosphatidyl ethanolamine, which are listed in Table I, differ in composition from those previously reported (24) since this fraction as previously isolated must have contained phosphoinositides, phosphatidyl serine, and lysophosphatidyl ethanolamine. For example, in the present report fatty acids with chain length less than 16 carbon atoms contributed only 2.9 mole % to the total fatty acids and 75 mole % of the latter were unsaturated. Nelson (24) found that 10% of the acids were of less than 16 carbon atoms and only 63% were unsaturated in the phosphatidyl ethanolamine fraction. Examination of the content of fatty acids of less than 16 carbons, and of the level of total unsaturated fatty acids of lysophosphatidyl ethanolamine, phosphatidyl serine, and phosphatidyl inositol, listed in Table I, reveals that mixtures of phosphatidyl

ethanolamine with the three other phospholipids just described would have a higher content of acids with less than 16 carbons and a lower content of unsaturated fatty acids than the phosphatidyl ethanolamine analyzed in the present study. This hypothesis might explain the discrepancy between Nelson's results (24) and those of the present study.

The content of the major fatty acids of sphingomyelin is in agreement with the results of other workers (16,24,37); however, there is some disagreement concerning the ratio of saturated to unsaturated acids. The 14 mole % of unsaturated acid is in contrast to the 9 mole % described by Hanahan (16) or the 36 mole % reported by Nelson (24), but agrees with the value reported by Sweeley (37) using plasma sphingomyelin that had been purified by alkali treatment rather than by column chromatography as in the present study. The fatty acid composition of the serum sphingomyelin isolated in the present study confirms the results obtained by Sweeley (37). Both Hanahan (16) and Nelson (24) found considerable amounts of linoleic and arachidonic acids in sphingomyelin, whereas Sweeley (37) reported none of these acids. The latter's results were confirmed in this study. Sweeley (37) attributed these discrepancies to the presence of lecithin in the sphingomyelin described by Nelson (24) or Hanahan (16).

Lysolecithin isolated in the present study contained more than twice the unsaturation found by Nelson (24). The composition of lysolecithin listed in Table I was confirmed by analysis of another source of human serum in this laboratory. Lysolecithin prepared enzymatically from serum lecithin by means of lecithinase A, has about one half the amount of unsaturated fatty acids of the lysolecithin isolated from serum.

The fatty aldehyde composition of the choline and ethanolamine plasmalogens is presented in Table II. The four major fatty aldehydes, myrist-, palmit-, stear-, and olealdehydes constituted about 80 mole % of total aldehydes in both the choline and the ethanolamine plasmalogen. The remainder was scattered among eleven other identified and two unknown aldehydes. Most of the aldehydes present in both choline and ethanolamine plasmalogens were saturated. Over half the total aldehydes in the choline plasmalogens could be accounted for by palmitaldehyde, whereas, a more even distribution of aldehydes was observed in the ethanolamine plasmalogen. The latter had a surprisingly high content of myristaldehyde.

TABLE II

Fatty Aldehyde Composition of Human Serum Phospholipids (Mole %)

	Plasmalogens	
Aldehyde	Choline	Ethanolamine
12:0	...	0.9
ISO 13:0	0.9	1.0
13:0	1.0	....
ISO 14:0	1.0	1.0
14:0	6.2	22.5
14:1	1.2	1.2
Unknown	...	1.3
15:0	0.4	2.5
ISO 16:0	4.5	3.2
16:0	51.2	16.9
16:1	2.2	1.8
Unknown	2.7	3.1
17:0	2.4	3.3
ISO 18:0	0.5	0.3
18:0	14.3	26.2
18:1	11.5	14.4
20.0	....	0.4
Saturated	82.4	78.2
Unsaturated	14.9	17.4
Unknown	2.7	4.4

The proportion of each individual phospholipid class in the serum total phospholipid is presented in Table III. The content of lecithin and sphingomyelin phosphorus is within the range of most recent reports for fasting individuals (6,14,24,26,28,30,32).

The proportion of lysolecithin phosphorus reported has varied widely, ranging from 3% (24) to 12% (26). The proportion of lysolecithin of 8.2% of the total lipid phosphorus found in the present studies is within the range of these observations. Likewise, the proportion of 4.2% for noncholine phospholipid phosphorus is in accord with the observations of most other workers (24,26,28,30,32). However, with the exception of the studies of Renkonen (30), the components of the noncholine fraction were not separated by other workers to a degree which permits comparison with the results of the present communication. Renkonen (30) reported somewhat higher levels of choline plasmalogen and phosphatidyl inositol than given in Table III. The same author isolated phosphatidyl ethanolamine and lysophosphatidyl ethanolamine in a ratio of 1:1 which is markedly different than the ratio of 3:1 found for this pair in the present study. Also, Renkonen (30) found that ethanolamine plasmalogen phosphorus was only 7% of all the phospholipids containing ethanolamine; whereas in the present study, the ethanolamine plasmalogen represented over 50% of the phosphorus of the phospholipids containing ethanolamine. The content of phosphatidyl inositol phosphorus is within the range of other reports (26,30).

TABLE III

Human Serum Phospholipids

Name	Phosphorus		Phospholipid (wt %)
	Percent [a]	Factor	
Lecithin	69.6	26.2	73.0
Choline plasmalogen	0.8	24.9	0.8
Lysolecithin	8.2	17.5	5.8
Sphingomyelin	17.2	23.4	16.2
Phosphatidyl ethanolamine	1.0	25.0	1.0
Ethanalamine plasmalogen	1.4	23.3	1.3
Lysophosphatidyl ethanolamine	0.3	16.0	0.2
Phosphatidyl serine	0.3	25.7	0.3
Phosphatidyl inositol	1.2	29.2	1.4

[a] Percent of total lipid phosphorus.

The fatty acid composition of these phospholipids is given in Table I, and the fatty aldehyde content of the plasmalogens in Table II.

The relative proportions of the individual phospholipids in the serum total phospholipids also are expressed in Table III on a weight % basis, using a factor for each individual phospholipid class which was calculated from the actual fatty acid and fatty aldehyde composition of that class. This factor ranged from 16.0 with lysophosphatidyl ethanolamine to 29.2 for phosphatidyl inositol. The average factor, weighed for percent phosphorus for the three major phospholipids: lecithin, sphingomyelin, and lysolecithin, is 24.9. The average factor, calculated in the same way for the serum total phospholipids, is 25.0 which is identical with the factor usually employed to convert serum lipid phosphorus to a weight basis.

On a weight basis, as seen in Table III, lecithin, sphingomyelin, and lysolecithin account for 95% of the total phospholipid. The ethanolamine containing phospholipids add up to 2.5% and the remainder is divided among phosphoinositol, choline plasmalogen, and phosphatidyl serine. As far as we are aware, this is the first time that serum phosphatidyl serine has been isolated as a separate phospholipid component and its fatty acid composition studied.

The large disproportionality of the content of the individual serum phospholipid classes causes difficulty in detecting on thin-layer plates phospholipids such as phosphatidyl serine and lysophosphatidyl ethanolamine. Both of these phospholipids have R_f values in the vicinity of lecithin, the level of which is several hundredfold that of either of these two cephalins. Fractionation of lipids on the columns involves the contact of phospholipids with adsorbents which might cause the breakdown of the original phospholipids and, thereby, produce artifacts. The decomposition of cephalin plasmalogen on silicic acid column to form lysocephalin has been reported (13) and breakdown of lecithin to lysolecithin on aluminum oxide column has been observed (29).

The question arises as to whether the lysophosphatidyl ethanolamine isolated in the present study was such an artifact. This phospholipid was detected initially in the effluent from the first silicic acid column used with the lipid extract (Fig. 1, Region B). The formation of lysophosphatidyl ethanolamine was not observed during the separation of the major part of the phosphatidyl ethanolamine from phosphatidyl inositol (Fig. 1, Region A) on an aluminum oxide column. Furthermore, this study and those of others (10) have shown that plasmalogen fatty acids are primarily unsaturated, and isolated lysophosphatidyl ethanolamine showed only 43 mole % unsaturated acid. In addition, a plasma phospholipase which catalyzes the conversion of phosphatidyl ethanolamine to the lyso compound has been described (39). Lysophosphatidyl ethanolamine has been detected in plasma and serum by paper chromatography (23,26) and determined by column chromatography (30). All these facts support the conclusion that lysophosphatidyl ethanolamine is a native constituent of human serum.

Other workers have presented evidence that lysolecithin is a naturally occurring lipid in human serum (6,14,24,26,28,30,32). The presence in such serum of an enzyme which transfers a fatty acid moiety from lecithin to cholesterol with formation of cholesterol esters and lysolecithin has been noted (9). A small part of lecithin (Fig. 1, major fraction B) was recovered by additional fractionation on an aluminum oxide column, but there was no lysolecithin detected in the eluted fraction. Since the formation of lysolecithin from lecithin depends primarily upon time of contact of lecithin with aluminum oxide (29), and lecithin was eluted quickly from the column as an initial sharp peak, decomposition of this part of the lecithin undoubtedly was prevented.

Finally, the observations made in this study are in agreement with the conclusion of Renkonen (30) that claims of the instability of phospholipids during column chromatography seem somewhat exaggerated, although the lability of these lipids under these conditions cannot be neglected.

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[Received Sept. 13, 1965]

Determination of the Specific Positions of *cis* and *trans* Double Bonds in Polyenes[1]

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ABSTRACT

A method is described for the determination of the positions and geometric configurations of double bonds in polyunsaturated fatty acids. The procedure consists of three steps:

1) Partial reduction of the double bonds with hydrazine under conditions which give high yields of monoenes.
2) Isolation of the *cis*- and the *trans*-monoene fractions by thin-layer chromatography (TLC) directly or in the form of their ozonide derivatives. In the former technique, selective argentation is employed, in the latter, silicic acid adsorption.
3) Determination of the structure of the monoenes via reductive ozonolysis.

The position of the double bonds is determined from the structures of the monoenes. Since the *cis*-monoenes are separated from the *trans*-monoenes the geometric configuration of each double bond is determined.

The method also provides a direct determination of the spacings of the internal double bonds and it may be employed for the determination of the structures of mixtures of fatty acids in conjunction with direct ozonolysis procedures. The various ramifications of the method are demonstrated on pure fatty acids and model mixtures thereof.

INTRODUCTION

ALTHOUGH FATTY ACIDS containing *trans* double bonds may be detected and determined quantitatively by infrared spectroscopy (1) or by gas-liquid chromatography (GLC) with capillary columns (2), these methods do not permit the determination of the specific positional isomers of *cis-trans*-polyenes. Neither do degradative methods for the determination of the structure of polyenes give the location of the specific positions of *trans* and *cis* double bonds.

Recently the course and mechanism of the homogeneous reduction of double bonds with hydrazine (3–10), cobalt carbonyl (11) and iron pentacarbonyl (12), and catalytic hydrogenation of several monoenoic fatty acids (13) were studied via determination of the positional and geometric isomer composition of the fatty acids at various stages in the reduction. In these studies the composition of the products of the reaction was determined by a combination of fractionation techniques and localization of the double bonds by the periodate-permanganate method (14,15). Scholfield et al. (15A) determined the geometric isomer composition of linolenate isomerized with selenium or nitrous acid, and Hopkins and Chisholm (16) elucidated the structure of *trans*-3, *cis*-9, *cis*-12, *cis*-15-octadecatetraenoic acid by a similar combination of procedures. Stearns and Quackenbush (17) described a novel technique for the determination of the positions of radioactive carbon atoms in unsaturated fatty acid chains based on a partial catalytic hydrogenation which effects the widest possible shift in double bonds, isolation of the partially reduced esters and determination of the radioactivity in the fragments given by reductive ozonolysis.

Our method for the determination of specific positions of *cis* and *trans* double bonds involves partial reduction with hydrazine without isomerism of double bonds, separation of *cis* and *trans* monoenoates, and location of double bonds by reductive ozonolysis. The reduction is carried out as near as possible to the maximum yield of monoenoic fatty acids. The principle of the method is to produce a monoenoic fatty acid for each double bond in the molecule. Isolation and determination of the structure of each monoenoic fatty acid enables one to elucidate the structure of the original molecule. The sequence of the steps in the procedure may be enumerated as: 1) partial hydrazine reduction, 2) esterification, 3) isolation of *cis*- and *trans*-monoenoate esters by TLC via selective argentation, and 4) determination of the position of the double bond in the isolated *cis*- and *trans*-monoenoate esters by reductive ozonolysis. The positions of the *cis* double bonds in the original esters are deduced from the structures of the isolated *cis* monoenoates and the positions of the *trans* double bonds are

[1] Presented at the AOCS Meeting, Houston, April 1965.

deduced from the structures of the isolated *trans* monoenoates.

EXPERIMENTAL

Materials

Highly purified methyl oleate, elaidate, petroselinate, vaccenate, nervonate, linoleate, linoelaidate, *cis*-11,*cis*-14-eicosadienoate, erucate, linolenate and arachidonate were obtained from The Hormel Institute. GLC analysis of these compounds indicated that they contained less than 1% impurities. Thin-layer chromatography (TLC) with Silica Gel G impregnated with silver nitrate, prepared as described by Barrett et al. (18), also showed that they were pure. Chromatoplates of the TLC analysis of a number of these compounds are shown in connection with various experiments in the results.

Procedure

Partial Reduction with Hydrazine. A sample of 5 to 100 mg of fatty acid is dissolved in 100 volumes of a 10% (V/V) methanol solution of hydrazine hydrate (Eastman Organic Chemicals) and stirred vigorously by means of a magnetic stirrer in the appropriate size test tube or Erlenmeyer flask. The reduction is continued until as near as possible to the maximum amount of monoene is produced. The degree of agitation and temperature are important factors that influence the rate of reduction of double bonds with hydrazine. Concentrations of monoenes of the order of about 50% can be obtained regardless of the degree of unsaturation of the parent fatty acid. We usually carry out the reaction at 36C and allow about 1.5 to 2 hr for dienes. Corresponding longer periods are required for more unsaturated fatty acids. The reaction may, of course, be speeded up by carrying it out at higher temperatures. A few pilot experiments should be made under a standardized set of conditions to establish the optimum reaction time for the production of about 50% monoene.

The reaction is stopped at the predetermined time and the fatty acids are converted to methyl esters by heating them with dry methanolic HCl (6% HCl w/w). For small samples, that is, of the order of 5 mg, the solution of partially reduced acids is added to about 3 ml of the dry methanolic HCl (6% w/w) in a 16 × 150 mm test tube with a constricted neck for sealing. After the tube is flushed with nitrogen, it is sealed with a torch and esterification of the acids is effected by heating the ampule in a boiling water bath for about 1 hr.

Larger samples are usually neutralized with acid, extracted into petroleum ether, dried with anhydrous sodium sulfate, recovered by evaporation of the solvent and then esterified with methanol.

Isolation of cis- and trans-monoenes. The solution of methyl esters is usually evaporated to near dryness, and dissolved in about 0.5 ml of low boiling petroleum ether for fractionation by TLC. As much as 10 mg of sample may be fractionated on a 20 × 20 cm chromatoplate coated with Silica Gel G (Merck, A. G., Darmstadt, Germany) or porous glass containing 10% calcium sulfate (Corning Glass Works, Corning, N.Y.) impregnated with silver nitrate and obtain a complete separation of the *cis*- and *trans*-monoene fractions. The separated bands are made visible under ultraviolet light by spraying the plate with 2,7-dichlorofluorescein, marked with a pencil or similar sharp object and scraped into a small (125 ml) separatory funnel containing about 60 ml of a mixture of ethyl ether and petroleum ether (1:1, v/v). The solution of esters and the adsorbent are shaken with about 40 ml of 1% aqueous HCl and then washed with distilled water several times. Finally, the solvent layer is separated, dried with anhydrous sodium sulfate and filtered. The solvent is evaporated and the residue of esters is reextracted with dry pentane. Traces of indicator which may still contaminate the sample do not dissolve in the dry pentane.

Figure 1 shows the TLC analyses of the products of the partial reduction of a number of model fatty acids (in the form of methyl esters) by the above technique. These results show that in addition to separation of the *cis*- and *trans*-monoenoate esters some separation of positional isomers contained in these fractions also occurs.

Preparation and Purification of Ozonides. The pentane solution of each group of esters is reduced in volume to about 1 ml, cooled to a temperature just above that where crystallization occurs and poured into 10 ml of pentane saturated with ozone at about −65C. The pentane solution of ozone is prepared as previously described (19). Ozonization is virtually instantaneous under these conditions and after a reaction time of about 1 min the excess ozone and dissolved oxygen are removed as the solution is evaporated to about 0.5 ml under reduced pressure by means of a water aspirator. The solution of ozonides is then applied to the base of a 20 × 20 cm chromatoplate coated with Silica Gel G, and developed with low boiling petroleum ether con-

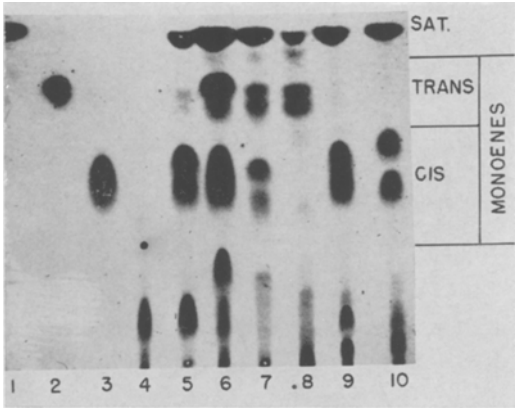

FIG. 1. Chromatoplate of the silver nitrate-TLC of reference methyl esters and products of the partial hydrazine reduction of model fatty acids via a solvent system of 0.75% methanol in chloroform: *1*, methyl stearate; *2*, methyl elaidate; *3*, methyl oleate; *4*, methyl linoleate; *5*, partially reduced methyl linoleate; *6*, partially reduced mixture of methyl linoleate and methyl *cis-9,trans*-12-octadecadienoate; *7*, partially reduced mixture of methyl linoleate and methyl linoelaidate; *8*, partially reduced methyl linoelaidate; *9*, partially reduced methyl linolenate; *10*, partially reduced methyl arachidonate.

taining 10% diethyl ether. The bands of separated ozonides are made visible by spraying the plate with 2,7-dichlorofluorescein and viewing it under ultraviolet light. Figure 2 shows a chromatoplate of the TLC of the ozonides of monoenoate, dienoate and trienoate esters under these conditions. The spots in Figure 2 were made visible by charring. Chromatography of the ozonides not only effects their purification for the subsequent reduction but also provides a means of separation of *trans, trans*-dienes which sometimes cannot be separated completely from *cis*-monoenes via silver nitrate-TLC.

Catalytic Reduction of Ozonides. Ozonides may be reduced in a variety of solvents. We usually use methylene chloride but ethyl chloride that has a boiling point of 12C may be employed for the analysis of very short chain fragments. For the reduction, the ozonides may be scraped from the chromatoplate directly into a 15 ml conical centrifuge tube containing a Teflon coated bar (10 × 3 mm, Cole Parmer Instrument Equipment Company) for magnetic stirring. Approximately 1 ml of solvent and 10–25 mg of Lindlar catalyst (20) are added to the tube that is then alternately evacuated and filled, first with nitrogen and then with hydrogen. During this operation the tube is immersed in a beaker with Dry Ice and acetone to cool it to about −70C to prevent evaporation of the solvent and premature reduction of the sample. Finally, the solution is maintained under about 2 lb of hydrogen pressure and stirred vigorously to give a very fine suspension of the catalyst throughout it. The temperature of the solution is raised to about 10C by placing the tube in a water bath. About ½ hr at 10C is allowed for the completion of the reduction, then the hydrogen atmosphere is replaced with nitrogen by means of a two-way stopcock and the products of the reaction are analyzed. Completion of the reaction may be determined by TLC using plates coated with Silica Gel G and a solvent system of 10% diethyl ether in low boiling petroleum ether.

The products of the reaction, the aldehydes and aldesters, are analyzed by GLC by the following procedure. A portion of the solution (or all of it, if the sample is small) containing the catalyst dispersed in it is withdrawn into the needle of a syringe (50 μl or 100 μl, depending on the size of the sample) and emptied into a small glass tube of about 25 × 2 mm (I.D.) loosely packed with glass wool to provide a large surface area for evaporation of the solvent. Most of the solvent evaporates in a minute or two at room temperature, especially when ethyl chloride is used. Then the tube is placed directly in the top of the column of a GLC instrument. The gas is turned off and the pressure released prior to introduction of the sample on the column to prevent blow out of part of the sample and loosening of the column packing. The tube is removed at the finish of the analysis by means of a small pair of tweezers. Most analyses may be carried out with a column packed with Chromasorb W as the inert support coated with 30% weight of silicone (14,21) and with the temperature programmed from 60 to 190C. When overlapping of aldehydes with aldesters is suspected (by the size and shape of the peak) an analysis may also be made on a column packed with 15% ethylene glycol succinate polyester. The temperature of the column in this analysis is also generally programmed from 60 to 190C in order to detect both the long and the short chain compounds.

The analysis of the products of the reductive ozonolysis was carried out with a Packard gas chromatograph. This instrument was equipped with dual columns and β-ionization detectors (150 mc tritium). Identification of the simple aldehydes was made by comparison

with a standard mixture of these compounds, with chain lengths from 3 to 12 carbon atoms, obtained from commercial sources. The aldesters were identified from a mixture of these compounds obtained by the reductive ozonolysis of a mixture of pure methyl petrolselinate oleate, vaccenate, erucate and nervonate. These esters yielded methyl aldehydates with chain lengths of 6, 9, 11, 13 and 15 carbon atoms, respectively.

The retention time of aldesters relative to aldehydes is much greater on a polyester column than on a silicone column. Thus any overlapping of these compounds with the silicone column can be readily distinguished by a second analysis on a polyester column. Generally, aldehydes have the same retention time as aldesters with three less carbon atoms in the chain on a silicone column. For example, nonanal generally has the same retention time as methyl adipaldehydate. The spread of the overlapping on ethylene glycol succinate polyester column generally is about seven carbon atoms in the chain. That is, overlapping might be expected between tridecanal and methyl adipaldehydate.

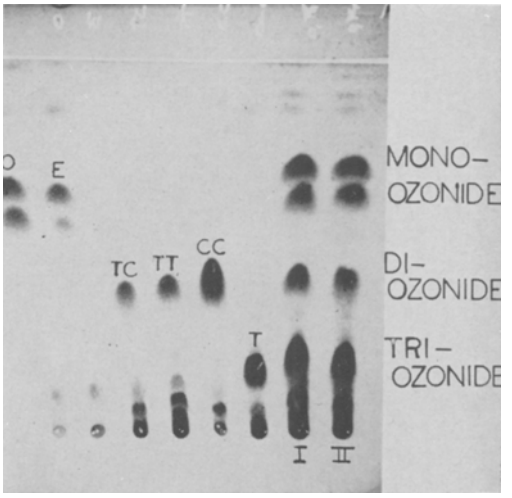

FIG. 2. Chromatoplate of the TLC of the products of the ozonization of methyl oleate (O), methyl elaidate (E), methyl *trans*-9,*cis*-12-octadecadienoate (TC), methyl *trans*-9,*trans*-12-octadecadienoate (TT), methyl linoleate (CC), methyl linolenate (T), I. mixture of O, TT, T. II. mixture of O, TC, T. Development with 10% ethyl ether in petroleum ether on preextracted Silica Gel G.

RESULTS AND DISCUSSION

In order to test the methods, it was applied to a number of fatty acids of known structures and certain mixtures of fatty acids. One such mixture, which demonstrated the method well, consisted of *cis*-11,*cis*-14-eicosadienoic acid and linoelaidic acid. This mixture of acids should yield *trans*-9-octadecenoic and *trans*-12-octadecenoic acids from linoelaidic acid, and *cis*-11-eicosenoic and *cis*-14-eicosenoic acid from *cis*-11, *cis*-14-eicosadienoic acid. Since the *cis*-monoenes are derived from the 20 carbon chain diene and the *trans*-monoenes are derived from the 18 carbon chain diene, the separation of the *cis*-monoenes from the *trans*-monoenes by TLC could be readily demonstrated by GLC as shown in Figure 3.

Should *trans,trans*-dienes not be completely separated from the *cis*-monoenes by argentation techniques, they may be separated in the form of their ozonides because diozonides can be readily separated from monoozonides via silicic acid adsorption as illustrated in Figure 2.

The analysis of the fragments obtained on the reductive ozonolysis of the *trans*- and *cis*-monoenoate fractions isolated by TLC is shown in Figure 4 (curve B and C, respectively). These analyses correspond to the GLC analysis of the parent monoenes (Fig. 3). The hexanal (6A, curve B, Fig. 4) and methyl dodecanoaldehydate (12AE, curve B, Fig. 4) arise from methyl *trans*-12-octadecenoate, the nonanal (9A, curve B, Fig. 4) and methyl azelaldehydate (9AE, curve B, Fig. 4) arise from methyl *trans*-9-octadecenoate constituting the two monoenes of the *trans*-monoene fractions. The hexanal (6A, curve C, Fig. 4) and the methyl tetradecanoaldehydate (14AE, curve C, Fig. 4) arises from methyl *cis*-14-eicosenoate, the nonanal (9AE, curve C, Fig. 4) and the undecanoaldehydate (11AE, curve C, Fig. 4) arise from methyl *cis*-11-eicosenoate constituting the two monoenes of the *cis*-monoene fraction.

As a demonstration of the method for the determination of internal double bond structure, it was applied to arachidonic acid. This acid should give four positional *cis*-isomers of eicosenoic acid on partial reduction with hydrazine, namely those with double bonds in the 5, 8, 11 and 14 positions. These, in turn, should give, on reductive ozonolysis of the corresponding methyl ester, one aldehyde and one aldester each corresponding to the position of the double bond.

The GLC analysis of the fragments of the reductive ozonolysis of the mixture of monoenes isolated via silver nitrate-TLC is also shown in Figure 4 (Curve A). In this analysis the following aldehyde and aldesters pair up to give the parent 20 carbon monoenes, 5AE and 15A, 8AE and 12A, 11AE and 9A, and 14AE

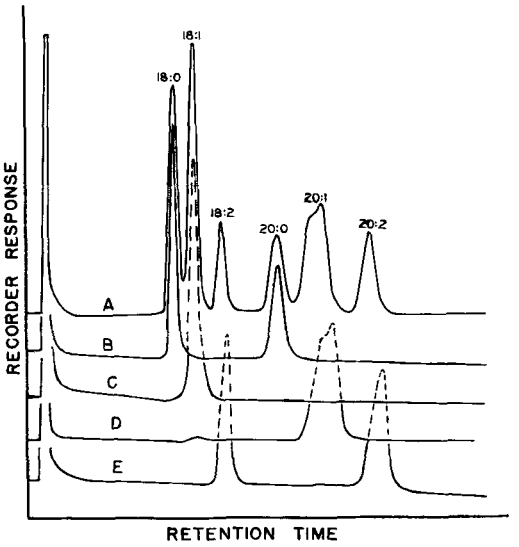

FIG. 3. Chromatograms of the GLC analysis of methyl ester of: (A) the products of the partial reduction of a 1:1 mixture of linoelaidic and *cis*-11,*cis*-14-eicosadienoic acids, (B) the saturated esters separated from (A), (C) the *trans*-monoene fraction separated from (A), (D) the *cis*-monoene fraction separated from (A), (E) the unreacted diene fraction separated from (A). Conditions: 15% ethylene glycol succinate polyester phase at 185C.

and 6A. These arise from the 5, 8, 11 and 14 eicosenoate isomers corresponding to the positions of the 4 double bonds in methyl arachidonate.

In order to demonstrate the method on a fatty acid containing both *cis* and *trans* double bonds in the same molecule, the method was applied to *cis*-9,*trans*-12-octadecadienoic acid. This acid was prepared from dehydrated methyl ricinoleate as described by Jackson et al. (22). Final purification was made by TLC employing plates coated with Silica Gel G impregnated with silver nitrate as described by Barret et al. (18). No geometric isomer impurities could be detected in the final preparation. However, a direct structural analysis of this sample as described by the authors (19) showed that it contained traces of an isomer with double bonds in the 8 and 11 positions. The results of the analysis of the *cis*- and the *trans*-monoene fractions obtained from this preparation are shown in Figure 5. These results showed that in addition to fragments expected from monoenes which originate from the major isomer, *cis*-9,*trans*-12-octadecenoate (methyl *cis*-9-, and methyl *trans*-12-octadecenoate), this sample contained a small amount of *cis*-8-,

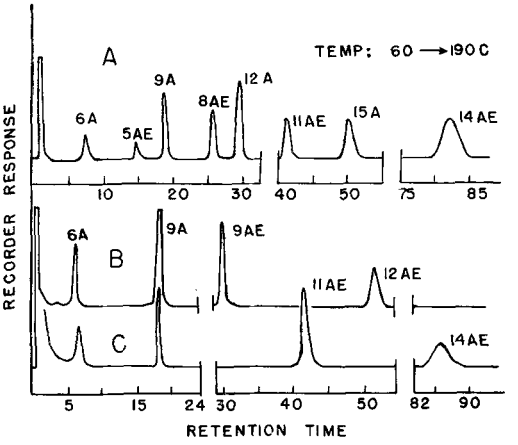

FIG. 4. Chromatograms of the GLC of the products of the reductive ozonolysis of A, the monoene fraction obtained from the partial reduction of methyl arachidonate. B and C are, respectively, the products of the reductive ozonolysis of the *trans*-monoene and *cis*-monoene fractions isolated from the partial reduction of a mixture of methyl linoelaidate and methyl 11,14-eicosadienoate. Conditions: 30% silicone phase temperature programmed 60–190C.

trans-11 and *trans*-8, *cis*-11 isomers as indicated by the near equal distribution of fragments from monoenes expected of this isomer (8, 11–18:2) in both the *cis*- and *trans*-monoene fractions. Since the original preparation was purified of *trans, trans*- and *cis, cis*-diene, it obviously could not contain any *trans, trans* or *cis, cis* isomers of either of the positional isomers. That minor amounts of positional isomers other than the expected 9, 12 isomer may be formed is indicated by recent studies on the dehydration of ricinoleic acid by Body and Shorland (23).

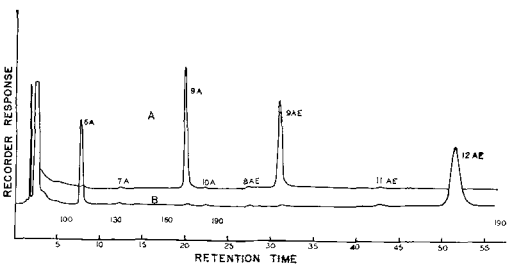

FIG. 5. Chromatograms of the GLC of the products of the reductive ozonolysis of the monoenes isolated from the partial reduction of methyl *cis*-9,*trans*-12-octadecadienoate preparation. A = *cis*-monoene fraction. B = *trans*-monoene fraction. Conditions: 30% silicone phase, temperature programmed from 60–190C.

Theoretically the method should be applicable to fatty acids with any type or combination of types of polyunsaturation, methylene interrupted, isolated or conjugated. The only factor militating against its unqualified use is the possibility that the reduction may be selective for certain double bonds. There apparently are some differences in the rate of reduction of double bonds in different types of structures with hydrazine, as for example, conjugated versus nonconjugated systems (4,9). However, only large differences in the rate of reduction of double bonds would have a significant effect on the method for identification purposes. In fact, selectivity of the reduction could work to advantage in some cases, but it could be a complicating factor in application of the method for quantitative analysis if it is not recognized. Quantitative analysis may be made by the usual reductive ozonolysis procedures described by us (19,21) employing the technique of quantification of the peak area by GLC reported by Ackman et al. (24). Since a reductive ozonolysis by the usual procedure can be carried out on an ultramicro scale (21), a number of advantages in addition to quantification accrue from the use of simple reductive ozonolysis and the procedure described here especially for the analysis of complex mixtures. For example, since double bonds nearest the carboxyl group always give an aldester fragment the number of fatty acids in a mixture can be determined from the number of aldesters by the usual method of reductive ozonolysis. The application of the two techniques provides the basis upon which complex mixtures of fatty acid esters can be determined because they provide information on the chain length of the parent acid, the positions of the two outer double bonds as well as the configuration of all of the double bonds.

ACKNOWLEDGMENT

Supported in part by USPHS Grant AM 05018 from the National Institutes of Health.

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[Received July 1, 1965]

An Electrostatic Precipitator for Preparative Gas-Liquid Chromatography

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ABSTRACT

The effect of the operating variables of electrostatic precipitators on the recovery and structure of methyl esters and related aerosol forming compounds collected in preparative gas-liquid chromatography was studied.

Aerosol formation was prevented by AC or DC voltages of 5000 to 12000 volts. AC was more effective than DC but caused changes in structure which were detectable by both thin-layer and gas-liquid chromatographic methods of analysis.

An apparatus of simple construction and operation was designed for the collection of methyl esters and its use demonstrated with several model compounds.

INTRODUCTION

ELECTROSTATIC PRECIPITATION (1) was first applied to preparative gas-liquid chromatography (GLC) for the collection of samples by Atkinson and Tuey (2). In the course of the development of this technique, modifications in the apparatus have been described by Kratz et al. (3), Thompson (4), Snelson (5) and Ross et al. (6). These workers have shown that aerosol formation which is the main factor resulting in low recoveries in preparative GLC can be broken by voltages of greater than 3400 volts.

Both AC and DC voltages have been employed in electrostatic precipitators and no specific recommendation regarding their use has been made except that AC has been reported to be more effective than DC in the prevention of aerosol formation.

Since high voltages are known to cause changes in the structures of organic compounds (7), we made a study of the operating variables of electrostatic precipitators for preparative GLC and designed a simple apparatus for the collection of methyl esters and related compounds.

EXPERIMENTAL

Materials

Hexadecane >99% purity was obtained from Matheson, Coleman and Bell. Highly purified (>99%) stearic acid, methyl oleate, methyl linoleate, methyl linolenate, methyl palmitate, palmityl alcohol, methyl laurate, capryl alcohol and caprylic acid were obtained from The Hormel Institute, Austin, Minnesota.

Apparatus

The gas chromatograph used throughout this work was an F & M Model 500 equipped with a thermal conductivity detector and 7 ft × ¼ in. column packed with 10% (w/w) ethylene glycol succinate polymer on 100–120 mesh Chromosorb P. Helium was used as the carrier gas at 75 ml/min. The column and block were operated at temperatures of 185 and 250C, respectively.

The electrostatic precipitator designed for this study is shown in Figure 1.

A schematic diagram of the apparatus is shown in Figure 2. It has two main features: 1) It is of very simple construction. 2) It is simple to operate as it can be quickly and easily disassembled. Some of the construction features of the apparatus are as follows: The inner electrode consists of a 35 cm × 1.5 mm *o.d.* steel wire inside a glass tube. The outer electrode consists of a 1/16 in. sheet of aluminum foil (28 × 13 cm) and it is wrapped around a glass tube (44 × 0.9 cm) and fastened by plastic tape. The distance between the two

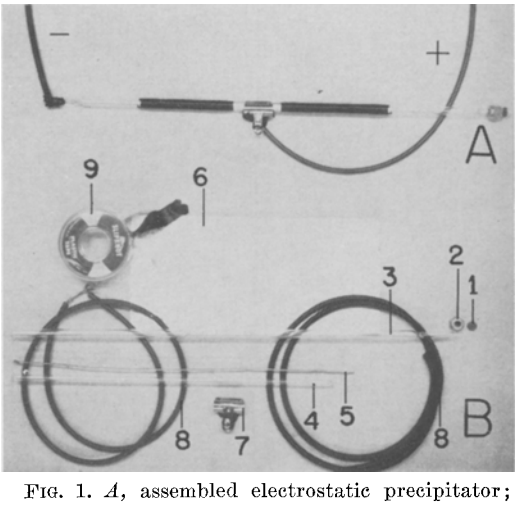

FIG. 1. *A*, assembled electrostatic precipitator; *B*, the component parts of A; *1*, silicone rubber disk; *2*, nut; *3*, outer glass tube; *4*, inner glass tube; *5*, inner electrode; *6*, aluminum sheet; *7*, clamp; *8*, insulated wires; *9*, plastic tape.

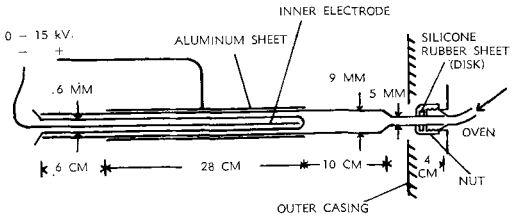

FIG. 2. Schematic diagram of electrostatic precipitator.

glass tubes is narrow (0.5 mm) to make the passageway for the aerosol narrow and at the same time provide a large surface area of electrode per volume of the passageway. The steel wire can be readily withdrawn from the tube within which it is placed and the wire from the transformed to the outer electrode can also be readily removed to permit the quick (few seconds) replacement of another collection unit A (Fig. 1).

For the collection of samples the outer tube of the unit is inserted through the septum of the outlet of the gas chromatograph until it comes into contact with the end of the column.

Experiments were carried out with both alternating and direct current. For the alternating current experiments the precipitator was connected to an AC transformer with an input of 115 v and an output of 15 kv (30 ma). The input voltage for the high voltage transformer was regulated by means of a variable transformer which made it possible to produce voltages from zero to 15,000.

DC voltages were obtained by means of a DC power supply with an input of 118 AC and an output of 15 kv at 1.5 ma DC (Model No. HV150-152M, Plastic Capacitors Inc., Chicago, Ill.). It was also connected to a variable transformer to provide a range of voltages.

RESULTS

In the first experiments the voltage required to prevent aerosol formation was determined by observation of the smoke or fog which emerged from the outlet of the precipitator when it was alternately turned on and off. The results for a variety of compounds of different structures are shown in Table I. In accordance with the observation of Kratz et al. (3) AC voltage was more effective in the prevention of aerosol formation than DC voltage.

Recovery Experiments

In order to eliminate spurious results from column bleed the amount of recovery was measured by analytical GLC using an internal standard. In this procedure the amount of sample collected was determined by dissolving it in a known volume of a standard solution of the compound used as an internal standard, and comparing the peak areas of the standard with that of the collected substance. The results of the recovery of several methyl esters via the electrostatic precipitation is compared to gradient cooling condensation which is superior to cold trap precipitation in Table II. The results (Table II) show that the recoveries of aerosol-forming compounds average about 96%. These experiments illustrated the application of the apparatus to analytical separations obtained with the conventional ¼ in. column. Essentially 100% recoveries may be obtained with large diameter preparative columns because the actual loss of material appears to be about the same, regardless of the amount of sample applied to the column.

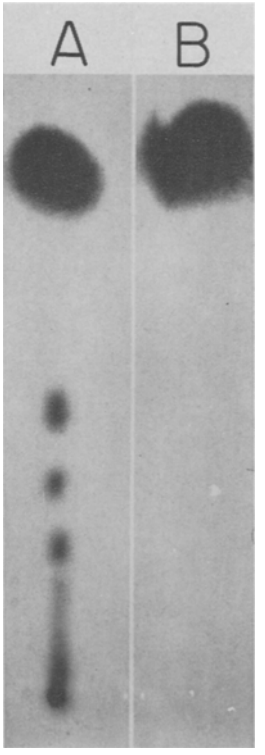

FIG. 3. Thin-layer chromatoplate, showing methyl oleate, treated with high voltage. *A*, the effect of AC high voltage; *B*, the effect of DC high voltage. Coating: Silica gel G, developed in a mixture of petroleum ether (30–60C), ethyl ether (90:10). Charred at 180C after spraying with 70% sulfuric acid (v/v), saturated with potassium dichromate.

TABLE I

Precipitation of Aerosol-Forming Compounds

Compound	AC voltage	DC voltage
Stearic acid	5500	12000
Methyl oleate	5500	8200
Methyl linoleate	5500	9500
Methyl linolenate	6800	11000
Hexadecane	6500	10200
Methyl palmitate	6100	12300
Palmityl alcohol	5700	7500
Methyl laurate	6100	10200
Capryl alcohol	5500	8200
Caprylic acid	5500	10600

Structural Alteration of Compounds

When direct current was employed, aerosol formation could be prevented without any evidence of structural alteration to the compounds.

Alternating current caused changes with all compounds. Changes could be detected with some compounds at voltages below that required to break aerosol formation. The alteration in structure was determined mainly by thin-layer chromatography (TLC). The chromatoplate in Figure 3 shows the analysis of methyl oleate collected via AC and DC voltages. In no case could alternating current be employed without evidence of some structural alteration of the collected compounds. In addition to nonvolatile products, gases such as methane, ethane are generally formed. The mechanism and products of discharge reactions of the type encountered in electrostatic precipitators using AC current will be described in more detail in a separate report by the authors (8).

DISCUSSION

Low recoveries of compounds encountered in preparative GLC because of aerosol formation may be greatly improved by the use of electrostatic precipitation techniques. With long-chain methyl esters no other devices are required to attain recoveries of the order of 95%, and when DC voltages are employed no alteration in the structure of the collected samples occurs. AC voltage caused changes in structure of methyl oleate via discharge reactions. The structural changes in this and related compounds may be readily detected by TLC. Polar compounds are formed as well as short-chain hydrocarbons which may be detected by GLC as demonstrated in further work from this laboratory (8).

TABLE II

Recovery of Methyl Esters via Electrostatic Precipitation Technique

Compound	Remark	% Recovery by only condensation	% Recovery by condensation and electrostatic precipitation
1 μl Methyl caprylate	No visible aerosol	87.0	90.1
1 μl Methyl oleate	Aerosol	88.6	96.1
1 μl Methyl arachidonate	Aerosol	79.1	96.3

In addition to the use of DC voltage there are certain other features which should be considered in the design of a precipitator for preparative GLC. These may be enumerated as follows:

1. Since high voltages are employed, the apparatus should be constructed to minimize the hazard of electrical shock.
2. The unit should permit the collection of multiples of samples in a short period of time.
3. The design should attain a gradient cooling of the emerging vapors before they enter the electrical field to decrease the amount and stability of the aerosol.
4. The apparatus should be simple to permit fast assembly and cleaning.
5. Finally, it should be pointed out that since discharge reactions generally give rise to polymers and highly volatile products (among others), GLC is not a good method for the detection of structural changes unless conditions are used to detect these types of compounds.

ACKNOWLEDGMENTS

Supported by ARS, USDA Grant No. 12–14–100–1653 (73) and by US Public Health Service, National Institutes of Health, Grant No. AM-05018.

One of us, L. Borka, is indebted to the Norwegian Research Council for Sciences and Humanities and the Alf Bjerckes Foundation, Oslo, Norway, for a travel fellowship.

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[Received Aug. 30, 1965]

Laboratory Contaminants in Lipid Chemistry: Detection by Thin-Layer Chromatography and Infrared Spectrophotometry and Some Procedures Minimizing Their Occurrence

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ABSTRACT

Many sources of contamination for lipid preparations exist in the laboratory. These contaminants can be detected using thin-layer chromatography (TLC) and infrared spectroscopy. Numerous components that are potential contaminants and can lead to false analyses were demonstrated by TLC in laboratory soaps, cleaners, hand creams and lotions, hair tonics, laboratory greases, floor waxes, oil vapors, tobacco smoke, hydrocarbon phases for gas-liquid chromatography, etc. Procedures preventing introduction of contaminants are presented including descriptions of equipment and precautions to eliminate or minimize contamination. These are useful in isolation of pure polar and nonpolar lipids.

INTRODUCTION

AN ACCURATE ANALYSIS of lipids from biological samples depends upon the rigorous elimination of artifacts and contaminants. Sources of contaminants are numerous and the ease with which they can be introduced into preparations is often overlooked. In our experience, this is particularly true when new personnel are being trained in the laboratory.

Lipids and other solvent soluble contaminants may come from silicone greases and other lubricants, wax pencils, hand lotions, hair preparations, rubber and cork stoppers, tobacco smoke, pump oils, detergents, floor waxes, chromatographic adsorbents, and from solvents themselves. Since it is impractical to remove completely all contaminant sources from the laboratory, the lipid chemist needs rapid methods to detect such contaminants and procedures which will minimize or prevent their introduction into preparations. This paper describes such methods and procedures.

MATERIALS AND METHODS

Methods of solvent evaporation and techniques for working under nitrogen have been described previously (1,2).

Collection of Laboratory Contaminants

Several preparations of some common classes of potential contaminant were obtained. Selection was based upon ready availability and common use. Thus five hair preparations, five hand creams and lotions, four laboratory greases, six hand (toilet) soaps, three floor waxes, two brands of wax marking pencils, four laboratory detergents, and several hydrocarbon phases for gas-liquid chromatography (GLC) were examined. The solutions for TLC (2–10 mg/ml) were prepared by weighing the solid or waxy preparations (without drying) and dissolving in chloroform/methanol 2/1. Complete solution of some samples required the addition of more methanol and/or the addition of a small amount of water. The liquid floor waxes were diluted directly with solvent to prepare spotting solutions.

Fresh rubber and cork stoppers were cut into small pieces and extracted at room temperature with chloroform/methanol 2/1 overnight. The clear filtrates were evaporated to dryness, weighed and dissolved in chloroform/methanol 2/1 for TLC.

Tobacco smoke from cigars and cigarettes was collected in chloroform/methanol 2/1. Solvent (250 ml) was placed in a 500 ml side-arm flask fitted with a solvent washed rubber stopper through which passed a glass tube with one end immersed in the solvent. The portion of the glass tube above the stopper was made approximately 10 in. long with three bends in it to facilitate trapping of droplets and bits of solid tobacco. Cigars and cigarettes were smoked in the usual manner without inhaling and the smoke was blown through the solvent layer. Clear yellowish-brown solutions were obtained, evaporated to dryness in a rotary evaporator, the residue weighed and dissolved in chloroform/methanol 2/1. Many other potential sources of contamination were examined but are not presented. An attempt is made only to present illustrations of common sources of problems and methods for study of others.

Qualitative TLC for Contaminant Identification

TLC was carried out with adsorbent composed of 9 parts of silicic acid (Silica Gel plain) mixed with one part of finely powdered magnesium silicate and the adsorbent was spread, heat-activated, and samples applied as described previously (3).

Developing solvents of graded polarity were used for TLC as shown in Table I. Many solvent ratios other than those shown are useful, but the seven systems used are satisfactory for comparisons of many products and general detection of potential contaminants. The presence of an acidic substance on TLC is frequently disclosed by comparing chromatographic runs using first a solvent containing acid then the same or similar solvent without acid. The presence of acid in a solvent depresses dissociation of acidic substances on TLC causing them to migrate as less polar compounds and places them in different positions on chromatograms relative to nonacidic substances.

TLC Spray Reagents

Substances separated by TLC were visualized using nonspecific spray reagents. Most chromatograms were sprayed with 55% sulfuric acid (by weight) containing 0.6% potassium dichromate (by weight) followed by heating at 180C to produce charred spots (3). Spraying with a 0.001% aqueous solution of rhodamine 6G and examination under short-wave ultraviolet light was found to be useful for visualizing substances that did not char or were volatilized by heat before charring. Elementary sulfur is soluble in lipid solvents and migration of sulfur on TLC is similar to that of hydrocarbons. Sulfur is detectable as a deep purple spot with rhodamine 6G spray (4). A 5% solution of phosphomolybdic acid in ethanol is useful as a spray reagent for many relatively volatile substances. After a chromatogram was sprayed, it was placed in an oven at room temperature and the oven temperature then allowed to rise gradually to about 120C. This results in the production of strong colors from volatile hydrocarbons (e.g., bitolyl) that are not visualized at all with the char spray and give only faint colors with rhodamine. Elementary sulfur gives a light blue color with the phosphomolybdate spray.

The three wide-range detection reagents did not disclose the presence of all substances on chromatograms. This could be demonstrated by using additional spray reagents such as saturated methanolic silver nitrate. Other spray reagents would undoubtedly disclose the presence of additional components in some samples.

TABLE I

Solvents for TLC

	Polarity of components	Example
n-Hexane	Low	Fig. 1
n-Hexane/diethyl ether 70/30	Med.	
n-Hexane/diethyl ether/glacial acetic acid 70/30/1	"	Fig. 2
Chloroform	"	
Chloroform/methanol 98/2	"	Fig. 3
Chloroform/methanol/H_2O 65/25/4	High	Fig. 4
Chloroform/acetone/methanol/-glacial acetic acid/H_2O 5/2/1/1/0.5	"	Fig. 5

Infrared Examination

The potassium bromide micro pellet technique was used as previously described (2) with samples of 100–300 μg weighed on a Cahn microbalance. Spectra were recorded on a Beckman IR-4 double beam instrument equipped with sodium chloride optics.

RESULTS AND DISCUSSION

Both TLC and infrared spectrophotometry are useful tools for detection of contaminants and impurities. TLC is used for the demonstration of solvent soluble organic compounds, while infrared examination has proved to be most useful for the detection of insoluble substances (adsorbents such as DEAE cellulose, etc.) and inorganic contaminants (silicic acid and silicates, other inorganic salts, silicone greases, etc.).

Results of TLC Studies of Potential Contaminants

Various substances which might contaminate biological lipid preparations were examined at different concentrations with different solvent systems and detection reagents. A series of TLC runs were made of some samples to illustrate and compare some of the major contaminants of different laboratory aids and cosmetics. Differences between competing commercial preparations were noted.

Fig. 1 shows the least polar components in some potential contaminants. The great similarity in composition of a common laboratory sealing wax, a GLC phase, and a waxy hair cream is shown by applications 1–3. The very large differences in some hair preparations is shown by samples 3 and 4 of Fig. 1 where it is demonstrated that one preparation is principally a relatively saturated type of hydro-

carbon, while another (sample 4) does not contain hydrocarbon at all. One hand cream preparation (application 6) was found to contain a large amount of hydrocarbon. Several relatively nonpolar components are also present in extracts of rubber stoppers (application 5) and cigar smoke (application 7).

Fig. 2 illustrates results with the hexane/diethyl ether/acetic acid system for TLC. Extracts of cork stoppers (application 1) contained many substances migrating in this system, extracts of rubber stoppers (application 2) migrated to the solvent front indicating the presence primarily of less polar substances. Numerous components from rubber tubing, cigarette and cigar smoke, and a floor wax (applications 3, 4, 5, and 6, respectively) were detected on chromatograms developed with this solvent. A popular skin cream (application 7) was found to contain triglyceride and free fatty acid.

Fig. 3 illustrates the resolution of moderately polar substances with chloroform/methanol 98/2 as the developing solvent mixture. Again, many components are separated as very discrete spots from an extract of cork stoppers (application 1). Similarly, many components of rubber stoppers and rubber tubing extracts, a floor wax, a laboratory grease for stopcocks and desiccators, a skin cream, and a hand lotion (applications 2 to 7) were separated by this solvent system.

Fig. 4 illustrates results with a chloroform/methanol/water mixture commonly used for TLC of polar lipids (cerebrosides, sulfatides, and phospholipids). Components from hand creams, soaps, detergents, and extracts of cork and rubber stoppers and rubber tubing are separated quite well with this solvent system. This behavior of tobacco smoke components (application 4) in this system as well as those illustrated in Fig. 1, 2 and 3 leaves little doubt that this potential contaminant gives rise to many components of widely varying polarity. It is readily apparent why exposure to tobacco smoke can give rise to a background color on TLC and why isolation of pure lipids from chromatograms is incompatible with exposure of TLC plates or samples to tobacco smoke at any time.

Chloroform/methanol/water is also very useful for the detection of ionic detergents in hand (toilet) soaps (application 3). Fatty acids migrate ahead of the more polar detergent.

Fig. 5 illustrates the chromatographic results with a polar, acidic solvent mixture. Fatty acids migrated to the solvent front and acidic substances in general tended to have higher R_f values in this system as compared to the neutral chloroform/methanol/water solvent. The anionic detergent component of soaps and laboratory glassware cleaning preparations shown in Figure 5 migrated in a way almost indistinguishable from the naturally occurring sulfatides of brain.

The results shown in Figs. 1 through 5 demonstrate that there are many potential sources in the laboratory from which lipid contaminants of all grades of polarity can be introduced into biological preparations.

Infrared Detection of Extraneous Substances

The use of infrared spectroscopic examination as a means for detecting small amounts of inorganic and organic adsorbents ("fines") in column chromatographic fractions has been reported earlier (1). Similar problems are encountered when TLC is used for isolation of lipids. This is most pronounced with polar lipids where chloroform/methanol/water mixtures are required for complete elution from adsorbent. These polar solvents, in contrast to chloroform, diethyl ether, petroleum ether, etc., for elution of less polar lipids, solubilize silicates and other salts. Fig. 6 illustrates typical findings when a developing solvent containing acetic acid is used. The spectrum is largely that of magnesium acetate produced by interaction of acid and magnesium silicate. The presence of lipid (sphingomyelin) in the eluate is largely obscured. Use of calcium sulfate as binder introduces calcium acetate and sulfate. Small amounts of silicates are also carried into the eluate. These substances can be removed by Sephadex column chromatography (5) and the spectrum of a pure lipid is then obtained as shown in Fig. 7.

PRECAUTIONS AND CONTROL MEASURES IN LIPID PREPARATION AND ANALYSES

The results presented above demonstrate the character of many potential contaminants. Experience has shown that most contaminants can be eliminated by following some simple precautions and control measures.

Solvents, Acids, and Bases

Even reagent grade solvents contain undesirable nonvolatile impurities (usually about 0.2 to 0.6 mg/100 ml). This nonvolatile residue can be removed by distillation from glass into glass containers. Nonvolatile impurities including hydrocarbons are present in the reagent grade chloroform and methanol available to us. Use of these solvents for column chroma-

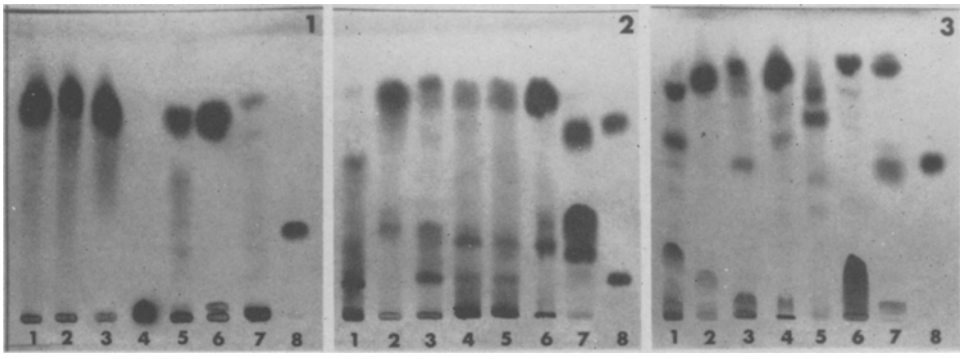

FIG. 1. TLC developed with n-hexane as solvent (char spray) to illustrate the least polar components in potential laboratory contaminants. The ubiquitous occurrence of hydrocarbons is illustrated in these comparisons. Applications were 1) 100 μg of a laboratory grease for stopcocks etc., composed primarily of hydrocarbon (compare with application 5 of Figure 3); 2) 100 μg of gas-liquid chromatographic stationary phase (Apiezon M), also largely hydrocarbon; 3) 100 μg of a waxy (semisolid) hair preparation composed almost exclusively of hydrocarbon; 4) 100 μg of a liquid hair preparation entirely devoid of hydrocarbon; 5) 100 μg of extract of rubber stopper showing more saturated hydrocarbon near the solvent front and many hydrocarbons of intermediate polarity within the body of the chromatogram; 6) 100 μg of a hand cream preparation composed largely of hydrocarbon (compare with application 1 of Figure 4 where the same preparation is shown); 7) 100 μg of solids collected from tobacco (cigar) smoke; 8) 20 μg of squalene as a reference compound.

FIG. 2. TLC developed with n-hexane/diethyl ether/glacial acetic acid 70/30/1 (char spray) to illustrate substances of intermediate polarity present in potential laboratory contaminants. Applications were: 1) 100 μg of cork stopper extract; 2) 100 μg of rubber stopper extract; 3) 100 μg of rubber tubing extract; 4) 100 μg of tobacco (cigarette) smoke components (compare with 5); 5) 100 μg of cigar smoke components (note similarity to 4); 6) about 200 μg of floor wax largely of hydrocarbon nature; 7) 600 μg of a popular hand cream preparation containing triglyceride (upper spot) and fatty acid (lower spot); 20 μg each of authentic triglyceride (upper spot) and cholesterol (lower spot).

FIG. 3. TLC developed with chloroform/methanol 98/2 (char spray) to illustrate moderately polar components in potential laboratory contaminants. Applications were 1) 100 μg of cork stopper extract; 2) 100 μg of rubber stopper extract; 3) 100 μg of rubber tubing extract; 4) about 200 μg of a floor wax preparation; 5) 100 μg of a laboratory grease not composed of hydrocarbon (compare with application 1 of Fig. 1); 6) 600 μg of a popular skin cream; 7) 600 μg of a popular hand lotion; and 8) 20 μg of cholesterol as a standard.

tography without distillation is usually not satisfactory since large elution volumes are not uncommon and minor components of samples may contain a significant weight of solvent residue that influences infrared spectrophotometric and other results. Although glacial acetic acid contains only a very small amount of residue that is not volatile under the mild conditions used in our work, it may yield a highly colored residue. The residue contributes an undesirable yellow or brown color to lipid fractions and may cause lipids to decompose rapidly.

Aqueous ammonia and ammonium acetate are used in our laboratory in DEAE cellulose column chromatography as components of eluting solvents. These compounds were selected because of their volatility under the conditions used for evaporation of solvents. Unfortunately, the best available grades of both have a nonvolatile residue. Commercial ammonia stored in glass vessels contains silicates and other substances. These difficulties are overcome by preparing these reagents as needed. Concentrated (28% by weight) aqueous ammonia is prepared by bubbling gaseous ammonia into freshly distilled water (cooled in an ice bath) until the proper weight is obtained (an excess can be adjusted by dilution with water). The ammonia is stored in plastic (polyethylene or Teflon) bottles and discarded after a few weeks if not used since nonvolatile materials may again appear on standing.

Residue free ammonium acetate is prepared by mixing residue free ammonia and redistilled acetic acid (residue free). This is conveniently done by mixing the required amounts into the chromatographic solvents.

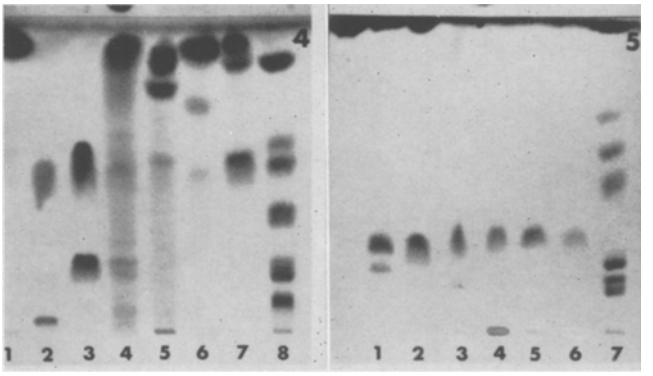

FIG. 4. TLC developed with chloroform/methanol/water 65/25/4 to illustrate the most polar components of potential laboratory contaminants. Applications were: 1) 100 μg of a popular cold (hand) cream (compare with application 6 of Figure 1 where the same preparation is shown); 2) 100 μg of another hand cream entirely devoid of hydrocarbon but containing fatty acid (compare with application 1); 3) 100 μg of a hand soap showing fatty acid (above) and detergent spot (below); 4) 200 μg of tobacco (cigarette) smoke components; 5) 100 μg of cork stopper extract; 6) 100 μg of rubber stopper extract; 7) 100 μg of rubber tubing extract; 8) 100 μg of normal human brain lipids showing cholesterol near the solvent front, 2 spots of cerebrosides then phosphatidyl ethanolamine, lecithin (mixed with other lipids) and sphingomyelin (mixed with other components).

FIG. 5. TLC developed with chloroform/acetone/methanol/acetic acid/water 5/2/1/1/0.5 (char spray) to illustrate detection of detergent in cleaning preparations. Free fatty acid migrates to the solvent front with this solvent mixture. Applications were: 1) 100 μg of a popular hand soap; 2) 100 μg of a second hand soap; 3) 100 μg of a third hand soap; 4), 5), 6) 600 μg each of three different laboratory glassware clearners, and 7) 100 μg of normal human brain lipid extract with the first spot being cerebroside with normal fatty acids, the second cerebroside with hydroxy fatty acids, and other spots from phosphatidyl ethanolamine, lecithin, and sphingomyelin (from above down).

Elimination of Laboratory Greases

We have used silicone greases as lubricants in the past, but these have now been eliminated for the most part by using Teflon stopcocks for many operations, desiccator rings of neoprene rubber or other inert material (Des-O-Rings, LaPine Scientific Company, Chicago, Ill.) and smoothly ground glass surfaces for lids of chromatography chambers. Even the occasional use of silicone grease for stopcocks of vacuum evaporation assemblies can be avoided by using plastic tubing and metal needle values. This eliminates the flow of grease into fractions and prevents freezing of stopcocks. Rotary evaporator flask joints are used dry without difficulty and, if necessary, are ground to fit tightly. All glassware is glass-stoppered and ground to fit, if necessary. Glass stoppers of 10 and 12 ml graduated centrifuge tubes used for storage of samples from TLC and column chromatography may be ground with 800 grit to fit very well without loss of interchangeability. After this grinding, only 1 to 2 ml of n-hexane will evaporate from a 10 ml tube at room temperature over a period of one year. The tight fit also prevents entrance of air and thus aids in preventing autoxidation and entrance of contaminants from air.

Dishwashing

All glassware in this laboratory is washed with detergent (Lakeseal, Peck's Products Co., St. Louis, Mo.) and rinsed with copious quantities of distilled water. Use of hand creams by dishwashers should be discouraged. It should be noted that even if most organic greases are eliminated from a laboratory, similar materials are usually still present in the form of marking pencils, etc. Glassware used in phosphorus analyses is washed in addition with a sulfuric acid-potassium dichromate cleaning solution. Glassware is oven-dried and stored with aluminum foil over the opening and over glass stoppers to prevent entrance of dust and other extraneous materials. Large vessels in particular are rinsed with chloroform and/or chloroform/methanol 2/1 just before use (and dried under a stream of nitrogen, if necessary). This removes a small coating that may accumulate on the vessels. The solid material rinsed from the vessels can be seen easily if the solvent is evaporated in a rotary evaporator, the small

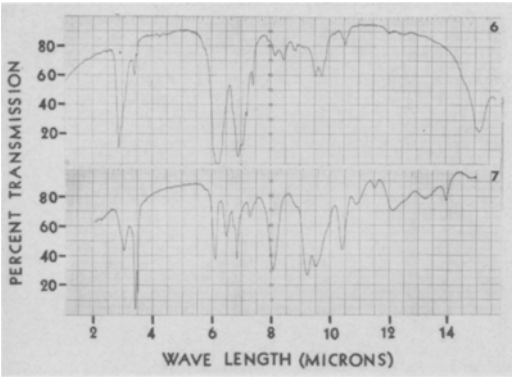

FIG. 6. Infrared spectrum of sphingomyelin eluted from a thin-layer chromatogram prepared with an adsorbent containing magnesium silicate and a solvent containing acetic acid. The spectrum from about 1.3% sample in KBr indicates the lipid preparation to have been heavily contaminated with magnesium acetate. Compare with Fig. 7 after removal of salt.

FIG. 7. Infrared spectrum of sphingomyelin isolated by TLC and freed of salt by passage through a small Sephadex column (5). This spectrum is almost identical to those prepared from the original sphingomyelin sample before TLC. Compare with Fig. 6 before cleanup by column chromatography.

solid residue becoming apparent as a ring around the evaporation flask.

Cork and Rubber Stoppers, Rubber Tubing

The use of cork and rubber stoppers is avoided as much as possible in this laboratory. Rubber stoppers are used only on vacuum rack assemblies used for removal of solvent prior to weighing (2). These rubber stoppers are first washed with detergent and solvent (chloroform and methanol) and may be coated with Teflon applied as a fine spray from a pressurized container. Cork stoppers may be coated in a similar fashion.

Rubber tubing is confined in most cases to uses where introduction into samples would not be a danger. Rubber tubing is replaced by plastic (polyethylene) tubing whenever practical to avoid introduction of rubber fragments and plastic to glass connections made with the appropriate Swagelok fitting.

Labels

Labelling of containers presents a number of problems. We have eliminated all conventional labels and substituted an appropriate strip of masking tape. Most varieties of this tape may be stripped off even after prolonged periods (unless heated) without leaving a weighable residue. This is convenient since a label can be transferred from one container to another as a sample is transferred. All common marking pencils and inks (other than lead pencils) have some lipid components that can appear as spots on chromatograms, and since they are soluble in solvents there is a tendency for them to run and the label to be spoiled. Lead pencil is very useful for marking on masking tape labels. These labels may be immersed in water, stored in a freezer, or even heated in an oven at a moderate temperature and still be legible. When covered with a clear tape (e.g., Scotch tape) pencil labels may be handled many times without becoming illegible from smearing.

Elimination of Airborne Contaminants

Contaminants introduced into the air outside of the laboratory can be controlled with filters, particularly charcoal filters. The most common fumes in the laboratory are from tobacco smoke, oil droplets from vacuum pump exhausts and gas-liquid chromatography effluents. Smoking should be prohibited in areas where adsorbents are being processed and TLC plates are exposed. Vacuum pump vapors can be removed by placement of pumps in or next to fume hoods. Without such precautions, a great deal of hydrocarbon can be distributed throughout a laboratory and hydrocarbon films deposited upon glassware. These deposits are frequently difficult to remove quickly by solvent washing.

Elimination of Impurities from Chromatographic Adsorbents

Elimination of impurities in TLC is accomplished by solvent wash just prior to chromatography. Adsorbents (silicic acid, magnesium silicate, DEAE cellulose) for column chromatography are washed carefully before use (1,2,6).

ACKNOWLEDGMENTS

Work supported in part by USPHS Grants NB-01847-07 and NB-03743 from the National Institute of Neurological Diseases and Blindness, and CA-03134-09 from the National Cancer Institute.

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[Received July 27, 1965]

Influence of Temperature on the Fatty Acid Pattern of Mosquitofish (*Gambusia affinis*) and Guppies (*Lebistes Reticulatus*)

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ABSTRACT

Adult male mosquitofish were adapted to 14-15C and 26-27C water temperature over a 14-day period and the fatty acids from their total lipids analyzed by gas-liquid chromatography.

Newly born guppies were raised at the same temperature for eight weeks and analyzed in the same way. Some fish in the warm water group were subjected to a sudden drop in temperature and the changes of the fatty acids studied after two and eight days, and after two and four weeks.

In all fish the tendency is toward higher unsaturation at lower temperature, but the acids involved in the change differ with the species of fish. A distinct difference is also obvious when guppies are raised at, or when they are adapted to the low temperature. The diet, too, influences the kind and amount of fatty acid synthesis and deposition.

INTRODUCTION

A CORRELATION BETWEEN environmental temperature and the fatty acid pattern has been reported by several authors for many different organisms.

Henriques and Hansen (1) reported that fat from subcutaneous tissue of swine kept at 10C was more highly unsaturated than that from animals kept at 30–35C.

Similar findings were reported by Fisher et al. (2) for hens and by Fraenkel and Hopf (3) for the phosphatides of blow-fly larvae.

For some microorganisms, the temperature effect varies quite widely and may even be reversed (4–6) but in most cases decreasing temperature is associated with a greater formation of unsaturated fatty acids (7,8).

For the mechanism of the temperature influence on the fatty acids of *E. coli*, Marr and Ingraham (9) proposed a direct effect on the relative rates of two or more enzymes involved in the synthesis of saturated or unsaturated fatty acids, and two indirect effects from a change either in growth rate or in the concentration of intermediates.

Kates and Baxter (10) found that lower temperature decreased the oleic acid but strongly increased the linoleic acid of mesophilic and psychrophilic yeasts of the *Candida* species, possibly because of a retarded oxidation rate of the linoleic acid at the lower temperature.

The effect of the environmental temperature on higher plants has been reported by Belehradek (11) and recently by Holton et al. (12) on the blue-green algae *Anacystis nidulans*. For this organism, the major change was a relative decrease of hexadecenoic acid.

According to Lewis (13), certain species of marine poikilothermic animals from arctic regions have lower proportions of the saturated fatty acids and higher palmitoleic acid than similar species from temperate water, possibly as a means of preservation of protoplasmic viscosity.

Farkas and Herodek (14) found the melting point of the lipid from planktonic copepods from Lake Balaton, Hungary to be somewhat lower than the environmental temperature during the entire year, due to an increase in the long-chain unsaturated fatty acids.

In an aquatic food-chain experiment Kayama et al. (15) fed brine shrimp, *Artemia salina* to guppies, *Lebistes reticulatus*, kept at different temperatures. The fish kept in warmer water showed an increased percentage of palmitic and stearic acid and a relative decrease of palmitoleic, oleic, and docosahexaenoic acid. From this experiment, in connection with the above-mentioned investigations by Holton et al. (12) on blue-green algae and by Farkas and Herodek (14) on crustacean plankton, it seems evident that there is an influence of the temperature on the composition of the lipids, possibly through the entire food chain.

Research on the effect of temperature differences particularly on the fatty acids of brain lipids of goldfish, *Carassius auratus L.*, by Johnston and Roots (16) also indicated the tendency to higher unsaturation with declining temperatures, especially with respect to the 18-

carbon acids and the long-chain polyunsaturated acids.

Preliminary to a study of the possible mechanism of the temperature effect on the lipid composition of fish, several species were investigated as possible experimental subjects.

In the experiments described in this paper two supposedly hardy species, mosquitofish, *Gambusia affinis*, and guppies, *Lebistes reticulatus*, were chosen, since it was planned to use rather wide temperature differences in order to elucidate the effect on the fatty acid composition. Moreover, in addition to adaptation of adult fish to different temperatures the objective was also to raise newly born fish under these same environmental conditions, in order to study differences between patterns of preformed fatty acids and those formed at certain temperatures *de novo*, where the progress of the changes in the various lipids as a function of time was also investigated.

EXPERIMENTAL AND RESULTS

For all analytical evaluations, the fatty acids of the total lipids from the whole fish were used. Analysis of the fatty acids by gas-liquid chromatography was performed with a Barber-Colman Model 10 apparatus with a 40 × 0.25 in. column of ethylene glycol succinate, 16.9% on gas chrom P, 80 to 100 mesh. All calculations of the mass peaks in the chromatograms were done by multiplication of the peak height by the peak width at half-height and do not consider possible trace amounts of hydroxy- and branched-chain fatty acids or any fatty acids with chain lengths greater than 22 carbon atoms. The other acids were calculated as percentages of the total.

Investigations on Mosquitofish, Gambusia affinis

Adult male mosquitofish, about 1 in. long, age unknown, were adapted to water of 14–15C and 26–27C, three fish for each experiment. The acclimatization was achieved by immersing the aquariums gradually over a three-day period into preset water baths. The fish were fed *ad libitum* with frozen brine shrimp once a day. In the food uptake of the fish in warm water no change could be observed during the adaptation period, while the fish in cold water ate very little for three days, and seemed to have a much lower, but steady uptake of food afterwards. All fish appeared to be in good health.

After 14 days, the acclimatization was assumed to be completed. The fish of each group were killed and weighed (543 mg for cold water fish, 744 mg for warm water fish) and the lipids extracted from the wet tissue in a blendor with chloroform:methanol 2:1 (v:v). After filtration of the extracts and drying of the solutions ($MgSO_4$), the solvents were removed on a rotary evaporator at 30C under reduced pressure. The remaining lipid extracts from the three fish of each group were saponified overnight at room temperature with 1 ml of 10% methanolic KOH, containing 5% water. The methanol was partially removed on a rotary evaporator at 30C under reduced pressure. After diluting the methanol with an equal volume of water, the unsaponifiable material was extracted three times with n-pentane. The combined solutions were reextracted once with water:methanol 1:1, and the aqueous layers combined with the proper soap solutions. After acidification with HCl, the free fatty acids were extracted four times with ether and the combined ether solutions washed with water until neutral and dried over $MgSO_4$. Evaporation of the ether yielded the free fatty acids, Table I, Column 1.

Investigations on Guppies, Lebistes reticulatus

Guppies seemed to adapt quickly to relatively low and high water temperatures, and an attempt was made to raise 1 or 2-day-old guppies at different temperatures. The time for the water of the aquariums to reach the preset temperature of the water bath (14–15C and 26–27C) was limited to one day. Two groups of fish were kept at each temperature setting, one of which at a given temperature was fed with trout chow diet (group A), the other, at the same temperature, with ether-extracted trout chow diet (group B). The latter diet contained little, if any, ether-extractable lipids. The unextracted diet contained 5.3% fatty acids, the analysis of which is shown in Table II. The guppies kept in warm water on a complete diet grew fastest of all the groups, while the ones at low temperature on an extracted diet grew very little and many died. The fish in the other two groups were intermediate in size.

After eight weeks, the fish were killed by placing them on dry ice and their total fatty acids isolated and analyzed as described above. The results are given in Table I, Columns 2 and 3.

Another group of guppies, raised on a complete trout chow diet for eight weeks in warm water, was forced to adapt rapidly (over a 1-day period) to water of 14–15C and left there for another four weeks. Samples of groups of three fish were taken after 2 and 8 days and again after 2 and 4 weeks. The changes

TABLE I

Gas Chromatographic Analysis of Methyl Esters from Total Lipids of Mosquitofish (Column 1) and of Guppies Raised on Complete Trout Chow Diet, Group A, (Column 2) and on Ether-Extracted Trout Chow Diet, Group B, (Column 3) (percent of total)

	Column 1		Column 2		Column 3	
Fatty acid	14–15C	26–27C	14–15C	26–27C	14–15C	26–27C
14:0	1.3	1.6	3.9	3.7	6.1	4.4
14:1	+	+	1.5	3.1	1.7	1.1
15:0	+	+	+	+	+	+
16:0	14.7	16.0	19.2	22.5	23.7	24.0
16:1	20.0	19.8	10.1	14.1	19.0	19.0
16:2	+	+	+	+	+	+
16:4	+	+	+	—	+	+
16:3 and/or 18:0	5.4	6.5	10.4	7.7	12.1	8.6
18:1	31.8	30.8	26.6	25.7	21.1	24.6
18:2	7.3	7.9	15.0	8.0	2.4	8.0
18:3	+	+	0.1	1.7	1.0	1.1
18:4 and/or 20:2	0.4	1.0	0.8	1.3	0.6	0.9
20:0	+	+	0.1	+	—	+
20:1	5.0	5.1	2.5	3.6	1.2	1.2
20:3	+	+	0.6	0.6	1.1	0.6
20:4	4.0	4.5	1.5	2.7	2.0	1.4
20:5 and/or 22:2	1.2	1.2	0.5	0.7	+	0.5
22:3	0.5	0.6	0.3	+	0.5	0.3
22:4	0.4	+	0.3	+	1.0	0.9
22:5	2.1	1.4	1.5	0.6	+	0.4
22:6	5.9	3.6	5.1	4.0	6.5	3.0

in the fatty acids were examined and are listed in Table III.

DISCUSSION

Mosquitofish

In the experiment with mosquitofish a distinct trend to higher unsaturation of the fatty acids at lower temperature is obvious, with the major changes in the decrease of palmitic and stearic acid, and in the increase of docosahexaenoic acid, less pronounced in oleic and docosapentaenoic acid. The decrease of linoleic and arachiodonic acids at lower temperature represents an unexpected and, at the present time, inexplicable phenomenon.

TABLE II

Gas Chromatographic Analysis of Methyl Esters from Trout Chow Diet (per cent of total)

Fatty acid	
14:0	1.8
14:1	+
15:0	—
16:0	18.5
16:1	5.4
16:2	—
16:4	—
16:3 and/or 18:0	10.4
18:1	27.5
18:2	25.0
18:3	5.3
18:4 and/or 20:2	1.8
20:0	+
20:1	+
20:3	—
20:4	1.7
20:5 and/or 22:2	+
22:3	—
22:4	—
22:5	+
22:6	2.6

Linolenic acid is present only in trace amounts at both temperatures, which gives rise to the questions of how mosquitofish increase the docosahexaenoic acid in cold water. The key to the answer is probably the food intake. In the experiments of Kayama et al. (15), brine shrimp, the diet of the fish during their four months' captivity, did not contain any acids with 22 carbon atoms. The increased amount of docosahexaenoic acid, therefore, was probably not deposited from the food, but must have been synthesized from a parent acid. Eicosapentaenoic acid, comprising 12% of the fatty acids of brine shrimp, and present to a rather small extent in mosquitofish could serve this function. This acid may also be the precursor for docosapentaenoic acid in the fish.

Guppies

For evaluation of the experiment with guppies, the consideration of the diet is equally important. These fish were raised on trout chow diet alone, one group on the complete diet (group A), the other on ether-extracted diet (group B). The two groups of fish reacted in quite different ways to the environmental temperatures as shown in Table I, Columns 2 and 3.

In comparing the fatty acids isolated from fish of all groups to the fatty acids of the diet, the considerable increase of acids with 14 and 16 carbon atoms, especially hexadecenoic acid, is of interest. The 18-carbon acids are generally lower, especially the unsaturated fatty acids, linoleic and linolenic. Part of this drop can

TABLE III

Gas Chromatographic Analysis of Methyl Esters from Total Lipids of Guppies Raised on Complete Trout Chow Diet in Warm Water and Adapted to Cold Water (per cent of total)

	26–27C	14–15C			
	8 Weeks	2 Days	8 Days	2 Weeks	4 Weeks
14:0	3.7	3.0	3.7	3.9	4.6
14:1	3.1	4.1	3.0	2.1	1.3
15:0	+	+	+	+	+
16:0	22.5	19.8	19.8	19.9	20.0
16:1	14.1	16.3	14.8	16.8	18.6
16:2	+	+	0.5	+	+
16:4	–	+	+	+	+
16:3 and/or 18:0	7.7	8.5	7.6	6.1	8.5
18:1	25.7	28.6	26.9	25.4	24.5
18:2	8.0	8.4	8.7	9.1	9.5
18:3	1.7	0.7	0.8	1.7	1.6
18:4 and/or 20:2	1.3	0.5	0.4	+	+
20:2	+	–	+	+	–
20:1	3.6	1.4	2.0	2.8	2.8
20:3	0.6	0.3	0.4	0.9	0.4
20:4	2.7	1.7	2.2	2.1	1.9
20:5 and/or 22:2	0.7	0.7	0.4	1.0	0.7
22:3	+	0.4	0.4	0.6	0.3
22:4	+	0.6	0.6	0.8	0.5
22:5	0.6	0.6	0.6	0.8	0.3
22:6	4.0	4.4	7.2	6.0	4.5

be explained by their conversion to the long-chain polyunsaturated acids, but the polyunsaturated acids in the food may also have been extensively oxidized.

In both the warm water groups, A and B, the levels of the long-chain polyunsaturated fatty acids and their 18-carbon precursors are very similar, suggesting that these unsaturated acids may be present in concentrations required for metabolic processes. In group B these acids could only be accumulated from the traces present in the diet. Nevertheless, the increase to approximately the same percentage as in group A, where they are offered in abundance with the food.

The picture for the low temperature groups appears to be quite different. In group A, the cold environment leads to an increase of linoleic and a decline of arachidonic acid, while in group B the opposite effect can be observed. The interchange between these two acids in both groups supports the findings of Reiser et al. (17), that fish, although they are able to convert 18-carbon acids to long-chain polyunsaturated acids, do not do so to any great degree if the precursors are offered in the diet at a level of 5% or higher, but do so readily at a level of 1%. This explanation may serve for the fact that in group A, with adequate dietary linoleic acid (25% of the fatty acid mixture), the response to colder water is mainly a deposition of a large amount of linoleic acid and a lower level of arachidonic acid. In group B, with only trace amount of dietary fat, the low temperature is accompanied by a relatively low level of linoleic and an increase of arachidonic acid.

For increasing the docosahexaenoic acid in cold water fish, the organism seems to use different parent acids in groups A and B. The intake with the diet must be considered as well, but the low level of linolenic acid in group A, considered in the light of an accumulation of docosapentaenoic and docosahexaenoic acid, leads to the assumption that linoleic acid is elongated and desaturated, as demonstrated by Kayama et al. (18).

In group B, the lower temperature does not affect the linolenic acid, but eicosapentaenoic and docosapentaenoic acids disappear almost completely in favor of docosahexaenoic acid.

The response of the organism to colder environment is expressed in group A particularly in the high percentage of linoleic acid, less pronounced but still obvious in the long-chain polyunsaturated acids, while in group B the emphasis is on docosahexaenoic acid and perhaps arachidonic acid. An explanation for these different responses to temperature variation cannot be offered at present, nor can the changes in the acids with 16 and 18 carbons be interpreted. The generally strong increase of the 16-carbon acids in comparison to the diet has already been mentioned. Stearic acid is markedly increased at lower temperature, as well in group A as in B. In the complete diet group A, lower temperature increases the oleic acid content and reduces palmitoleic acid; in the fat-free diet group B, oleic acid is decreased while palmitoleic acid remains constant.

The picture is different in many ways when guppies, raised for eight weeks in warm water on complete trout chow diet, are forced to acclimatize to cold water rather rapidly (over one day, temperature difference 12C). The findings may be interpreted in terms of an emergency adaptation in the fatty acid pattern, which enables the organism to maintain its functions, obviously involving some fatty acids more than others (Table III). Myristic and palmitic acids drop off, as one would expect, but the stearic acid content rises, just as in the above-described experiments. Marked increases occur in myristoleic, palmitoleic and oleic acid, smaller in linoleic acid. Arachidonic acid drops, as does linolenic acid, while docosahexaenoic acid increases slightly. The effects on the long-chain polyunsaturated fatty acids and the EFA resemble to a large degree the pattern in the fish group A raised in cold water, although on a much smaller scale.

After the rapid changes during the first two days, the adaptation of the system becomes gradually completed during the ensuing period. Thus, some major acids like linolenic, and docosahexaenoic acid return slowly but steadily almost to their original warm water levels. Palmitic acid remains constant after the quick drop in the first two days, while myristic, palmitoleic and linoleic acids increase steadily and the percentages of myristoleic, oleic, eicosadienoic and arachidonic acids decrease. The pentaenoic acids did not change very much during the entire experiment. After four weeks in cold water the organism had, more or less, eliminated the quick changes of all unsaturated acids except the continuously increasing palmitoleic and linoleic acids and the decreasing oleic acid. It seems, then, that differences depend on whether the fish have lived all their lifetime in cold water, or whether they were raised in warm water and adapted to a cold environment.

Furthermore, there are obviously profound differences in the species of fish examined. This might be due partly to the nature of the fish, but to a larger extent also to the diet which will influence the rate of synthesis and deposition of fatty acids. Deposition of fatty acids offered in the diet has been confirmed by several experiments (19,20), as has the synthesis of more highly unsaturated longer chain fatty acids from certain types of precursors (18,21). Synthesis and deposition are overlapping processes, the ratios of which seem to depend largely on the levels at which certain acids are offered. If the food effect could be neglected, the lipid pattern of a particular species of fish should, at a given temperature, always result in a unique fatty acid composition, which is certainly not the case.

ACKNOWLEDGMENT

Mosquitofish supplied by B. Waldron, Health Department, Los Angeles, Calif.

Supported in part by Contract AT(04–1)GEN-12 between the Atomic Energy Commission and the University of California.

Supported in part by Public Health Service Research Career Award No. GM-K6-19, 177 from The Division of General Medical Sciences, National Institutes of Health.

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[Received Aug. 20, 1965]

Fatty Acids of *Lindera umbellata* and Other Lauraceae Seed Oils [1,2]

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ABSTRACT

Seed kernel oils of seven species of Lauraceae were examined and the fatty acid composition of six of these was determined. The oil of *Lindera umbellata* had 4% of *cis*-4-decenoic, 47% of *cis*-4-dodecenoic, and 5% of *cis*-4-tetradecenoic acid in the total fatty acids. Positive identification of these acids was made and new derivatives were prepared. Possible routes of biosynthesis are discussed.

Oils from the other species did not contain more than a trace of unsaturated C_{10}-C_{14} acids. Their major acids were capric and lauric with varying amounts of unsaturated C_{18} acids.

INTRODUCTION

Seed oils of the Lauraceae (laurel) family are noted for their generally high content of lauric acid, although there is considerable variation in the amount among the various species. Oils of the genus *Lindera* of this family are of particular interest because of the occurrence of C_{10}, C_{12}, and C_{14} monounsaturated acids in some species (1–3). These acids, which have the double bond at the 4-position, are not known to occur in the seed oils of any other plant family and may be of special significance in the study of biosynthesis of unsaturated fatty acids.

In the present work, the oils of three species of *Lindera* and four species of other genera were examined, with special regard to the possible occurrence of C_{10}-C_{14} unsaturated acids. The oil of *L. umbellata,* not studied previously, was found to have 4-dodecenoic acid as its major acid.

PROCEDURE AND RESULTS

Preliminary data are given in Table I. The classification into tribes is that of Engler (4). The oils of *Lindera benzoin, Cinnamomum, Sassafras,* and *Umbellularia* have the low refractive indices and low iodine values typical of Lauraceae oils. *Laurus nobilis* has a large proportion of unsaturated C_{18} acids, which accounts for the higher iodine value and refractive index. The oils were converted to methyl esters and analyzed by gas-liquid chromatography (GLC). In addition, the esters of *Lindera umbellata* and *L. benzoin* were examined by fractional distillation to confirm the identity and proportions of the various acids.

Lindera umbellata

L. umbellata oil had a relatively high iodine value (71.4) for oils of this family but the infrared (IR) and ultraviolet (UV) spectra had no unusual features (no conjugated, *trans*- or $\alpha\beta$-unsaturation). GLC of the methyl esters (before and after hydrogenation) showed the presence of monoenoic C_{10}, C_{12}, and C_{14} acids. The esters were separated according to chain length by distillation and the individual monoenoic acids were isolated or concentrated by low-temperature crystallization of the fractions.

4-Decenoic Acid. The unsaturated C_{10} acid was examined by nuclear magnetic resonance (NMR). The spectrum had a strong peak at 7.6 τ, characteristic of the protons at positions 2 and 3 of a Δ^4 unsaturated acid (5,6). In conjunction with the multiplet for the double bond protons at 4.65 τ, this established the position of the double bond at Δ^4. Absence of any *trans* absorption in the IR showed that the double bond is *cis*. Hydroxylation of the acid by alkaline permanganate (7) gave a lactone, judged to be 5-hydroxydecano-γ-lactone because of the known ease of formation of γ-lactones. It had IR maxima at 3480(s) cm^{-1} (OH stretching) and 1790 (vs) cm^{-1} (C = O stretching in γ-lactone). These results identify the C_{10} acid as *cis*-4-decenoic acid.

4-Dodecenoic Acid. The monoenoic C_{12} acid was treated with alkaline permanganate, giving *erythro*-4,5-dihydroxylauric acid. Oxidative splitting of the dihydroxy acid by von Rudloff's method (8) gave octanoic acid, identified by GLC (as its methyl ester), showing that the original double bond was at Δ^4. The monoenoic acid is thus *cis*-4-dodecenoic acid. Two new derivatives were prepared, viz., *threo*-4,5-dihydroxylauric acid and 4,5-dioxolauric acid.

4-Tetradecenoic Acid. The monoenoic C_{14} acid had the typical NMR spectrum of a Δ^4-unsaturated acid, as described above for 4-decenoic acid (Fig. 1). Hydroxylation by alkaline permanganate gave *erythro*-4,5-dihy-

[1] Issued as NRC No. 8928.

[2] Presented at the AOCS Meeting, Cincinnati, October 1965.

TABLE I
Seed Oils of Lauraceae

Tribe and species	Oil in seed [a] wt. %	n_D^{25}	Acid value	Iodine value [b]
Laureae				
Lindera umbellata Thunb. syn. *Benzoin umbellatum* Rehd.	52.1[c]	1.4620	0.6	71.4
Lindera praecox Blume syn. *Parabenzoin praecox*	27.4[d]	1.4594	....	24.7
Lindera benzoin Blume	51.3[c]	1.4553	3.4	13.9
Laurus nobilis L.	19.1[d]	1.4735	15.3	78.3
Cinnamomeae				
Cinnamomum camphora Nees and Eberm.	39.0[c]	1.4525	2.2	3.4
Sassafras albidum Nees	55.8[d]	1.4533	....	6.7
Unclassified				
Umbellularia californica Nutt.	56.8[d]	1.4533	1.0	5.1

[a] Air-dry basis
[b] Wijs
[c] Whole seed
[d] Kernel

droxymyristic acid. The original acid was therefore *cis*-4-tetradecenoic acid.

There was no indication of palmitic or stearic acid in the chromatogram of the total esters but there were small peaks corresponding to octadecenoic and octadecadienoic acids. The octadecenoic acid was identified as oleic acid by conversion to 9,10-dihydroxystearic acid.

The total fatty acid composition, calculated from the GLC curve, was as shown in Table II.

Lindera benzoin

A portion of the methyl esters (70 g) was distilled through a spinning band column. The distilled fractions were almost completely saturated. The iodine value of the first C_{12} fraction was 3.2, suggesting the presence of a little dodecenoic acid. However, the amount would not exceed 0.1% of the total acids. The results of the distillation were in accord with the GLC data.

L. benzoin is native to Canada and the United States and is fairly common in the southern part of Ontario, Canada. It is a shrub, growing to 10 ft in height. The fruit is a drupe, about 3/8 in. in diameter, borne in clusters. It is a potential source of oil of the coconut oil type.

Other Species

The total composition of the fatty acids of the oils, determined by GLC, is given in Table III. No more than a trace of unsaturated C_{10}-C_{14} acids was detected in any of the oils except that of *Lindera umbellata*.

EXPERIMENTAL

Seeds were obtained from established commercial sources. UV spectra were determined in cyclohexane, IR spectra in carbon disulfide, and NMR spectra in deuterochloroform with tetramethylsilane as internal standard. Iodine values were obtained by the Wijs method (30 minutes). GLC was carried out with a diethyleneglycol succinic acid polyester as liquid phase, at temperatures from 125–200C using a thermal conductivity detector. Petroleum ether refers to the fraction of bp 30–60C.

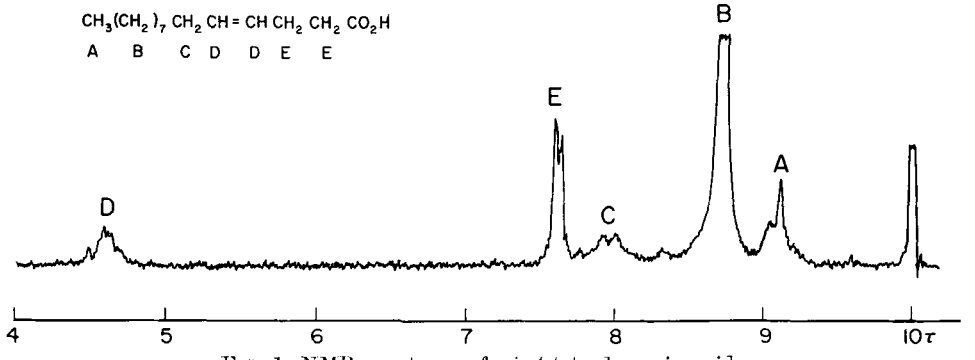

FIG. 1. NMR spectrum of *cis*-4-tetradecenoic acid.

TABLE II
Fatty Acids of Lindera Umbellata

Acid	Weight percent	
	Saturated	Unsaturated
Capric	3	
Lauric	29	
Myristic	3	
Palmitic	0	
cis-4-Decenoic		4
cis-4-Dodecenoic		47
cis-4-Tetradecenoic		5
Oleic		6
Octadecadienoic		3
	35	65

The classification of species in Lauraceae is said to be "much in need of revision" (9). The species names given here are believed to be those currently in use. Specimens of the *Lindera* seeds have been deposited in the Herbarium, Plant Research Institute, Department of Agriculture, Ottawa, Canada.

Large seeds and those with adhering fruit flesh were hulled and the hulls or shells were discarded. Small seeds with a clean, hard coat were ground as received. The finely ground kernels or seeds were extracted in a Soxhlet with petroleum ether. The oil yield and properties of the oils are given in Table I. Methyl esters were prepared by transesterification with hydrogen chloride catalyst, except where noted.

Lindera umbellata

The methyl esters were submitted to GLC at column temperatures 185C, 162C, and 138C. The hydrogenated esters were examined in the same way.

The chromatogram obtained at 162C showed a series of doublet peaks corresponding to C_{10}, C_{12}, and C_{14} acids. One peak of each doublet was identified as the straight-chain saturated acid ester by comparison of the retention times with those obtained from methyl decanoate, laurate, and myristate, respectively. The other peak of each doublet had the appropriate retention time for esters of monoenoic C_{10}, C_{12}, and C_{14} acids. After hydrogenation of the mixture, the chromatogram showed only three peaks in this region representing esters of the saturated C_{10}, C_{12}, and C_{14} acids.

Palmitic and stearic acids were absent. There were small peaks indicating octadecenoic and octadecadienoic acids.

A portion of the oil (70 g) was saponified, the unsaponifiable matter (1%) was removed, and the acids were cooled to −45C in acetone. Crystals were removed and the acids from the filtrate (30.4 g, iodine value 107.4) were converted to methyl esters and distilled under reduced pressure through a spinning band column at 0.6 mm pressure.

Fractions were collected at 57–59C (esters of C_{10} acids), 80–81C and 81-82C (esters of C_{12} acids), and 100–105C (esters of C_{14} acids). The C_{10} fraction was examined by NMR and by hydroxylation as described above.

The first C_{12} fraction was largely monounsaturated (10.1 g, iodine value 113.5); the iodine value of methyl dodecenoate is 119.5. A portion was saponified and treated with alkaline permanganate for 45 sec at 0C. The precipitate was washed with petroleum ether and crystallized from ethyl acetate, giving *erythro*-4,5-dihydroxylauric acid, mp 105.5–106.0C. Komori and Ueno reported mp, 102C (3).

Anal. Calcd. for $C_{12}H_{24}O_4$: C, 62.03; H, 10.41; equiv. wt. 232.3. Found: C, 62.15; H, 10.19, equiv. wt. 231.8.

Another portion of *cis*-4-dodecenoic acid (15 g) was hydroxylated by performic acid by the method of Swern and co-workers (10).

TABLE III
Composition of the Fatty Acids

Species	Fatty acid, wt. % of total acids										
	8:0[a]	10:0	10:1	12:0	12:1	14:0	14:1	16:0	18:0	18:1	18:2
Lindera umbellata	0	3	4	29	47	3	5	0	0	6	3
Lindera praecox	tr[b]	46	0	31	tr	5	0	2	0	12	4
Lindera benzoin	0	42	0	47	tr	3	0	1	0	4	3
Laurus nobilis	0	tr	0	42	0	2	0	9	0	36	11
Cinnamomum camphora	tr	47	tr	47	tr	1	0	tr	0	3	2
Umbellularia californica	0	21	tr	70	tr	2	0	tr	0	5	2

[a] The figures represent chain length and number of double bonds.
[b] tr = trace.

The crude product was a lactone (IR max at 1760 cm^{-1}). It was dissolved in a slight excess of 3 *N* sodium hydroxide and the solution was acidified dropwise by 1 *N* hydrochloric acid. The solution was extracted several times with ether during and after the acidification. After removal of the ether, the product (5.2 g) was washed with petroleum ether and crystallized from ether-petroleum ether to give *threo*-4,5-dihydroxylauric acid, mp 61–63C. Its IR spectrum showed no evidence of lactone.

Anal. Calcd. for $C_{12}H_{24}O_4$: C, 62.03; H, 10.41. Found: C, 62.13; H, 10.26.

Erythro-4,5-dihydroxylauric acid was treated with N-bromosuccinimide by the method of Cramp and co-workers (11). The product was crystallized from methanol, giving 4,5-dioxolauric acid, pale yellow crystals, mp 97.5–98.0C, λ_{max} 274 mμ.

Anal. Calcd. for $C_{12}H_{20}O_4$: C, 63.13; H, 8.83. Found: C, 63.14; H, 8.85.

A C_{14} fraction was saponified and treated with alkaline permanganate for 45 secs at OC., as described above, giving *erythro*-4,5-dihydroxymyristic acid, m.p. 110.0–110.5C.

Anal. Calcd. for $C_{14}H_{28}O_4$: C, 64.57; H, 10.84. Found: C, 64.36; H, 10.98.

The residue from the distillation of the total esters was saponified and the acids (6.9 g) were crystallized from acetone at −25C. The filtrate was cooled to −40C when it deposited crystals of monoenoic acid (1.6 g). This acid was treated with alkaline permanganate, giving *erythro*-9,10-dihydroxystearic acid, mp and mixed mp 129–130C.

Other Species

The fatty acid composition of each of the oils was calculated from the GLC curves by measurement of peak areas. The results, corrected according to the method of Horrocks, Cornwell and Brown (12), are given in Table III. Each figure is the mean of measurements from three chromatograms.

There were very small unidentified peaks in some of the chromatograms which may have arisen from branched or odd-numbered carbon acids. These were seen in the analysis of the esters of *Laurus nobilis* (possible C_{15} acid) and *Lindera benzoin* (possible C_{11} acid).

The results for *Laurus nobilis* were similar to those of Collin (13), who examined the kernel oil by ester distillation. The analysis of *Umbellularia* agrees with that of Noller and co-workers (14) for the saturated acids. Noller did not examine the unsaturated acids. The results for *Cinnamomum* were qualitatively the same as those of Narang and Puntambekar (15) but the ratio of capric to lauric acid was much higher in our sample. *Sassafras albidum* oil was analyzed recently by Earle and co-workers (16) and the present work confirms his results. There was no unsaturated C_{10}, C_{12}, or C_{14} acid.

The three *Lindera* species had not been examined quantitatively. However, an excellent study of *L. benzoin* oil was made by Caspari in 1902 (17), in which he identified capric, lauric and oleic acids as the major components.

DISCUSSION

The seed oil of *Lindera umbellata* is unusual in having *cis*-4-dodecenoic acid as the major fatty acid, as well as small amounts of *cis*-4-decenoic and *cis*-4-tetradecenoic acids. Thus it resembles the oils of *L. obtusiloba* (2,3) and *L. hypoglauca* (1). The oils of *L. benzoin* and *L. praecox* are strikingly different since they contain no more than a trace of C_{10}-C_{14} unsaturated acids (Table III). Similarly, the examples of other genera of Lauraceae included in this work have only a trace of these unusual acids.

Δ^4-Unsaturated acids were discovered in seed oils and characterized by Tsujimoto, Toyama, Komori and Ueno (1–3). So far, the acids are known to occur only in two genera of Lauraceae, viz. *Lindera* and *Litsea,* and not at all in the seed oils of any other family. Acids with double bonds at Δ^4 are not common in nature, although they occur (mostly with multiple double bonds) in fish oils, in certain lower plant forms, and in the leaves and flowers of some plants, e.g. 4-decenoic acid in the volatile oil of hops (*Humulus*) (5).

Fatty Acids and Taxonomy

The differences in fatty acid composition may be of value in classifying Lauraceae species. On the basis of the few species studied so far, three types of seed kernel oils are evident: (a) those whose acids are mainly C_{10} and C_{12} saturated, e.g. *Lindera benzoin, L. praecox, C. camphora,* and *U. californica;* (b) those whose acids are mainly saturated medium-chain (C_{12}) and unsaturated long-chain (C_{18}), e.g. *Laurus nobilis;* (c) those whose acids are mainly medium-chain saturated and unsaturated (C_{10}-C_{14}), e.g. *Lindera umbellata.*

Biosynthesis

Occurrence of the homologous series of three monoenoic acids, each with the double bond at Δ^4, indicates that the mechanism of biosynthesis

must be distinctly different from the ordinary process of synthesis in seeds. A series of three or more monoenoic acids in a seed oil is usually considered to be formed by chain-lengthening at the carboxyl end so that the double bond moves away from the carboxyl group as the chain length increases.

There are several possible routes for formation of the three Δ^4 acids in *L. umbellata*. Shortening or lengthening of the chain at the methyl end could conceivably occur, although this seems unlikely. It is more probable that the mechanism involves condensation of a 4-carbon acid with a unit of 6, 8 or 10 carbon atoms to form the Δ^4 acids.

ACKNOWLEDGMENTS

Advice on botanical names by W. G. Dore. NMR spectra by M. Lesage. Microanalyses by H. Seguin.

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[Received Sept. 27, 1965]

Glycolipids of *Briza spicata* Seed [1]

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ABSTRACT

The seeds of *Briza spicata* contain 20% of lipid that is semisolid and quite unusual in character. This lipid contains 49% digalactosylglycerides, 29% monogalactosylglycerides, and consequently little, if any, conventional triglycerides. The predominant fatty acids present are palmitic, oleic, and linoleic. Partial resolution of the galactosylglycerides on the basis of fatty acid composition was achieved by countercurrent distribution.

INTRODUCTION

THIS PAPER WILL discuss the isolation and characterization of galactose-containing glycolipids of the seed of *Briza spicata* Sibth. et Sm., a member of the Gramineae or grass family. Grasses of the genus *Briza* are known by the common name quaking grass.

Galactosylglycerides have been found in a wide variety of plant sources—including wheat and other cereal grains (3,4,8), red clover (20), alfalfa (12), runner bean leaves (16), spinach and lettuce leaves (1,11)—and in certain microorganisms—including *Anameba variabilis* (7) and *Chlorella pyrenoidosa* (12). Carter and coworkers (3) conducted the first intensive study of these galactosylglycerides on material they isolated from the benzene-soluble portion of wheat flour. They isolated two different types of galactosylglycerides—one that yielded β-D-galactopyranosyl-1-glycerol on alkaline hydrolysis, and another that afforded α-D-galactopyranosyl-1, 6-β-D-galactopyranosyl-1-glycerol. Throughout the present paper, these two galactosides will be referred to simply as monogalactosylglycerol and digalactosylglycerol.

EXPERIMENTAL

General Methods

Gas-liquid chromatographic (GLC) analyses were carried out as described by Miwa et al. (10). Methyl esters were prepared with 1% methanolic sulfuric acid; reflux periods of 2–3 hr were employed.

Paper chromatographic analyses were carried out on Whatman No. 4 paper with the descending technique. Two solvent systems were used, pyridine-1-butanol-water (3:2:1.5) (3) and ethyl acetate-pyridine-water (12:5:4) (17). Spots were visualized with 5% ammoniacal silver nitrate or with ninhydrin (0.1% w/v in acetone). Papers were heated in an oven at 110C for 5–10 min after they had been sprayed with the appropriate reagent.

Thin-layer chromatography (TLC) was carried out on plates coated with Silica Gel G (according to Stahl). Three solvent systems were used: 1-butanol-acetone-water (4:5:1) (14), chloroform-methanol-water (65:25:4) (20), and isobutyl ketone-acetic acid-water (8:5:1) (9). Spots were visualized either by charring with 50% sulfuric acid or by viewing plates under UV light after spraying with dichlorofluorescein solution (0.2% w/v in ethanol).

IR spectra were determined with an Infracord Model 137 spectrophotometer on 1% chloroform solutions.

Amino acid analyses were carried out by the automatic procedure of Spackman, Stein, and Moore (18) with a Model MS Beckman Spinco instrument.

Isolation of Lipid

Coarsely ground seeds (26.1 g) of *Briza spicata* Sibth. et Sm. were extracted 64 hr in a Soxhlet apparatus with petroleum ether (bp 30–60C). The solvent was evaporated in vacuo, and 5.1 g of semisolid residue was obtained. Elementary analyses of the crude lipid showed 0.94% N, 0.51% S, and 0.47% P.

Countercurrent Distribution

Petroleum ether extract of *Briza* seed (3.80 g) was subjected to a 200-tube CCD (countercurrent distribution) in a Craig-Post apparatus with hexane/90% methanol as the solvent system. The weight distribution obtained is shown in Figure 1. Considerable difficulty with emulsions was encountered. The apparent peaks obtained in CCD, and the weight percent they represented of the total, were as follows: A-1, 6.6%; A-2, 41.8%; B-1, 12.3%; B-2, 16.7%, and C, 22.6%. These were examined by TLC and IR. Elementary analyses of material from selected fractions under the major peaks gave the following results: A-1 (tubes 1–9), 2.80% N; A-2 (tubes 21–24), 1.46% N; A-2 (tubes 26–29), 0.71% N, 1.02% S, 0.47% P, 61.9% C, 9.42% H.

[1] Presented at the AOCS meeting in Houston, April 1965.

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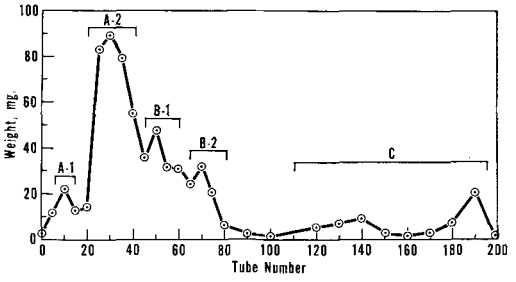

FIG. 1. CCD of *Briza spicata* seed lipid.

Characterization of Fraction A-1

A-1 (0.023 g) was refluxed 1 hr with 10% hydrochloric acid. The resulting hydrolysis mixture was extracted with petroleum ether. Evaporation of petroleum ether extracts afforded 0.011 g of free acids; these were converted to methyl esters in the usual way. Evaporation of the acidic solution in vacuo afforded 0.017 g of water-soluble hydrolyzate. This was examined by paper chromatography using pyridine-butanol-water solvent. This same acid hydrolyzate was also subjected to a complete amino acid analysis.

A portion of A-1 was dispersed in chloroform and extracted three times with 1 *N* hydrochloric acid. Most of the lipid was recovered from the chloroform layer, which was dried over sodium sulfate. The IR spectrum of this recovered material showed substantial reduction of the concentration of amide groups, but removal was incomplete. Amino acid analysis on the lipid fraction showed the presence of most of the common amino acids.

Characterization of Fraction A-2

Fraction A-2 was hydrolyzed with barium hydroxide essentially as described by Weenink (21); 0.123 g of the lipid was refluxed 3 hr in 25 ml of saturated barium hydroxide. Insoluble barium soaps were removed by centrifugation and filtration. The filtrate was neutralized with 0.1 *N* sulfuric acid; precipitated $BaSO_4$ was removed by centrifugation and/or filtration. The clear filtrate afforded 0.055 g of syrup upon evaporation in vacuo; this was examined by TLC and by paper chromatography.

The syrupy product was refluxed 2 hr in 10% aqueous hydrochloric acid. The hydrolysis mixture was evaporated to dryness in vacuo, and the resulting hydrolyzate was chromatographed both by TLC and on paper.

The saponification-acetylation procedure of Holla, Horrocks, and Cornwell (6) was applied to confirm the presence of a glycerol moiety. A-2 (0.032 g) was dissolved in 30 ml of methanol. To this solution was added 10 ml of methanol in which 25 mg of sodium had been dissolved, and the resulting mixture was refluxed 2 hr. Methanol was removed in vacuo, and the residue was refluxed 1 hr with 10 ml of water. Then were added 70 ml of acetic anhydride and 70 ml of xylene. The mixture was refluxed 6 hr, then evaporated to dryness in vacuo. The semisolid residue was triturated with chloroform. Combined chloroform washings were evaporated; the residue (0.006 g) was shown by GLC analysis to have an equivalent chain length (ECL) (10) on an Apiezon L column of 15.6, identical with that of triacetin.

Characterization of Fractions B-1 and B-2

Fraction B-1 (0.136 g) was hydrolyzed with saturated barium hydroxide as described in the preceding section. Upon evaporating the neutralized aqueous phase in vacuo, 0.054 g of syrup was obtained. The insoluble barium soaps were converted to methyl esters.

Fraction B-2 was hydrolyzed with barium hydroxide in the same manner as A-2. Hydrolysis of 0.142 g of B-2 afforded 0.048 g of syrup or foam. For acid hydrolysis, 0.022 g of this syrup was refluxed 2 hr in 10% hydrochloric acid. The hydrolysis mixture was evaporated to dryness in vacuo, and the residue was chromatographed both by TLC and by paper.

The diethyl dithioacetal derivative of the sugar in the acid hydrolyzate was prepared, essentially by the method of Zinner (23). The crude derivative had mp 134–138C. Recrystallization from water afforded a specimen having mp 138–140C, undepressed on admixture with authentic D-galactose diethyl dithioacetal, mp 140–142C [lit. mp, 140–142C (22)].

A portion of the presumed monogalactosylglycerol from alkaline hydrolysis of B-1 was triturated repeatedly with warm water. The combined portions of aqueous solution were evaporated to dryness in vacuo. This residue was crystallized from methanol-ether (3) after seeding with authentic monogalactosylglycerol. A small yield of solid, mp 132–138C, was obtained; this mp was undepressed on admixture with authentic monogalactosylglycerol having approximately the same mp.

RESULTS

Examination of the total *Briza* lipids by TLC revealed two major components along with some minor ones, mostly of higher R_f. In isobutyl

ketone-acetic acid-water, the major spots had R_f values of 0.33 and 0.52 (Fig. 2); in chloroform-methanol-water, they had values of 0.62 and 0.92. The IR spectrum of this lipid showed maxima at 2.95 μ (OH), 5.75 μ (ester), 6.05 μ (amide), and 9.03 μ (ether).

Separation of the lipid into several fractions was achieved by CCD (Fig. 1). Fractions A-1 (see Fig. 2) and A-2 were shown by TLC to correspond to the major component having the lower R_f. Fractions B-1 and B-2 had R_f values that corresponded to the faster-moving major component in TLC. The IR spectra of these various fractions (Fig. 3) showed some differences. There was a prominent amide peak (6.05 μ) in the spectrum of A-1 which was greatly diminished or absent in the other fractions. The hydroxyl absorption of B-1 was strong, but less so than that of A-1 and A-2. The IR spectrum of B-2, not shown, was similar to that of B-1.

The acid hydrolyzate from Fraction A-1 was found to contain practically all the amino acids commonly found as protein constituents. Thus it appeared that A-1 contained the same polar lipid found in A-2 mixed with a protein or polypeptide that is difficult to separate from it. Since the amide-containing material was not completely removed by acid extraction (see Experimental), some lipopeptide, such as was found in wheat flour (3,4), may be present.

Hydrolysis of fractions A-2, B-1 and B-2 with aqueous barium hydroxide afforded their fatty acid constituents as barium soaps together

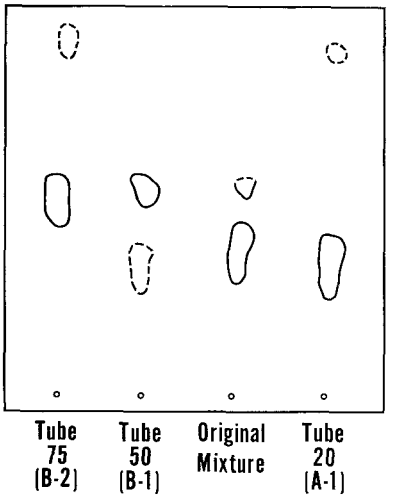

FIG. 2. TLC comparison of crude *Briza spicata* seed lipid mixture with derived CCD fractions. The solvent system used was isobutyl ketone-acetic acid-water (8:5:1). Distinct spots are indicated by solid lines, and faint spots by broken lines.

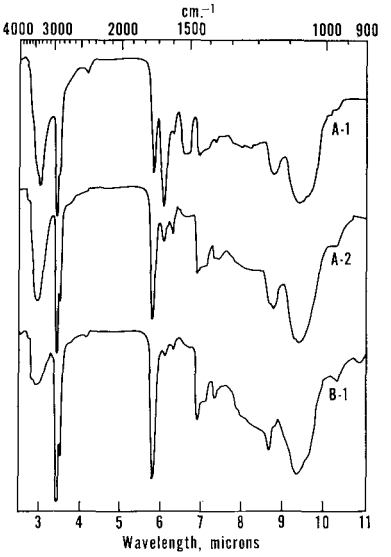

FIG. 3. IR spectra of selected fractions from CCD of *Briza spicata* seed lipid, run as 1% chloroform solutions.

with highly polar, water-soluble hydrolysis products.

When the polar hydrolysis product from A-2 was examined by TLC and by paper chromatography, spots were visualized having the same R_f as authentic digalactosylglycerol. This material was not obtained in crystalline condition. Paper chromatography and TLC of the products of acid hydrolysis of this water-soluble fraction revealed the presence of galactose and glycerol. The presence of glycerol in the water-soluble moiety from A-2 was confirmed by the saponification-acetylation procedure of Holla, Horrocks, and Cornwell (6). Thus it seems highly probable that Fraction A-2 consists of acyl derivatives of the same digalactosylglycerol originally characterized by Carter and co-workers (3,4).

The water-soluble hydrolysis product obtained by barium hydroxide treatment of B-1 or B-2 had the same R_f values as monogalactosylglycerol when examined by paper chromatography and TLC. This hydrolysis product was obtained in crystalline form; its melting point was undepressed upon admixture with authentic monogalactosylglycerol. Further hydrolysis with aqueous acid yielded components with chromatographic mobility values corresponding to galactose and glycerol. The properties (mp and mixed mp) of the diethyl dithioacetal derivative of the sugar proved that it was D-galactose. Thus it was shown with reasonable certainty that B-1 and B-2 are acyl derivatives of monogalactosylglycerol.

TABLE I

GLC Analyses of Methyl Esters from *Briza spicata* Seed and Selected CCD Fractions (area percent of methyl esters)

Acid [a]	Original extract [b]	A-1 (tubes 11–19)	A-2 (tubes 26–29)	B-1 (tubes 46–49)	B-1 (tubes 51–54)	B-2 (tubes 66–69)
C_{16} S	14.8	19.9	18.4	17.0	12.7	5.6
C_{16} I	1.0	1.5	1.7	2.0	1.9	0.1
C_{18} S	0.6	0.4	1.0	1.6	0.3	....
C_{18} I	49.5	35.5	39.5	47.2	47.6	86.1
C_{18} II	27.7	38.5	34.4	29.9	29.1	8.2
C_{18} III	3.4	3.4	2.9	2.5	6.7	....

[a] In this column, Roman numerals indicate the number of double bonds in unsaturated acids; S indicates a saturated acid.
[b] Data of F. R. Earle and co-workers.

TLC results suggested that the least polar portion of material obtained by CCD ("C" in Fig. 1) contained some ordinary triglycerides mixed with more polar materials. Fraction C was not investigated further.

Fatty acids from the various fractions were shown by GLC analyses to be mainly palmitic, oleic, and linoleic acids (see Table I).

DISCUSSION

The lipid portion of *Briza spicata* seed obtainable by petroleum ether extraction has been shown to contain galactosylglycerides as its predominant constituents. Some ordinary triglycerides may be present, but not more than 22% of the total. Together, the digalactosylglyceride fractions A-1 and A-2 account for a maximum of 49% of the total seed lipid of *B. spicata*, while the monogalactosylglyceride fractions B-1 and B-2 comprise a maximum of 29%.

Partial fractionation of the mono- and digalactosylglycerides on the basis of their fatty acid composition was achieved by the CCD procedure. The general pattern of differences in fatty acid composition (Table I) was in accord with that observed in separations of conventional triglycerides. Other factors being equal, increasing unsaturation or shortening chain lengths of a triglyceride would be expected to *lower* its mobility in a hexane-methanol system (5). Although it separated the mono- and digalactosides fairly effectively, the CCD treatment had certain disadvantages in that it produced a large number of fractions that were similar, but had silightly different properties.

The fatty acid compositions of the various CCD fractions are generally similar. An exception is Fraction B-2, which differs markedly from the others in having a great predominance (86.1%) of oleic acid. Each of the others contains substantial amounts of palmitic, oleic, and linoleic acids, and is qualitatively similar to the mono- and digalactosylglycerides of wheat flour (4). In contrast, galactosylglycerides from certain leaf lipids have been found to contain linolenic acid almost to the exclusion of other fatty acids (16,21).

The ratio of di- to monogalactosylglycerides found in *Briza spicata* seed was about 1.7:1. In other cases where such a proportion has been determined, the di- to monogalactosylglyceride ratio was 2:1 in *Chlorella pyrenoidosa* (12), 1:4 in *Phaseolus multiflorus* leaf lipids (16), and 1:2.1 in *Trifolium pratense* leaf lipids (21).

To our knowledge, *Briza spicata* is the first example of a plant whose seed lipids contain more galactosylglycerides or other glycolipids than ordinary triglycerides.

ACKNOWLEDGMENTS

Specimens of mono- and digalactosylglycerol furnished by M. Kates, National Research Council, Ottawa; GLC analyses by J. W. Hagemann; amino acid analyses by J. E. Peters; elementary analyses by B. Heaton and A. Dirks; *Briza spicata* seeds identified and supplied by Q. Jones, Crops Research Division, ARS, USDA.

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[Received May 11, 1965]

Absorption of Di- and Triglycerides by Intestinal Slices in vitro

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ABSTRACT

The uptake by hamster intestinal rings of labeled 1,3-diolein and triolein in bile salt emulsions was studied.

About 6% of triolein was taken up from emulsions containing glycerides and fatty acid in 6 mM sodium taurodeoxycholate. Lesser uptake was noted when triolein was emulsified with lecithin, cholesterol and bile salt; lowest uptake (3%) was observed from triolein-lecithin-cholesterol emulsions prepared without bile salt.

Absorption of 1,3-diolein from bile salt emulsion was greater and acylation to triglyceride was observed.

Diglycerides and triglycerides in small quantity may be absorbed intact from a micellar phase.

RECENT INVESTIGATIONS have suggested that lipid micellar solutions may be the form in which fat penetrates the intestinal mucosa (1). The absorption of labeled fatty acids and/or monoglycerides was faster from micellar solutions of conjugated bile salts than from albumin solutions; the uptake of triolein from triglyceride emulsions was slow (2). Mixed bile salt emulsions may be prepared which simulate the oil-micellar phase system of the fat content of the small intestine during digestion (3). Gel filtration of such mixtures of glycerides, fatty acid and bile salt yielded data concerning their relative partition into micellar and oil phases (4). Appreciable amounts of diolein were distributed into the micellar phase, as were small but quantifiable amounts of triolein. The possibility that diglyceride and triglyceride in micellar dispersion might be able to penetrate the small intestinal mucosa was therefore investigated. The uptake by hamster intestinal rings of diolein and triolein in bile salt emulsions was studied.

MATERIALS AND METHODS

Sodium taurodeoxycholate was synthesized (5). Triolein, 1,3-diolein, 1-monoolein, and oleic acid were obtained from commercial sources described in detail elsewhere (4). Cholesterol was obtained from Distillation Products Industries. Lecithin prepared from egg phosphatides according to Hanahan, was obtained from Vitrum, Stockholm, Sweden.

(9,10-^{3}H) Oleic acid and (1-^{14}C) oleic acid were obtained from The Radiochemical Centre, Amersham, England. ($^{3}H_3$) Triolein and ($^{14}C_2$) 1,3-diolein were synthesized using labeled oleoyl chlorides. ($\alpha\alpha$,$^{14}C_2$,β^{3}H) Triolein was synthesized from the oleoyl chlorides (6).

Emulsions were prepared by sonication. The determined amount of lipid, labeled or unlabeled, in organic solvent was added to a test tube. The solvent was evaporated and Krebs-Ringer buffer (pH 6.3) with 6 mM sodium taurodeoxycholate was added. One-minute exposure to ultrasonic waves was employed, using volumes of 5 to 10 ml. One emulsion was prepared without bile salt using lecithin as stabilizer. A similar emulsion was prepared in 6 mM sodium taurodeoxycholate.

Rings were prepared from everted hamster small intestines as described previously (2,7). Incubations were carried out in 1 ml of emulsion containing 127–190 mg wet weight of tissue and 10 μmoles glucose. Incubations were in duplicate at 37C in a shaking water bath in an atmosphere of 100% O_2. Incubation times in various experiments were 5, 15, 30 and 60 min. All emulsions were pregassed with O_2 for 1 hr before adding the tissue to preweighed vessels. Emulsions were stable throughout the experimental period.

After incubation, the medium was poured off and the tissue washed with 2 ml buffer with or without bile salt, according to the composition of the incubation medium. The tissue was homogenized and extracted in 10 ml chloroform-methanol (2:1, v/v) to which 4 ml 0.15 M KH_2PO_4 was added. A portion of the emulsion originally added to the incubation flasks, and the medium after incubation combined with the wash-solution from each flask, were extracted with 18 ml chloroform-methanol (2:1, v/v). After standing overnight in the cold, the lower phase was dried with Na_2SO_4, was filtered and was evaporated to dryness. The lipids were then separated by thin-layer silicic acid chromatography and their radioactivity assayed in a scintillation spectrometer. The details of these procedures have been described (2,7,8). Mean

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recovery of activity from medium and tissue was 92% of that added.

There was no microscopic evidence of cell destruction, and the mucosal membrane potential was maintained in intestinal slices exposed to 6 mM taurodesoxycholate over periods of several hours (9). These results are in accordance with those of Dawson and Isselbacher (10) who found no evidence of mucosal damage at the end of one hour's incubation of rat small intestine when taurodesoxycholate was in the medium (5×10^{-3}M).

RESULTS AND DISCUSSION

Incorporation of glycerides and fatty acid into a bile salt emulsion yields a system containing oil and micellar phases. In the absence of bile salt no micellar phase exists in triolein emulsions stabilized with lecithin at pH 6.3. The partition of various lipids between the micellar and oil phases differs. Column elution experiments with 60 ml gel bed, Sephadex G-100, of lipids emulsified in and eluted with 6 mM sodium taurodeoxycholate, indicated that monoglyceride and fatty acid were recovered completely in the micellar phase, about 30% of diolein was in micellar dispersion, with about 3% of triolein distributed into the micellar phase (4).

The first step in the process of fat absorption takes into account distribution between the lipids of the cell wall and the medium (2,10–13). Uptake from the intestinal lumen into the mucosal cells may mean adsorption or on permeation into the membrane or penetration into the cell. Factors such as the physical form of the substance, dependent on rapid phase transitions in the medium, e.g., micellar solubility, the nature of the cell membrane, lipid solubility in the membrane, avidity of cell binding sites may be determinants. Uptake has been demonstrated to be reversible with exchange occurring between the tissue and the medium (13). These processes are not dependent upon metabolic energy but involve physical phenomena. The complexities of this step in absorption may be elucidated by investigations in vitro.

The next step includes active phenomena of chemical modifications within the cell. These biosynthetic processes require energy, are slower and may be rate-limiting in systems in vitro. The final step requiring passage out of the mucosal cell into the central lacteal via the formation of chylomicrons is dependent on protein synthesis (14). This may be the limiting step in the overall absorption process in the intact organism.

The uptake of triolein by small intestinal slices was compared at various concentrations in emulsions prepared without and with sodium taurodeoxycholate and with lecithin or fatty acid-glycerides included. In all instances a small uptake of triglyceride was demonstrated (Tables I,II). Uptake on a molar basis per 100 mg tissue at 1 hr was lowest from an emulsion prepared without bile salt and stabilized by lecithin and a small amount of cholesterol. Uptake was 50% greater when 6 mM sodium taurodeoxycholate was included in the preparation of the emulsion. The highest triolein uptake was observed from an emulsion containing 6 mM sodium taurodeoxycholate and 5.4 mM diolein, 5.4 mM monoolein and 10.8 mM oleic acid. Variable results were obtained when the time-course of triglyceride absorption was followed. Even excluding very high results obtained at 30 min with one set of incubations (Experiments 2,3) compared to those at 30 min in Experiment 4, there was a rapid uptake of triglyceride in the first 5 min which de-

TABLE I

Uptake of Triolein from Various Suspensions

Expt.[a]	Triolein concn [b] mM	Other lipids [c] μMoles	Uptake mμMoles/100 mg slices 60 min	5 min	15 min	30 min
1	25.0	L 1.25 C 0.62	686	.....	.....	.....
2	12.5	L 0.62 C 0.31	521	588	533	1301
3	11.2	DO 5.4 MO 5.4 OA 10.8	630	847	704	1562
4	13.0	DO 5.4 MO 5.4 OA 10.8	754	.....	.....	732

[a] In Expts. 2–4, 6 μmoles sodium taurodeoxycholate were included in the emulsion, while the emulsion in Expt. 1 was prepared without bile salt.

[b] In Expts. 1–3 (^{3}H) triolein was used. In expt. 4 ($\alpha\alpha$,-$^{14}C_2$,β-^{3}H) triolein was used.

[c] C, cholesterol; L, lecithin; DO, diolein; MO, monoolein; OA, oleic acid.

TABLE II

Uptake by Hamster Intestinal Slices of Lipids in Micellar Solutions or Emulsions

Solutes [a]	Physical form	Uptake/100 mg	
		%	mμmoles
Oleic Acid-monoolein (2)	Micellar solution	36.0	342
Triolein-monoolein-oleic acid	Bile salt emulsion	5.7	630
Triolein-lecithin-cholesterol	Bile salt emulsion	4.2	521
Triolein-lecithin-cholesterol	Emulsion	2.7	686
Triolein (2)	Emulsion	1.7	16
Diolein-tri-, monoolein, oleic acid	Bile salt emulsion	6.9	461

[a] Labeled compound is italicized.

creased for the next 25 min of incubation and remained unchanged at 1 hr.

Uptake of triolein was low in comparison to the uptake of oleic acid and monoolein from micellar solutions. Slices of hamster small intestine took up about six times more lipid on a molar basis per 100 mg when incubated with 0.6 mM oleic acid and 0.3 mM monoolein in 2.4 mM sodium taurodeoxycholate. The differences in the degree of uptake of monoglycerides vs. di- and triglycerides may be correlated to the marked difference in degree of micellar dispersion of the lipids (4). Absorption apparently parallels partition into the micellar phase. This is further supported by the increased absorption of triolein from the mixed bile salt emulsion compared to that from an emulsion without bile salt. The uptake of micellar solution of fatty acid and monoglyceride was similarly doubled in comparison to a suspension of these lipids in albumin (2).

It is concluded that triolein penetrated the intestinal mucosa and that the uptake occurred largely intact. Evidence for this was provided in the experiment using double-labeled triglyceride by the similar ratios $^{3}H:^{14}C$ in that triglyceride which penetrated (14.7) compared to the ratios in triglycerides in the original medium (14.6) and in the medium poured off at the end of incubation (14.8). That portion of total activity recovered as triglyceride also remained constant in original and final incubation media and tissue. No appreciable amount of radioactivtiy (less than 5%) was recovered in lower glycerides or fatty acid at any time of incubation. In short, with the absence of evidence of hydrolysis of triolein in the incubation medium and the recovery of the activity in tissue triglycerides with unchanged $^{3}H:^{14}C$ ratio it was assumed that triolein was taken up intact.

Uptake of 1,3-diolein from a bile salt-fatty acid-glyceride emulsion was of similar magnitude on a molar basis per 100 mg tissue as that of triolein (Table II). There was an increase of 52 mμmoles diolein taken up at 60 min compared to 30 min. At both 30 and 60 min there was evidence of acylation of diglyceride to triglyceride. Triglyceride activity was 53 mμmoles at 60 min compared to 20 mμmoles at 30 min. At both time intervals 88 to 95% of the tissue activity was in diglyceride. The portion of activity recovered as diglyceride was constant in the original and final incubation media, with no recovery of appreciable activity in other lipid classes. Without evidence that hydrolysis occurred in the medium it was assumed that diglyceride was taken up intact and then acylated. This acylation provided evidence for entry of 1,3-diolein into the cell. The relatively low degree of acylation in these experiments may be related to the lesser activity of diglyceride transacylase in the presence of 1,3-diglycerides compared with 1,2-diglycerides (15). The latter are the natural products of pancreatic lipolysis which favors hydrolysis of primary ester bonds (16).

From these experiments we can conclude that the quantitative contribution to fat absorption of intact diglyceride or triglyceride absorption is small. A larger difference in uptakes might have been anticipated from comparison of the micellar distribution of diolein (31%) with that of triolein (2%) when mixed bile salt emulsions were partitioned into micellar and oil phases by gel filtration (4).

In summary, monoglyceride and fatty acid absorption is determined by rapid and extensive micellar dispersion in bile salt mixed micelles which permits uptake by the brush border membrane in a process enzymically and energetically independent and reversible. Specifically how this penetration occurs is unknown. The manner in which lipids leave the membrane is also unknown and may require energy. Once entry into the cell has occurred a continual biosynthetic process of acylation to triglyceride occurs which requires energy and has a lesser capacity than the system of membranous uptake (2). Lesser uptake of diglycerides and triglycerides may be determined by their lesser ability to enter the micellar phase. The experi-

mental results do provide further evidence for the entrance of di- and triglycerides into the intestinal mucosa in the form of small aggregates.

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ACKNOWLEDGMENTS

Synthesis of a number of compounds used in this investigation by L. Krabisch; technical assistance, Mrs. G. Björklund. Work was carried out during the tenure by one of us (E.B.F.) of a USPHS Special Fellowship; additional financial support by USPHS Grant No. H-5302, the Swedish Medical Research Council and the "Svenska Margarinindustrins Förening för Näringsfysiologisk Forskning."

[Received Oct. 27, 1965]

Determination of the Structure of Lecithins[1]

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ABSTRACT

A method is described for the determination of the classes of lecithins in terms of unsaturated and saturated fatty acids based on a total fatty acid composition, the composition of the fatty acids in the β-position, and the amount of disaturated class determined via mercuric acetate adduct formation. The accuracy of the method was determined on lecithins of known composition and the method was applied to lecithins isolated from milk serum and egg lipids, safflower and soybean oils.

INTRODUCTION

THE POSITIONAL arrangement of fatty acids in lecithin may be determined by the methods described by Privett and Blank (1), Moore and Williams (2), or in the form of triglycerides to which they may be converted by dephosphorylation and re-esterification of the 1,2-diglyceride analogues (3,4). The method of Privett and Blank employs a combination of reductive ozonolysis and quantitative thin-layer chromatography (TLC). The method developed by Moore and Williams (2) is based on a difference in the rate of hydrolysis of the different lecithin classes by phospholipase A, and that no disaturated lecithins are hydrolyzed during the time the measurements are made.

Procedures involving the conversion of lecithins to triglycerides require that no acyl migration of fatty acids nor fractionation occur during the preparation of the triglycerides. These methods are long and, as yet, have not been fully developed and tested, but since they permit the application of the complete array of techniques of triglyceride analysis, especially fractionation by selective argentation, they should provide a highly detailed analysis. Kaufmann et al. (5) have reported the direct fractionation of lecithins by selective argentation by means of TLC which should eliminate the need to convert the sample to triglycerides but apparently the fractionation by this method has not been sufficiently refined to be made the basis of an analytical method.

Described here is a simple method for the determination of the positional arrangement of fatty acids in lecithins in terms of classes of unsaturated and saturated fatty acids; that is, 1,2-disaturated; 1-saturated — 2-unsaturated — 1-unsaturated; 2-saturated and 1,2-diunsaturated.

EXPERIMENTAL

Materials

Highly purified lecithins were obtained from safflower seed oil, soybeans, milk serum and chicken eggs. The safflower lecithin was obtained from the product prepared by the degumming of crude safflower seed oil and was supplied to us through the courtesy of John Kneeland of the Pacific Vegetable Oil Company, Richmond, California. The soybean and egg lecithin were prepared from commercial samples of these compounds obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. Milk serum lecithin was isolated from milk serum supplied to us through the courtesy of the Rice Lake Creamery, Rice Lake, Wisconsin. The same general procedure was used for the isolation and purification of lecithins from each of these sources. The total lipid or lecithin concentrate, as the case may be, was fractionated by DEAE cellulose column chromatography as described by Rouser et al. (6). In this procedure the neutral lipids are separated first by elution with chloroform. Then a fraction containing the lecithin is eluted with 9:1 chloroform:methanol. The lecithin is isolated from this fraction by means of the silicic acid-silicate water column chromatographic technique described by Rouser et al. (6). In this procedure the column is prepared by slurrying the silicic acid in chloroform:methanol, 1:1, and ammonium hydroxide (10 ml of NH_4OH per 50 g of silicic acid). The methanol, water and excess NH_3 are washed out of the column with chloroform. One fraction containing such compounds as the ceramides and sterols is eluted first with 4:1 chloroform:methanol containing 0.5% water. Then the lecithin is eluted with a 4:1 ratio of chloroform:methanol containing 1.5% water and recovered. The lecithins prepared in this way were pure by TLC and infrared spectral analysis, but except for milk serum these samples are not necessarily representative of the source because some fractionation may

[1] Presented at the AOCS Meeting, Houston, April 1965.
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be expected in the course of the preparation of the commercial concentrates.

Lecithin of the disaturated class was prepared from the sample isolated from commercial soybean lecithin by catalytic hydrogenation in a mixture of 3:7 ethyl ether:alcohol with a platinum catalyst at 50 lb pressure and room temperature. The final product was purified by the silicic acid-silicate water chromatographic technique as described above (6). Gas-liquid chromatography (GLC) of the constituent acids of this preparation as methyl esters showed only saturated fatty acids.

Methyl pentadecanoate and other methyl esters used as reference standards for GLC and TLC were obtained from The Hormel Institute.

Methods

Gas-liquid chromatography (GLC) was carried out with an F & M Model 609 flame ionization instrument equipped with a ¼ in. × 7 ft column packed with 10% ethylene glycol succinate polyester on 100–120 mesh Gas-Chrom P (Applied Science Laboratories, State College, Pa.), and a carrier gas flow of 60 ml/min at 185C.

General Procedure

Three separate analyses are carried out on the purified samples of lecithin: 1) a total fatty acid composition; 2) the composition of the fatty acids distributed in the β-position; and 3) the amount of disaturated class in the sample.

Determination of the total fatty acid composition is carried out by GLC on the methyl esters which are prepared by heating about 2 mg of the sample sealed in a glass ampule in a nitrogen atmosphere with 3 ml of 6% dry HCl in methanol for 2 hr in a boiling water bath.

The composition of the fatty acids distributed in the β-position is determined by first carrying out an enzymatic hydrolysis of the sample with phospholipase A as described by Robertson and Lands (7). The fatty acids which are liberated from the β-position by this enzyme are isolated by preparative TLC with chromatoplates coated with chloroform-extracted Silica Gel G, esterified and analyzed by GLC as described above.

The saturated class of lecithins is determined as follows. About 5 mg of sample is treated with 1 ml of a saturated methanol solution of mercuric acetate as described by Mangold (8) in a 10 × 75 mm test tube. After the sample has reacted for 24 hr, a small piece of fine glass wool is pushed down into the test tube. A clear supernatant layer of liquid rises above the glass wool plug and is withdrawn by means of a syringe. This solution is then applied to a chromatoplate, coated with chloroform-extracted Silica Gel G and chromatographed in 70:30:4, chloroform:methanol:water. The unreacted (disaturated) lecithins are readily separated from the other lecithin classes which form adducts with mercuric acetate, as illustrated in Figure 1.

Quantitative analysis of the relative amount of disaturated class is made by means of an internal standard as follows. The bands (on a preparative plate) of the disaturated class and the other classes unseparated at the origin on the plate are scraped into individual glass test tubes restricted for sealing. A small but known amount (0.1 mg) of methyl pentadecanoate dissolved in 3 ml methanol containing 6% (w/w) of dry HCl is added to each tube. The tubes are then flushed with nitrogen, sealed with a torch and heated for 2 hr in a boiling water bath to convert the constituent fatty acids of the lecithins to methyl esters. The methyl esters are recovered in the usual way and analyzed by GLC. By comparison of the peak areas of the fatty acids of the two samples in relation to those given by the internal standard in the two samples, the percentage of the disaturated class is determined.

RESULTS AND DISCUSSION

The data on fatty acid composition of the four lecithins are presented in Table I. As

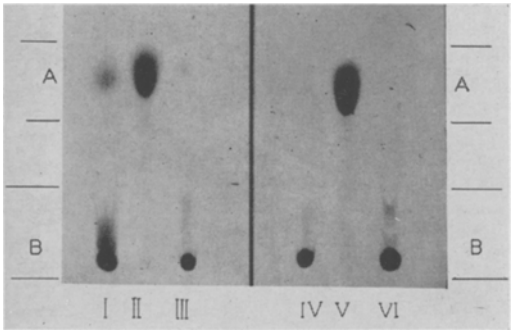


FIG. 1. Silica Gel G coated chromatoplate showing the separation of disaturated lecithins from unsaturated lecithins in the form of mercury acetate adducts: *A*, disaturated spots; *B*, mercury adducts; *I*, milk serum; *II*, hydrogenated soybean; *III*, egg; *IV*, soybean; *V*, hydrogenated soybean and *VI*, safflower. Solvent: 70:30:4 chloroform:methanol:water, spots visualized by charring.

TABLE I

Fatty Acid Analysis of Total Lecithins and Composition of Fatty Acids Distributed in the β-Position (% wt)

	Egg		Soybean		Safflower		Milk	
Fatty Acid[a]	Total	β-Position	Total	β-Position	Total	β-Position	Total	β-Position
14:0							3.0	3.9
15:0							1.1	1.2
16:0	33.0	1.4	14.7	4.8	15.1	2.5	27.8	19.5
17:0							1.1	0.8
18:0	15.4		5.2	1.2	4.8	0.3	11.0	3.0
15:1							0.3	0.5
16:1	2.1	1.0					2.6	3.4
17:1							tr	0.8
18:1	31.7	59.8	13.5	12.7	10.0	11.0	41.4	50.1
18:2	17.8	37.8	61.2	75.1	70.1	86.2	9.6	13.1
18:3			5.4	6.2			2.1	2.9
20:3							tr	0.8

[a] Identified by carbon number; shorthand designation.

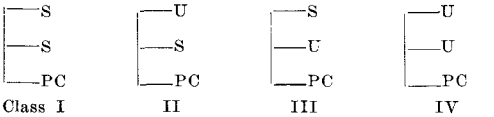
shown in Figure 1 the amount of the disaturated class of lecithin in the samples of soybean, safflower and egg was too small to be measured. The amount of disaturated lecithin in the milk serum sample was 12.7%.

For calculation of the class composition the percent of saturated fatty acids of the total fatty acids and the percent of saturated fatty acids in the β-position of the total fatty acids (Table I) are totalled. These values for egg are 48.4 and 0.7, respectively; for soybean, 19.9 and 3.0, respectively; for safflower seed, 19.9 and 1.4, respectively; and for the milk serum sample 44.0 and 14.2, respectively. It should be noted that percentages reported in Table I for the fatty acids distributed in the β-position should be divided in two in order to convert them to percentages of the total fatty acids in the molecule. The calculation of the class composition of milk serum lecithin from these data and the amount of disaturated class which is 12.7% in this case as illustrated in Table II. The amount of the disaturated class, 12.7%, was obtained from a direct analysis as described above. Since 12.7% of the total fatty acids are present in this class,

TABLE II

Analysis of Milk Lecithins

	Total	β-Position
% Sat.	44.0	14.2



Class I II III IV

I	=	12.7%
II	= (14.2 − 6.35) × 2	15.7%
II + III	= (44.0 − 12.7) × 2 = 62.6	
III	= 62.6 − 15.7	46.9%
IV	= 100 − (12.7 + 62.6) =	24.7%

it is evident that it contributes one half of this value or 6.35% to the percentage of the saturated fatty acids distributed in the β-position in the total sample. The remainder of the saturated fatty acids distributed in the β-position, i.e., (14.2–6.35) 7.85%, must originate from class II because this is the only other class having saturated fatty acids in the β-position. Since the amounts of saturated and unsaturated fatty acid in class II are equal, the total amount of this class is twice the value of the saturated fatty acids or (2 × 7.85) 15.7%.

The amount of class III is obtained by first calculating the combined amount of II and III and then subtracting the amount of II. Since classes II and III contain all of the saturated fatty acids except those found in the disaturated saturated class, the saturated fatty acids in these classes consist of 44.0 − 12.7 = 31.3% of the total fatty acids. The percent of the total of these classes is twice 31.3% because the amount of saturated and unsaturated fatty acids in these classes are equal. Thus, as shown in Table IV, the amount of class II and III = 62.6%, and the amount of class III = 46.9%. Class IV is determined by subtracting the amounts of all three classes from 100% and is 24.7% in this case.

Since lecithins of known structure were not available for an analysis, the validity of the method was determined by the analysis of three mixtures of known composition as shown in Table III. These mixtures were prepared by adding a known amount of fully hydrogenated soybean lecithin to safflower lecithin. Since the safflower lecithin contained no measurable amount of the saturated class, the composition could be changed in a very specific and known manner. Moreover, the analysis of these mix-

TABLE III

Analysis of Safflower Lecithin Containing Added Hydrogenated Soybean Lecithin

Class α,β	Original sample	Mix No. 1		Mix. No. 2		Mix No. 3	
		Calc.	Found	Calc.	Found	Calc.	Found
SS	—	13.2	14.0	21.8	22.5	40.0	40.0
SU	37.0	32.4	29.8	29.6	27.2		
						22.8	22.8
US	2.8	1.4	2.4	1.2	2.2		
UU	60.2	53.0	53.8	47.4	48.1	37.2	37.2

tures gave a direct method for checking the validity of the method for the determination of the disaturated class. Table III shows that there was very close agreement between the known and the found values. In general, the error in the values that may be expected should be about the same as that for a determination of fatty acid esters by GLC because the analytical values are based essentially on this method analysis.

The application of the method to the data on the four samples of natural lecithins is summarized in Table IV. In accordance with the general findings of others (9), the unsaturated fatty acids are found preferentially esterified in the β-position. The results do not determine if there is any preferential association of specific fatty acids. Information of this type will have to await further developments in the analytical methods, specifically methods which permit the breakdown of the components in each class.

Only milk serum contained a measurable amount of the disaturated class of the lecithins examined although traces of this class were detected in the samples of egg and soybean lecithins. Since plant lecithins are generally highly unsaturated, the amount of the disaturated class is generally small. The maximum possible amount of disaturated lecithin can be calculated from the amount of saturated fatty acids in the β-position. For example, in the case of egg lecithin, since only 1.4% of the fatty acids distributed in the β-position were saturated, the amount of the disaturated class could be no more than 1.4%. Since the disaturated class can be neglected in many cases, the method can be applied to much data on lecithins already in the literature because it requires information on only the total fatty acid composition and the composition of the fatty acids distributed in the β-position.

TABLE IV

Analysis of Natural Lecithins

Class α,β	Egg	Soybean	Safflower	Milk serum
SS	tr	—	tr	12.7
SU	95.4	33.8	37.0	46.9
US	1.4	6.0	2.8	15.7
UU	3.2	60.2	60.2	24.7

ACKNOWLEDGMENT

This work was supported in part by USPHS grant HE 05735 from the National Institutes of Health and by a grant from the Special Dairy Industry Board of the National Dairy Council.

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[Received July 1, 1965]

Characterization of Fatty Acids from Root and Shoot Lipids of *Capsicum* Species

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ABSTRACT

Lipids were extracted from the roots and shoots of four species of the *Capsicum* (pepper) genus and separated into three fractions: triglycerides; free fatty acids, mono- and diglycerides; and phospholipids. The component fatty acids were determined by subjecting the methyl esters to gas-liquid chromatography. The predominate fatty acids obtained were palmitic (16:0) and linoleic (18:2), with lesser amounts of linolenic (18:3), stearic (18:1), and oleic (18:0). Differences existed in the neutral lipid fractions which might be of value from taxonomic interests; however, the phospholipids from each of the species and plant parts did not differ so greatly. A comparison of the amount of unsaturated fatty acids in the phospholipid fractions indicates that differences exist which might be of value in determining the relative sensitivity of the several species to chilling temperatures.

INTRODUCTION

CHARACTERIZATION OF FATTY ACIDS from different *Capsicum* species, a small but extremely interesting genus, has not been reported before. Wheaton (14) in a study of the relationship between fatty acid composition and sensitivity to chilling temperatures of plants from several genera included the genus *Capsicum;* however, no species designations were defined. These findings indicated that the major fatty acids present in the root phospholipids were of a similar nature to those present in most plant species. Palmitic and linoleic were the major fatty acids, and palmitoleic, stearic, oleic, and linolenic were present in lesser amounts. Marion and Dempsey (11) reported that differences existed in the fatty acid content of *C. annuum* depending on whether the lipid was derived from the seed, fruit wall, or placenta.

The fatty acid composition of different plant species is of interest from two standpoints: as an aid in taxonomic classification of species or botanical varieties on the basis of similarities or dissimilarities; and as a possible criterion for evaluating sensitivity of species toward injury at chilling temperatures (temperature below about 10C but above freezing). McNair (9) in his comprehensive review, indicated a relationship between the area of origin of a species and its fatty acid composition. In general, species having an origin in mild, warm climates favored the formation of saturated fatty acids and unsaturated fatty acids with one double bond, while species from colder climates formed unsaturated fatty acids with two or three double bonds. Since the *Capsicum* species had their origin in the more tropical areas, one would predict on this basis their lipid composition would contain the more saturated fatty acids in comparison with those genera having their origin in the colder climates. A relationship between the amount of unsaturation found in the fatty acid composition of membrane fractions from various species and the sensitivity of those species to chilling temperatures has been reported recently by Lyons et al. (7). As a rule, those species which were resistant to chilling temperatures contained more unsaturated fatty acids, while those sensitive to chilling temperatures contained the more saturated fatty acids. From a taxonomic standpoint, different coniferous species have been distinguished on the basis of the fatty acid composition of their pollen (2), and Eglinton et al. (3) have used a characterization of the hydrocarbon content of leaves to classify plant species.

The main purpose of the results reported herein is to fully characterize the fatty acids from different *Capsicum* species and to ascertain if differences exist which might be useful in either a taxonomic classification or in the determination of the relative sensitivity of these species to chilling temperatures.

TABLE I

Recovery of *Capsicum* Lipids from Column Fractionation

	Mg/g fresh weight[a]				
	Total lipid	Triglycerides	Mono- and Diglycerides	Phospholipids	Per cent Recovered
Roots	4.48	3.08	0.57	0.65	96.0
Hypocotyl	3.75	1.97	0.74	0.67	90.0

[a] Average of the four species studied.

TABLE II

Comparison of Triglyceride Fatty Acids from Root and Shoot of *Capsicum* Species

Fatty acid[b]	Relative retention[c]	Per cent by weight of total fatty acid content[a]							
		C. frutescens		C. annuum		C. pendulum		C. sinense	
		Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
	.51	2.18	.....	.63	.....	1.32	.....	.....	.....
14:0	.55	.97	.46	.69	.....	1.80	2.04	2.70	.....
	.60	1.09	.73	.11	.25	.69	.36	.68	4.09
	.66	.37	.....	.45	.53	.97	.....	.42	.95
	.74	.42	.....	1.03	.....	.65	1.76	2.78	.....
	.81	.....	.....	.29	.....	.33	.97	1.01	1.76
	.87	.25	1.14	.42	.....	.38	.53	.41	2.04
	.97	1.99	.....	.....	13.49	18.69	.....	.....	.....
16:0	1.00	15.49	21.77	25.59	11.21	16.39	43.23	36.33	44.85
16:1	1.16	2.24	.79	1.73	.50	2.78	3.10	3.71	.....
17:0	1.24	.....	.....	.85	.....	.....	.....	1.55	4.70
	1.34	.72	.86	.99	.26	.78	2.57	2.66	1.83
	1.57	1.53	1.48	.43	.....	.91	4.13	2.28	1.58
18:0	1.80	6.73	11.31	6.47	3.43	6.54	10.98	10.99	3.93
18:1	2.04	10.12	7.48	6.96	6.72	7.71	8.07	10.81	15.65
18:2	2.52	49.27	48.51	47.51	58.06	35.25	17.96	20.70	16.05
	2.84	.....	.....	.....	.....	1.25	.94	1.02	2.58
18:3	3.22	4.06	5.47	5.29	5.55	3.55	3.34	1.94	.....
	3.53	2.56	.....	.56	.....	.....	.....	.....	.....
Total weight per cent of C_{16} and C_{18} unsaturated acids		65.69	62.25	59.76	70.83	49.29	32.47	37.16	31.70
Total weight per cent of C_{14}, C_{16}, and C_{18} saturated acids		23.19	33.54	32.75	14.64	24.73	56.25	50.02	48.78
Ratio (unsaturated/saturated)		2.83	1.85	1.82	4.84	1.99	0.58	0.74	0.65
Double bond index[d]		1.23	1.21	1.10	1.40	0.92	0.57	0.62	0.48

[a] Averages of 2 or 3 preparations from each tissue.
[b] The ratio shown is the number of carbon atoms to the number of double bonds in the molecule.
[c] Retention time relative to palmitic (16:0) as 1.00.
[d] Double bond index (DBI): the summation of weight per cent of each acid multiplied by the number of double bonds it contains per molecule and divided by 100.

MATERIALS AND METHODS

Plant Material

Seeds of *Capsicum frutescens, C. annuum, C. pendulum,* and *C. sinense*[1] were soaked in running tap water for six hours, spread on moist paper toweling, germinated, and grown in the dark at 30C. At the end of 10 days, roots and hypocotyls were harvested and stored frozen until preparation of the lipid extracts.

Lipid Extraction

Lipids were extracted by the method of Bligh and Dyer (1), modified for extraction on a smaller scale. Six grams of tissue were homogenized for 2 min in a Waring blendor with 10 ml of chloroform, 20 ml of methanol, and 2.6 ml of water (assuming 90% water in tissue). The homogenate was added to a separatory funnel containing 10 ml of water, the blendor cup washed with 10 ml of chloroform, thus giving a chloroform:methanol:water ratio 2:2:1.8. The lower chloroform phase containing the lipids was withdrawn and evaporated under nitrogen to give a final volume of 200 to 400 μl.

Column Fractionation

After extraction, the lipids were fractionated by silicic acid chromatography using the micromethod described by Lis et al. (6). Columns (125 × 8 mm) were packed with one gram of silicic acid, washed with 2 ml portions each of absolute methanol, redistilled dry acetone, redistilled dry ethyl ether, and redistilled petroleum ether. The silicic acid was conditioned prior to use by heating for 12 hr at 110C.

The lipid extract was placed on the columns and eluted with 15 ml portions of each of the following solvents: 1) 4% ethyl ether in petroleum ether—yielding the triglycerides; 2) 50% ethyl ether in petroleum ether—yielding the free fatty acids, mono- and diglycerides; and 3) absolute methanol—yielding the phospholipids. The fractions were evaporated to dryness under nitrogen for saponification and subsequent preparation of methyl esters by the methods of Marco et al. (10). Between 88%

[1] The species designations *C. pendulum* and *C. sinense* have been considered in a recent thesis by Eshbaugh (4) to be *C. baccatum* var. *pendulum* and *C. chinense*, respectively.

TABLE III

Comparison of Mono- and Diglyceride Fatty Acids from Root and Shoot of *Capsicum* Species

Fatty acid[b]	Relative retention[c]	Per cent by weight of total fatty acid content[a]							
		C. frutescens		C. annuum		C. pendulum		C. sinense	
		Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
	.51	4.41	.....	.....	.....	1.25	.....	.....	.23
14.0	.55	1.04	2.09	2.27	.45	1.76	2.76	2.69	2.07
	.60	1.71	.38	.45	.68	2.20	.78	2.04	.39
	.66	.51	1.70	.40	.....	1.59	.33	.99	.19
	.74	.92	.88	1.20	.29	.....	2.41	2.08	1.11
	.81	.....	.48	.81	.08	.....	1.03	1.31	.51
	.87	.45	.51	.28	.28	2.77	.....	.....	.49
	.97	.....	5.87	.....	10.16	12.10	.39	3.96	.....
16:0	1.00	16.64	21.71	20.08	13.34	10.97	32.17	22.92	28.29
16:1	1.16	2.22	1.86	1.85	.77	4.03	2.73	4.20	2.16
17:0	1.24	.....	1.45	.34	.....	.....	1.32	.....	.70
	1.34	1.04	.....	.58	.30	.....	1.43	1.20	.68
	1.57	1.07	1.27	.33	.34	5.42	1.02	1.52	1.19
18:0	1.80	7.05	6.89	3.80	4.13	5.55	8.48	4.78	5.26
18:1	2.04	7.57	6.19	6.62	5.55	12.85	6.02	8.98	5.57
18:2	2.52	45.87	45.08	50.24	60.26	24.30	32.98	33.51	43.75
	2.84	1.95	.....	.76	.....	..	1.39	2.38	.62
18:3	3.22	3.56	3.63	9.99	3.37	15.20	4.75	6.02	6.76
	3.53	3.99	.....	.....	..		.....	1.43	.....
Total weight per cent of C_{16} and C_{18} unsaturated acids		59.22	56.76	68.70	69.95	56.38	46.48	52.71	58.24
Total weight per cent of C_{14}, C_{16}, and C_{18} saturated acids		24.73	30.69	26.15	17.92	18.28	43.41	30.39	35.62
Ratio (unsaturated/saturated)		2.39	1.85	2.63	3.90	3.08	1.07	1.73	1.64
Double bond index[d]		1.13	1.09	1.39	1.38	1.12	0.89	0.98	1.15

[a,b,c,d] See legend under Table II for information pertaining to these superscripts.

and 96% of the lipids extracted were recovered from the column fractionation. Minor differences in the amount of lipids from the several species were observed and the average recovery for each fraction is shown in Table I.

Gas Chromatography of Fatty Acids

Gas chromatographic analyses of the methyl esters were made on a Loenco chromatograph with a 4-filament thermal conductivity detector connected to a Honeywell Electronik 1 mv strip chart recorder. The analytical column was 8.5 ft of 0.25 in. O.D. copper tubing, packed with 20% diethylene glycol succinate (DEGS) on 60/80 mesh firebrick. The operating conditions were: temperature 205C; helium flow rate, 80 ml/min; and filament current 110 ma. These conditions gave good separation of stearic, oleic, linoleic, and linolenic acids, which were of particular interest. Peaks were identified by comparing 1) their retention times with those of a known mixture of 7 esters of purified fatty acids, 2) a semilogarithmic plot of relative retention time against chain length or degree of unsaturation for the various components, and 3) relative retention times with published data obtained under comparable conditions (5). Calculations were made in a manner similar to that of Smith (13).

RESULTS

The fatty acid composition of the triglyceride fraction for the four *Capsicum* species studied is shown in Table II. The predominant fatty acids present were palmitic and linoleic with lesser amounts of linolenic, stearic, and oleic. Considerable variation existed in the relative amounts of the major fatty acids in the triglyceride fraction from these species as well as between the root and shoot within a species. The major trends in this fraction occur in the higher unsaturated acids where the linoleic and linolenic content of *C. frutescens* and *C. annuum* is considerably greater than that found in the other two species. Using either the double bond index (DBI) or the ratio of unsaturated/saturated fatty acids, this difference in the amount of unsaturation between *C. frutescens* and *C. annuum* and the others becomes readily apparent. In each case the ratio, or the DBI, reflects a greater amount of unsaturation in the root than in the shoot, except in *C. annuum* where the reverse is true. In fact, the ratio of unsaturated to saturated in this instance is twice that of any other sample. It is interesting to note the occurrence of a relatively large amount of an unidentified fatty acid with a relative retention of 0.97 in the shoot of *C. annuum* and the root of *C. pendulum*. This

TABLE IV

Comparison of Phospholipid Fatty Acids from Root and Shoot of *Capsicum* Species

Fatty acid[b]	Relative retention[c]	Per cent by weight of total fatty acid content[a]							
		C. frutescens		C. annuum		C. pendulum		C. sinense	
		Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
	.51	.....	.....	.....	.....	.06	.26	.59	.....
14:0	.55	.51	.82	.85	.47	.....	.27	1.23	.....
	.60	.31	.28	.21	.20	.07	.32	.45	.....
	.66	.27	.12	.33	.10	.....	.09	.94	.27
	.74	.85	.67	.55	.30	.49	.71	.94	.82
	.81	....	.17	.19	.....	.....	.....	.....	.....
	.87	.34	.45	.28	.18	.68	.49	.91	.79
	.97	.....	.....	.....	.....	.....	.....	.....	.....
16:0	1.00	18.96	18.47	22.83	19.07	20.41	25.43	21.13	25.33
16:1	1.16	.88	1.17	1.32	.38	1.18	.76	1.73	.62
17:0	1.24	.....	.31	.....	.50	.....	.63	.....	.95
	1.34	.84	.84	1.23	.46	1.32	1.39	.92	1.12
	1.57	.55	1.26	.47	1.01	1.19	1.75	1.06	1.99
18:0	1.80	5.83	6.61	4.98	6.55	5.47	7.68	3.43	6.33
18:1	2.04	2.51	3.50	3.17	2.03	2.53	2.27	7.43	2.42
18:2	2.52	62.00	54.95	59.91	60.40	58.77	52.26	51.59	52.78
	2.84	.69	...	.....	.....	.....	.44	.....	.....
18:3	3.22	5.48	10.38	3.68	8.34	7.83	4.54	5.68	3.46
	3.53	.....	.....	.....	.....	.....	.73	1.97	2.49
Total weight per cent of C_{16} and C_{18} unsaturated acids		70.87	70.00	68.08	71.15	70.31	59.83	66.43	59.28
Total weight per cent of C_{14}, C_{16}, and C_{18} saturated acids		25.30	25.90	28.66	26.09	25.58	33.38	25.79	32.30
Ratio (unsaturated/ saturated)		2.80	2.70	2.37	2.73	2.72	1.79	2.57	1.84
Double bond index[d]		1.44	1.45	1.35	1.48	1.45	1.22	1.29	1.19

[a,b,c,d] See legend under Table II for information pertaining to these superscripts.

occurred in these two samples in an amount equal to that of palmitic while it occurred only to a very small per cent or not at all in any other sample.

The mono- and diglyceride fatty acids and the free fatty acids obtained in fraction 2 again demonstrate a high degree of variation between species and between plant part (Table III). While the amounts vary between this fraction and that of the triglycerides, the relative distribution of the unsaturated and saturated is the same in these two fractions. The unidentified fatty acid in this fraction (fraction 2) at relative retention of 0.97 occurs in either the root or shoot in each of the species, in contrast to the results indicated for the triglyceride fraction (fraction 1).

Table IV presents the results of the fatty acid analysis of the phospholipids (fraction 3) and is of particular interest since the phospholipids are primarily associated with the subcellular membranes. In general, there is considerably less variation between the root and shoot of each species in this fraction than existed in the other two fractions. The root and shoot of both *C. frutescens* and *C. annuum* exhibit fatty acid content of a similar nature. *C. pendulum* on the other hand, has nearly 10% less unsaturated fatty acids in the shoot than in the root and *C. sinense* has 6% less in the shoot than root. This large difference in the amount of unsaturated fatty acids is reflected in the ratio of unsaturated to saturated acids, and in the DBI.

DISCUSSION

The fatty acid composition of *Capsicum* species indicates there are considerable differences between species, plant part, and lipid fraction from which they were extracted. The phospholipid fraction yielded results for root tissue similar to that reported by Wheaton (14). Composition of *C. annuum* seeds, fruit wall, and placenta reported by Marion and Dempsey (11) indicated differences in linoleic and linolenic between various plant parts, and in general, the results from the root and shoot reported here are more closely related to composition of the seed than either the fruit wall or placenta. Newman (12) has recently demonstrated the difference in fatty acid composition of chloroplast fractions which resulted from having grown plants in various nutrient solutions. However, since the tissues used in these studies were grown in the dark and were thus etiolated, the analyses were not confounded by

the presence of the complex chloroplast lipids.

Lyons et al. (7) have demonstrated the relationship between the amount of unsaturation present in the fatty acids of mitochondrial membranes and the sensitivity of the species to chilling temperatures. The species with the higher amount of unsaturated fatty acids were more resistant to chilling temperatures than those with the more saturated fatty acids. The *Capsicum* species have been shown to be sensitive to chilling temperatures and the ratio of unsaturated/saturated fatty acids found here corresponds to those observed for the sensitive species reported previously (14). While the differences in phospholipid fatty acids between plant parts and between species appear slight in some instances, it has been shown with mixtures of fatty acids that a difference of less than 5% in the amount of unsaturated fatty acid in the mixture can result in a 20C difference in the temperature which causes solidification of that mixture (8).

Differences existed in the neutral lipid fraction (fraction 1 and 2) which might be useful in identifying these species; however, the phospholipids (fraction 3) from each of these species and plant parts did not differ so greatly as to make these analyses useful as a general taxonomic tool in characterization of species difference in the *Capsicum* genus.

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[Received Nov. 23, 1964]

Long-Chain Fatty Acids Containing Ether Linkage. I. The Antibacterial and Fungicidal Activities of Some New β-Alkyloxypropionic Acids and Their Methyl Esters

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ABSTRACT

β-Alkoxypropionic acids and their methyl esters were made with alkoxy groups ranging from C_4H_9O to $C_{18}H_{35}O$: R-O-$CH_2CH_2COOH(CH_3)$. Methyl esters and acids were also made with one and with two oxyethylene groups between the alkoxy group and the propionic acid group: $RO(CH_2\ CH_2\ O)n\text{-}CH_2\ CH_2\ COOH(CH_3)$. The compounds were tested against *Staphylococcus aureus* and against *Penicillium* for growth inhibition. The optimum size of the alkoxy group appears to be R = $C_{12}H_{25}$. Oxyethylene groups enhanced the activity against *S. aureus,* but had relatively little effect against *Penicillium.*

INTRODUCTION

ON THE ANTIBACTERIAL and fungicidal activities of the straight-chain aliphatic fatty acids, many publications are available. It was confirmed that the C_8 to C_{12} acids in these series were more effective against the common test organisms than the higher members. In particular undecylenic acid appears to be active against some fungi. Sodium propionate is also a well-established commercial fungicide, being used in bread, pickles, some fruits and vegetables.

The combination involving germicides and surfactants has generally been used in addition to the germicidal surfactants. There have been many interesting studies on the influence of the surfactants upon the bactericidal and fungicidal action of the active compound. In their study of deodorant soaps, Linfield et al. (1) found that only nonionic detergents, particularly nonionic detergents of the polyoxyethylene ester series, exhibit a peculiar potentiating effect upon certain germicidal agents in relatively low level. Further it would be expected that the combination in one molecule of surface activity plus specific germicidal radicals should produce a large inhibiting effect against microorganisms, as in anacordic acid (2), alkyl benzoylacrylic acid (3), S-alkyl thiomalic acid (4), and polyoxyethylated pentachlorophenol (5).

This report is concerned with the antibacterial and fungicidal activities of β-alkyloxypropionic acids containing several oxyethylene radicals in an alkyl chain and their methyl esters. In the French patent (6) it has been shown that the long chain carboxylic acids containing ether linkages was excellent as an improver of mercerization lyes. Also β-octadecyloxypropionic acids as an emulsifying agent has been obtained by Fieser et al. (7), from octadecyl alcohol and methyl acrylate. Terentev et al. (8) have synthesized β-butoxypropionic acid, 3-(2-butoxy)-ethoxypropionic acid by the cyanoethylation of the corresponding alcohols and hydrolysis of those products. Recently some new patents concerning the continuous process of the preparation of alkyl β-alkyloxypropionate from aldehyde or acetal and ketene have been published independently (9).

EXPERIMENTAL PROCEDURES AND DATA

Materials

The starting alcohols were purified carefully by fractional distillation of commercially available alcohols in vacuo and their purities were checked with gas-liquid chromatography. Their boiling points, molecular weights and elemental analysis data were summarized in Table I with those of 2-oxyethyl-, and 2-(2-oxyethyl)-oxyethyl derivatives of individual alcohols. The latter were obtained by fractionally distilling the adducts, prepared in the conventional base-catalyzed procedure from the corresponding alcohols and ethylene oxides, through a 60 cm electrically heated column packed with glass helices fitted to a 1 liter flask and the vacuum equipment.

The Preparation of β-Alkyloxypropionic Acids

β-Alkyloxypropionic acids were prepared according to Terentev et al. (8) by the following scheme:

$$R(OCH_2CH_2)nOH + CH_2 = CHCN$$
$$\rightarrow R(OCH_2CH_2)nOCH_2CH_2CN$$
$$\rightarrow R(OCH_2CH_2)nOCH_2CH_2COOH$$
n: 0, 1 and 2

For members of the compounds listed in the Tables II–V, the following general synthetic method was applied.

TABLE I

The Constants of Starting Alcohols: $R-(OCH_2CH_2)n-OH$

R	$(OCH_2CH_2)n$ OH	BP C/mm Hg	Mol wt Found[a]	Mol wt Calcd.	C% Found	C% Calcd.	H% Found	H% Calcd.
	n = 0	115–7.2	.....	.....	.....	.....	.....	.....
C_4H_9	1	62–7/1	116.5	118.2	61.12	60.99	11.76	11.94
	2	85–90/1	164.1	162.2	59.40	59.24	11.03	11.19
	n = 0	154.1–4.8	103.5	102.2	.....	.....	.....	.....
C_6H_{13}	1	75–8/0.5	151.3	146.2	65.85	65.72	12.28	12.41
	2	101–7/0.5	191.4	191.3	63.32	63.14	11.58	11.66
	n = 0	193.2–4.5	130.4	130.2	.....	.....	.....	.....
C_8H_{17}	1	94–6/1	174.8	174.3	69.12	68.92	12.60	12.73
	2	122–6/1	222.3	218.3	66.15	66.03	11.86	12.01
	n = 0	60–4/3	132.9	130.2	.....	.....	.....	.....
C_8H_{17}[b]	1	99–104/3	176.9	174.3	69.40	68.92	12.61	12.73
	2	125–30/3	221.4	218.3	66.23	66.03	11.95	12.01
	n = 0	231.8/2.6	159.5	158.3	.....	.....	.....	.....
$C_{10}H_{21}$	1	115–22/1.5	204.3	202.3	71.84	71.24	12.68	12.95
	2	143–5/1.5	251.7	246.4	68.96	68.26	12.12	12.27
	n = 0	126–31/7	186.7	186.3	.....	.....	.....	.....
$C_{12}H_{25}$	1	138–48/4	231.8	230.3	73.12	72.99	13.05	13.13
	2	163–70/4	280.1	274.3	70.16	70.29	12.78	12.54
$C_{14}H_{29}$	n = 0	133–5/3	215.4	214.3	.....	.....	.....	.....
$C_{16}H_{33}$	0	158–9/5	245.4	242.4	.....	.....	.....	.....
$C_{18}H_{35}$[c]	0	177–82/3	263.5	268.5	.....	.....	.....	.....

[a] Calculated from hydroxyl value.
[b] 2-Ethylhexyl.
[c] Oleyl, IV: Found 90.4, Calcd. for $C_{18}H_{35}OH$, 94.5.

Cyanoethylation. A mixture of 1.0 mole of alcohol and 0.5% of potassium hydroxide was placed in a four-necked flask provided with a mechanical stirrer, a dropping funnel, a thermometer and a reflux condenser. The contents were mixed thoroughly, then 4.0 moles of acrylonitrile were added from a dropping funnel at a rate such that temperature did not exceed 40C. The mixture was stirred for an hour at 80C after all the acrylonitrile had been

TABLE II

The Physical Properties of β-Alkyloxypropionitriles as Formulated in General $R(OCH_2CH_2)nOCH_2CH_2CN$

R	$(OCH_2CH_2)n$	bp C/mm Hg	N% Found	N% Calc.
	n = 0	79.2–9.8/8	11.14	11.01
C_4H_9	1	130–2/15	8.26	8.18
	2	127–9/1	6.63	6.51
	n = 0	82–4/0.5	9.13	9.02
C_6H_{13}	1	117–22/1.5	7.62	7.03
	2	139–42/0.5	5.59	5.76
	n = 0	103–4/1	7.73	7.64
C_8H_{17}	1	128–31/1	6.77	6.16
	2	165–72/1	5.38	5.16
	n = 0	108–13/5	7.79	7.64
C_8H_{17}[a]	1	132–5/3	6.19	6.16
	2	165–8/4	5.30	5.16
	n = 0	122–6/1	6.61	6.63
$C_{10}H_{21}$	1	148–53/1	5.87	5.49
	2	170–5/1	4.72	4.68
	n = 0	154–8/4	5.92	5.85
$C_{12}H_{25}$	1	160–75/1.5	5.12	4.94
	2	165–75/0.5	4.39	4.28
$C_{14}H_{29}$	n = 0	163.5–4.5/1	5.40	5.24
$C_{16}H_{33}$	0	185–90/1	5.07	4.94
$C_{18}H_{35}$[b]	0	185–90/1	4.55	4.28

[a] 2-Ethylhexyl.
[b] Oleyl.

TABLE III

The Physical Properties of Methyl β-Alkyloxypropionates as Formulated in General $R(OCH_2CH_2)nOCH_2CH_2COOCH_3$

R	$(OCH_2CH_2)n$	bp C/mm Hg	n_D^{20}	D_4^{20}
	n = 0	57–61/4	1.4152	0.9451
C_4H_9	1	102–7/1.5	1.4320	0.9617
	2	131–5/1	1.4368	0.9820
	n = 0	98–103/3	1.4219	0.9389
C_6H_{13}	1	114–8/1	1.4326	0.9600
	2	145–9/1.5	1.4381	0.9805
	n = 0	139–44/3	1.4293	0.9134
C_8H_{17}	1	130–7/1.5	1.4365	0.9436
	2	161–8/2	1.4415	0.9654
	n = 0	84.5–9/2	1.4277^{25}	0.9151
C_8H_{17}[a]	1	125–30/2	1.4348^{25}	0.9440
	2	178–81/5	1.4392^{25}	0.9677
	n = 0	125–8/1	1.4345	0.9058
$C_{10}H_{21}$	1	147–52/1	1.4398	0.9275
	2	178–83/2	1.4434	0.9483
	n = 0	135–8/1	1.4368^{25}	.....
$C_{12}H_{25}$	1	167–72/2	1.4396^{25}	.....
	2	204–10/2	1.4445^{25}	.....
$C_{14}H_{29}$	n = 0	150–3/1	1.4323^{45}	.....
$C_{16}H_{33}$	0	167–72/1	1.4372^{45}	.....
$C_{18}H_{35}$[b]	0	183–5/1	1.4470^{45}	.....

[a] 2-Ethylhexyl.
[b] Oleyl.

TABLE IV

The Analysis Data of Methyl β-Alkyloxypropionates as Formulated in General $R(OCH_2CH_2)nOCH_2CH_2COOCH_3$

R	$(OCH_2CH_2)n$	Sap. V		C%		H%	
		Found	Calcd.	Found	Calcd.	Found	Calcd.
	n = 0	360.7	350.2	59.99	59.98	10.12	10.07
C_4H_9	1	280.1	274.7	58.85	58.80	9.93	9.87
	2	234.9	226.0	58.10	58.05	9.84	9.74
	n = 0	300.2	298.1	63.86	63.80	10.65	10.71
C_6H_{13}	1	242.0	241.6	62.30	62.05	10.20	10.41
	2	197.0	203.1	60.92	60.86	10.12	10.21
	n = 0	257.3	259.4	66.77	66.64	11.22	11.18
C_8H_{17}	1	213.8	215.5	64.68	64.60	10.88	10.84
	2	176.8	184.4	63.61	63.14	10.50	10.60
	n = 0	257.3	259.4	66.70	66.64	11.24	11.18
C_8H_{17}[a]	1	216.0	215.5	64.90	64.60	10.70	10.84
	2	183.6	184.4	63.61	63.14	9.96	10.60
	n = 0	229.6	229.7	68.91	68.83	11.53	11.51
$C_{10}H_{21}$	1	196.0	194.6	65.98	66.65	11.13	11.19
	2	173.1	168.8	65.02	64.99	10.85	10.92
	n = 0	204.6	206.0	70.60	70.56	11.60	11.84
$C_{12}H_{25}$	1	175.8	177.3	68.35	68.28	11.33	11.47
	2	153.2	155.7	66.40	66.59	11.09	11.19
$C_{14}H_{29}$	n = 0	185.8	186.7	71.83	71.95	12.00	12.08
$C_{16}H_{33}$	0	167.2	170.8	73.02	73.12	12.23	12.27
$C_{18}H_{35}$[b]	0	158.8	158.2	74.01	74.52	11.88	11.94

[a] 2-Ethylhexyl.
[b] Oleyl, IV: Found 71.0, Calcd. for $C_{22}H_{42}O_3$, 71.3.

added, then made acidic with acetic acid, and the products were distilled in vacuo through an efficient packed column as mentioned above. Table II gave the boiling points of the prepared nitriles with the elemental analysis data of them.

Hydrolysis and Methylation. Each β-alkyloxypropionitrile was hydrolysed with concen-

TABLE V

The Physical Properties of β-Alkyloxypropionic Acids: $R(OCH_2CH_2)nOCH_2CH_2COOH$

R	$(OCH_2CH_2)n$	bp C/mm	Neutr. V.		C%		H%	
			Found	Calcd.	Found	Calcd.	Found	Calcd.
	n = 0	117–8/6	378.6	383.9	58.02	57.52	9.87	9.65
C_4H_9	1	141–5/1	297.5	295.0	56.95	56.83	9.60	9.54
	2	169–71/1	243.6	239.5	56.48	56.40	9.49	9.47
	n = 0	143–5/2	328.3	322.1	62.25	62.05	10.44	10.41
C_6H_{13}	1	171–3/1	253.2	257.1	60.88	60.54	10.20	10.16
	2	199–204/1	209.0	213.4	59.72	59.40	10.01	9.97
	n = 0	26.3–6.8 [c]	282.6	277.4	66.77	65.32	11.22	10.96
C_8H_{17}	1	165–70/1.5	230.1	227.8	64.00	63.43	10.71	10.64
	2	190–2/1	195.8	193.3	62.18	62.05	10.53	10.42
	n = 0	139–9.5/2	275.2	277.4	66.88	65.32	11.00	10.96
C_8H_{17}[a]	1	168–9/2	236.4	227.8	63.90	63.43	10.72	10.64
	2	199–201/1	200.1	193.3	62.24	62.05	10.50	10.42
	n = 0	40.5–1.0[c]	241.5	243.6	67.75	67.79	11.25	11.38
$C_{10}H_{21}$	1	18.8–9.0[c]	200.8	204.5	64.92	65.67	11.00	11.02
	2	27.8–8.0[c]	175.8	176.2	64.01	64.13	10.70	10.80
	n = 0	52.0–2.5[c]	209.3	217.2	69.08	69.73	11.75	11.70
$C_{12}H_{25}$	1	29.5–31.0[c]	183.3	185.5	67.02	67.74	11.28	11.37
	2	35.5–6.5[c]	159.6	152.0	65.11	65.87	11.15	11.06
$C_{14}H_{29}$	n = 0	61.0–1.5[c]	189.8	195.9	70.95	71.28	11.90	11.96
$C_{16}H_{33}$	0	66.7–7.7[c]	175.0	178.4	72.32	72.56	12.15	12.18
$C_{18}H_{35}$[b]	0	.....	160.1	164.8	73.88	74.07	11.66	11.84

[a] 2-Ethylhexyl.
[b] Oleyl (purified by molecular distillation), IV: Found 72.5, Calcd. for $C_{21}H_{40}O_3$, 74.5.
[c] Melting point.

trated hydrochloric acid to the corresponding β-alkyloxypropionic acid in the usual way, and the latter was purified as methyl ester. The general synthetic methods was: A part of β-alkyloxypropionitrile was placed in a flask provided with a reflux condenser and a dropping funnel, then four parts of concentrated hydrochloric acid (sp gr 1.18) was added dropwise from a funnel. The mixture was heated for one hour at 100C. After cooling, ammonium chloride separated was removed. The filtrate was concentrated in the vacuum film evaporator to obtain a viscous syrup. Excess of methyl alcohol was added to the syrup and the mixture was refluxed for one hour. The excess of methyl alcohol was removed and the methyl β-alkyloxypropionate was distilled carefully in vacuo. Their physical properties were determined and shown in Table III.

Also the saponification values and elemental analysis data of those methyl esters are given in Table IV.

Hydrolysis of Methyl Esters. The β-alkyloxypropionic acids were obtained by the hydrolysis of the corresponding methyl esters with potassium hydroxide in water, then were purified by distillation in vacuo and/or recrystallization from ethyl alcohol. They are new compounds except 3-butoxy-, 3-hexyloxy-, 3-octyloxy-, 3-decyloxy-, 3-dodecyloxy-, 3-((2-ethylhexyl) oxy)-, and 3-((2-butoxy) ethoxy)-propionic acids. Their physical properties were determined and given in Table V.

Infrared Spectrum

The infrared spectra of the β-alkyloxypropionitriles, β-alkyloxypropionic acids, and methyl β-alkyloxypropionates were determined in order to confirm their structures and those of 3-(2-(2-(octyloxy) ethyloxy) ethyloxy)-propionic series are pictured for example (Fig. 1). The characteristic absorption are as follows: ca. 720, ca. 1470, and ca. 2900 cm^{-1}, the absorption of $-CH_2-$; at 1390 cm^{-1}, the absorption of CH_3; at ca. 1120 and 1350 cm^{-1}, the absorption of $-CH_2-O-CH_2-$; and in the nitriles, at 2250 cm^{-1}, the absorption of C≡N; in the acids, the absorption of COOH appeared at 1200 and 1725 cm^{-1}; further in the methyl esters, at ca. 1165, ca. 1430, and 1750 cm^{-1}, the characteristic -COO- absorptions of methyl esters were observed.

Antibacterial and Fungicidal Study

The antibacterial and fungicidal evaluations were carried out by a dilution method. Aliquots of a definite concentration of the sample solu-

TABLE VI

The Minimum Concentration (γ/ml) of β-Alkyloxypropionic Acid (I) and Methyl β-Alkyloxypropionates (II) Which Show Inhibition of the Growth of the *Staphylococcus aureus* 209P and P.408 — 701

Tested microbes	R	n	C_4H_9			C_6H_{13}			C_8H_{17}			C_8H_{17}[c]			$C_{10}H_{21}$			C_{12} H_{25}			C_{14} H_{29}	$C_{16}H_{33}$	$C_{18}H_{35}$	Comparison
			0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	0	0	
S[a]	I[c]		10^3	>10^3	10^3	10^3	10^3	500	500	500	500	10^3	>10^3	500	500	100	100	100	50	50	500	500	>10^3	>10^3	>10^3 [g]
	II[d]		>10^3	>10^3	10^3	10^3	10^3	10^3	500	500	10^3	10^3	500	10^3	500	100	100	500	50	50	10^3	>10^3	>10^3	>10^3	500 [h]
P[b]	I		10^3	>10^3	10^3	500	10^3	>10^3	500	500	500	500	500	500	500	100	100	10^3	100	100	>10^3	>10^3	>10^3	>10^3	>10^3 [g]
	II		>10^3	>10^3	>10^3	>10^3	>10^3	10^3	10^3	500	500	10^3	10^3	500	10^3	500	500	10^3	500	500	>10^3	>10^3	>10^3	>10^3	10^3 [h]

[a] *Staphylococcus aureus* 209P. [b] *Penicillium* 408–701. [c] I = $RO(CH_2CH_2O)nCH_2CH_2COOH$. [d] II = $RO(CH_2CH_2O)nCH_2CH_2COOCH_3$. [e] 2-Ethylhexyl. [f] Oleyl. [g] Propionic acid. [h] Methyl laurate.

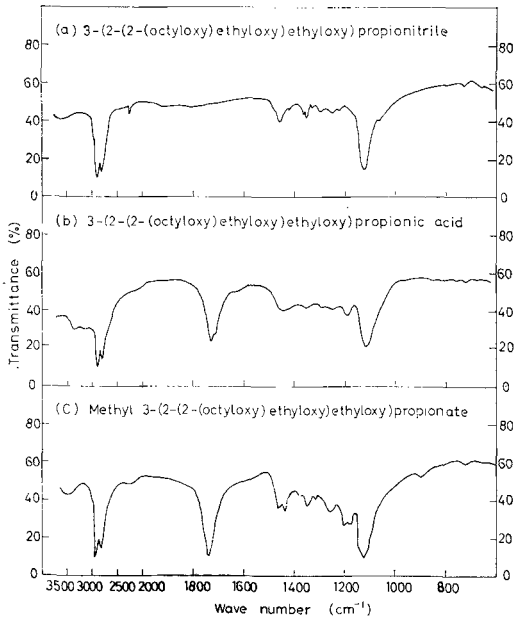

FIG. 1. Infrared spectra of the 3-(2-(2-(octyloxy) ethyloxy) ethyloxy) propionitrile, propionic acid, and its methyl ester.

tions were added to measured amounts of liquid agar media, which contained 1.0% of bouillon, 1.0% of peptone, and 0.25% of sodium chloride (for *Staphylococcus*) or 1.0% of peptone and 2.0% of glucose (for *Penicillium*). The surface of the agar medium was streaked with a standard loop containing a culture of the *S. aureus* strain No. 209 P or *Penicillium* strain No. 408–701. After an incubation period of 24 hr (S.) or one week (P.) at 37C the tubes were examined visually, and the conditions of growth were recorded. The results of these experiments are summarized in Table VI with that of evaluation on propionic acid and methyl laurate.

DISCUSSION

Table VI showed that the β-dodecyloxypropionic acid series were the most active against the *Staphylococcus* baccilli, and the β-decyloxypropionic and β-octoxypropionic acids were more active than the other alkyloxypropionic acid groups. In these acids, the increase of the ether radicals increased their antibacterial activities except octyloxypropionic acid. In the free acids, dodecyloxypropionic acids, especially those which had two or three oxyethylene linkages, were the most active against the *Staphylococcus*. Following the dodecyloxy-series, the decyloxypropionic, octyloxypropionic, and tetradecyloxypropionic acid groups were active. Further, in the methyl esters of these acids, the introduction of oxyethylene linkages in the molecule seems also to increase their bactericidal activities. And it was recognized that free fatty acids were more active than their methyl esters. In the study on the *Penicillium* inhibition, β-decyloxypropionic acid and dodecyloxypropionic acid groups were active, whereas the activities of methyl β-octoxypropionates were rather weak in the ester series. In these results, it was shown that there was no relation between the contents of the oxyethylene groups and the fungicidal activities up to three ether linkages. It seems that the *Penicillium* may be too much resistant against those acids to show the effect of the ether bonds in the molecule.

Generally the antibacterial or fungicidal activities of β-alkyloxypropionic acid and methyl β-alkyloxypropionate are dependent on the existence of the octyl, decyl and dodecyl radicals.

ACKNOWLEDGMENTS

Support in part by grants from the Asahi Glass Foundation for the Contribution to Industrial Technology. Alcohols from Nippon Soda Co., Ltd., and Kao Soap Co., Ltd.; acrylonitrile from Nitto Chemical Industries Co. Ltd. Elemental analysis by Mr. S. Nakada, and biological evaluations by Mrs. S. Iriyama.

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[Received Aug. 6, 1965]

Lipid Peroxidation in Rat Tissue Homogenates: Interaction of Iron and Ascorbic Acid as the Normal Catalytic Mechanism

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ABSTRACT

Iron and ascorbic acid appear to be the normal catalytic components responsible for the lipid peroxidation reaction in aerobically incubated rat tissue homogenates. The amounts of each present in the catalytically-active fractions of rat liver, brain, testis, and kidney are appropriate to explain the lipid peroxidation reaction measured. Utilization of ascorbic acid as part of the normal catalytic mechanism is indicated by the following: The catalytic activity of the tissue soluble phase occurs only in the small molecule fraction eluted from Sephadex, and ascorbic acid occurs only in this fraction; the extent of catalysis by the small molecule fractions of the soluble phases from several tissues is proportional to their ascorbic acid content; and pH effect on lipid peroxidation is the same for both soluble-phase and ascorbic acid catalysis. Utilization of iron as part of the normal catalytic mechanism is indicated by EDTA inhibition studies and by measurements of pH effects. Previous studies have demonstrated the lack of catalytic activity by cations other than iron for the lipid peroxidation reaction in homogenates. Lipid peroxidation is inhibited at high tissue concentration and the inhibition is due to components occurring in the large molecule fraction of the soluble phase.

INTRODUCTION

THE LIPID PEROXIDATION reaction in vitro has been correlated with several important biological problems including radiation damage (1,2), mitochondrial swelling and lysis (3,4), vitamin E deficiency (5,6), and tumor formation (7,8). In spite of the possible widespread significance of this peroxidation reaction, no changes in the concentrations of the tissue components responsible for lipid peroxidation have been reported for any of these experimental conditions. This is due, presumably, to the lack of quantitative measurements of these components in normal tissue homogenates. These measurements are also necessary to understand the differences noted in the extent of lipid peroxidation in various tissue homogenates such as brain, liver, kidney, and testis (1). Testis was examined in the present study as an example of tissue whose homogenates undergo little or no lipid peroxidation during aerobic incubation, whereas brain, liver, and kidney were selected as examples of those whose homogenates undergo considerable peroxidation.

The amount of lipid peroxidation in aerobically incubated tissue homogenates depends on the tissue type and concentration (1,9), the buffer concentration, and the pH (9). Certain aspects of this reaction have been extensively studied using homogenates (10), mitochondria (3,11–13), microsomes (9,14,15), and pure unsaturated lipids (16–18). Studies using a wide variety of exogenous materials have demonstrated both an enzymic (14) and a nonenzymic (3,11) lipid peroxidation in microsomes and mitochondria. Iron acts as the catalyst in both the enzymic (19,20) and nonenzymic (11) reactions. Extensive in vitro studies have led to the establishment of an iron-ascorbic acid catalytic mechanism for nonenzymic lipid peroxidation (11,12). However, the quantitative measurements of tissue iron and ascorbic acid, needed to establish their role as the normal catalytic mechanism present in homogenates, have not been carried out. This mechanism has been inferred from the many experiments using exogenous materials. The amounts of iron and ascorbic acid present in the catalytically active homogenate fractions of liver, brain, testis, and kidney, as well as the amounts of each required for the observed lipid peroxidation, are reported in this study.

MATERIALS AND METHODS

Tissue Preparation

Liver, brain, kidney, and testis were obtained from bled Sprague-Dawley rats (6-month-old males) and homogenized either in buffer (0.01 M phosphate, pH 7.0, containing 0.15 M NaCl) or in sucrose (0.25 M) solutions. Homogenates were centrifuged at 1,000 X *g* for 10 min and the pellets discarded. The 1,000 X *g* supernatant fraction was centrifuged at 10,000 X *g* for 10 min. The pellet was washed twice and saved as the mitochondrial fraction. The 10,000

X *g* supernatant fraction was centrifuged at 105,000 X *g* for 60 min. The pellet was washed once and saved as the microsomal fraction. The 105,000 X *g* supernatant fraction was saved as the soluble phase. The soluble phase was further separated into large (S-1) and small (S-2) molecular fractions by passage through Sephadex G-50 (21). Elution was at 90 ml/hr through a 25 X 2.5 cm column using a Milton Roy Minipump.

Chemical Procedures

Protein concentrations were determined colorimetrically using Folin-phenol reagent according to the method of Lowry et al. (22) using bovine serum albumin as the standard. Ascorbic acid was measured colorimetrically using 2,6-dichlorophenolindophenol solution according to the method of Roe (23). Sulfhydryl groups were measured colorimetrically using 5,5′-dithiobis-(2-nitrobenzoic acid) solution according to the method of Ellman (24) using glutathione as the standard. Total iron in microsomal fractions was determined colorimetrically by the Galbraith Laboratories, Knoxville, Tennessee. Lipid peroxidation was measured colorimetrically by the thiobarbituric acid (TBA) test (1). Following incubation at 37C for 90 min the contents of each vessel were poured into test tubes containing 1 ml of 30% trichloroacetic acid. One milliliter, of 0.75% TBA was added. Following 15 min of boiling, and subsequent cooling, the samples were centrifuged 10 min at 2,000 rev/min and the optical density of the supernatant fraction was read at 530 mμ. All spectrophotometric measurements were carried out using a Beckman DU spectrophotometer. The ethelenediamine-tetraacetate (EDTA) used was Fisher reagent grade.

RESULTS

The normal tissue components responsible for lipid peroxidation in aerobically incubated rat tissue homogenates are distributed in both the soluble and the particulate fractions. Each fraction was analyzed separately following its isolation by centrifugation.

Analysis of the Soluble Fraction

Small amounts of soluble phase from liver, brain, and testis catalyzed lipid peroxidation in aerobically incubated suspensions of brain microsomes, whereas larger amounts inhibited the reaction (Fig. 1). The same activities F-1 were noted when suspensions of liver, testis, or kidney microsomes were substituted for

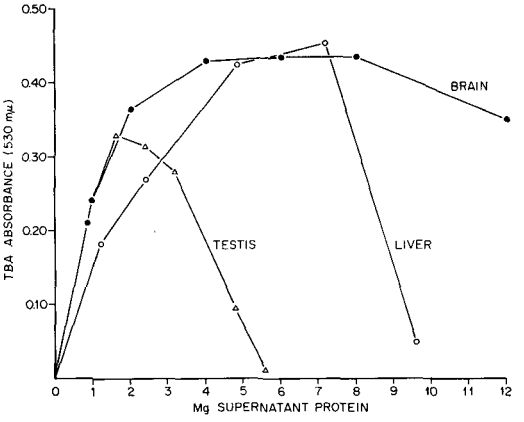

FIG. 1. Lipid peroxidation in rat brain microsomes following addition of soluble phase from liver, brain, and testis. Soluble phase containing the amount of protein indicated was added to 20 ml flasks containing 0.36 mg microsomal protein. Final volume was brought to 4.0 ml with 0.01 M phosphate buffer, pH 7.0, containing 0.15 M NaCl. TBA absorbance was measured following 90 min of incubation at 37C.

those of brain in the incubation mixture, although the extent of peroxidation was different. Testis supernatant inhibited at lower protein concentrations than did liver, and both inhibited at lower concentrations than did brain supernatant. The ascorbic acid contents of uniformly diluted soluble phases from liver, brain, and testis were similar in spite of large differences in protein and sulfhydryl content (Table I). The ratio of ascorbic acid to protein in the soluble phase of liver was below that of brain and testis, and the catalytic activity at low protein concentrations was correspondingly reduced.

The catalytic activity of the soluble phase is dialyzable and elutes with the fraction containing small molecules (S-2) when passed through columns of Sephadex G-50 (9). All of the ascorbic acid of liver and brain soluble phase also elutes with the S-2 fraction (Fig. 2). Soluble protein elutes only in the S-1 fraction, and sulfhydryl groups are distributed in both fractions. The sulfhydryl content of liver S-2 was considerably greater than that of brain S-2. The catalytic activity and ascorbic acid elution pattern of the soluble phase of testis was similar to that of liver and brain, and the sulfhydryl content of the S-2 fraction was intermediate.

The catalytic activity of the soluble S-2 fraction of liver increased with increasing concentration and was proportional to its ascorbic acid content (Fig. 3). The extent of peroxida-

TABLE I

Composition and Activity of Rat Tissue Homogenates and Microsomal Fractions

	Liver	Brain	Testis	Kidney
Homogenate composition [a]				
Protein (mg/ml)	35	10	13	....
Ascorbic acid (μg/ml)	105	80	80	....
Sulfhydryl (μg/ml)	1160	220	440	....
Microsome composition [b]				
Iron (μg)	106	29	34	21
Protein (mg)	65	42	50	75
Iron protein (μg/mg)	1.63	0.69	0.68	0.28
EDTA inhibition of microsomal lipid peroxidation [c]				
TBA absorbance (530 mμ) [d]	0.46	0.42	0.42	0.37
Microsomal protein (mg) [e]	0.4	0.4	1.2	0.9
Microsomal iron ($M \times 10^{-6}$) [f]	2.90	1.25	3.70	1.13
EDTA inhibition ($M \times 10^{-6}$) [g]	3.0	3.0	5.0	3.0

[a] Homogenates made up 1:3 (w/v).
[b] Values are units/gram wet weight of each microsomal fraction.
[c] Microsomes aerobically incubated with 10^{-4} M ascorbic acid; measured as TBA absorbance.
[d] Measured following 90 min of aerobic incubation at 37C.
[e] Total microsomal protein in each incubation vessel.
[f] Total microsomal iron in each incubation vessel; calculated from iron/protein ratios.
[g] Minimum concentration required to completely inhibit the peroxidation reaction.

tion increased rapidly with concentration up to 5×10^{-5} M (35 μg/4 ml) and remained constant up to 1.5×10^{-4} M. Peroxidation decreased at higher concentrations and at 6.0×10^{-4} M was one-half that noted at 1.5×10^{-4} M. Therefore, the inhibition of lipid peroxidation by the high concentrations of liver and testis soluble phase (Fig. 1) was not due to their ascorbic acid concentrations. Glutathione, in concentrations up to 10^{-3} M, had no effect on lipid peroxidation in brain microsome suspensions either in the presence or in the absence of 1.5×10^{-4} ascorbic acid. The sulfhydryl content of the liver S-2 fraction which catalyzed lipid peroxidation was far below this value.

Further evidence for the role of ascorbic acid in soluble phase catalysis was provided by examining lipid peroxidation in several microsomal systems catalyzed by soluble phase S-2 fractions and by ascorbic acid. The extent of lipid peroxidation catalyzed by liver and

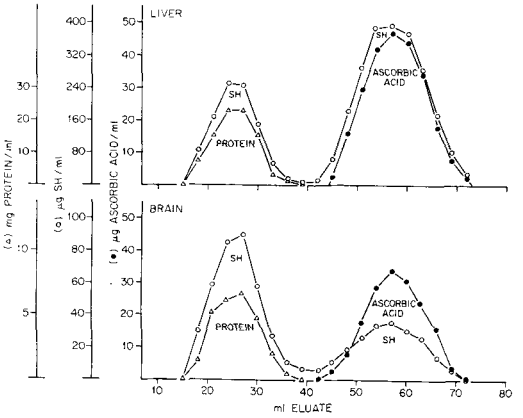

FIG. 2. Sephadex G-50 separations of soluble-phase protein, sulfhydryl, and ascorbic acid. Eight ml of soluble phase prepared from liver and brain homogenates (1:3 w/v) was passed through 25 x 2.5 cm columns using phosphate buffer (0.01 M, pH 7.0, containing 0.15 M NaCl) as eluant. All determinations were carried out on 3 ml samples collected in a Technicon Fraction Collector, and results are expressed as concentrations per milliliter. The peak eluting at 27 ml is referred to as S-1 and the one eluting at 57 ml as S-2.

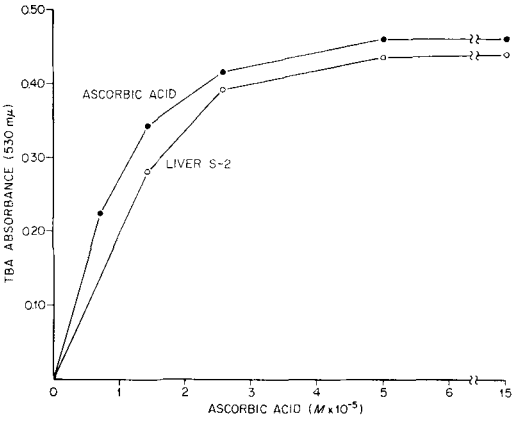

FIG. 3. Lipid peroxidation in rat-brain microsomes following addition of ascorbic acid or soluble S-2 fraction of liver. S-2 fraction was collected from Sephadex G-50 as described in legend of Figure 2, and the ascorbic acid content was adjusted for comparative purposes by diluting with buffer. Each flask contained 0.36 mg of brain microsomal protein in a final volume of 4.0 ml.

brain S-2 fractions was similar to the extent noted with equivalent concentrations of ascorbic acid when liver, brain or testes microsomes were used (Table II). The catalysis was similar in spite of the fact that the extent of peroxidation differed for each of the microsomal suspensions used.

Lipid peroxidation in rat brain microsomes catalyzed by ascorbic acid increased as the pH was lowered from 8.0 to 5.5 and decreased below pH 5.0 (Fig. 4). The general shape of the pH response curve was similar when liver S-2, containing equivalent amounts of ascorbic acid, was substituted for ascorbic acid in the reaction mixture. The same pH response curve was also noted when brain soluble S-2 was used or when liver microsomes were substituted for brain microsomes in the incubation mixture.

Analysis of the Particulate Fraction

Lipid peroxidation occurs when microsomes or mitochondria from rat liver, brain, testis, or kidney are aerobically incubated with ascorbic acid (25). The nitrogen, protein, and iron contents of the microsomal fraction from these four tissues are summarized in Table I. The iron/protein ratio was lowest in kidney (0.28) and highest in liver (1.63). The extent of lipid peroxidation in microsomal suspensions did not correlate with their iron content since peroxidation was similar in brain and testis in spite of the threefold difference in the amount of iron present (Table I). However, inhibition studies using EDTA indicated that the iron present was sufficient to catalyze peroxidation to the extent observed. The differences in EDTA/Fe ratios required for inhibition by EDTA indicate that the total iron measurement is only an approximate measurement of the catalytic iron available. Inhibition is never observed at EDTA/Fe ratios less than one. In all cases, the concentration of iron present in the incubation mixture was considerably below the concentrations reported to cause interference with the TBA test for lipid peroxidation (17). Results of EDTA inhibition studies on mitochondrial fractions from liver, brain, testis, and kidney were similar to those on microsomal fractions.

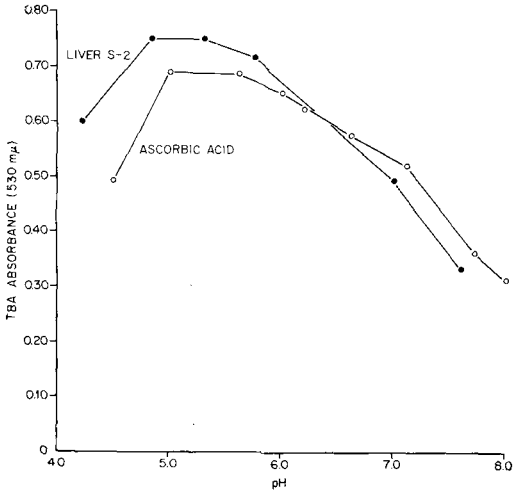

FIG. 4. Effect of pH on lipid peroxidation in rat brain microsomes catalyzed by 3.0×10^{-5} M ascorbic acid and by the liver soluble S-2 fraction containing equivalent amounts of ascorbic acid. Each flask contained 0.40 mg microsomal protein in a final volume of 4.0 ml. TBA absorbance was measured following 90 min of incubation at 37C.

TABLE II

Catalysis of Lipid Peroxidation by Soluble S-2 Fractions of Liver and Brain

Tissue microsomes	Microsomal protein [a] mg	TBA absorbance (530 mμ) Liver S-2 [b]	Brain S-2 [b]	Ascorbic acid (3×10^{-5} M)
Liver	0.6	0.63	0.58	0.60
Brain	0.3	0.31	0.30	0.32
Testis	1.0	0.27	0.28	0.30

[a] Total protein content of the microsomal fractions in each incubating vessel.

[b] The volume of the S-2 fractions added was adjusted to give a final ascorbic acid concentration of 3×10^{-5} M.

DISCUSSION

Nonenzymatic and enzymatically linked lipid peroxidation reactions have been described in isolated rat liver mitochondria and microsomes (9,11,14,20). The enzyme reaction involving oxidation of $TPNH_2$ in the presence of ADP requires Fe^{2+} as a catalyst, and the enzyme mechanism appears to involve the reduction of FE^{3+} to Fe^{2+} by $TPNH_2$, a reaction facilitated by ADP (20). The nonenzymatic reaction also requires Fe^{2+} as catalyst, but several reducing compounds, including ascorbic acid, can be used to reduce Fe^{3+} to Fe^{2+} (3,11). Results of the present study indicate that the nonenzymatic reaction is responsible for lipid peroxidation in dilute rat liver, brain, testis, and kidney homogenates and that ascorbic acid and iron are the normal catalysts. Ascorbic acid is indicated for the following reasons: the appropriate amount of ascorbic acid is present in the soluble phase of each tissue to explain the extent of peroxidation observed; the distribution of soluble-phase catalytic activity is similar to that of ascorbic acid; the catalytic activity of supernatant fractions is proportional to their ascorbic acid content regardless of the microsomal test

system used; and the pH effect on lipid peroxidation is similar for both ascorbic acid and soluble-phase catalysis.

The catalytic role of iron for both chemically and enzymatically induced lipid peroxidation has been well documented (9,11,19,20,26). The present study indicates that iron is the normal catalyst for peroxidation in tissue homogenates and their fractions. Sufficient iron is present in tissue particulates to explain both the extent of lipid peroxidation observed and the inhibition of peroxidation by EDTA. The concentration of iron in the microsomal suspensions used was 1.1 to 3.7 $\times$ 10^{-6} M, and the concentration of EDTA required for inhibition of lipid peroxidation was 3 to 5 $\times$ 10^{-6} M. Although iron itself in concentrations above 5 $\times$ 10^{-5} M gives added color in the TBA test for lipid peroxidation (17), the concentrations measured in the present study do not. High concentrations of iron result in an immediate peroxidation of the lipid, which increases only slightly with further incubation, whereas low concentrations of iron, in the presence of ascorbic acid, result in a more gradual peroxidation (3,11). The iron in tissue particulates therefore reacts catalytically rather than by interference with the TBA test itself. The catalytic role of iron in these homogenates has been clearly documented in studies of serum inhibition of lipid peroxidation (10). The inhibition was due to transferrin, the iron-binding protein of serum, and was completely abolished when the transferrin was saturated with iron (10). It is unlikely that other divalent cations act as catalysts for peroxidation in homogenates. For example, Co, Cu, Ba, Zn, and Ca do not catalyze lipid peroxidation in homogenates (9), as many of them do in purified lipid emulsion (18). In fact, most divalent cations are inhibitory when added to homogenates. Co and Cu inhibit at low concentrations, whereas large amounts of Zn and Ca are required to inhibit (9). The mechanism of inhibition by divalent cations is not known, although competitive inhibition of iron catalysis has been suggested (9). The catalytic role of iron is also suggested by the pH optimum for catalysis (18). This iron-ascorbic acid catalytic mechanism does not require separate substrates and precursors for the enzymatic and nonenzymatic reactions—a requirement previously suggested (27). Differences in the inhibitors for these two reactions could be due to specific interference with the Fe^{3+} reduction mechanism rather than in the initial phases of the peroxidation reaction.

The TBA reaction used to measure lipid peroxidation is specific for the higher unsaturated fatty acids such as linolenic, arachidonic, and hexaenoic acids (28). Rat liver mitochondria contain large quantities of arachidonic acid (29), and microsomes contain not only large amounts of arachidonic but also have significant amounts of docosahexaenoic acid (30). Peroxidation of these acids could explain the TBA reaction of these particulates. In fact, a decrease in both of these microsomal fatty acids did occur following microsomal exposure to hyperbaric pressure, $TPNH_2$ and ADP. The reduction was considered consistent with the hypothesis that lipid peroxidation of these acids had occurred (30).

The catalytic role of iron and ascorbic acid as an important nonenzymic mechanism for lipid oxidation (31,32), hydroxylation (32) and peroxidation (11) reactions is well established. Many of these reactions, including peroxidation, are also enzymatically catalyzed. The present investigation indicates that the lipid peroxidation reaction occurring in aerobically incubated rat tissue homogenates is normally catalyzed by the nonenzymic mechanism involving iron and ascorbic acid. The catalytic scheme is suggested as the model for examining the mechanisms responsible for the changes in lipid peroxidation noted under various experimental conditions (5–7). Quantitative studies of iron, ascorbic acid, and inhibitor concentrations, as well as measurements of the unsaturated lipid profile of cell particulates, are needed to establish the mechanisms responsible for these changes.

ACKNOWLEDGMENTS

Technical assistance by Mrs. J. Furumoto.

Research sponsored jointly by AEC AT (11-1)-34 Proj. 49, and the U.S. Atomic Energy Commission under contract with the Union Carbide Corp.

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[Received Nov. 29, 1965]

Quantitative Determination of Unsaturation in Oils by Using an Automatic-Titrating Hydrogenator

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ABSTRACT

A procedure was developed to adapt an automatic-titrating hydrogenator to the rapid determination of unsaturated carbon-carbon bonds in seed oils. Its utility as a research tool for detecting unusual types of unsaturation was demonstrated by analysis of 35 oils. When the hydrogen-iodine value of an oil determined by the hydrogenator differed significantly from the iodine value by the Wijs method, the presence of unsaturation such as acetylenic or conjugated double bonds was indicated. For repetitive analysis of samples of the same oil, or of oils having nearly the same extent of unsaturation, the hydrogenator can successfully accomodate injection of a new sample every 2 to 5 min. Possible utility of the method for monitoring samples from a processing plant is apparent.

INTRODUCTION

A RAPID, PRECISE METHOD for quantitative determination of unsaturation in organic compounds via hydrogenation was reported by Brown and co-workers (3). Their method utilizes a catalyst prepared by in situ treatment of platinum salts with sodium borohydride, in situ generation of hydrogen from sodium borohydride, and a valve at the tip of a buret that automatically introduces standardized sodium borohydride solution into the reaction mixture only as long as hydrogenation is proceeding.

Our initial attempts to apply the apparatus (Brown Hydroanalyzer No. 601, Delmar Scientific Laboratories, Maywood, Ill.) and published procedures to the analysis of oils were not successful. Modifications were then made to overcome the difficulties encountered, and the procedure finally adopted is described in detail in this report. Preliminary results of this investigation have been reported (10).

PROCEDURE

Apparatus

The hydrogenator was assembled as shown in Figure 1. A 250-ml, 24/40 ₸, round-bottomed flask, modified by adding a polytetrafluoroethylene stopcock at the bottom and a drying tube at the top, was placed upon the reservoir of the 5-ml buret to increase capacity (A, B in Fig. 1). The drying tube contained sodium hydrate asbestos absorbent and anhydrous magnesium perchlorate (Anhydrone, Dehydrite) (11). A millimeter scale (I) was used at

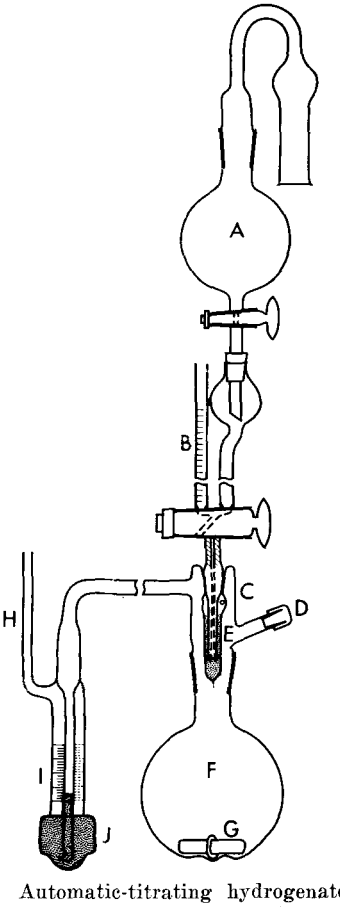

FIG. 1. Automatic-titrating hydrogenator. A) 250-ml $NaBH_4$ reagent reservoir; B) 5.0-ml precision-bore buret with polytetrafluoroethylene stopcock; C) 12/30 ₸ seal of the automatic valve assembly; D) 6-mm rubber serum septum; E) mercury-filled automatic valve; F) dimple-bottomed reaction flask; G) polytetrafluoroethylene-coated stirring bar with collar; H) sidearm of bubbler; I) millimeter scale on front and back of outer tube to avoid parallax in pressure reading; J) mercury pool.

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the bubbler to aid reading the gas pressure within the flask. Parallax was avoided by splitting the scale vertically and placing it in front and back of the bubbler tube. The side arm (H) of the bubbler was extended 15 cm to prevent loss of mercury droplets during the catalyst generation process.

The reaction flask (F) was either a 250-ml or 500-ml, 24/40 ₸, round-bottomed flask modified to allow high-speed stirring with a magnetic stirrer. A 2-in. polytetrafluoroethylene-coated stirring bar (G) with a polytetrafluoroethylene collar, 1.7 cm O.D., was placed within a dimple (or crater) in the bottom of the flask; this arrangement allows extremely rapid spinning of the bar without loss of magnetic connection between the bar and the activating rotor. Cold water was circulated in the 15-cm diameter glass dish (provided with the apparatus but not shown in the figure) to maintain the temperature of the bath at 25C.

Selection of Syringes and Needles

After experimenting with a large array of syringes and needles, the following were considered best suited for each step:

Glacial Acetic Acid. Glass syringe, 10 ml in 0.25-ml subdivisions; stainless-steel barrel holder and needle lock; 2-in. hypodermic needle, 25-gauge (0.020 in. O.D., 0.010 in. I.D.), 17-degree bent point; acid poured into syringe for filling.

Octene-1 Standard. Precision glass syringe, 100 μl in 1-μl subdivisions; stainless-steel barrel; fixed, 2-in., 28–22-gauge sheathed needle.

Oils. Glass syringe, 1 ml in 0.01-ml subdivisions; stainless-steel barrel holder and needle lock; 3-in. 19-gauge, regular point, hypodermic needle for filling the syringe and a needle for injection as described for glacial acetic acid.

Generation of Catalyst

The stirring bar (G) and 1.00 g of Darco K-B activated carbon were placed in the 250-ml round-bottomed flask (F) with the dimpled bottom, followed by 10.0 ml of fresh, practical-grade diglyme (diethylene glycol dimethyl ether or *bis*(2-methoxyethyl) ether) and 2.0 ml of 0.50 M chloroplatinic acid (10.36 g of H_2PtCl_6-$6H_2O$ in a 40.0-ml solution of anhydrous, distilled isopropanol). In a 125-ml Erlenmeyer flask, 0.76 g of $NaBH_4$ (99% pure) was dissolved in 20.0 ml of diglyme. This turbid 1.0 M solution was added dropwise to the round-bottomed flask (F) with gentle stirring. The Erlenmeyer flask was rinsed with 10.0 ml of diglyme, and another 10.0-ml portion was used to wash down the contents adhering to the walls of the round-bottomed flask. Five milliliters of anhydrous, distilled isopropanol were added, and the flask was secured to the assembled system. After a drop of diglyme was placed at the top of C to seal and prevent freezing of the joint, the system was gently stirred for 5 min. Then the stirring rate was increased slightly and the 10-ml glass syringe was used to add 5.0 ml of glacial acetic acid (dropwise at first).

Standardization of $NaBH_4$ Solution

The sodium borohydride (99% pure) was first dissolved in fresh, practical-grade diglyme and diluted tenfold with dry, distilled isopropanol. For 100-mg oil samples, 0.05 M solution was the most suitable and was prepared by dissolving 0.832 g (22.0 mmoles of borohydride in 40.0 ml of diglyme and diluting with 360 ml of isopropanol. The slightly turbid solution was allowed to stand at least 24 hr to permit flocculation of particles to provide facile filtration and a crystal-clear reagent. The clear, filtered solution was placed in the 250-ml dropping funnel and introduced into the buret (B). The stopcock was opened to allow downward flow and a rubber bulb was used to apply pressure at the top of the buret to force the solution to fill the automatic valve (E). The buret was refilled, the stopcock closed, and the stirrer stopped. With the 1-ml glass syringe, 500 μl of crambe oil (6) was injected through the septum (D) on to the center column that housed the automatic valve (E). The stopcock was opened and the stirrer was started at a slow rate to allow a gentle flow of the solution (5 ml/min).

When uptake of hydrogen had ceased, the buret was refilled only to the 2.30-ml mark and opened again to allow a small quantity of reagent to drip into the flask until the mercury seal was restored. The pressure within the flask at this point was usually identical to the end-point pressure resulting from hydrogenation of 100 μl of 1-octene. This artificial adjustment of pressure substituted for one or more injections of 100-μl samples of 1-octene in the process of obtaining an accurate end-point pressure. Initiation of a reaction at its end-point pressure eliminated any correction for difference in pressure before and after the reaction. After the artificially adjusted pressure (read at I in mm Hg below atmospheric

pressure) was noted, the buret was refilled to 0.00 ml and the stopcock closed.

The 100-μl precision glass syringe, described above, was filled with 1-octene (pure grade, 99 mole percent minimum, 100% by GLC) and weighed to 0.1 mg, the sample injected into the system, the buret opened, and the empty syringe weighed. When no change in buret reading was observed over 2 min, the volume of solution used and the pressure of the system were recorded. The process was repeated until three consecutive runs showed no variation in the final pressure reading. Deviations in weight of injected 1-octene were generally kept to $\pm$ 0.1 mg from the mean and were never allowed to be greater than $\pm$ 0.3 mg from the mean.

Taking into consideration the vapor pressures of isopropanol, diglyme, and glacial acetic acid at 25C and correcting for free space equivalent of the sample and $NaBH_4$ solution volumes, the molarity of the $NaBH_4$ solution was calculated from the formula:

$$M = \frac{\frac{\text{mg octene}}{448.8\ \text{mg/mmole}} - 0.009\ \frac{\text{mmole}}{\text{ml}}\ (\text{ml octene} + \text{ml } NaBH_4)}{\text{ml } NaBH_4}$$

The factor for free space equivalent, 0.037 mmole/ml, is valid for the ranges 740–760 mm atmospheric pressure and 20–30C reaction flask temperature.

Hydrogenation of Oils

The determination of degree of unsaturation in oils was carried out as described in the previous section with the exception of the choice of syringe and syringe needles. The oil was drawn into the 1-ml glass syringe with a 19-gauge needle but weighed and injected with a 25-gauge needle. This arrangement permitted rapid injections of oil with sample weights reproducible to within 1.0 mg. Use of 19- or 20-gauge needles for injection was avoided because of excessive tearing of the septum, causing air seepage, plus inability to retain the oil quantitatively during insertion through the septum.

Adjustment of pressure within the flask before the initial injection of any particular oil was done when the iodine value of the oil was known; but when unknown, one or two extra injections were made before three runs with identical end-point pressure were obtained.

The degree of unsaturation of an oil determined by hydrogenation has conventionally been expressed as hydrogen-iodine value (HIV) (7) for ready comparison with iodine value (I.V.) (1):

$$\text{HIV} = \frac{\text{mmoles } H_2 \text{ consumed}}{1\ \text{g of oil}} \times 25.4\ \frac{\text{cg } I_2}{\text{mmole}} = \frac{101.6\ [(\text{ml } NaBH_4 \times M\ NaBH_4) + 0.009\ (\text{ml } NaBH_4 + \text{ml oil})]}{\text{g oil injected}}$$

RESULTS

Evaluation of Procedure

Published procedures (3) were not applicable to soybean or crambe oils when the oil was injected without prior dilution with an appropriate solvent. Only two or three 100-μl samples could be handled and the time required per determination was 20 min to an hour. The end point was indefinite and this led to poor reproducibility of results. To analyze 50 injections of crude soybean or a similar oil at rates of 2 to 5 min per determination for each preparation of hydrogenating system, modifications were made by systematically evaluating each variable in the system.

The variables, investigated individually or in combinations, were catalyst concentration, catalyst solvent, amount of activated carbon per flask, reaction flask size, solvent for sodium borohydride, sodium borohydride concentration and amount for catalyst generation, sodium borohydride concentration for titration of oil, rate of addition of sodium borohydride to platinum salt during catalyst generation, acid for the dual purpose of destroying excess sodium borohydride during generation of catalyst and for generation of hydrogen during titration, preparation of nonturbid sodium borohydride reagent and its storage, solubilizing agent for oils, stirring rate during titration, and oil sample size. The optimal conditions observed for the variables were incorporated in the standardized procedures given above.

Dioxane and diglyme were equally efficient as solvent for sodium borohydride and the hydrogenation system, but diglyme was chosen for the studies in this report. Ethyl acetate, isopropanol, ethanol, ethylene glycol monobutyl ether, and dimethylformamide were unsuitable as solvents, especially when used for hydrogenation of triglyceride oils. In catalyst generation, the volume of liquid was necessarily kept at a minimum and stirred gently for efficient uptake of the liberated hydrogen gas. When chloroplatinic acid was added to the borohydride, generation of platinum catalyst was not so efficient as when borohydride was added to the acid. Addition of diglyme alone or diglyme and isopropanol to the system was not so effective as when diglyme, isopropanol,

and crambe oil were added to increase the solubility of the oils and to maintain an unchanging molarity of the borohydride reagent throughout the series of injections in a single flask. Except where 75 or more injections were made in a 500-ml flask, the 250-ml, round-bottomed, dimpled, 24/40 ℻ flask was used as standard equipment.

Concentrated hydrochloric acid invariably attacked the syringe needle and fittings, and the dissolved metallic ions rapidly poisoned the catalyst. Glacial acetic acid did not attack the syringe and was more compatible with the oils than was concentrated HCl. Syringes and needle sizes were selected according to the demands of each operation, ranging from the accurate, but rather expensive, 100-μl syringe for the 1-octene standard to the inexpensive, but precisely reproducible, 1-ml syringes for oils. Filling the syringe with oil required a needle at least of 19 gauge and for injection no larger than 25 gauge. The later did not tear the septum even after 75 injections through the same opening. It also allowed removal of all traces of air within the syringe without subsequent loss of oil during the injection process. Injection and withdrawal of the oil-filled syringe showed essentially no change in weight (0.1 mg or less per trial for three trials).

Stirring rate became an important factor when the number of injections increased. The dimple in the bottom of the flask, and the large-collared stirring bar combined to provide efficient stirring until the flask was almost completely filled. The extremely high stirring rate allowed ample dispersion of gas bubbles throughout the liquid and formed essentially a vertical vortex. With this arrangement the hydrogenator could be used for repetitive analysis of samples of the same oil, or of oils having nearly the same extent of unsaturation, by injection of a 100-μl sample every 2 to 5 min. For soybean oil, having an iodine value of 132, the mean HIV found for 50 injections (following an initial injection for end-point pressure adjustment) was 132.4, the range of values was 130 to 134, and the standard deviation was 0.7. The results indicate the possible utility of the method for monitoring samples from a processing plant.

Initiation of a reaction at its end-point pressure eliminated any correction for difference in pressure before and after the reaction. Attainment of end-point pressure of an oil, which differed from that of the immediately preceding sample, was facilitated by either removing hydrogen gas from the side arm with a pipette filler bulb or adding a small quantity of $NaBH_4$ reagent from the buret. A helpful guide in estimating the end-point pressure of any oil can be prepared by plotting the pressure reading versus buret reading during titration of the first oil. Since these points show a linear relationship, they are then plotted against HIV of the oils. This correlation would be altered for a subsequent run if mercury were lost from the seal during disassembly of the apparatus. The mercury trapped within the buret tip should be released before complete withdrawal of the buret. Without end-point pressure adjustment, HIV deviated appreciably (up to 20%) especially when the oil greatly differed in HIV from that of the immediately preceding oil.

Statistical evaluation of the method showed that when end-point pressure adjustments were made, the effect of an oil upon another which followed immediately was not significant. However, a trend was observed in the HIV of an oil, increasing in value as the position in the order of injection increased. This undesirable trend was greatly diminished by injecting 0.5 ml of crambe oil (I.V. 90) before standardization of the NaBH reagent with 1-octene. The statistical evaluation also showed no significant differences associated with sample size: 50, 100, 200, or 300 μl. Therefore, 100 μl was chosen for the sample size, and 0.05 M was the concentration of the $NaBH_4$ reagent most appropriate for handling the sample. The standard deviation was 1.4 I.V. units and relative precision was 1.0%. When the reagents were scaled down to one tenth of the proportions given in the procedures, 8-μl samples of methyl oleate or a similar ester recovered from thin-layer chromatographic plates were analyzed with good precision and moderate accuracy (HIV of methyl oleate 91, 91; calculated 86). The HIV obtained in this manner gave a good measure of the number of double bonds in the ester. The samples were readily recovered quantitatively for subsequent analyses, such as GLC, for carbon skeleton determination. Reagent-grade xylene (I.V. = 1), often used as solvent for oils, showed no hydrogen uptake.

Hydrogen-Iodine Values of Oils

HIV of 35 seed oils are listed in Table I. Wijs I.V. by the AOCS Method (1) and HIV calculated for mixed triglycerides in the oil based on gas-liquid chromatography of derived methyl esters (GLC-HIV) are also listed for comparison. Unusual fatty acid components in

TABLE I

Hydrogen-Iodine Values (HIV) of Seed Oils, with Iodine Values (I.V.) by AOCS Procedures and HIV as Calculated from GLC for Comparison

Plant Source	Common name	Unusual fatty acids in oil	HIV	I.V. (AOCS)	HIV (GLC)
Acanthosyris spinescens		Acetylenic	244	147	250
Aleurites fordii	Tung	α-Eleostearic	232	159	235
Arachis hypogaea	Peanut		98	98	101
Brassica juncea	Mustard		118	114	116
Brassica napus	Rapeseed		108	103	106
Carthamus tinctorius	Safflower		107	97	99
Ceiba accuminata	Kapok	Sterculic	89	87	91
Chilopsis linearis	Desert willow	Conjugated polyenoic	160	140	165
Crambe abyssinica	Crambe		88	90	93
Cuphea llavea	Red-white-and blue flower		10	13	10
Daucus carota	Carrot		90	102	90
Dimorphotheca sinuata	Cape marigold	Dimorphecolic	146	126	147
Euphorbia lagascae		Vernolic	88	88	88
Glycine max	Soybean		132	132	136
Gossypium species	Cottonseed		110	113	115
Helianthus annuus	Sunflower		134	134	136
Helichrysum bracteatum	Strawflower	Coronaric, crepenynic, dimorphecolic, helenynolic	136	117	131
Leonotis nepetaefolia	Lion's ear	Laballenic	110	91	103
Lesquerella lasiocarpa	Bladderpod	Lesquerolic	84	84	81
Lesquerella lescurii	Bladderpod	Densipolic	137	137	123
Limnanthes douglasii	Meadowfoam	*Cis*-5-mono, *cis,cis*-5,13-dienoic $>C_{18}$	78	85	84
Linum usitatissimum	Linseed		183	180	184
Lithospermum tenuiflorum	Gromwell	Octadecatetraenoic	226	224	227
Lunaria annua	Honesty	Nervonic	79	79	80
Olea europaea	Olive		83	84	83
Onguekoa (Ongokea) gore	Isano	Isanic, isanolic	336	146	369
Osyris alba		Ximenynic	206	113	203
Parinarium species	Cutia	α-Eleostearic	108	90	83
Petroselinum crispum	Curled parsley	Petroselinic	94	106	104
Ricinus communis	Castor		84	84	80
Sesamum indicum	Sesame		102	108	110
Sterculia foetida	Java olive	Sterculic	82	84	...
Thalictrum dipterocarpum	Yunnan meadowrue	Isolated *trans*	178	161	187
Vernonia anthelmintica	Indian ironweed	Vernolic	90	99	88
Ximenia americana	Hog plum	Ximenynic, ximenynolic	106	82	94

the oils are listed after the botanical and trivial names of their source.

Acanthosyris, Onguekoa (Isano), and *Osyris* species, which contain large percentages of acetylenic acids, showed quantitative uptake of 2 moles of hydrogen per acetylenic linkage. As expected, the Wijs method showed uptake of only 1 mole of halogen per acetylenic linkage.

Aleurites (tung), *Chilopsis* (desert willow), *Dimorphotheca* (Cape marigold), and *Parinarium* (Cutia) species contain conjugated dienoic or trienoic acids, or both, which absorb hydrogen quantitatively but resist quantitative addition of halogens across all double bonds. All of these oils, except *Parinarium*, gave HIV in good agreement with GLC-HIV. The low GLC-HIV for *Parinarium* resulted from the low percentage (8%) of the conjugated trienoic acid ester that was eluted and detected by the gas chromatograph. UV analysis of conjugated triene (4) showed *Parinarium* to contain 18% α-eleostearic acid. If this value were incorporated into the composition from GLC, the GLC-HIV would be 103. The values HIV = 108 and I.V. = 90 correspond to a composition which has 21% eleostearic acid. As illustrated above, observation of differences in HIV and I.V. is a valuable means of screening oils with acetylenic or conjugated unsaturation.

Oils with low I.V., e.g., *Cuphea* with I.V. = 13, were analyzed with moderate accuracy, and small quantities of oils with common unsaturated acids have been analyzed with comparable accuracy. Oils with a high degree of nonconjugated unsaturation, e.g., *Linum* (linseed) and *Lithospermum* (Gromwell), showed good agreement in HIV, I.V., and GLC-HIV.

No hydrogenolysis of hydroxy substituents was observed in the oils of *Dimorphotheca, Lesquerella* (bladderpod), Isano, or *Ricinus* (castor), all of which are rich (38–83%) in hydroxy fatty acids. The HIV indicated no excess uptake of hydrogen that could be attributed to hydrogenolysis. *Lesquerella lescurii* had identical HIV and I.V., which however differed substantially from the GLC-HIV. The difference cannot be explained by merely a low detector response from GLC to the densipolic (hydroxy dienoic) (9) constituent. By GLC the methyl esters of this oil have identical

compositions under four widely differing experimental conditions. The discrepancy between observed and GLC-calculated HIV must therefore be due to some highly unsaturated component in the oil which is not detected by GLC.

Oxirane substituents were also unaffected by the HIV procedure as seen in *Euphorbia* and *Vernonia*, both of which are rich in 12,13-epoxyoleic acid. The procedure has been successfully applied to the hydrogenation of several grams of *Vernonia* oil with almost quantitative recovery of the saturated epoxy fatty acids (5). Agreement between HIV and I.V. in *Sterculia* would indicate the hydrogenation of the cyclopropenoic sterculic acid to be essentially free of a ring-opening reaction.

The HIV of *Helichrysum* (strawflower) oil is in good agreement with the GLC-HIV calculated from the reported composition of the oil (8). This oil contains fatty acids with epoxy, conjugated enynic, conjugated dienolic, or conjugated enynolic constituents. *Ximenia* (hog plum) also contains conjugated enynic and conjugated enynolic acids, but the HIV was somewhat greater than expected from results by GLC. The observed HIV suggests a percentage of hydroxyximenynic approximately double the 6% deduced from fragmentation products of GLC.

Leonotis (lion's ear) has been reported (2) to contain 16% allenic acid, which may account for the difference between HIV and I.V. Addition of halogens across an allenic linkage appears to be limited to 1 mole. *Carthamus* (safflower), *Daucus* (carrot), *Limnanthes* (meadow foam), *Petroselinum* (curled parsley), and *Thalictrum* (Yunnan meadowrue) all contain unusual fatty acids or a high percentage of a single common acid and also showed discrepancies between HIV and I.V. that could not be explained.

ACKNOWLEDGMENTS

Suggestions on the procedure by H. C. Brown; guidance in oil preparation, F. R. Earle; Wijs I.V. determinations, Mrs. M. H. Rawls.

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[Received Aug. 10, 1965]

Quantitative Gas Chromatography of Sterols in the Free Form

STEROLS ARE USUALLY analyzed by gas-liquid chromatography (GLC) of a suitable derivative such as the trimethylsilyl ether. However, formation of the derivative is time-consuming and can become a serious limitation when it is necessary to analyze numerous samples. We investigated the possibility of analyzing sterols in the free form with commercially available packing material since this would not only save time in sample preparation, but eliminate the tedious treatment of the support to prevent adsorptive losses of sterol on the column.

A quantitative separation of a sterol mixture (Fig. 1) was obtained with XE-60, a nitrile silicone. Of particular interest is the complete separation of cholesterol from desmosterol, its monounsaturated analog. Such double bond selectivity is a useful property of the nitrile silicones (1). In addition, the thermal stability of the liquid phase in glass columns permitted continuous use for over 60 days without appreciable loss of resolution or accumulation of residue on the flame detector.

Another useful liquid phase is the dimethypolysiloxane, JXR. This polymer is similar to SE-30, but is more thermally stable. A quantitative separation of sterol classes can be quickly achieved with this liquid phase, but it is incapable of completely resolving the cholesterol/desmosterol pair (Table I).

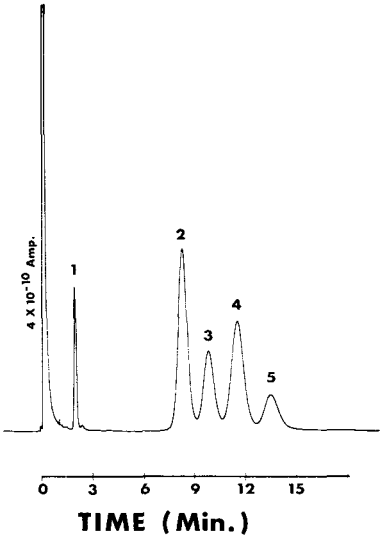

FIG. 1. Chromatogram of mixed sterols in the free form. Analysis conducted on a Barber-Colman Model 15 converted to use with a hydrogen flame ionization detector. Sample consisted of 0.5 μg mixed sterols in 1.0 μl $CHCl_3$. 3% XE-60 at 225C.

TABLE I

Retention Times of Sterols Chromatographed in the Free Form[a]

Sterol	Relative retention time[b]		
	JXR	XE-60	
	240C	215C	225C
Cholesterol	1.93	4.76	4.20
Desmosterol	2.08	5.66	4.94
Stigmasterol	2.74	6.71	5.80
β-Sitosterol	3.17	7.95	6.80

[a] Operating Conditions: JXR and XE-60 both as 3% w/w coating on 100/120 mesh Gas-Chrom Q (Applied Science Laboratories, State College, Pa.) packed into 6 ft × 4 mm (I.D.) Pyrex columns. Nitrogen used as carrier gas at pressure required for above elution time at temp. indicated. Flame gases adjusted for maximum response.

[b] Cholestane = 1.00 with following adjusted retention times: JXR at 240C, 2.76 min; XE-60 at 215C, 3.33 min and 225C, 1.86 min.

The column packing procedure is very critical to quantitative GLC of free sterols. Vibration of the column to settle the packing should be absolutely avoided as this fractures particles, exposing adsorptive sites. Suction should also be avoided to prevent the introduction of air-borne contaminants whose pyrolytic effluent contributes to baseline noise at high sensitivity. Instead, the combination of gentle tapping and inert gas at a pressure of 15–20 psig is used to settle the packing.

Proper conditioning is also a critical factor for quantitative results. The schedule shown in Table II is a modification of the procedure described by Vandenheuvel and co-workers (2) for conditioning JXR and was used to prepare the columns for this study.

TABLE II

Conditioning Schedule of Liquid Phases for Free Sterol Analysis by Gas Chromatography

24-Hour period after packing	Temperature[a]		Gas pressure
	XE-60	JXR	
1	100C	100C	10 psig
2	150	175	10
3	215	250	10
4	230	280	0
5	225	240	35

[a] Programmed temperature at 0.5C/min.

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ACKNOWLEDGMENT

Supported in part by USPHS grants NB-04116 and NB-04277.

[Received Dec. 2, 1965]

Removal of Water-Soluble Contaminants from Lipid Extracts of Heart

A study of lipids of beef and human hearts disclosed the presence of an unusually large amount of nonlipid contaminants in chloroform-methanol extracts. These contaminants made direct separation of the lipids by DEAE cellulose or other types of column chromatography difficult and procedures for removal of contaminants prior to lipid class separations were investigated. The procedure of Folch et al. (1) for washing chloroform-methanol extracts with KCl was compared with the Sephadex column procedure of Siakotos and Rouser (2).

Freshly ground ventricle was extracted with 19 volumes of chloroform/methanol 2/1 per gram of tissue, filtered, and washed (1). Each Folch extract was washed with one fifth its volume of 0.74% aqueous KCl and then twice with chloroform/methanol/water 3/48/47 containing 0.37% KCl. After each wash, phases were separated by brief centrifugation.

Extracts for direct Sephadex column chromatography were prepared by homogenizing with the following solvents and volumes expressed as ml/g wet weight of tissue: chloroform/methanol 2/1 three times (20, 10, and 10 volumes) followed by chloroform/methanol 7/1 saturated with 28% aqueous ammonia (10 volumes). In one case beef heart was also extracted with 10 volumes of chloroform/methanol 1/2 prior to extraction with the ammonical solvent mixture as a check for completeness of extraction of lipid.

The total solids recovered in the lipid extracts and the results of Sephadex column chromatography are shown in Table I. Sephadex column chromatography demonstrated the presence of 40–50% water soluble contaminants in the unwashed extracts. The extract washed according to Folch et al. still contained 8% nonlipid contaminants. Some lipid (about 2% of the total) was present in the upper methanol-water phase after washing since from 5.16 g of beef heart 3.15 mg of lipid was obtained in Fraction 1 by Sephadex column chromatography compared to 158.0 mg of lipid in Fraction 1 from the lower phase. The Sephadex column procedure is thus superior to the wash procedure for quantitative separation of lipids and contaminants.

TABLE I

Composition of Lipid Extracts of Beef and Human Hearts

Sample	Fresh wt, g	Lipid ext. wt, mg	Sephadex column fractions[a] (% total recovery) 1	2	3	4
Beef heart 1[b] (unwashed)	20.15	1202	59.81	2.08	3.85	34.26
Beef heart 2 (unwashed)	20.48	1120	50.09	4.17	1.43	44.31
Beef heart 1 (washed)	5.16	172	92.00	3.28	0.55	4.10
Human Heart (unwashed)	19.19	862	57.37	3.07	1.30	38.25

[a] Column 2.4 (*i.d.*) × 30 cm with a flow rate of 3 ml/min.

Solvents were: fraction 1, chloroform/methanol 19/1 saturated with water (500 ml); fraction 2, 5 parts chloroform/methanol 19/1 plus 1 part glacial acetic acid plus 23 ml of water per liter (500 ml); fraction 3, 5 parts of cholorform/methanol 9/1 plus 1 part glacial acetic acid plus 40 ml of water per liter (1000 ml); fraction 4, methanol/water 1/1 (1000 ml). Protein in the extracts was not eluted from the column but did not interfere with reuse of columns.

[b] Extracted with chloroform/methanol 1/2 in addition to other solvents (see text).

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[Received Oct. 18, 1965]

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[Received Oct. 18, 1965]

Altered Fatty Acid Distribution of Glycerophosphatides Induced by Acetolysis

IT WAS SHOWN RECENTLY that individual molecular species of phospholipids can be analyzed by fractionation of the corresponding diglyceride acetates or ceramide acetates (1). Originally both acetolysis and degradation with phospholipase C were recommended, but additional work has shown that acetolysis induces changes in the positional distribution of fatty acids in lecithin, phosphatidyl ethanolamine and phosphatidyl serine. This isomerization was detected by comparison of acetolysis products with parent phosphatides and with diglyceride acetates produced by enzymic hydrolysis and subsequent acetylation at 20C. Hydrolysis of the original phosphatides with phospholipase A of *Crotalus adamanteus,* and pancreatic lipase degradation of the diglyceride acetates produced by the enzymic method revealed similar positional distributions of fatty acids, but acetolysis appeared to have caused some shifting of fatty acids between the positions 1 and 2 of glycerol.

Typical experimental data appear in Table I; the figures in lines 2 and 3 reveal the change that took place in the "fatty acid population" on C-2 during acetolysis of egg lecithin. It is as if one fifth of the C-2 acids had been substituted by the C-1 acids. Acetolysis was carried out by heating 20 μmoles of egg lecithins with 12 ml of acetic anhydride-acetic acid (2:3) mixture for 16 hr at 143C. The 1,2-diglyceride acetates formed were freed from small amounts of contaminating 1,3-diglyceride acetates by preparative thin-layer chromatography, and finally the pure sample was partially hydrolyzed with pancreatic lipase (2), and the fatty acid composition of the resulting monoglycerides was analyzed by gas-liquid chromatography. The fourth line in Table I shows analogous results obtained from 1,2-diglyceride acetates prepared from the same sample of egg lecithins by degradation with phospholipase C and subsequent acetylation. In this case the fatty acids on C-2 were the same as in the parent lecithin.

TABLE I

Positional Distribution of Fatty Acids in Egg Lecithin and 1,2-Diglyceride Acetates Derived Thereof

	Relative amounts of principal fatty acids			
	16:0	18:0	18:1	18:2
1. Parent lecithin C-1 position	74	26	0	0
2. Parent lecithin C-2 position	3	0	68	23
3. 1,2-Diglyceride acetates (acetolysis) C-2 position	19	6	51	20
4. 1,2-Diglyceride acetates (enzymic procedure) C-2 position	3	1	72	23

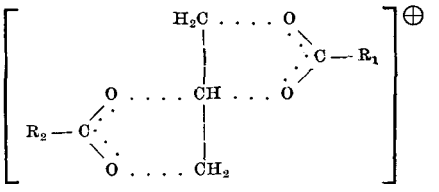

FIG. 1. A cyclic intermediate suggested to explain the observations on acetolysis of glycerophosphatides.

The mechanism of the rearrangement of fatty acids is not known. However, the reaction appears to be intramolecular since no "hybridization" of fatty acids was observed when dipalmitoyl and dioleylphosphatidyl ethanolamines were acetolyzed together (1). A cyclic intermediate (originally proposed by Dr. P. Olavi I. Virtanen, University of Oulu, Finland) like that pictured in Figure 1 would explain most of our findings so far; attack to C-3 of this intermediate would give the original arrangement of fatty acids, attack to C-1 would cause a shift of the two fatty acids *in the same direction* along the glycerol molecule and thus lead to their apparent "crossing over." The formation of the 1,3-diglyceride acetates would follow from an attack to C-2.

Since acetolysis cannot be recommended for analytical dephosphorylations it must be replaced by phospholipase C hydrolysis followed by acetylation of the diglycerides. By using phospholipase C from *Bacillus cereus* (3) we have prepared unisomerized 1,2-diglyceride acetates from phosphatidyl ethanolamines, phosphatidyl serines and phosphatidyl inositols and there is no doubt that the list can eventually be extended to include most natural glycerophosphatides.

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[Received Nov. 17, 1965]

Phospholipids of the Human Lens

DEVELOPMENT OF A procedure for phospholipid analysis by two dimensional thin-layer chromatography (TLC) of lipids (1) and phosphorus analysis of spots provided us with a convenient micromethod with which to determine human lenticular phospholipid composition. Previous work established that the lens of the human eye contains approximately 2 mg of phospholipid and that sphingomyelin is the major component (2,3), although the total phospholipid mixture was only partially resolved. With the superior resolution of two dimensional TLC, the mixture could be more completely defined.

Three human lenses were obtained at autopsy within one hour post mortem. Each lens was examined by an ophthalmic pathologist and found to be free of gross evidence of pathological change. The lenses were individually extracted and the polar lipids separated by TLC. The phosphorus-containing spots were then analyzed by the procedure of Rouser et al. (1). Figure 1 is a typical chromatogram showing the phosphorus-containing and blank areas that were aspirated from the plate for analysis. The results for each lens are summarized in Table I. The very high level of sphingomyelin, the relatively large proportion of phosphatidyl inositol, and the low level of lecithin are relatively unusual features since lecithin is usually a major component and sphingomyelin and phosphatidyl inositol minor components of tissue extracts. The phospholipids probably play an important role as structural components of the cellular membranes of the lens fibers.

TABLE I

Phospholipid Composition of the Lens of the Normal Human Eye

Component	% of Phospholipid phosphorus		
	Lens (1)	Lens (2)	Lens (3)
Sphingomyelin	70.7	69.5	64.6
Phosphatidyl inositol	12.7	12.7	10.9
Phosphatidyl ethanolamine	8.6	8.4	10.0
Lecithin	0.5	2.4	3.0
Phosphatidyl serine	2.6	3.0	4.2
Phosphatidic acid	4.7	3.9	7.2

FIG. 1. Two dimensional TLC of a typical sample of lipids from a normal human lens. Developing solvents were chloroform/methanol/water 65/25/4 in the vertical direction and n-butanol/acetic acid/water 60/20/20 in the horizontal direction. Spots were localized with a sulfuric acid-potassium dichromate char spray.

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[Received Oct. 8, 1965]

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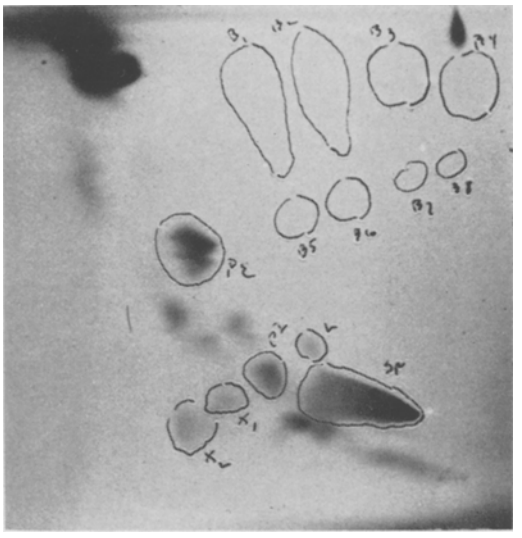

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[Received Oct. 8, 1965]

LETTERS TO THE EDITOR

An Unsolved Problem of Triglyceride Analysis

Sir: A complete chemical description of a fat can only be given after analytical separation of the fat into all its triglyceride species (triglycerides containing the same three fatty acids), followed by analytical resolution of the species into their positional isomers. With our present methods this is still an impossible task even with the simplest natural fats. There are three difficulties of which only two can, in principle, be overcome.

First, there is the complexity of many fats; a fish oil, for instance, may well contain thousands of different triglycerides. This is a problem of improvement of existing methods, and of endurance, and it is not in principle insoluble.

The second problem is posed by "critical pairs." Triglycerides can be separated according to carbon number by gas chromatography, according to unsaturation by $AgNO_3$,-silicic acid, and according to both structural features by "reverse phase chromatography," but species containing equal numbers of carbon atoms and double bonds cannot be *preparatively* separated. *Analytical* separation, however, is in many cases possible through chemical modification. The critical pair 18:1-18:1-18:0 and 18:2-18:0-18:0, for instance, could be separated after oxidation to the dibasic and monobasic triglycerides.

The third problem, insoluble with present methods, might be called the "three acid problem." After a single species containing the acids A, B, and C has been isolated by chromatographic methods, we want to know the proportions of the six possible isomers: ABC, ACB, BAC, BCA, CAB, CBA. Pancreatic lipolysis and stereospecific analysis will give the fatty acid composition 1, 2, and 3. Fictional percentages are given in Table I for the purpose of demonstration. We know, then, that ABC and ACB, together, make up 60% of the species. ACB, however, cannot be higher than 10%, the total concentration of B in position 3. ABC, therefore, amounts to 50–60% of the triglycerides; there may be up to 10% ACB, or none at all: the analysis does not tell. It can be easily tested that any ACB value from 0 to 10, together with a value for ABC complementary to 60, will satisfy the analytical finding.

TABLE I

		Fatty acid		
		A	B	C
Position	1	60	30	10
	2	10	60	30
	3	30	10	60

The imaginary analysis of Table I presents a favorable example insofar as at least the major triglyceride can be quantitated with reasonable accuracy. If an analysis should show 33% of each acid in each position, then even the *presence* of any one of the six isomers can no longer be ascertained, although this isomer might be the major one with 33%.—Triglyceride species with two fatty acids are easily analyzed: Only three isomers are possible, AAB, ABA, and BAA, and the percentage of B in positions 3, 2, and 1 gives immediately the ratio of isomers.

To solve the "three acid problem" one further independent analytical datum is required: the ratio of isomers in one isomeric pair. A stereospecific analysis yields four independent data, e.g., in the table, A-1, A-2, B-1, B-2. With these data the others are given since columns as well as lines must add up to 100. Two analytical data, e.g., A-1 and A-2, will give us the proportions of three isomeric pairs,

		Triglyceride		
Position	1	[A A]	B C	B C
	2	B C	[A A]	C B
	3	C B	C B	A A

but the ratios of isomers in these three pairs are independent of each other. We therefore need three more independent data; but only two more are available at present through stereospecific analysis.

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[Received Nov. 17, 1965]

Preparation of Pure Methyl Esters by Counter Double Current Distribution [1]

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ABSTRACT

Counter double current distribution with continuous stills for solvent and product recovery and an acetonitrile-hexane solvent system is a convenient method for preparative isolation of individual fatty methyl esters. Preparations of pure methyl linoleate from safflower esters and a methyl arachidonate concentrate from hog liver lipids are described.

INTRODUCTION

In a previous paper (4) we reported the use of countercurrent distribution (CCD) with an acetonitrile-hexane solvent system for the isolation of pure methyl esters of several fatty acids. This procedure has proved useful and has been used routinely at our Laboratory for the preparation of methyl linoleate and methyl linolenate.

In 1963, Post and Craig (3) presented a new procedure, called counter double current distribution (CDCD), which has certain advantages over CCD for preparative work. Our use of this CDCD procedure for the preparation of methyl linolenate and the operation of continuous stills for solvent and product recovery has been described in a recent paper (2). The present paper reports the application to some other fatty acid esters.

EXPERIMENTAL

The operation of the 25-tube CDCD apparatus and solvent recovery stills has been described previously (2). At each equilibration stage, 50 ml of lower phase solvent was added. Based on partition coefficients of the esters and on preliminary CDCD runs, 10 ml volumes of upper phase were chosen for the methyl linoleate preparations reported here. Esters were fed to an intermediate tube (Table I) as 1 ml portions of a 1:2 ester:hexane mixture at each equilibration stage. Approximately 250 transfers were required to reach steady state conditions in the apparatus. For maximum recovery of product approximately 250 transfers may be run after the feed is stopped to remove nearly all the esters from the apparatus.

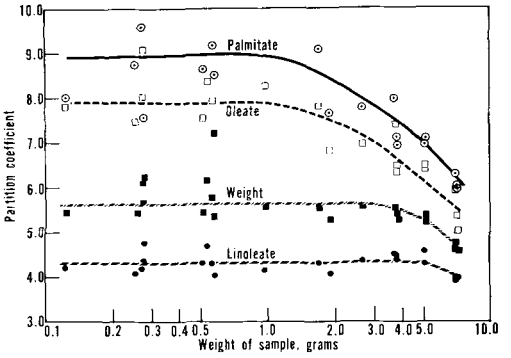

FIG. 1. Effect of concentration on partition coefficients of corn methyl esters. Total weight of corn methyl esters dissolved in 10 ml of upper hexane phase and 50 ml of lower acetonitrile phase plotted against partition coefficients. Solid squares: Partition coefficients of corn oil methyl esters (concentration in upper layer/concentration lower layer). Solid circles: Partition coefficients of methyl linoleate. Open squares: Partition coefficients of methyl oleate. Open circles: Partition coefficients of methyl palmitate.

Preliminary work on the preparation of methyl linoleate was carried out with corn methyl esters. To determine the effect of concentration on partition coefficients, corn methyl esters were partitioned between 10 ml of hexane upper layer and 50 ml of acetonitrile in a separatory funnel. Esters recovered from each layer were weighed and analyzed by gas chromatography (Fig. 1).

In Table I data for several runs with corn methyl esters and one with safflower methyl esters are shown; all were made with 10 ml portions of upper phase. Highest purity

TABLE I

Preparation of Methyl Linoleate by Counter Double Current Distribution

Methyl esters	Feed		Composition of extract, %			Recovery of Lo, %
Run	Tube	Wt Esters, mg	O	Lo	Ln	
Corn 1	10	355	2.0	94.0	4.0	75
Corn 2	12	346	0.2	97.8	2.0	73
Corn 3	12	172	0.7	95.7	3.6	79
Corn 4	14	334	0.3	95.1	4.6	68
Safflower	12	335		100.0		85

See Table II for key to abbreviations.

[1] Presented at AOCS Meeting, Houston, April 1965.
[2] No. Utiliz. Res. Dev. Div., ARS, USDA.

TABLE II

Counter Double Current Distribution of Corn Methyl Esters. Composition of Effluent Fractions, %

Sample	Wt.	P	S	O	Lo	A	Ln
Original corn methyl esters		12.3	1.9	28.0	55.0	1.2	1.6
Hexane phase	58.3	20.6	3.3	47.9	25.9	1.6	0.8
Acetonitrile phase	41.7	.....	....	0.2	97.8	....	2.0

P = palmitate, S = stearate, O = oleate, Lo = linoleate, A = arachidate, and Ln = linolenate.

linoleate was obtained with the conditions used for corn methyl esters, run 2, and for the single run on safflower methyl esters. Decreasing the feed rate in corn methyl esters run 3 did not increase linoleate purity. For corn methyl esters, run 2, Table II contains more detailed data and Figure 2 shows the concentration of esters in CDCD tubes at the steady state condition.

In our previous paper (4) we described the separation of a 90% methyl arachidonate concentrate from hog liver lipids by CCD. A portion of these same hog liver lipids containing 9% arachidonate was fractionated by CDCD with 50 ml portions of acetonitrile lower layer and 20 ml portions of hexane and with the feed at tube 7. Nearly all the arachidonate was recovered as a 76% concentrate from the acetonitrile phase.

DISCUSSION

In CDCD both upper and lower phase solvents move in opposite directions from tube to tube. The mixture to be fractionated, which is fed into one of the intermediate tubes, is separated into two parts in the two solvent streams from the instrument. Thus CDCD, although it operates in discrete stages, resembles a continuous countercurrent separation process. CCD separates a complex mixture into many fractions, whereas CDCD gives only two, but for much preparative work this number is sufficient. Since CDCD utilizes the instrument tubes more efficiently, fewer are needed and a greater throughput is accomplished. After steady state conditions are obtained, the composition of the two fractions will remain constant so that less attention and fewer analyses are required. CDCD does require large amounts of solvents, but the continuous solvent and product recovery stills (2) used with a volatile two-component system, such as hexane and acetonitrile, make possible unattended automatic operation and reduce the inventory of flammable solvents.

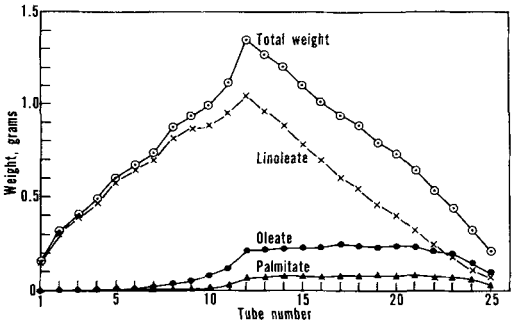

FIG. 2. Distribution pattern of corn methyl esters in CDCD apparatus. Feed is at tube 9. Lower layer moves toward decreasing tube numbers. Upper layer moves toward increasing tube numbers.

The conditions recommended for preparation of methyl linolenate (2) (20 ml upper phase, 50 ml lower phase, with feed at tube 9) seem close to optimum, and the reported yield of 57% has consistently been equaled or exceeded in subsequent preparations.

For methyl linoleate 10 ml upper phase and 50 ml lower phase with feed at tube 12 gave the best results. Linoleate prepared from our corn methyl esters always contained a small amount of linolenate. This is expected from the results of Beadle et al. (1) who found small amounts of linolenic acid in all samples of corn oil. From safflower methyl esters which were essentially free of linolenate, pure linoleate was obtained.

For best operation of CDCD the concentration of esters in the instrument must be low enough so that partition coefficients remain constant. Figure 1 shows that with the solvent volumes used for linoleate preparation the partition coefficient for linoleate is constant until about 5 g of corn methyl esters are present per tube. However, partition coefficients for oleate and palmitate begin to decrease with about 1.5 g corn oil esters per tube.

A similar behavior was previously noted (2) for linoleate in the presence of linolenate. Thus, it seems that the most unsaturated esters acts as a third solvent component, increasing the solubility of less unsaturated esters in the acetonitrile phase and causing the difference in partition coefficients to become less.

As shown in Figure 2, the weight of esters in tube 9, the feed tube, is 1.35 g, which is in the range for satisfactory operation of the instrument. Under these conditions, with 22 transfer stages per hour, about 30 g of methyl linoleate can be obtained in an 8 hr day.

Since methyl arachidonate has one more

double bond but two more carbons than linolenate, the two esters should have approximately the same partition coefficient. Therefore, to prepare an arachidonate concentrate, the same solvent ratio as for linolenate was used. It was known from previous work (4) that small amounts of more highly unsaturated esters would be found in the acetonitrile phase and that additional purification would be necessary to obtain pure arachidonate. Because of this fact, the feed was moved from tube 9 as with linolenate to tube 7 to give a higher recovery of arachidonate. Nearly all the arachidonate was recovered as a 76% concentrate, which is a more suitable material for further purification by CCD, distillation, or other methods than the original esters, which contained only 9% arachidonate.

Thus, CDCD with automatic solvent recovery stills is a useful preparative method for isolation of pure methyl esters and of concentrates for further purification. In addition to linoleate, linolenate and arachidonate, the method should have general applicability for isolation of esters of many other fatty acids that are difficult to obtain in sufficient volume for laboratory use.

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[Received Nov. 22, 1965]

A Simple, Rapid Micromethod for the Determination of the Structure of Unsaturated Fatty Acids via Ozonolysis[1]

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ABSTRACT

A micromethod for the localization of double bonds in unsaturated fatty acids via ozonolysis employing pyrolytic cleavage of ozonides in the presence of a hydrogenation catalyst is described. Cleavage of the ozonides is carried out in a gas-liquid chromatographic instrument in a small glass tube, containing the catalyst, inserted in the top of the column opposite the input heaters at 225C. Ozonides of methyl esters of straight chain unsaturated fatty acids are cleaved through the action of the catalyst to aldehyde fragments which are swept simultaneously into the column for analysis.

The double bond positions are deduced from the chain length of the fragments. The method is demonstrated on methyl oleate, linoleate, linolenate and arachidonate.

INTRODUCTION

THE DETERMINATION of the structure of unsaturated fatty acids via ozonolysis methods is usually carried out by hydrolytic (1–3) or reductive (1,4–6) cleavage of the preformed ozonides. Hydrolytic cleavage yields acids; reduction of ozonides gives aldehydic fragments. The positions of the double bonds are deduced from the chain length of the fragments. This paper shows that aldehydes are essentially the exclusive products of the pyrolysis of the ozonides of straight chain unsaturated fatty acids esters under standardized conditions in the presence of a hydrogenation catalyst, and a method is described for the determination of the structure of unsaturated fatty acids based on this reaction.

EXPERIMENTAL

Materials

Highly purified methyl oleate, linoleate, linolenate and arachidonate were obtained from The Hormel Institute. Methyl oleate contained a small amount of methyl linoleate, and methyl linoleate contained a small amount of methyl oleate. The methyl linolenate contained 12% *trans* unsaturation expressed as methyl elaidate by infrared analysis but was >99% 9,12,15-octadecatrienoate.

The methyl arachidonate, as obtained, was approximately 92% pure and, therefore, was further purified by passage through a column packed with silicic acid impregnated with silver nitrate as described by Privett and Nickell (7). The final product was also about 99% pure as determined by gas-liquid chromatography (GLC).

The preparation of the Lindlar catalyst has been described (10). The palladium on charcoal hydrogenation catalyst was obtained from the American Platinum Works, Newark, N. J.

GENERAL PROCEDURE

Ozonization

From 10 to 100 μg of methyl esters is ozonized in highly purified pentane as previously described (5). In this procedure approximately 5 ml of a 0.03 M ozone solution in pentane is prepared by bubbling oxygen containing about 2% ozone through the purified pentane at about −65C for about 10 min. The ozone is generated by passing pure oxygen through an ozone generator similar to that described by Bonner (8). The sample is dissolved in about 2 ml of pentane, cooled to about −65C, or to a temperature just above which crystallization of the sample occurs, and added to the solution of ozone. The ozone solution may also be added to the solution of the sample. In either case, the final solution should have a blue color which indicates an excess of ozone. Ozonization of common fatty acid esters is virtually instantaneous and thus, after ½ to 1 min, the excess ozone and the oxygen are removed by bubbling purified nitrogen through the solution as it warms up to room temperature. Finally, the solution is evaporated to about 0.1 to 0.2 ml under reduced pressure.

Pyrolysis of Ozonides and Simultaneous Analysis of Fragments of Reaction

The pyrolysis is carried out in a Pyrex glass tube (25 mm × 2 mm I.D.) coated on the inside

[1] Presented at the AOCS Meeting, Cincinnati, October, 1965.

with about 3 mg of catalyst. The tube or several tubes are coated by dipping them in a fine slurry of the catalyst in methylene chloride. The slurry is made by shaking vigorously 50 mg of catalyst containing 5% anhydrous calcium sulfate as a binder per 100 μl of methylene chloride in a small test tube. When the coating was applied with a slurry of this composition, about 3 mg of catalyst was deposited on the inside of each tube. A slow stream of argon or helium is passed through the tubes to evaporate the methylene chloride. After the solvent is evaporated, the pentane solution of the ozonides or a portion of it, depending on such factors as the sensitivity of the GLC instrument and the number of fragments expected, is introduced by means of syringe onto the inside wall of a coated tube. The pentane evaporates as it is applied, spreading the sample over the entire surface of the catalyst on the inside of the tube. The last traces of pentane are removed by passing a slow stream of argon or helium through the tube. The pyrolysis and analysis are carried out in a single operation with a Packard gas chromatograph equipped with dual columns and β-ionization detectors. Coiled glass columns (6 ft × ¼ in.) packed with 30% silicone (5,9) or 10% ethylene glycol succinate polyester phase on Chromosorb W supports were used for the analysis of the products of the reaction. The instrument was operated with an argon carrier gas flow of 60 ml/min at 60C, electrometer setting generally of 3×10^{-8} amps, in conjuction with a Honeywell Dual Channel Model Electronik 17 recorder with a 1 sec response, 1 mv full chart deflection. Under these conditions about 20 μg of a single compound will give a full chart peak but by decreasing the electrometer settings the sensitivity may be increased 100-fold. The design of the Packard instrument is such that it permits the insertion of the glass tube containing the sample directly into the top of the column. Introduction of the sample by this technique has been used previously by the authors (9). The argon gas leading to the gas chromatograph is momentarily turned off while the glass tube (pyrolysis chamber) is placed in the top of the column opposite the input heaters. As soon as the glass tube is placed in the column the silicone-rubber plug is replaced in the top of the column and the argon is turned on again. The entire operation only takes a few seconds. The pyrolysis takes place as the glass tube containing the sample rises to 225C, the temperature at which the input section of the column is heated. Since most fatty acids generally give both long- and short-chain aldehydes, the GLC analysis is carried out by temperature programming starting at about 60C. Thus, whereas the input temperature of the instrument is 225C, the column temperature is only 60C at the start of the operation. The column temperature is then programmed at 5C per min to 190C. The separation of model mixtures of aldehydes on the silicone column and aldester and aldehydes on an ethylene glycol succinate polyester phase (Applied Science, State College, Pennsylvania) are shown in Figure 1. Generally, a complete analysis of the aldehydes and aldester fragments can be obtained on a silicone column, but there may be overlapping of short-chain aldesters with aldehydes masking some components or making the identification of some of the peaks difficult. In general, peaks for aldehydes will coincide closely with aldesters of 3 less carbon atoms in the chain. For example, nonanal and methyl adipaldehydate are very difficult to separate on a silicone column. Should there be any suspicion of overlapping of peaks or should there be any question of the identification of the peaks, a second analysis should be performed on a polyester column. The retention times of the aldesters relative to aldehydes are much greater on a polyester column than on a silicone column (Fig. 1) and, thus, a second analysis on a polar phase permits the resolution of compounds which overlap on the silicone column.

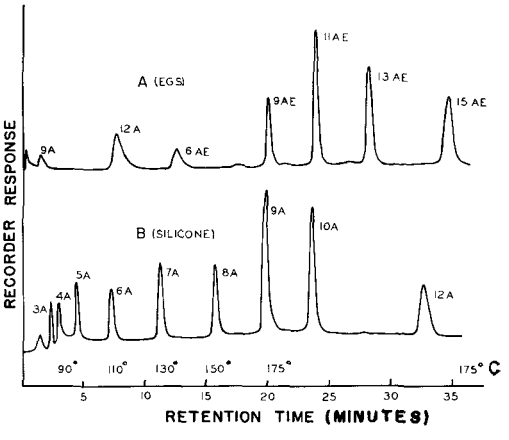


FIG. 1. GLC of: (A) Standard mixture of aldesters with 15% ethylene glycol succinate polyester phase. (B) Aldehydes with a 30% silicone phase. Temperature programmed from 60 to 190C. In shorthand designations: A = simple aldehydes, AE = aldester and the number designates the chain length.

RESULTS AND DISCUSSION

The pyrolysis of the ozonides of fatty acid esters in the presence of the hydrogenation catalyst gave only aldehyde fragments as illustrated in Figure 2. This figure shows the fragments obtained from the application of the method to mono, di, tri and tetra unsaturated fatty acid esters of known structure. The identification of the fragments was made by comparing the results of the analysis of the same esters by reductive ozonolysis (5) and comparison with standards that were available. Nonanal and hexanal were collected and identified as their 2,4-dinitrophenylhydrazine derivatives by TLC by comparison of their R_f values with that of standards (11,12). In all of the analyses in Figure 2, the fragments were identified as those expected from cleavage of the ozonides into aldehyde fragments. No fragments were detected that indicated double bonds in positions that did not exist. The peak 9A (nonanal) in the GLC tracing from methyl linoleate originated from methyl oleate that was the impurity in this preparation. The peak 6A (hexanal) in the GLC tracing from methyl oleate (Fig. 3) originated from the methyl linoleate impurity in that compound. Since both these esters, as well as linolenate, have the double bond proximal from the carboxyl group in the 9 position they yield the same aldester (methyl azelaaldehydate).

Malonaldehyde, which is the theoretical product of methylene-interrupted polyunsaturated fatty acids, linoleic, linolenic and arachidonic acid, generally gives either two peaks or one askewed peak which overlaps the peak for 3A (propanal). The peak for this compound (malonaldehyde) emerges just before propanal with the silicone column and can be clearly observed in all of the polyunsaturated fatty acid esters in Figure 2. When a small peak or odd-shaped peak is observed with a retention time corresponding to propanal it should be suspect as probably being due to malonaldehyde. The peak in this area in the tracing from methyl arachidonate (Fig. 2), for example, may be attributed to this compound for this reason and because there are no other fragments in this tracing that could match up with the propanal to give a 20 carbon chain isomer with the double bond in the 17 position. In accord with this view is the strong peak for propanal when this compound is a true fragment, as in the case of methyl linolenate (Fig. 2).

The results of the pyrolysis of the ozonides of methyl oleate and linolenate in the absence of the hydrogenation catalyst are shown in Figure 3. In addition to the expected aldehyde fragments, several other compounds (artifacts) were produced. Also, it should be noted that the amount of the artifacts increased considerably with the increase in the degree of unsaturation from one to three double bonds in the original esters. It is well known that the substituent groups strongly influence the course of the pyrolysis reaction. Accordingly, the formation of greater amounts of artifacts should be expected from the pyrolysis of the ozonides of polyunsaturated fatty acid esters than monounsaturated methyl esters.

Two major artifacts formed in the pyrolysis of the ozonides of fatty acid esters under the

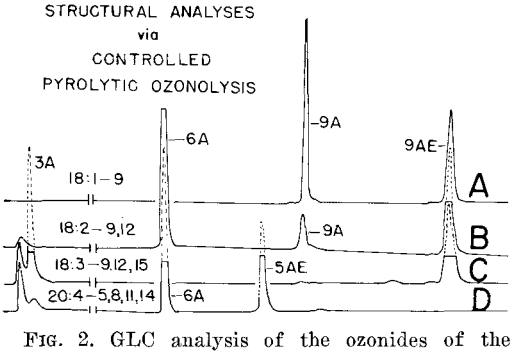


FIG. 2. GLC analysis of the ozonides of the controlled pyrolysis (in the presence of the Lindlar catalyst): (A) Methyl oleate. (B) Methyl linoleate. (C) Methyl linolenate. (D) Methyl arachidonate.

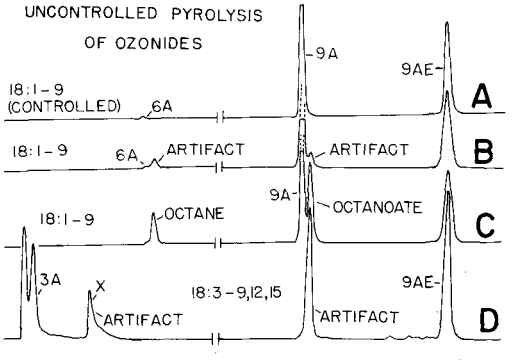
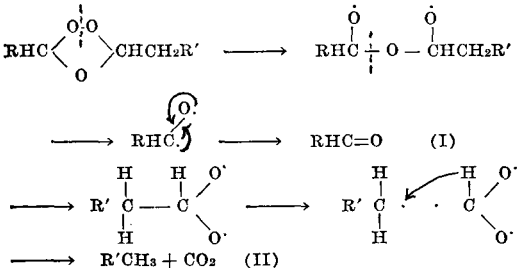


FIG. 3. GLC analysis of the products of the pyrolysis of the ozonides of: (A) Methyl oleate controlled (in the presence of the Lindlar catalyst). (B) Ozonide of methyl oleate uncontrolled (C) Ozonide of methyl oleate uncontrolled plus added octane and methyl octanoate as reference standards. (D) Ozonide of methyl linolenate uncontrolled.

conditions employed here were shortchain methyl esters and hydrocarbons. The unidentified peak (x) in the chromatogram of the analysis of products of the pyrolysis of methyl linolenate (Fig. 3) is probably due to an acid as indicated by the askewed nature of the peak and because the pyrolysis of ozonides generally gives acids as well as hydrocarbons and shortchain methyl esters (13–16).

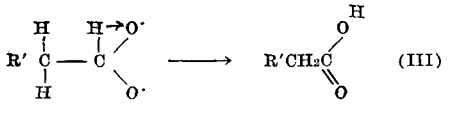
The identification of the methyl octanoate and octane among the products of the pyrolysis was made by GLC by comparison with reference standards of these compounds on both a polyester and silcione phase. They were also analyzed in an admixture with the controlled pyrolysis of the ozonide of methyl oleate as illustrated in tracing C (Fig. 3).

The formation of acids, esters and hydrocarbons together with aldehydes in the uncontrolled pyrolysis may be represented by the following reactions:



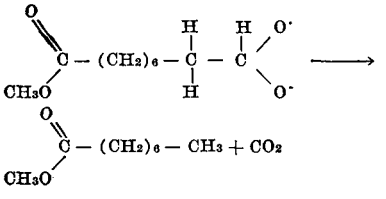
Where R′ is the proximal end of the molecule, $R'CH_3$ is an ester, and where R′ is from the terminal end of the molecule, $R'CH_3$ is hydrocarbon.

Likewise, mono- and dibasic acids may be formed by the following reactions:

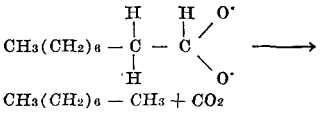


In addition to the above reactions, intermolecular reactions could occur to yield still other products as well as aldehydes and acids.

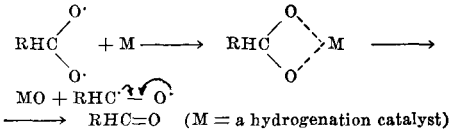
The formation of methyl octanoate from the pyrolysis of the ozonides of methyl oleate and linolenate can be explained on the basis of reaction II above, as follows:



Likewise, the formation of octane from the pyrolysis of methyl oleate ozonide may be explained as follows:



The function of the hydrogenation catalyst to provide quantitative yields of aldehydic fragments may be to effect a simple reduction of the ozonide or the dioxygen radical by virtue of adsorbed active hydrogen that may remain on the catalyst in the course of its preparation. However, this possibility seems to be remote in view of the manner in which the catalyst was treated and since it gave no reduction of ozonides at room temperature in an atmosphere of nitrogen. It appears that the catalyst reacts directly with the ozonides in accordance with the following mechanism:



Other techniques, of course, may be employed with different equipment whereby the pyrolysis may be effected in the injection port or flash evaporator of the GLC instrument so as to permit a simultaneous analysis of the fragments. Presumably a standard pyrolysis unit could also be used for the reaction-analysis operation.

The advantage of the pyrolysis method of ozonolysis is that it is fast and simple; no solvents are used for the cleavage reaction and the number of manipulations is reduced to a minimum. The method is easily applicable on a microgram scale as none of the sample is lost and there are no solvents to interfere with the GLC analysis of the aldehyde fragments. Since the only products of the reaction are aldehydes and there is no loss, fractionation or alteration of the products, the method should permit the quantitative analysis of mixtures of unsaturated fatty acid esters that cannot be analyzed readily by other means.

ACKNOWLEDGMENT

Supported in part by USPHS, NIH Grant No. AM-05018.

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[Received Oct. 27, 1965]

Metabolism of ^{14}C-Labelled Oleic Acid, Erucic Acid and Nervonic Acid in Rats[1]

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ABSTRACT

1-^{14}C-Oleic acid, 2-^{14}C-erucic acid and 2-^{14}C-nervonic acid were administered to rats by tail-vein and the distribution of radioactivity in liver lipids was determined at intervals from 15 min to 6 hr after injection. High levels of activity were found after short time intervals which were mainly associated with triglycerides in the case of oleic acid and with free fatty acids in the case of erucic acid and nervonic acid. The activity in these lipids decreased with time and was later exceeded by that in more polar lipids. In rats given erucic acid or nervonic acid, sphingolipids were more highly labelled than glycerophosphatides. Nervonic acid showed little tendency to form a complex with serum albumin and erucic acid complexed less readily than palmitic acid.

INTRODUCTION

ERUCIC ACID (Δ^{13}-docosenoic acid) is a major component fatty acid of rapeseed oil and seed oils of other Cruciferae (1–3). Its metabolism in mammalian tissues is of interest because rapeseed oil is widely used as an edible oil (4), and because feeding rapeseed oil to experimental animals produces various effects such as growth inhibition (5) and altered cholesterol metabolism (6), which appear to be due to its erucic acid content. Nervonic acid (Δ^{15}-tetracosenoic acid) is of little interest as a dietary component, but further information on its metabolic properties is desirable because of its occurrence in mammalian tissue lipids (3,7) and its close chemical relationship to erucic acid.

Earlier studies in our laboratory (6), involving the use of ^{14}C-labelled fatty acids administered either by mouth or by tail vein injection, showed that erucic acid and nervonic acid gave lower levels of radioactivity in tissue lipids than palmitic acid. Significant differences in rate of excretion of radioactivity in respiratory CO_2 were also observed. The present report deals with the distribution of radioactivity among lipid classes of liver at different time intervals after administration of labelled erucic acid and nervonic acid by intravenous injection. Oleic acid, which belongs to the same homologous series, was also included in these studies.

EXPERIMENTAL

Male Sprague-Dawley rats of the Holtzman strain raised in our laboratory and weighing 170–200 g were used in these experiments. They were maintained on fox chow and were not fasted prior to administration of the labelled fatty acids.

Oleic acid labelled in the 1-position and erucic acid and nervonic acid labelled in the 2-position were obtained from Merck and Co. Ltd., Montreal, and were purified prior to use by chromatography on acid-treated Florisil (8). Their specific activities were 7.5, 0.19 and 0.25 mc/mmole, respectively. Aliquots of the sodium salts containing approximately 1×10^6 counts per minute were administered by tail vein in 1 ml of saline solution.

Animals were killed at intervals from 15 min to 6 hr after administration of the labelled acids. The livers were removed, ground in a Virtis homogenizer in the presence of chloroform-methanol (2:1) and the extracts washed with 0.2 volumes of water as described by Folch et al. (9). The lipids were then chromatographed on 12 g columns of acid-treated Florisil, eluting with 75 ml each of chloroform, chloroform-methanol 95:5, 90:10, 75:25, 50:50 and methanol (10). Neutral lipids and free fatty acids were eluted with chloroform and were subsequently separated further by rechromatographing the chloroform fraction on Florisil (11). Ceramide was eluted with chloroform-methanol 95:5 and phosphatidyl ethanolamine with the 75:25 mixture. Phosphatidyl choline and sphingomyelin were present in both the chloroform-methanol 50:50 and methanol fractions. Radioactivity was measured by plating aliquots of column fractions in duplicate and counting in a D-47 gas-flow counter with Micromil window (Nuclear-Chicago). The results shown in Table I were obtained by counting aliquots ranging from 1/10th to 1/50th of the total volume of fractions from the column, diluted so that aliquots of 0.5 ml to 1 ml could be plated in each case,

[1] Presented at the AOCS Meeting in Houston, April 1965.

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TABLE I

Distribution of Radioactivity in Rat Liver Lipids Separated by Column Chromatography[a]

Time after injection	Triglyceride	Sterol	Free fatty acid	Ceramide	Phosphatidyl ethanolamine	Phosphatidyl choline and sphingomyelin
			Oleic			
¼ hr	13.2	2.0	1.7	0.4	2.2	2.9
½ hr	10.9	1.8	1.6	0.5	3.7	4.6
1 hr	7.1	0.8	1.0	0.3	2.8	4.3
2 hr	4.1	0.6	1.3	0.5	1.7	4.5
4 hr	2.2	0.3	0.5	0.3	0.9	2.7
6 hr	0.6	0.1	0.2	0.1	1.1	2.1
			Erucic			
¼ hr	5.8	1.2	18.0	1.7	0.6	1.5
½ hr	5.0	0.6	7.5	2.1	0.9	1.6
1 hr	2.2	1.9	1.2	2.0	1.0	1.5
2 hr	2.1	1.5	0.6	1.5	2.0	2.5
4 hr	1.1	1.2	0.4	0.8	1.9	2.7
6 hr	0.4	0.7	0.2	0.5	0.8	2.1
			Nervonic			
¼ hr	-[b]	-[b]	42.7	2.7	0.1	0.7
½ hr	0.9	0.6	24.4	4.4	0.3	0.7
1 hr	0.5	0.5	8.5	6.4	0.4	1.3
2 hr	0.3	0.5	4.0	4.7	0.4	2.3
4 hr	0.5	0.5	0.5	1.2	0.6	2.9
6 hr	0.3	0.8	0.4	1.3	0.7	3.5

[a] Results are expressed as percent of total radioactivity administered to rats in the form of 1-^{14}C-oleic acid, 2-^{14}C-erucic acid or 2-^{14}C-nervonic acid. Amounts given were approximately 1×10^6 cpm in each case. Fractions which never contained more than 1% of the radioactivity injected are omitted from the Table. Most results are averages for 2 or 3 rats.

[b] Not measured.

depending upon level of radioactivity and the amount of lipid present. Aliquots used gave counting rates which were nearly always greater than 100 counts per minute above background and which were commonly several hundred to several thousand per minute. The amount of lipid plated was never sufficient to cause significant errors due to self-absorption.

The identity of radioactive components was investigated further by rechromatographing aliquots of column fractions on thin-layer plates and scanning for radioactivity with an Actigraph II Model C100 B (Nuclear-Chicago). A slit width of ⅛ in., scan speed of 3 in. per hour, time constant of 20 sec and count-rate range of 500 counts per minute for full-scale deflection were used. After being scanned, the plates were sprayed with sulfuric acid and charred to determine the positions of separated lipids. Solvent systems petroleum ether-ether-acetic acid, 60:40:1, and chloroform-methanol-water, 65:20:3, were used for neutral lipids and phospholipids, respectively. The ceramide fraction was also chromatographed in chloroform-methanol, 65:6, chloroform-acetic acid-methanol, 94:10:2, and chloroform-methanol-acetone, 4:1:1.

The extent to which erucic acid and nervonic acid were metabolized and the radioactive carbon incorporated into other fatty acids was determined by converting the fatty acids of the liver lipids to methyl esters, separating them by gas chromatography and analyzing the effluent for radioactivity. The methyl esters were prepared by refluxing with methanol to which was added 10% (v/v) of acetyl chloride (12). Gas-liquid chromatography was carried out on an F & M Model 500, using a 6 ft × ¼ in. column packed with 5% SE-30 on siliconized Diataport W (80–100 mesh) with a column temperature of 230C and a helium flow rate of 80 ml/min. The effluent from the detector was passed through a Packard Tri-Carb Combustion Furnace, Model 325, maintained at 800C and a Packard Flow Detector, Series 317. The radioactivity was recorded with a Packard Tri-Carb Flow Monitor, Series 320E, using a linear range of either 1000 or 3000, a time constant of 10 and a voltage of 750.

RESULTS

Distribution of Label from Fatty Acids in Lipid Classes of Liver

The distribution of radioactivity in rat liver lipids at different time intervals after tail-vein injection of the labelled fatty acids is shown in Table I. The triglyceride fraction contained high levels of activity at short time intervals after administration of oleic acid but was less active after administration of erucic acid and contained relatively little activity when ner-

vonic acid was given. Conversely, the free fatty acid fraction was highly radioactive after administration of nervonic acid or erucic acid but contained much less activity when oleic acid was given. The radioactivity in the free fatty acid fraction was highest and persisted longest in rats given nervonic acid. The sterol fraction was the only other neutral lipid fraction which contained appreciable amounts of radioactivity and in it the levels seemed to be generally higher with rats given erucic acid.

The liver lipids of rats injected with nervonic acid showed a peak of activity in the ceramide fraction, which reached a maximum about an hour after administration of the labelled acid and then declined in parallel with the decline in free fatty acid activity. The main radioactive component appeared to be ceramide on the basis of comparison in several thin-layer chromatographic systems (13) with an authentic sample provided through the courtesy of H. E. Carter. This fraction was also labelled to some extent by erucic acid but contained very little activity when oleic acid was administered.

The amount of label in the phosphatidyl ethanolamine fraction decreased with increasing chain-length of fatty acid administered and the peak of activity was reached at later time intervals with longer-chain acids. The latter finding was also noted in the lecithin-sphingomyelin fraction. In this fraction, radioactivity from nervonic acid was found mainly in sphingomyelin whereas that from erucic acid was distributed more evenly between lecithin and sphingomyelin (Fig. 1). None of the label from oleic acid was found in sphingomyelin, in confirmation of earlier studies (14).

Further evidence on the nature of the phospholipid components labelled by erucic acid and nervonic acid was obtained by subjecting aliquots of the different fractions to mild alkaline hydrolysis as described by Dawson (15) and then rechromatographing them on columns of acid-treated Florisil. The results were consistent with the identification of radioactive phospholipids based on column and thin-layer chromatography.

Gas-liquid radiochromatography of fatty acid methyl esters from the free fatty acid fractions labelled by erucic acid and nervonic acid indicated that these fatty acids were deposited largely unchanged in the liver. Some of the label was present, however, as shorter chain compounds. The radioactivity in the ceramide fraction was associated with nervonic acid when labelled nervonic acid was administered and it also appeared to be associated with nervonic acid when labelled erucic acid was given. The latter finding suggests that chain-lengthening of erucic acid may have occurred prior to incorporation into the ceramide fraction.

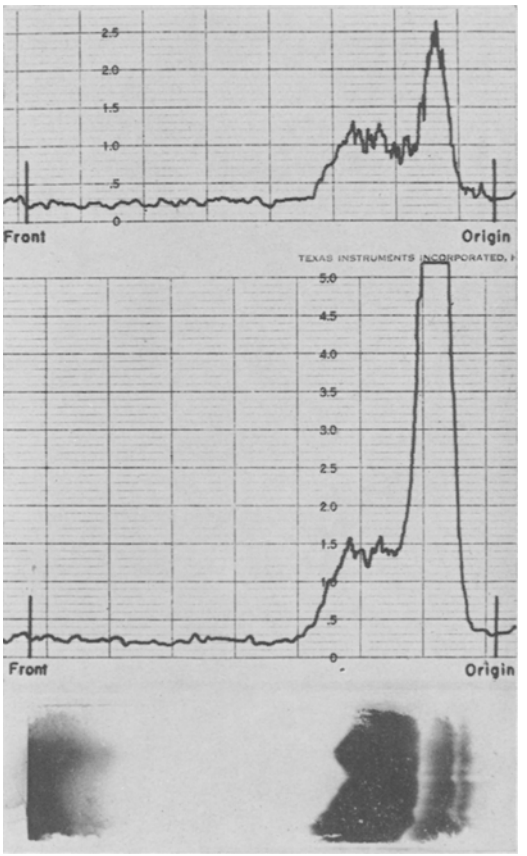

FIG. 1. Tracings obtained by scanning thin-layer chromatograms of lecithin-sphingomyelin fractions for radioactivity. *Upper chart*, rat injected with erucic acid; *lower chart*, rat injected with nervonic acid. The chromatogram shows, from left to right, a broad lecithin band by a narrower sphingomyelin band.

Affinity of Erucic Acid and Nervonic Acid for Serum Albumin

The common C_{16} and C_{18} fatty acids form a complex with serum albumin and the so-called "free fatty acids" of serum are transported in this form. Gatt showed, however, that lignoceric acid (24:0) failed to form a complex (16) and it seemed of interest to determine the behavior of erucic acid and nervonic acid in this regard. The labelled fatty acids, neutralized with dilute NaOH, were added to rat serum to give a fatty acid-albumin ratio of approximately 1:1 and the mixture was subjected to

paper electrophoresis in 0.05 M sodium barbital buffer at pH 8.5. The strip was scanned for radioactivity in the Actigraph scanner and then stained with bromphenol blue. The results (Fig. 2) showed that about half of the label from erucic acid migrated with the albumin fraction and the remainder stayed at the origin. Nervonic acid behaved more like lignoceric acid in that most of the activity remained at the origin. Radioactive palmitic acid migrated entirely with the albumin fraction when added to rat serum and subjected to electrophoresis under the same conditions.

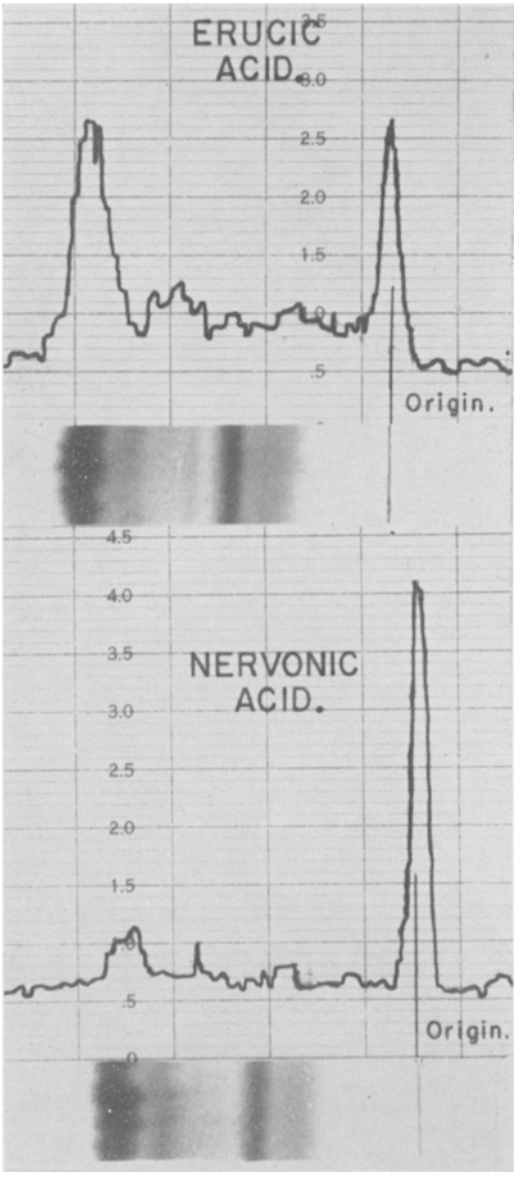

FIG. 2. Charts showing distribution of radioactivity following paper electrophoresis of labelled fatty acids mixed with rat serum.

DISCUSSION

The distribution of radioactivity in rat liver lipids was found to be different for each of the three fatty acids used in these experiments. Nervonic acid, and to a lesser extent erucic acid, were deposited in the liver as free fatty acids and were metabolized more slowly than oleic acid (Table I). Similar findings have been reported by Gatt (16) for lignoceric (tetracosanoic) acid, which accumulates to an even greater extent in the free fatty acid fraction of liver and persists longer in that form. Göransson (17) also noted that 4% of the radioactivity from injected arachidic (eicosanoic) acid was associated with liver free fatty acids whereas almost none of the label from palmitic acid was found in that fraction. It appears that as chain-length increases, fatty acids are metabolized more slowly and accumulate to a greater extent as free fatty acids. This tendency seems to be more marked with saturated than with monounsaturated fatty acids.

Earlier studies in our laboratory showed that dietary erucic acid stimulated incorporation of labelled acetate into cholesterol by rat liver slices (18) and increased fecal cholesterol excretion in the absence of dietary cholesterol (19). It is therefore of interest that the level of radioactivity in the sterol fraction was higher for a longer period of time after administration of labelled erucic acid than after administration of either of the other labelled acids (Table I).

The accumulation of radioactivity from nervonic acid in alkali-stable polar lipids is consistent with the fact that it normally occurs in tissue lipids as a component of sphingolipids. The relatively rapid rise and fall of radioactivity in the ceramide fraction suggests that ceramide is an intermediate in the formation of sphingomyelin, which accumulates radioactivity at a slower rate. It should be noted, however, that recent evidence indicates that the ceramide in liver is mainly in the *erythro* configuration and there is still some uncertainty whether a ceramide of this configuration can serve as an intermediate in the formation of other sphingolipids or whether it represents a metabolic end-product (13).

Labelled erucic acid contributed somewhat less radioactivity to liver polar lipids, in agreement with earlier findings that dietary erucic

acid is not readily incorporated into phospholipids (20). The amount of erucic acid incorporated as such into polar lipids may in fact be less than indicated by the level of radioactivity since gas-liquid radiochromatography indicated that it had undergone chain-lengthening before being incorporated into the ceramide fraction. Because of such transformations, the distribution of radioactivity cannot be relied on to give a true picture of the distribution of the fatty acid administered. However, in the present experiments, radioactivity present in the free fatty acid fraction of liver at short time intervals after administration of erucic acid or nervonic acid was found to be largely in the form of the unaltered fatty acid. Evidence has also been obtained in other laboratories that oleic acid is not converted to other fatty acids to an appreciable extent in mammalian tissues (21) and the preferential incorporation of oleic acid into triglycerides is in accord with previously reported results (22).

Electrophoresis of the labelled fatty acids mixed with rat serum showed that their affinity for serum albumin decreased with increasing chain-length (Fig. 2) in agreement with results from other laboratories (16,17,23,24). Under the conditions of our experiments, most of the radioactive material which did not migrate with albumin remained at the origin. Gatt obtained a similar result with lignoceric acid (16) but other reports indicate a tendency for longer-chain fatty acids to associate with the globulin fraction (17,23).

ACKNOWLEDGMENTS

Technical assistance by H. E. Pedersen and by M. W. Edmonds, G. J. Tevaarverk and E. D. Ralph. Assistance with the electrophoretic studies by J. B. D. Derrick. Assistance and equipment for the gas-liquid radio analyses of fatty acid esters by Frances McElroy, F. B. Palmer and K. P. Strickland of the Department of Biochemistry.

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[Received Nov. 5, 1965]

Mass Spectrometry of Lipids. I. Cyclopropane Fatty Acid Esters[1]

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ABSTRACT

A method was developed for the almost quantitative conversion of unsaturated esters (from monoenes to tetraenes) to cyclopropanes using diiodomethane and a highly active zinc-copper couple. These derivatives are sufficiently volatile for GLC analysis and *cis* and *trans* isomers can be distinguished by this technique. Equivalent chain lengths of the cyclopropane derivatives were measured on polar and nonpolar phases. The mass spectra of the monocyclopropane compounds are very similar to those of the parent unsaturated esters. Those of dicyclopropanes, however, are quite distinctive so that the original structure of the ester can be deduced. Polycyclopropanes give complex spectra which are difficult to interpret in terms of the position of the original double bonds.

INTRODUCTION

ISOMERIC UNSATURATED fatty acid esters, differing in the position of the olefinic center, have identical mass spectra because of the mobility of double bonds under electron impact (1). One of the principal unsolved problems of mass spectrometry as applied to lipid chemistry, therefore, is to find some derivative of a double bond which has a unique mass spectrometric fragmentation pattern to allow the original structure of the ester to be determined. Ideally, this derivative should be sufficiently volatile to be readily eluted from a GLC column, even when prepared from a polyene. The method of preparation should be simple and quantitative so that it can be applied either to small amounts of purified esters or preferably to mixed methyl esters prepared from tissue lipids to allow a GLC mass spectrometer combination to be used. Of the existing methods, deuterohydrazine reduction (2) can only be applied to pure monoenoic esters giving mass spectra which are difficult to interpret. Oxidation to an epoxide, followed by rearrangement to a pair of ketones (3), probably is not quantitative and gives a mixed product too polar for ordinary GLC analysis if more than one double bond is present in the original acid.

The simplest nonpolar symmetrical compound which can be prepared from an olefin is a cyclopropane, so it was decided to investigate methods of preparing such derivatives in the hope that they might have distinctive mass spectra. The reaction of an olefin with diiodomethane and a zinc-copper couple has become a general method for the synthesis of stereochemically pure cyclopropanes since it was first described by Simmons and Smith (4). The reaction is a bimolecular process involving the formation of a stable organozinc intermediate which then transfers a methylene group to a double bond (5,6).

$$2\ CH_2I_2 + 2Zn \longrightarrow (I\ CH_2)_2\ Zn \cdot ZnI_2$$

$$2\ -CH{=}CH- + (I\ CH_2)_2\ Zn \cdot ZnI_2 \longrightarrow$$

$$2\ -\underset{\diagup}{CH}\ \ \underset{\diagdown}{CH}- + 2ZnI_2$$

The yields, however, were not particularly good until LeGoff (7) described the preparation of a much more active zinc-copper couple. A slight modification of LeGoff's method enabled us to achieve quantitative conversion of monoenoic through tetraenoic unsaturated esters to cyclopropanes. A series of cyclopropane esters were prepared and their gas chromatographic and mass spectrometric properties investigated.

A method of synthesizing cyclopropane acids in high yield is also desirable because these have now been found as major components in the lipids of a number of bacterial species (8). They also occur as minor components of certain seed oils (9,10) along with cyclopropene acids. The metabolism of cyclopropane acids in the rat has recently been discussed (11).

EXPERIMENTAL

Materials

Diiodomethane (Fisher Scientific Co.) was redistilled and stored over copper metal before use. Zinc dust was Mallinckrodt analytical reagent grade and cupric acetate monohydrate was Fisher's reagent grade. Most of the methyl esters of the unsaturated fatty acids were supplied by The Hormel Institute, though the methyl linoleate isomers were prepared synthetically as part of another project.

Preparation of the Zinc-Copper Couple

To vigorously stirred nearly-boiling glacial acetic acid (10 ml) in a 25 ml 2-necked flask was added zinc dust (2.0 g). After 1 min,

[1] Presented at the Symposium on Spectrometry Applied to Lipids, AOCS Meeting, Cincinnati, October, 1965.

cupric acetate monohydrate (0.4 g) in hot glacial acetic acid (10 ml) was added and the mixture stirred approximately 1 min until the blue color disappeared. The hot supernatant liquid was decanted and the couple washed thoroughly with glacial acetic acid (5 × 20 ml) and then with anhydrous ether (5 × 20 ml). Fresh couple was always prepared immediately before use and no attempt was made to store it.

Preparation of the Cyclopropane Derivatives

The general procedure for the preparation of the cyclopropanes was as follows, taking the cyclopropane derivative of methyl linoleate as an example. A condenser and dropping funnel were fitted to the flask containing the zinc-copper couple in anhydrous ether (10 ml). To this was added a solution of diiodomethane (4 ml and methyl linoleate (0.2 g) in ether (5 ml) at such a rate that the solution was kept at reflux by the heat of reaction. When the addition was complete, the solution was refluxed overnight under nitrogen. At the end of this time, the ether solution was decanted from the unchanged couple and washed 3 times with cold hydrochloric acid (1N) and 3 times with water before drying over sodium sulfate. The ether was removed and the excess diiodomethane distilled off at 100C at 0.5 mm Hg. To remove polar by-products, the crude material was chromatographed on a column of florisil (20 cm × 1 cm) and the required cyclopropane ester eluted with 100 ml of a petroleum ether: ether mixture (70:30). Traces of unchanged unsaturated esters were removed by preparative TLC on silica gel plates (0.5 mm thick) impregnated with silver nitrate (12) with petroleum ether:ether (90:10) as the solvent system. The other cyclopropane esters were prepared in a similar manner, the amounts of the various reagents being adjusted according to the number of double bonds in the ester and the quantity of ester available. GLC analysis showed that 95–100% reaction occurred in all cases and overall yields of 70–80% were obtained.

Gas Chromatography

Gas chromatographic separations were made on an 8 ft × ¼ in. glass column packed with 20% ethylene glycol succinate (EGS) and 2% phosphoric acid on Gas-Chrom P (80–100 mesh) in a gas chromatograph with a β-ionization detector, at a temperature of 180C and argon flow rate of 70 ml/min. A Beckman GC-2A instrument with flame ionization detector was also used with a 6 ft × ⅜ in. aluminum column packed with 20% Apiezon L (Ap L) on Gas-Chrom P (80–100 mesh) at a temperature of 220C and helium flow rate of 50 ml/min. Relative retention times were recorded as equivalent chain lengths (ECL) (13).

Mass Spectrometry

The mass spectra were obtained on a Hitachi Perkin-Elmer RMU-6D single focusing instrument. A liquid injection system was used with a suboven heating the sample to 160C to produce the vapor. Spectra were produced at a standard ionization potential of 80eV and then at the lowest voltage which gave a countable spectrum, usually 6–12eV.

Elemental Analyses

Elemental analyses of some of the cyclopropane derivatives, selected at random, were carried out and found to be satisfactory. Clark Microanalytical Laboratories, Urbana, Illinois, performed the estimations.

RESULTS AND DISCUSSION

GLC of the Derivatives

The cyclopropane derivatives of all the unsaturated esters, even those of the tri- and tetraenoic esters, were sufficiently volatile to be readily examined by GLC. The ECLs of each ester on both polar and nonpolar columns (EGS and Ap L) were determined and are recorded in Table I. The average increment in ECL over that of the normal saturated ester is approximately +1.5 on EGS or +0.9 on Ap L per *cis* cyclopropane ring in the molecule. For a *trans* cyclopropane ring, it is approximately +0.85 on EGS or +0.55 on Ap L. The

TABLE I

Cyclopropane Derivatives and Equivalent Chain Lengths On EGS and Apiezon L

Derivative of:	ECLs: EGS	ApL
Me 10-undecenoate	13.70	11.20
Me palmitoleate	17.57	16.85
Me oleate	19.46	18.77
Me elaidate	18.80	18.52
Me petroselinate	19.56	18.93
Me petroselaidate	18.92	18.57
Me vaccenate	19.57	18.88
Me 11-eicosenoate	21.31	20.85
Me erucate	23.38	22.80
Me nervonate	25.54	24.86
Me linoleate	21.07	19.60
Me *trans,trans*-linoleate	19.90	18.95
Me 4,7-octadecadienoate	20.90	19.68
Me 5,8-octadecadienoate	20.83	19.56
Me 10,13-octadecadienoate	21.05	19.63
Me *trans,trans*-9,11-octadecadienoate	20.02	18.12
Me linolenate	22.95	20.58
Me arachidonate	25.68	23.01
Me hydnocarpate	20.05	19.19

cyclopropane derivatives of related *cis* and *trans* fatty acid esters, e.g., methyl oleate and elaidate or methyl *cis,cis*-linoleate and *trans, trans*-linoleate, are readily separable on both polar and nonpolar GLC columns, so the reaction has potential as a method of estimating *trans* double bonds. The derivative from the single conjugated ester, methyl *trans,trans*-9,11-octadecadienoate has remarkably low ECL values, particularly on Ap L.

Mass Spectrometry

Mass spectra were recorded for each ester at both high and low ionization potentials. Larger amounts of sample are necessary for low voltage spectra, but these are often remarkably distinctive and emphasize the primary or larger fragments and minimize secondary degradations. For some substances measurement of spectra at intermediate voltages facilitated counting and indicated the approximate appearance potentials of certain unique peaks in the spectra.

Monocyclopropanes

The mass spectra of the cyclopropane derivatives of methyl oleate and elaidate have already been described by Wood and Reiser (11). They noted no differences in the spectra of the two isomers which, in turn, were very similar to those of the parent esters. Our findings are similar and our interpretation of the spectra differs only in minor details. In Figure 1, the mass spectra of methyl oleate and its cyclopropane derivative at 80eV are compared. The only difference immediately apparent is that the molecular weight of the derivative is 14 more than that of the parent ester. The characteristic peaks described by Stenhagen et al. (1) for monounsaturated esters are found in both, i.e., at m/e = M-32 (loss of methanol from the ester function), m/e = M-74 (loss of the ester group plus one carbon from the chain) and m/e = M-116 (loss of the ester group plus four carbons from the chain). The hydrocarbon peaks at the low end of the spectra are also virtually indistinguishable. When the relative intensities of the ions corresponding to $(C_nH_{2n+1})^+$, $(C_nH_{2n})^+$, $(C_nH_{2n-1})^+$, $(C_nH_{2n-2})^+$, and $(C_nH_{2n-3})^+$, in the spectra of both were plotted against number of carbons and compared, almost no difference was found. The low voltage spectra are equally similar. Peaks which might be expected by fragmentation on either side of the cyclopropane rings at m/e = 113 and 197 or m/e = 153 and 157 are no more prominent in the spectrum of the cyclopropane derivative than in that of the parent ester.

The spectra of the other monocyclopropane esters are likewise similar to those of the parent monoenoic esters at both high and low ionization potentials. Also, geometrical isomers cannot be distinguished. The only exception is the

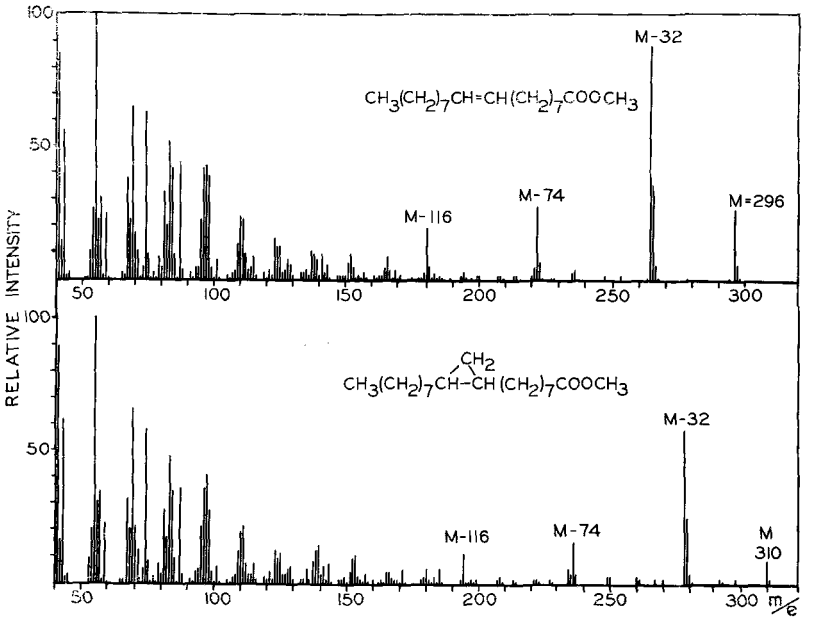
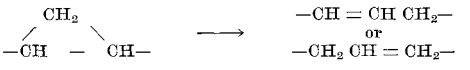


FIG. 1. Mass spectra of methyl oleate and its cyclopropane derivative at 80eV.

low voltage (8eV) spectrum of the cyclopropane derivative of methyl erucate, in which m/e = 226 or M-100 is the base peak and is probably formed by the loss of a rearrangement ion including the ester group and three carbons from the chain.

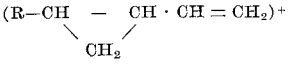
To explain the similarities between the spectra of the monocyclopropanes and those of the monoenoic esters from which they were derived, we postulate an immediate and complete cleavage between the two carbon atoms in the cyclopropane ring which originally constituted the double bond. This gives, in effect, a monounsaturated ester, one carbon atom longer, i.e.:



This then exhibits the mass spectral fragmentation pattern expected of a monoene. Thus, the position of the cyclopropane ring cannot be deduced.

Dicyclopropanes

The mass spectra of the cyclopropane derivatives of the dienoic esters are much more distinctive, particularly those of the series containing the vinyl methylene rhythm. In Figure 2, the spectra of the derivative from methyl *trans,trans*-linoleate at high (80eV) and low (6eV) ionization potentials are compared. This compound was chosen for illustrative purposes because it contains all the features of interest noted in the spectra of the other esters of this type. At 80eV, the base peak is at m/e = 149, and this peak is the base peak for all these diene derivatives with the exception of the cyclopropane derived from methyl *cis,cis*-linoleate itself, in which it is insignificant. The most prominent peaks occur at m/e = 224 (especially at low voltages) and at m/e = 192 and 138. The molecules, therefore, appear to cleave preferentially to give fragments with two carbon atoms attached to a cyclopropane ring. A completely analogous pattern is found with the other dicyclopropanes. In the high and low voltage spectra of the cyclopropane derivative of methyl 10,13-octadecadienoate, there are very distinctive peaks at m/e = 238 and 124. In those of the 5,8 isomer, there are prominent peaks at m/e = 168, 194 and 248; and in those of the 4,7-isomer, large peaks are found at m/e = 154 and 208. In each case, ions of the form:



appear to be particulary stable though, of course, the ions may not remain in this form but may exist as conjugated or cyclic structures. If, in fact, the presence of a grouping of this kind does confer stability on an ion,

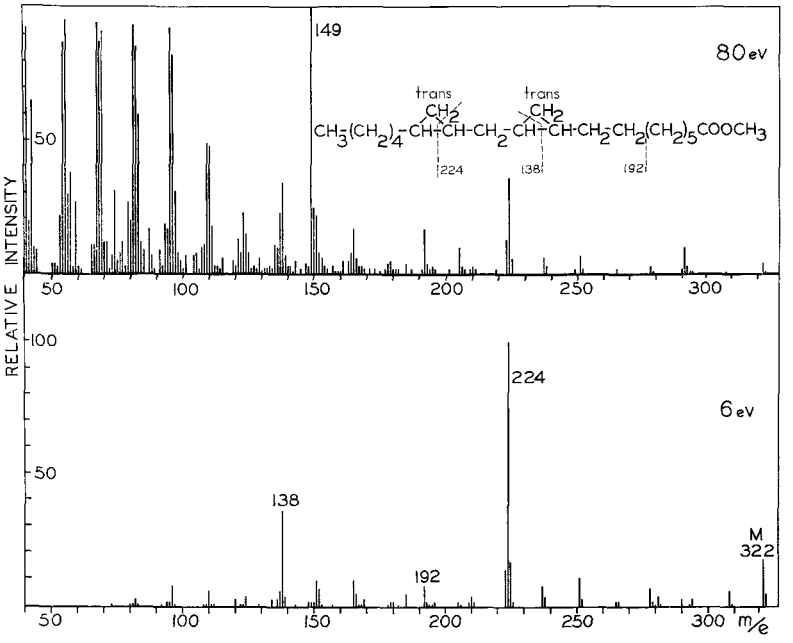
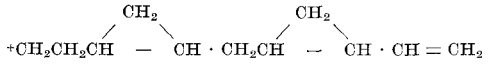


FIG. 2. Mass spectra of the dicyclopropane derivative of methyl *trans,trans*-linoleate at 80eV and at 6eV.

it might be postulated that the peak at m/e = 149 is equivalent to:



though, possibly some rearrangement occurs.[2] Formation of this ion requires a double cleavage of the molecule, so it would be expected at high rather rather than low ionization potentials. In a study of the effect of variation of the ionizing voltage on this ion, it remained the base peak down to about 20eV when it began to decline sharply until at about 10eV it virtually disappeared.

The mass spectra of the cyclopropane derivative of the conjugated ester, methyl *trans, trans*-9,11-octadecadienoate, also has some unusual features (Fig. 3). At a high voltage (80eV), little of interest is apparent at higher mass numbers though, at the other end of the spectrum, the peaks at m/e = 45, 59 and 73 are much larger than usual. These are not particularly significant in the spectra of the parent ester or of any of the other dienes or their derivatives. They must contain at least one oxygen atom, but it is difficult to see how the presence of cyclopropane rings influences the disruption of the carboxyl end of the molecule. In the low voltage spectrum (6eV), the base peak is the parent ion, which may imply that the two cyclopropane rings rearrange readily to give a conjugated diene, for in the low voltage spectrum of the ester from which this was derived, the parent ion is the only one found. However, a large peak occurs at m/e = 266, corresponding again to cleavage giving two carbons attached to a cyclopropane ring. There are also peaks which could be attributed to cleavage at nearly every bond in the rings.

[2] Evidence for a strong conjugative effect of cyclopropyl groups with adjacent carbonium ion centers or double bonds has recently been obtained by nuclear magnetic resonance spectrometry. See Deno, N. C., H. G. Richey, Jr., J. S. Liu, D. N. Lincoln and J. O. Turner, J. Am. Chem. Soc. *87*, 4533 (1965), and Pittman, C. V., Jr., and G. A. Olah, Ibid. *87*, 5123 (1965).

Polycyclopropanes

The mass spectra of the cyclopropane derivatives of polyunsaturated esters are much less predictable than the spectra of the dicyclopropanes. This can be seen from Figure 4 where the mass spectra of the cyclopropane derivative of methyl arachidonate at high and low ionization potentials are compared. In the high voltage spectrum, the only unusual feature is a prominent peak at m/e = 270 which probably results from some rearrangement after cleavage between the 4th and 5th carbons in the original chain. At low voltages, the base peak is at m/e = 266, corresponding to a break between the 3rd and 4th rings in a manner contrary to the theory that the most stable ions are those

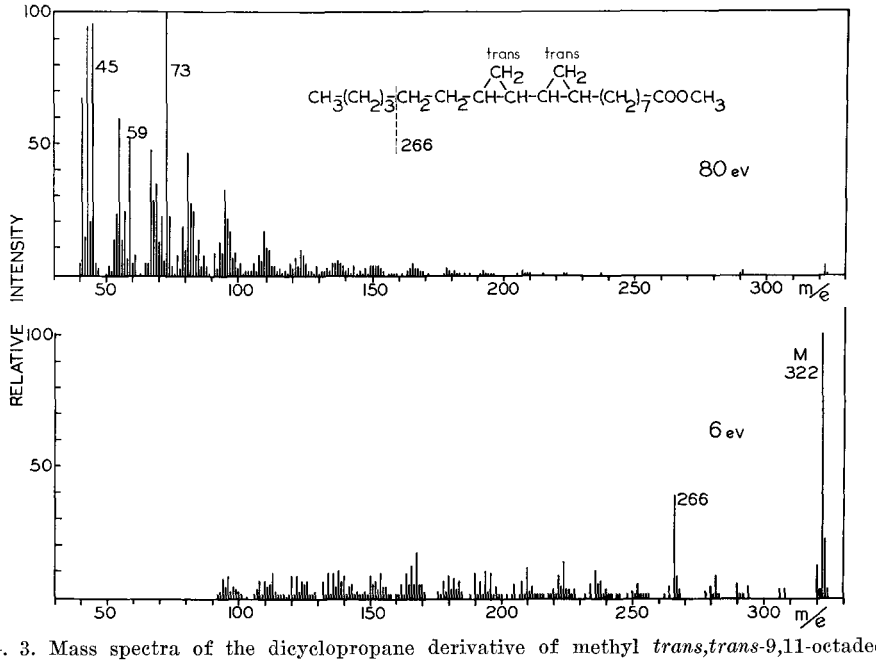


FIG. 3. Mass spectra of the dicyclopropane derivative of methyl *trans,trans*-9,11-octadecadienoate at 80eV and 6eV.

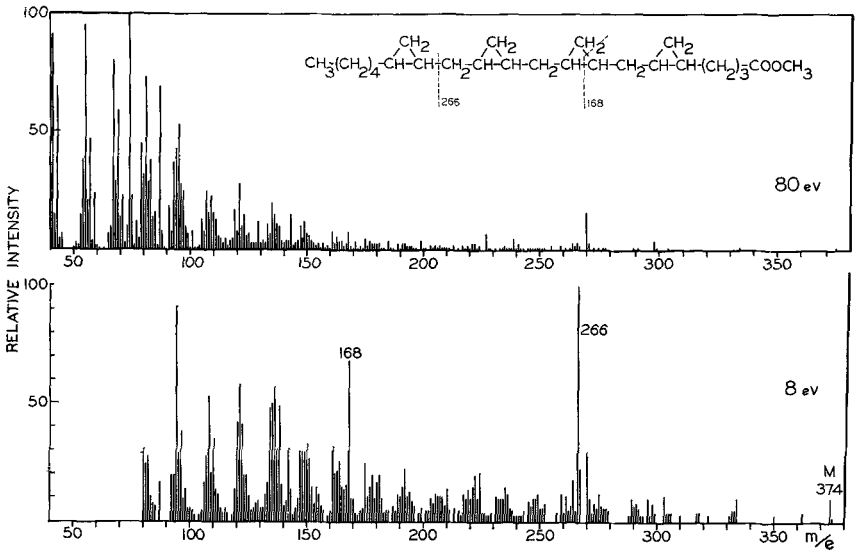


FIG. 4. Mass spectra of the tetracyclopropane derivative of methyl arachidonate at 80eV and 8eV.

with two carbon atoms attached to a cyclopropane ring. There is a large peak at m/e = 168, resulting from fragmentation in the second ring in the prescribed manner, though there are also peaks which could be interpreted as due to cleavage at almost any position in the rings or the chain.

The spectra of the cyclopropane derivative of methyl linolenate are only slightly less complicated than those of the tetracyclopropane compound above. At 80eV, there are no prominent peaks of high mass number. In the low voltage spectrum (6eV), however, the base peak is at m/e = 224, corresponding to cleavage in the second ring in the expected manner; though the next largest peak, at m/e = 136, must result from a break between the 1st and 2nd rings contrary to expectations. In fact, there is a large number of other significant peaks which could occur by fragmentation at any of the bonds in or between the cyclopropane rings.

Mass spectra of all the parent esters and the cyclopropane derivatives listed in Table I have been measured and are available upon request.

CONCLUSION

The reaction of unsaturated esters with diiodomethane and the highly active zinc-copper couple, described above, provides a useful synthesis of cyclopropane esters in high yields. These derivatives are sufficiently volatile to be eluted from a GLC column under moderate conditions and *cis*- and *trans* isomers are easily distinguished.

The method did not furnish a derivative which could be used to locate double bonds in all unsaturated esters by mass spectrometry. Monocyclopropanes have mass spectra very similar to those of the parent esters. Dicyclopropanes from dienoic esters with a single methylene group between the double bonds have unique fragmentation patterns which allow the positions of the original double bonds to be determined. This may possibly be extended to conjugated dienes though if the double bonds were separated by more than one methylene group this might not hold. Polycyclopropanes give complex spectra which are difficult to interpret in terms of the position of the cyclopropane rings, though the reaction could at least be used to confirm the number of double bonds in the molecule.

In the mass spectra of the di- and polycyclopropanes, there are a number of peaks for which the assistance of high resolution mass spectrometry will be necessary before the structures of the ions which they represent can be discussed with any confidence.

ACKNOWLEDGMENTS

Preparation of cyclopropane derivatives by W. E. Brugger; operation of mass spectrometer by Herbert Hayes. The methyl *trans*, *trans*-9,11-octadecadienoate was provided by Wilma Schneider of the Northern Regional Laboratory, Peoria, Illinois, and methyl hydnocarpate was received from D. Rebello, University of Bombay, India.

Supported in part by PHS Grant No. HE 03559 from National Institutes of Health.

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Reactions of Dimethyl Sulfoxide with Sulfonate Esters of Fatty Alcohols. I. Synthesis of Higher Saturated and Unsaturated Fatty Aldehydes[1]

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ABSTRACT

Long-chain saturated fatty aldehydes (C_{10} to C_{18}), as well as the C_{18} unsaturated aldehydes (oleyl, linoleyl, and linolenyl), were synthesized in good yields by the selective oxidation of the sulfonate esters of the corresponding alcohols with dimethyl sulfoxide in the presence of sodium bicarbonate. Chromatographic procedures for the isolation of the pure aldehydes from the reaction mixtures are described. The purity of the aldehydes was ascertained by thin-layer chromatography, melting points of their 2,4-dinitrophenyl hydrazones, infrared spectra and other physical methods.

INTRODUCTION

STUDIES INVOLVING the isolation of aldehydes from the aldehydogenic lipids of various tissues and their characterization by gas-liquid chromatography necessitated the preparation of several fatty aldehydes to serve as standards.

Generally the aldehydes are prepared from the corresponding carboxylic acids via a derivative which is subsequently reduced (1). However, the available methods are usually either not suitable for the synthesis of aliphatic aldehydes or, alternatively, are restricted to saturated aliphatic aldehydes. Unsaturated aliphatic aldehydes are generally synthesized by Grundmann's method (2) or by the acyloin condensation reaction (3). In the former, an acid chloride is converted to the acetoxy ketone via the diazoketone. The glycol obtained by reduction of the keto group and simultaneous hydrolysis is cleaved by lead tetraacetate to yield the aldehydes containing the same number of carbon atoms as the starting material. By employing suitable modifications of this method, Mangold (4) prepared unsaturated fatty aldehydes in yields of approximately 30%. In the acyloin condensation method, the glycols are produced by reduction of the acyloins and cleaved by lead tetraacetate. Gauglitz and Malins (3) prepared polyunsaturated fatty aldehydes from fish oil fatty acids by this procedure. Although these two methods yield pure aldehydes, they involve a number of steps and the yields are rather low.

An alternative route for the synthesis of long-chain fatty aldehydes lies in the controlled oxidation of the corresponding alcohols. The reagents often used to oxidize alcohols to aldehydes are: chromium ion in sulfuric acid or pyridine (5); manganese dioxide (6); aluminum isopropoxide in acetone (7); and lead tetraacetate (8). The application of these reagents for the selective oxidation of long-chain fatty alcohols, especially the polyunsaturated ones, needs further investigation.

The use of dimethyl sulfoxide as a solvent and oxidizing reagent has steadily increased since it became commercially available. In 1959, Kornblum, Jones and Anderson (9) reported a simple procedure for the selective oxidation of benzylic tosylates and a short-chain alkyl tosylate to aldehydes in yields of 75% by dimethyl sulfoxide. The use of this reagent for the synthesis of oleyl and elaidyl aldehydes by oxidation of the corresponding tosylates was recently reported from this laboratory (10).

This paper describes the synthesis of long-chain saturated and polyunsaturated fatty aldehydes by oxidation of the tosylates and mesylates of the corresponding long-chain alcohols. Thin-layer chromatography (TLC) of the crude aldehydes revealed the presence of two minor by-products. One of them was identified as the α-olefin with the same number of carbon atoms as the alcohol. Methods for the isolation of the aldehydes from the oxidation mixture are described.

EXPERIMENTAL

Materials

The methyl esters of saturated fatty acids (>99% pure) were obtained from The Hormel Institute inventory of fatty acid derivatives and reduced with $LiAlH_4$ to the corresponding alcohols (11). Oleyl, linoleyl and linolenyl alcohols (>99% pure) were also obtained from this source. Dimethyl sulfoxide was obtained

[1] Presented at the AOCS Meeting in Houston, April, 1965.

from a commercial source (Matheson, Coleman and Bell) and used without purification.

Preparation of Tosylates and Mesylates

The alcohols were converted to the tosylates with *p*-toluenesulfonyl chloride in pyridine by Tipson's method (12), and to the mesylates with methanesulfonyl chloride in pyridine as described by Baumann and Mangold (13). The purities of the tosylates and mesylates were checked by ascending thin-layer adsorption chromatography on Silica Gel G; they were found to be free from alcohols. The plates were developed with petroleum ether-diethyl ether (90:10, v/v) for tosylates and with petroleum ether-diethyl ether (70:30, v/v) for mesylates.

Oxidation of Tosylates and Mesylates by Dimethyl Sulfoxide

The oxidation of the tosylates and mesylates was accomplished by heating them with a mixture of sodium bicarbonate and dimethyl sulfoxide at 160C for 5–10 min. Efforts to produce the aldehydes by the same reaction at room temperature for 24 hr or by using dimethyl sulfoxide-acetic anhydride mixture as recently reported (14) were unsuccessful. The oxidations of palmityl tosylate and mesylate are selected for detailed description below, because the identification of one of the by-products, 1-hexadecene, in this case, rather than the unsaturated hydrocarbon from the corresponding unsaturated alcohols, was relatively easy.

In a 100 ml round-bottom ground-neck flask fitted with a condenser was placed 2.1 g palmityl tosylate, 1.0 g sodium bicarbonate and 10 ml dimethyl sulfoxide. Nitrogen was kept bubbling through the reaction mixture. The flask was immersed in a bath preheated at 165–170C. A vigorous reaction started in 1 min. After 5–10 min the reaction mixture was cooled rapidly to room temperature and poured into ice water. The precipitated material was extracted with ethyl ether, and the ether extract was washed once with oxygen-free water, dried over anhydrous Na_2SO_4 and filtered. All operations were conducted in a nitrogen atmosphere. Yield of crude palmityl aldehyde, 1.02 g.

Palmityl mesylate (2.27 g) on oxidation by the same procedure yielded 1.3 g of crude palmityl aldehyde.

Figure 1 shows the thin-layer chromatogram of the products of oxidation of palmityl tosylate and mesylate on Silica Gel G. The plate was developed with petroleum ether-ethyl ether (90:10, v/v) and the spots were made visible by spraying with 50% sulfuric acid and subsequent charring. In addition to the main oxidation product, palmityl aldehyde, two other reaction products obtained in minor quantities were also seen in each case. Small amounts of the starting material, as well as palmityl alcohol, were also present as impurities.

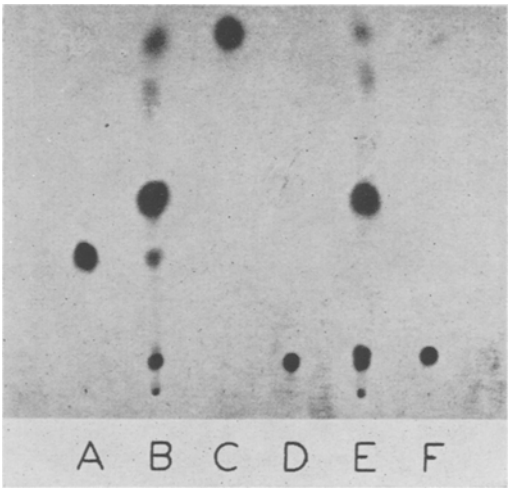


FIG. 1. Thin-layer chromatogram of oxidation products of palmityl tosylate and mesylate and reference compounds. Samples were: A) palmityl tosylate; B) oxidation products of palmityl tosylate (top to bottom; 1-hexadecene, unidentified compound, palmityl aldehyde, -tosylate and -alcohol); C) 1-hexadecene; D) palmityl alcohol; E) oxidation products of palmityl mesylate (top to bottom; 1-hexadecene, unidentified compound, palmityl aldehyde, -mesylate and -alcohol); F) palmityl mesylate.

Isolation of the Aldehyde as 2,4-Dinitrophenylhydrazone

The 2,4-dinitrophenylhydrazone of palmityl aldehyde was obtained from the reaction mixture (1.02 g) as described by Johnson (15) and recrystallized from 95% ethanol. Yield, 1.75 g.

Isolation of the Aldehyde by Column Chromatography

There is a large difference in distance of travel on the plate between the mesylate and the aldehyde. The difference in migration rates of the tosylate and the aldehyde is smaller than that of the mesylate and the aldehyde. This was also found to be the case with the system Silica Gel G/benzene. This indicated that the free aldehyde might be obtained pure by column chromatography if the mesylate rather than the tosylate were used as the starting material, and such was found to be the case.

The reaction product (1.3 g) was crystallized from 50 ml of petroleum ether (bp 30–60C) at −10C. This procedure removed the minor oxidation products. The unreacted mesylate and the alcohol could not be removed by crystallization techniques. However, they could be removed by a simple column chromatographic procedure. Silicic acid (Mallinckrodt, 100 mesh, chromatographic grade) was prepared for chromatography as described by Hirsch and Ahrens (16) without grinding the commercial product. Twenty grams of silicic acid was made into a slurry with petroleum ether (bp 30–60C), poured into a water-jacketed column (250 × 18 mm) and settled by frequent tapping. Nitrogen was kept bubbling through the slurry when the column was being prepared. Dissolved oxygen was removed from solvents by bubbling with nitrogen. The crude aldehyde (0.5 g) was dissolved in 5 ml of petroleum ether (bp 30–60C), applied on the column and washed with another 5 ml of the solvent. The solution was allowed to flow in without pressure until the liquid had been completely adsorbed. The aldehyde was then eluted with 250 ml of petroleum ether containing 5% diethyl ether. Most of the palmityl aldehyde appeared in this fraction and was recovered by evaporation of the solvents. The eluant can also be collected in 10 ml fractions and each fraction monitored for the presence of impurities by TLC. Alternatively, the aldehyde could be eluted from the column by benzene alone; 0.32 g of the crude aldehyde in benzene solution was applied on top of the column and the column was eluted with 300 ml of benzene. Evaporation of the solvent yielded 0.23 g of pure aldehyde. Further prolonged passage of benzene eluted the mesylate and the alcohol.

The yields and physical properties of the aldehydes and their 2,4-dinitrophenyl hydrazones are listed in Table I.

Identification of By-Products

The minor oxidation by-products obtained from both the tosylate and the mesylate seen in Figure 1 were isolated by preparative TLC. Chromatoplates, 8 × 8 in., were coated with 0.5 mm layers of Silica Gel G, which was repeatedly extracted with ethyl ether to remove any impurities. A solution of 100 mg of the crude reaction product in hexane was applied along a straight line about 1 cm from the edge of each plate and the plate was developed with petroleum ether-ethyl ether (90:10, v/v) for 45 min. The relevant portions of the adsorbent containing the impurities were scraped off the chromatoplate and were extracted with ethyl ether. Another plate was developed without any sample and a patch of the adsorbent equal in area to that of the sample was extracted to serve as a blank. The substances were recovered by evaporation of the ether in amounts of approximately 2% each of the crude aldehyde.

One of the by-products was identified as 1-hexadecene on TLC by comparison with a known standard. The material was also identified as 1-hexadecene by GLC using a Beckman GC-4 apparatus equipped with a hydrogen flame detector and a 6 ft column, ⅛ in. O.D., containing 20% EGS on 80–100 mesh Gas-Chrom P by correlating retention time data for a standard sample of 1-hexadecene. The identity of the olefin was further substantiated by oxidizing it with the permanganate-periodate reagent of Von Rudloff (17). The expected oxidation product, n-pentadecanoic acid, was recovered and methylated with methanolic HCl and the methyl ester identified by GLC. The remaining oxidation by-product could not be identified with certainty.

Purity of the Aldehydes

The purity of the aldehydes was ascertained by TLC, melting points of their 2,4-

TABLE I

Yields and Melting Point of Aldehydes and 2,4-Dinitrophenyl Hydrazones (DNPH'S)

Starting material	% Yield of DNPH	% Yield of Aldehyde	MP. C[a] of DNPH Observed	MP. C[a] of DNPH Literature (Ref.)
Decanol	61	63	104 – 105	104.2–104.8 (23)
				108 (24)
Lauryl alcohol	65	62	105 – 106	105 –105.6 (23)
Myristyl alcohol	66	65	106.5–107.5	108.5–109 (23)
				108 (24)
Palmityl alcohol	68	65	107 – 108	108 (24,25)
Stearyl alcohol	70	67	108 – 109	110 (24)
Oleyl alcohol	64	69	66 – 67	68 (24)
				65.5– 66.5 (4)
Elaidyl alcohol	72	68	92.5– 93.5	90 (2)
Linoleyl alcohol	62	64	50 – 51	42.5– 43 (4)
Linolenyl alcohol	60	63	42 – 43	40.0– 40.5 (4)

[a] Melting points uncorrected.

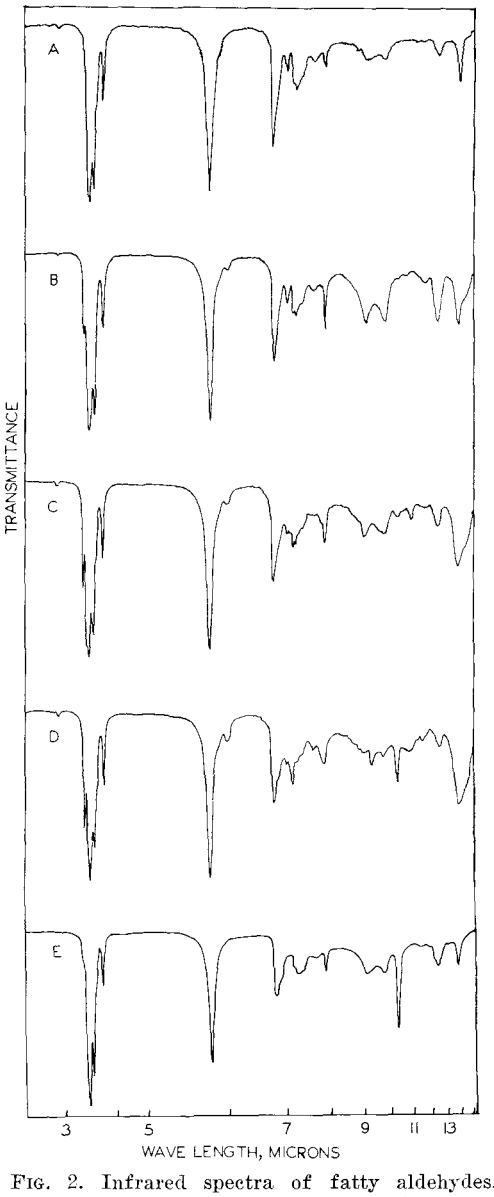


FIG. 2. Infrared spectra of fatty aldehydes. A) Stearaldehyde; B) oleyl aldehyde; C) linoleyl aldehyde; D) linolenyl aldehyde; E) elaidyl aldehyde. Solution spectra in 0.1 mm cell using 10% solutions of A-D and 8% solution of E in CS_2 (2.0 to 4.2 μ, 5 to 6.1 μ and 7.2 to 15.0 μ) and in tetrachloroethylene (4.2 to 5.0 μ and 6.1 to 7.2 μ).

dinitrophenylhydrazones (Table I), IR spectra and other physical methods. The purified aldehydes yielded single spots when analyzed by TLC using Silica Gel G/petroleum ether-ethyl ether (90:10, v/v) system. The aldehydes were also recovered from the plates and characterized as their 2,4-dinitrophenyl hydrazones.

Figure 2 shows the IR spectra of the pure aldehydes in CS_2 and C_2Cl_4 solutions between 2 and 15 μ. Bands characteristic of the aldehyde groups, 3.7 μ (2700 cm^{-1}) and 5.78 μ (1730 cm^{-1}) are present in all of the IR spectra. Bands characteristic of *cis* double bonds, 3.3 μ (3030 cm^{-1}) and 6 μ (1667 cm^{-1}) are seen in the spectra of oleyl, linoleyl and linolenyl aldehydes. No *cis* to *trans* isomerization occurred in the preparation of oleyl and linoleyl aldehydes. Linolenyl alcohol contained 10–15% of its unsaturation in the *trans* form. The same amount of *trans* unsaturation is found in the linolenyl aldehyde. Elaidyl aldehyde exhibited a strong absorption band at 10.3 μ (970 cm^{-1}).

TLC did not reveal the presence of any corresponding acids in the aldehydes. UV spectroscopy showed no conjugation in the linoleyl and linolenyl aldehydes. The purity of the aldehydes was also established by GLC. A Beckman GC-2A gas chromatograph equipped with a hydrogen flame detector and 12 ft aluminum column, 0.25 in. O.D., packed with Gas-Chrom R 30–60 mesh, and coated with 20% β-cyclodextrin acetate was used for the analysis. Temperature of the column was 230C and helium was the carrier gas. Each aldehyde exhibited a single peak when analyzed gas chromatographically. Figure 3 shows their separation when a hexane solution of the mixture was injected. Studies on the behavior of the aldehydes and their dimethyl acetals on stationary liquid phases commonly used in the GLC of fatty acid methyl esters are in progress.

Discussion

No satisfactory and universally applicable method has been described in the literature for the oxidation of long-chain saturated and unsaturated fatty alcohols to the corresponding aldehydes. These aldehydes are generally

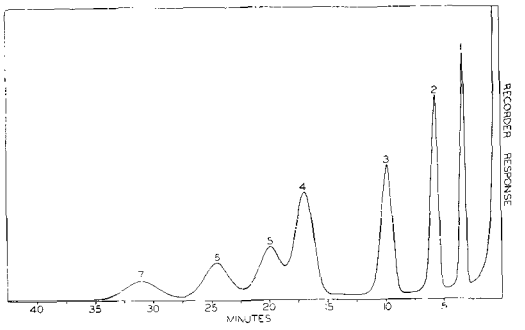
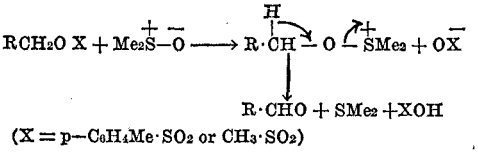


FIG. 3. GLC separation of fatty aldehydes. For experimental details, see text. 1) lauryl-; 2) myristyl; 3) palmityl-; 4) stearyl-; 5) oleyl; 6) linoleyl; 7) linolenyl-aldehyde.

synthesized from the derivatives of carboxylic acids and the synthesis involves a number of steps. The selective oxidation of the tosylates and mesylates of long-chain fatty alcohols by dimethyl sulfoxide has greatly facilitated the preparation of polyunsaturated fatty aldehydes in good yield. Under the conditions described, this reagent does not attack the double bond oxidatively nor cause any conjugation or *cis, trans* isomerization, unlike other reagents often used to oxidize alcohols to aldehydes.

The main product of the oxidation, the aldehyde, may be isolated as the 2,4-dinitrophenylhydrazine derivative if the free aldehyde is not immediately desired and converted directly to the dimethyl acetal as described by Mahadevan et al. (18) or to the free aldehyde by the method of Keeney (19) and Schogt et al. (20). However, the free aldehyde may also be obtained pure by a simple chromatographic procedure.

The mechanism suggested for this reaction (21) involves initial nucleophilic attack by dimethyl sulfoxide followed by collapse of the intermediate to aldehyde and dimethyl sulfide as shown below:



(X = p–$C_6H_4Me \cdot SO_2$ or $CH_3 \cdot SO_2$)

In the above reaction involving primary alkyl tosylates, elimination reactions giving rise to olefins have not been reported. On the other hand, with the sulfonic esters of secondary alcohols, olefins are formed readily under the same conditions (22). Generally the products of oxidation of primary alcohol sulfonates are isolated as their 2,4-dinitrophenylhydrazones and little effort has been made to detect the presence of olefins during the reaction. We have found that although aldehydes are the major products of the above reaction, olefin formation also does occur albeit to a small extent (2%). This observation has been made possible by the application of newer sensitive techniques of TLC and GLC. Although the sulfonate esters used as starting materials were free of alcohols, alcohols invariably were one of the minor products from the reactions. They might arise from the anhydrous reaction mixtures or by hydrolysis of some intermediate species during working up.

The reactions of dimethyl sulfoxide with long-chain alkyl halides and the sulfonate esters of hydroxy fatty acids under various experimental conditions are being investigated, which might hold great promise in the field of synthetic lipid chemistry.

ACKNOWLEDGMENTS

Supported in part by PHS Research Grant HE 02772 from the National Heart Institute, Public Health Service Assistance in the determinations of infrared spectra by Jacques Chipault and Werner Deutsch.

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[Received Feb. 17, 1966]

The Structure of the Glycerides of Ergot Oils[1]

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ABSTRACT

The oils from sclerotia or from suitable mycelial cultures of *Claviceps purpurea* (ergot) contain up to 44% of ricinoleic acid but no free hydroxyl groups. This is due to the presence of, besides normal triglycerides, tetra-acid, penta-acid and hexa-acid triglycerides. These contain respectively one, two and three ricinoleic acids esterified to glycerol, these in turn being acylated at their hydroxy groups with normal long-chain fatty acids. By suitable complementary use of TLC, GLC and lipase hydrolysis techniques, the proportions, compositions and structures of these novel triglyceride classes were determined. Four types of positional specificities in fatty acid combinations could be shown by our procedures. These are discussed and, on the basis of our results, some tentative proposals as to possible biosynthetic mechanisms are advanced.

INTRODUCTION

ERGOT OIL, THE GLYCERIDE oil present in the sclerotia of *Claviceps purpurea,* normally contains about 35% of a hydroxy acid. Matthes and Kürscher (1) considered this acid to be identical with ricinoleic acid, which is D-(+)-12-hydroxy-*cis*-9-octadecenoic acid, and this structure was unequivocally confirmed by Bharucha and Gunstone (2). Marqués and Rodríguez (3) concluded from acetyl values and optical rotations before and after hydrolysis of the oil and from vacuum distillation results that a proportion of the ricinoleic acid is further acylated at the hydroxyl group. This conclusion was also drawn by Bharucha and Gunstone (2).

Because our laboratory is currently studying the biosynthesis of ricinoleic acid by *Claviceps sp.* (4) it was considered desirable to determine the structures of the lipids containing ricinoleic acid. This paper describes the procedures used to separate the various components of ergot oils and to determine their structures. The complete analysis of the neutral lipid components of one sclerotial oil is recorded as are less detailed analyses of three mycelial oils of *Claviceps purpurea.*

[1] Dedicated to the late Prof. T. P. Hilditch, and presented to the Symposium on Analysis of Natural Fat Triglycerides, AOCS Meeting, Houston, April 1965.

EXPERIMENTAL PROCEDURES AND RESULTS

Materials

Sclerotia of *Claviceps purpurea* which had been harvested from rye and, in one case, from wild spartina grass were generously provided by P. G. Mantle, Biochemistry Department, Imperial College, London. Mycelia of *Claviceps sp.* were grown in still culture from samples also supplied by Dr. Mantle.

The sclerotia were ground up in a small coffee grinder and immediately extracted with chloroform-methanol (2:1) at room temperature. Mycelia were homogenized in the same solvent in a Waring blendor. Water-soluble impurities were removed by washing the chloroform-methanol extract with physiological saline according to the method of Folch et al. (5). Sclerotial and mycelial samples gave 20–30% of lipid, on the basis of their dry weight.

Reference Compounds

As this paper will show, the novelty of ergot is that all the ricinoleic acid moieties present have their hydroxyl groups acylated with one of the common long-chain fatty acids. Such compounds, for brevity, are termed estolides and suitable estolides of known structure were synthesized for use as reference and test compounds. Thus, standard mono-, di-, and triestolide triglycerides were prepared by acylation with palmitoyl chloride of the triglycerides containing one, two and three ricinoleic acid groups, isolated from castor oil by preparative TLC. Estolide methyl ester standard, i.e. methyl 12-0-palmitylricinoleate, was similarly prepared from methyl ricinoleate and palmitoyl chloride, and estolide 2-monoglyceride standard was derived by lipase hydrolysis from the synthetic triestolide triglyceride.

Fatty Acid Composition of Ergot Oils

Methyl esters from whole oils, from individual triglyceride fractions and from components from lipase hydrolysates were prepared by saponification with 10% methanolic KOH and esterification of the recovered acids with diazomethane or with methanol-sulfuric acid. This procedure was found to be necessary when TLC of the methyl esters prepared from a series of ergot oils by conventional transmethylation with methanol-benzene-H_2SO_4 mixture (20:10:1) re-

vealed incomplete cleavage of the estolide ester bonds, even after 1–2 hr reflux.

Fatty acid analyses were carried out by GLC of the methyl esters on two columns. The proportion of ricinoleate relative to the normal fatty acid esters was determined on a column of SE-30 stationary phase (15%, w/w) on chromosorb W, using an argon ionization detector. Under these conditions methyl ricinoleate was eluted as a symmetrical peak with a carbon number (6) of 19.5. Since our detector was found to give a low response for ricinoleate, relative to normal fatty acids, it was calibrated with pure standard ester mixtures. A correction factor for ricinoleate of 1.39 was obtained which has been included in all calculations of compositions reported below. The proportions of normal fatty acids were then obtained by a second analysis on a polyethylene glycol adipate (20%, w/w on Celite) column connected to a flame ionization detector.

The total fatty acid compositions of a number of sclerotial and mycelial ergot oils, determined in this way, are listed in Table I. There is relatively little variation in composition among the sclerotial oils we have examined despite their differing countries of origin. The mycelial oils, on the other hand, show a considerable variation in their content of ricinoleic acid and an inverse variation in oleic and, particularly, linoleic acids.

Structure and Analysis of Ergot Lipids

Conventional TLC of the various ergot oils showed a major component which migrated with or very slightly slower than reference triglyceride samples. In addition there was always a relatively minor amount of free sterols, of which, presumably, ergosterol is the most important constituent, and varying though generally minor proportions of polar lipids. These included phosphatidyl ethanolamine, phosphatidyl choline and phosphatidyl inositol, but since their composition will be the subject of another communication from our laboratory they will not be considered further here. In the oils prepared by us there were few or no free fatty acids or glycerides containing free hydroxyl groups. Some ergot oils from commercial sources which we examined, however, had considerable proportions both of free acids and of hydroxy triglycerides or partial glycerides but we consider that these most probably arose from enzymatic and chemical hydrolysis during their preparation and/or storage.

By multiple development TLC with a relatively nonpolar solvent (5–10% diethyl ether in hexane) we obtained a fractionation of the major "triglyceride" component of ergot oil into a component corresponding exactly with standard normal triglycerides and three progressively more polar components, as shown in Figure 1, samples 2 and 3. That these were not acetylated ricinoleyl triglycerides, which initially had seemed a possibility, was readily apparent by comparison with mono-, di- and triacetoxy triglycerides prepared by acetylating castor oil. These ergot components, however, did migrate respectively with the reference mono-, di and triestolide triglycerides (samples 4, 5 and 6; Figure 1). TLC, therefore, suggested that the hydroxyl groups of ricinoleic acid in ergot oil were in combination with long-chain fatty acids, i.e. as estolides, and also that mono, di- and triestolide triglycerides as well as normal triglycerides were present.

These four fractions were isolated by preparative TLC after applying the sample as a

TABLE I

Fatty Acid Composition (moles %)[a] of a Selection of Sclerotial and Mycelial Ergot Oils

	14:0	16:0	16:1	18:0	18:1	18:2	OH-18:1
Sclerotia[b]							
S1 (U.S.A.)	0.7	28.0	3.7	6.4	19.6	17.4	24.1
S2 (U.K.)	0.2	26.0	4.8	4.1	24.1	13.2	27.5
S3 (U.K.)	0.1	19.9	6.5	4.3	22.5	14.3	32.3
S4 (Spain)	1.2	25.1	4.3	4.6	20.0	9.8	34.9
S5 (Rumania)	0.4	22.9	3.1	5.4	17.0	15.8	35.5
Mycelia[c]							
M1	0.2	19.5	6.6	3.3	38.0	32.4	.....
M2	4.6	20.0	4.6	5.1	40.9	18.9	5.9
M3	1.0	23.4	6.0	3.6	30.1	14.5	21.5
M4	0.6	24.2	5.4	7.2	23.5	10.0	29.1
M5	0.5	22.7	6.0	2.3	18.7	8.0	41.8

[a] Compositions are listed as moles % in all tables to simplify further calculations from the experimental data.

[b] The countries of origin of the various samples of sclerotia are included in parentheses. All sclerotia had been harvested from rye except for S3 which had been parasitic on *Spartina*.

[c] M2, M3 and M4 were different subcultures of the same strain. Details of variations in fatty acid compositions of mycelial cultures of *Claviceps* sp. with differing culture conditions will be described elsewhere.

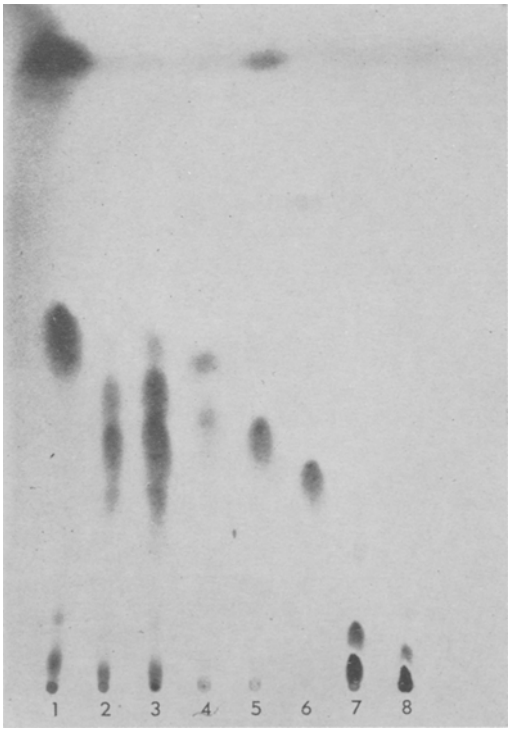

FIG. 1. Thin-layer chromatogram of an ergot oil and known reference glycerides. Samples are *1*—corn oil (+ hydrocarbon contamination at front); *2* and *3*—different loads of ergot oil (S4); *4*—normal triglycerides + monoestolide triglycerides; *5*—diestolide triglycerides; *6*—triestolide triglycerides; *7*—acetylated castor oil; *8*—castor oil. Samples *4*, *5* and *6* were obtained by palmitoylation of the relevant castor oil triglyceride fractions—see text. The plate was developed three times with ether-hexane (7.5:92.5) and spots were made visible by spraying with 25% H_2SO_4 and charring.

streak with the Desaga automatic sample applicator, developing three times as before and locating the separated bands under U.V. light after spraying with dichlorofluorescein. An attempt was made to determine the fatty acid: glycerol ratio of each of these fractions by the procedure of Horrocks and Cornwell (7). This involves hydrogenolysis of the glycerides with $LiAlH_4$, acetylation of the reduction complex with acetic anhydride and GLC analysis of the fatty alcohol acetates and glycerol triacetate so formed. Even with pure standard triglycerides we did not obtain quantitative or reproducible yields of triacetin and we were thus unable to obtain the fatty acid: glycerol ratios for the ergot oil fractions. [Our impression that this procedure of Horrocks and Cornwell is not as straightforward as it appears from the original description (7) seems to have been confirmed by the more recent publication of an improved method by the same authors (8)]. However, both GLC and TLC analysis of the products of this procedure from the four ergot oil fractions showed glycerol triacetate to be present in each case accompanied only by normal fatty alcohol acetates and also by ricinoleyl alcohol diacetate in the three more polar fractions.

Thus each fraction from the ergot oil was a triglyceride. Furthermore, in the products from the three abnormal triglyceride fractions the molar ratio of total normal alcohol acetates to ricinoleyl alcohol diacetate was close to 3.0, 1.5 and 1.0, respectively, giving a further indication that these were respectively mono-, di- and triestolide triglycerides (i.e. tetra-acid-, penta-acid-, and hexa-acid-triglycerides). The possible triglyceride components of ergot oils are therefore as summarised diagramatically in Figure 2 (cf. also Fig. 4).

The relative proportions and the fatty acid compositions of these four triglyceride classes in a typical sclerotial ergot oil (Oil S4, Table I; samples 2 and 3, Fig. 1) were determined. The classes were separated as before by multiple development preparative TLC and to each fraction, eluted from the adsorbent with pure diethyl ether, was added the same amount of pure methyl heptadecanoate. The total fatty acid composition of each fraction was obtained by GLC after hydrolysis and esterification and, by use of the added heptadecanoate as internal standard, the proportion of each triglyceride class in the original oil was readily computed. The results for this oil are given in Table II

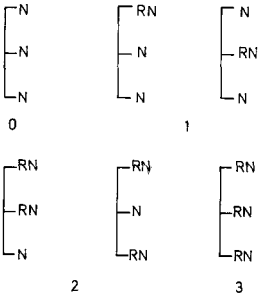

FIG. 2. Schematic representation of the triglyceride structures present in ergot oils; R = ricinoleic acid moiety and N = normal unsubstituted acid moiety. The numbers *0*, *1*, *2* and *3* correspond to the fraction numbers listed in Tables II, IV and V and denote, in the terminology used in this paper, normal triglycerides, monoestolide triglycerides, diestolide triglycerides and triestolide triglycerides respectively.

and included in this table are the molar ratios of normal esters to ricinoleate for each of the estolide triglyceride fractions. These ratios are again close to 3.0, 1.5 and 1.0, respectively for the fractions designated as mono-, di- and triestolide triglyceride, providing further evidence that these proposed structures are correct. The close agreement between the directly determined fatty acid composition of the whole oil and that calculated from the compositions and relative proportions of the four fractions attests to the reasonable accuracy of our analytical procedures.

The results of this class analysis are listed for only one sclerotial oil but a comparison of this oil with another two sclerotial oils and three mycelial oils on TLC is shown in Figure 3. The oils for this illustration were deliberately chosen to show the effect of a considerable range of ricinoleic acid content (36% to 6%) on the relative amounts of the four triglyceride classes.

Interestingly, this variation in ricinoleate content is apparently not mirrored in any great variation in relative amounts of the mono-, di- and triestolide triglycerides, these three being present in approximately the same relative proportions even in sample 7 which contains only 6% ricinoleate. The obvious variation is in the proportion of the normal triglyceride fraction relative to the total estolide triglycerides and the possible significance of this will be considered below in the discussion.

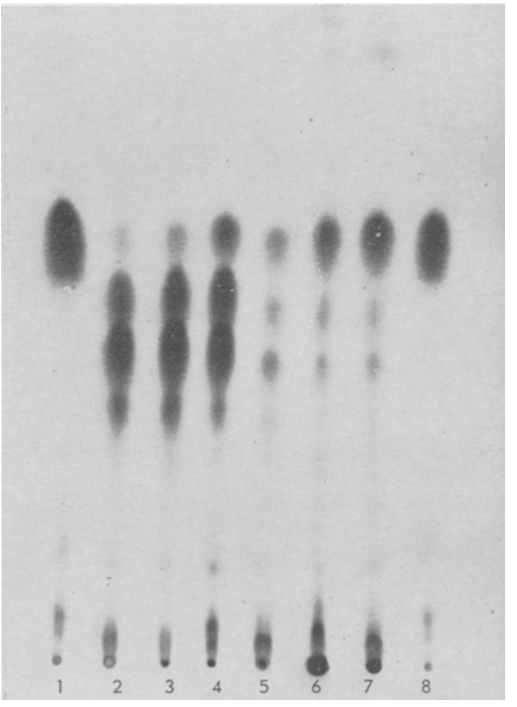

FIG. 3. Comparison of the glyceride compositions of some ergot oils by TLC. The samples are (see Table I): *1* and *8*—corn oil; *2*—S5; *3*—S4; *4*—S3; *5*—M4; *6*—M3; *7*—M2. The plate was developed three times with ether-hexane (7.5:92.5) and the spots were made visible by spraying with 25% H_2SO_4 and charring.

Lipase Hydrolysis Studies

Having shown that the ricinoleate containing lipids comprised mono-, di- and triestolide triglycerides, there remained still the questions of possible specificity of the acid or acids esterified to ricinoleate, of possible positional specificity of estolides in their attachment to glycerol in the mono- and diestolide classes, as indicated in Figure 3, and of possible specificity of the fatty acids combined with ricinoleate attached to the 2-position of glycerol as compared to those combined with ricinoleate in the 1- and 3-positions. In the di- and triestolide triglycerides there was the additional possibility that, instead of there being two or three ricinoleic acids each acylated with a normal fatty acid and each attached directly to glycerol, as in Figure 2, there might be polyestolide residues attached to glycerol as illustrated in Figure 4.

TABLE II

Analysis of the Glyceride Fractions of an Ergot Oil (S4)

Fraction[a]	Proportion (wt. %)	Composition (moles %) 14:0	16:0	16:1	18:0	18:1	18:2	OH-18:1	Ratio[b]
0	10.2	1.9	26.0	9.8	17.4	36.1	8.8	.....	
1	19.6	1.7	31.0	4.8	7.0	22.3	8.4	24.9	3.02
2	47.4	1.6	26.7	4.1	5.7	15.8	5.7	40.4	1.48
3	22.8	1.5	20.9	4.2	3.2	14.0	8.5	47.7	1.09
Total composition—calculated		1.6	26.2	4.8	6.6	18.7	7.2	34.9	
Total composition—determined		1.2	25.1	4.3	4.6	20.0	9.8	34.9	

[a] Glyceride fractions are numbered in decreasing order of mobility on TLC and correspond to the structures depicted in Figure 2.

[b] Molar ratio of total normal fatty acids to ricinoleic acid.

Lipase hydrolysis experiments were undertaken in the hope of answering these various questions.

The action of pancreatic lipase on synthetic standard estolide triglycerides and on various ergot oils gave hydrolysis of the primary glycerol ester bonds in the usual way with practically no concomitant hydrolysis of estolide ester bonds, provided the reaction was not allowed to proceed for too long. Chemical hydrolysis and transmethylation reactions also gave faster cleavage of glycerol esters than of estolide esters but the specificity was less pronounced and less easy to control than in lipolysis so that this latter procedure was our method of choice.

Lipase hydrolysis of total ergot oil samples was effected by the semimicro procedure of Luddy et al. (9), with some minor modifications. To 50 mg samples of oil weighed into 2 ml screw-cap vials was added 9 mg of pig pancreatic lipase (Koch-Light Laboratories Ltd., Colnbrook, Bucks., England; which had been thoroughly extracted with acetone and diethyl ether), 1.0 ml of 1 M tris buffer (adjusted to pH 8), 0.1 ml of 22% calcium chloride solution and 0.25 ml of 0.1% bile salts solution. The vials were tightly capped and shaken at room temperature on a bench vertical-action shaker at ca. 2000 oscillations per minute. A shaking time of 12 min gave maximal conversion to free acids and monoglycerides with minimal hydrolysis of estolide ester linkages, as shown by TLC. At the end of this period, the contents of each vial were acidified, transferred quantitatively into glass stoppered reaction tubes and thoroughly extracted with 5 ml of diethyl ether. Each ether extract was washed several times with water, using Pasteur pipettes to withdraw the lower aqueous layers, concentrated to about 1 ml and, after addition of 0.5 ml methanol, reacted with an ethereal solution of diazomethane. This simple procedure minimizes losses and results in a mixture of predominantly methyl esters and monoglycerides which is more readily separable by TLC than when free acids are present.

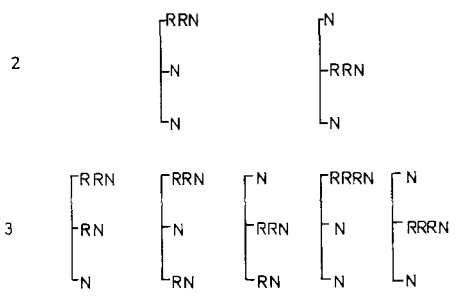

FIG. 4. Schematic representation of possible polyestolide triglycerides which might be present in di- and triestolide triglyceride fractions of ergot oils; *R*—ricinoleic acid moiety and *N*—normal unsubstituted acid moiety.

Using the synthetic reference materials again, it was found that not only could normal and estolide methyl esters be separated and normal and estolide monoglycerides be separated but, by a double development procedure, both of these resolutions could be effected on a single plate, as illustrated in Figure 5.

The esterified total product mixture from lipase hydrolysis of 50 mg of ergot oil, applied as a streak to a single 200 × 200 × 0.25 mm plate, could be quantitatively separated in this way and the four desired components isolated pure. To the normal and estolide monoglyceride fractions, which were eluted from the adsorbent with ether-methanol (1:1), was added the same amount of methyl heptadecanoate as internal standard. The isolated fractions were then saponified, esterified and analyzed as before on both SE-30 and PEGA columns. By reference to the internal standard, the relative proportions of the two monoglyceride fractions could be estimated thus giving the relative amounts of normal and estolide fatty acids attached to the 2-position of glycerol in the original oil. The results of this type of analysis of one sclerotial and three mycelial ergot oils are summarized in Table III.

In no case was there any evidence on TLC of the presence of either polyestolide methyl esters or polyestolide monoglycerides which would arise from the types of glycerides represented in Figure 4. These would be expected to differ in TLC migration behavior from monoestolide esters and monoestolide monoglycerides to the same extent that these compounds differ from normal methyl esters and normal monoglycerides. Additional evidence that such polyestolide species do not exist in ergot oils is provided by the molar ratios of normal esters: ricinoleate for the various estolide ester and estolide monoglyceride fractions. These ratios, included in Table III, are in all cases close to 1.0 whereas if appreciable proportions of polyestolides were present these ratios would be correspondingly less than 1.0. The conclusion that the polyestolide glycerides of Figure 5 were not present was finally verified by lipase hydrolysis and analysis of the individual separated triglyceride classes.

The four triglyceride classes of oil S4 were isolated in 5–10 mg amounts by multiple development preparative TLC using, in this case,

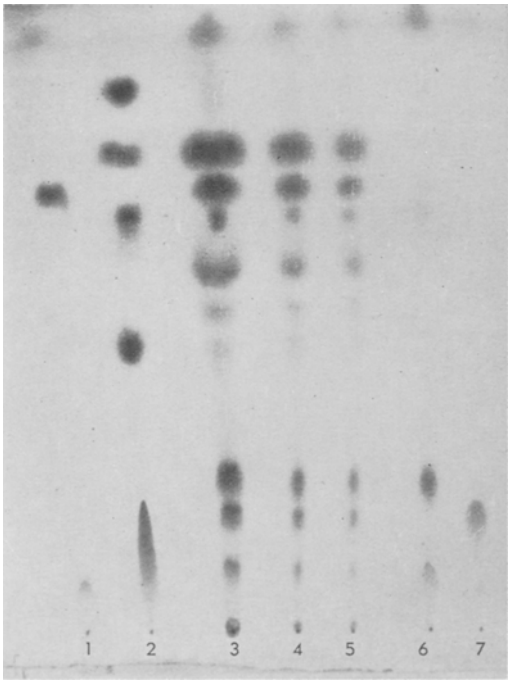

FIG. 5. Thin-layer chromatogram of esterified lipase hydrolysate of an ergot oil and known reference compounds. Samples are: *1*—synthetic estolide methyl ester; *2*—mixture of, from the bottom, oleic acid, cholesterol, triolein, methyl oleate and cholesterol oleate; *3*, *4* and *5*—different loads of lipase hydrolysate of an ergot oil after esterification with diazomethane; components are, from the bottom, origin, monoricinolein, monoglyceride, estolide monoglyceride, sterol + methyl ricinoleate, diglyceride, mono- and diestolide diglycerides, residual triglyceride, estolide methyl ester, methyl ester; *6*—synthetic estolide monoglyceride; *7*—monoglyceride. The chromatogram was developed twice, first for 12 cm with ether-hexane (80:20) and second for 18 cm with ether-hexane (10:90) and the spots were located by spraying with 25% H_2SO_4 and charring.

Note: Better separations of the desired components than those illustrated were achieved by a first development for 8 cm with pure ether followed by 18 cm development with ether-hexane (10:90). The residual di- and triglyceride fractions were then together in one band at the first development solvent front.

1 mm thick layers. Each fraction was subjected to lipase hydrolysis using the microtechnique of Luddy et al. (9), but with our modifications described above, and the esterified hydrolysis products were separated preparatively by TLC as before. Heptadecanoate was added in equal amounts to the estolide monoglyceride and normal monoglyceride fractions derived from the mono- and diestolide triglycerides and each fraction was analyzed by GLC. The results are summarized in Table IV.

The normal ester:ricinoleate molar ratio was again close to or greater than 1.0 for all the estolide fractions. This and the fact that the lipase hydrolysate of the triestolide triglyceride fraction comprised only estolide esters and estolide monoglycerides, with no detectable amount of normal esters or monoglycerides, completely rule out the possibility that any significant quantity of the polyestolide structures of Figure 4 are present in ergot oil.

DISCUSSION

The work described above has shown conclusively that the ricinoleic acid of both sclerotial and mycelial ergot oils is present in the lipids combined with glycerol and with the 12-hydroxyl group not free, as in castor oil, but further acylated by normal nonsubstituted fatty acids. Besides normal triglycerides of the common fatty acids, these ergot oils contain mono-, di- and triestolide triglyceride fractions (i.e., tetra-acid, penta-acid and hexa-acid triglycerides). In the di- and triestolide triglyceride fractions it is possible to envisage the presence of the polyestolide structures summarized in Figure 4. However, although some of the estolide ester and estolide monoglyceride products from the lipase hydrolyses had normal acid to ricinoleic acid molar ratios of less than 1.0, we were unable to find any evidence of polyestolide residues in these products by TLC nor could we detect any normal methyl esters or normal monoglycerides in the lipase hydrolysate of the triestolide triglyceride fraction. We conclude, therefore, that none of such polyestolide compounds are present in ergot oils.

The structures of the ricinoleic-containing glyceride compounds of ergot oils apparently differ considerably from the hydroxy acid glycerides of kamala oil (*Mallotus phillipinensis* seed oil), which is the only other example so far reported of a glyceride oil containing hydroxy acids acylated at the hydroxyl group by other long-chain acids. The hydroxy acid of kamala oil is 18-hydroxy-*cis,trans,trans*-9,11,13-octadecatrienoic acid known as kamlolenic acid, and, according to Achaya and co-workers (10,11), this acid is a component of four, very specific triglyceride classes. These workers claim that two of these triglyceride types have normal acids on both the 1- and 3-positions of glycerol and differ in having in the 2-position either kamlolenic acid or what may be termed as "dikamlolenic acid," i.e. one kamlolenic acid acylated at its hydroxyl group with another

TABLE III

Analysis of Lipase Hydrolysis Products from Total Glycerides of Some Ergot Oils

Sample	Fraction[a]	Composition (moles %) 14:0	16:0	16:1	18:0	18:1	18:2	OH-18:1	Ratio[b]		% of 2-position
S4	ME	1.5	36.8	7.7	12.5	25.9	15.5	.....			
	MG	4.1	11.2	0.2	0.4	43.0	41.1	.....			44.0
	EME	0.1	27.4	0.3	3.1	13.8	4.4	49.7	1.01		
	EMG	2.7	20.0	2.7	2.8	13.8	6.9	51.1	0.96		56.0
	EME-N	0.2	54.5	0.6	6.2	27.4	8.7	.....			
	EMG-N	5.5	40.9	5.5	5.7	28.2	14.1	.....			
M2[c]	ME	0.5	29.9	6.6	8.2	37.9	16.9	.....			
	MG	0.7	4.1	2.0	0.1	71.2	21.9	.....		74.4	Corr. 86.3
	EME	1.8	24.4	4.7	2.0	16.9	0.2	50.0	1.00		
	EMG	2.1	22.6	4.6	3.4	28.9	3.6	34.8	1.87	25.6	13.7
	EME-N	3.6	48.8	9.4	4.0	33.8	0.4	.....			
	EMG-N (corr.)	2.8	23.3	4.8	6.3	52.8	9.9	.....			
M3	ME	0.7	24.8	6.8	9.7	38.1	19.9	.....			
	MG	1.1	8.0	4.8	7.7	50.7	27.7	.....			69.9
	EME	1.2	21.3	3.5	7.1	10.6	6.2	50.1	1.00		
	EMG	1.0	17.7	4.8	5.6	12.8	9.0	49.2	1.03		30.1
	EME-N	2.4	42.7	7.0	14.2	21.2	12.4	.....			
	EMG-N	2.0	34.8	9.4	11.0	25.2	17.7	.....			
M4	ME	1.4	53.4	6.9	6.7	21.8	9.8	.....			
	MG	0.5	6.3	3.0	2.8	54.6	32.8	.....			58.4
	EME	0.5	19.6	3.7	6.3	13.2	3.6	53.0	0.89		
	EMG	0.7	22.4	3.2	5.3	12.4	3.3	52.5	0.90		41.6
	EME-N	1.1	41.7	7.9	13.4	28.1	7.7	.....			
	EMG-N	1.5	47.2	6.7	11.2	26.1	6.9	.....			

[a] Coding is as follows: ME = normal methyl esters; MG = normal monoglycerides; EME = estolide methyl esters; EMG = estolide monoglycerides; EME-N = normal fatty acids combined in estolide methyl ester fraction; EMG-N = normal fatty acids combined in estolide monoglyceride fraction.

[b] Molar ratio of total normal fatty acids to ricinoleic acid.

[c] The EMG fraction from the lipolysis of this oil has clearly not been quantitatively separated from the MG fraction. The EMG-N values listed have been corrected for this contamination on the assumption that the molar ratio of normal fatty acids to ricinoleic acid should be 1.00 and that the proportion of the MG fraction present in the EMG fraction has the same composition as the MG fraction isolated. The values for the proportions of the 2-position esterified with normal and estolide fatty acids have been corrected on the same basis.

kamlolenic acid. The other two triglyceride types are also claimed to have, respectively, these kamlolenic and "dikamlolenic" acid moieties in the 2-position and to have, instead of normal acids in the 1- and 3-positions, what may be termed "dikamlolenic acid estolide" moieties, i.e., one kamlolenic acid acylated with another kamlolenic acid whose hydroxyl group in turn is acylated with a normal acid. The four triglyceride classes considered by Achaya et al. to comprise kamala oil are therefore, respectively, triacid-, tetraacid-, heptaacid- and octa-acid- triglycerides each having one free hydroxyl group and each being symmetrical about the 2-position.

Our work has proved that ergot oils show none of these absolute structural and positional specificities claimed for kamala oil glycerides. However, our studies were designed to detect four possible kinds of positional specificities in fatty acid composition which could be exhibited by ergot oils and a greater or lesser degree of specificity of all four types has been demonstrated by our results.

Considering these four possible specificities in turn, there is first the question of specificity of the acids attached to the hydroxyl group of ricinoleate to form estolides. From comparison of the compositions of estolide-forming acids, listed as EME-N and EMG-N in Tables III and IV, with the acids esterified directly with glycerol, i.e., fractions ME and MG, in the corresponding oils or glyceride fractions it is evident that there is a considerable difference. Thus, the normal acids combined as estolides are in general considerably less unsaturated than those attached directly to glycerol and, in particular, have considerably less linoleic acid.

The second question is of possible differences in composition between the normal fatty acids in estolides attached to the 2-position of glycerol and in those attached to the 1- or 3-positions. Comparing the corresponding EMG-N and EME-N values in Tables III and IV there is apparently some specificity, although rather more tenous in this case. The normal acids of estolides attached to the 2-position are, in general, somewhat more unsaturated than those of estolides attached to the 1- and 3-positions of glycerol.

The third question of specificity is the more conventional one of the composition of normal acids attached to the 2-position of glycerol compared to those occupying the 1- and 3-

TABLE IV

Analysis of Lipase Hydrolysis Products from Glyceride Fractions of an Ergot Oil (S4)

Glyceride[a]	Fraction[b]	Composition (moles %) 14:0	16:0	16:1	18:0	18:1	18:2	OH-18:1	Ratio[c]	% of 2-position
0	ME	2.4	48.8	6.6	11.1	25.1	5.9	.....		
	MG	3.2	12.0	4.3	17.9	50.3	12.3	.....		100.0
1	ME	1.9	44.5	6.4	15.0	23.7	8.5	.....		
	MG	2.0	9.9	3.7	7.6	45.0	31.8	.....		53.3
	EME	1.0	32.1	1.3	2.8	11.2	2.6	49.0	1.04	
	EMG	0.7	16.0	3.3	12.7	18.2	2.4	46.7	1.14	46.7
	EME-N	2.0	62.9	2.5	5.5	22.0	5.1	.....		
	EMG-N	1.3	30.0	6.2	23.8	34.1	4.5	.....		
2	ME	2.2	65.6	5.1	4.7	14.3	8.2	.....		
	MG	3.0	31.7	5.0	6.3	30.1	24.0	.....		40.6
	EME	0.6	21.3	2.8	9.3	11.1	3.4	51.4	0.95	
	EMG	0.8	22.1	3.0	4.5	15.0	3.9	50.6	0.98	59.4
	EME-N	1.2	43.8	5.8	19.1	22.8	7.0	.....		
	EMG-N	1.6	44.7	6.1	9.1	30.4	7.9	.....		
3	EME	1.2	16.0	3.0	3.7	13.1	11.4	51.5	0.94	
	EMG	1.2	21.2	3.5	4.0	15.1	11.2	43.8	1.28	100.0
	EME-N	2.5	33.0	6.2	7.6	27.0	23.5	.....		
	EMG-N	2.1	37.7	6.2	7.1	26.9	19.9	....		

[a] The glyceride fractions correspond to those in Table II.
[b] The coding is the same as in Table III.
[c] Molar ratio of total normal fatty acids to ricinoleic acid.

positions. Again from Tables III and IV, it is evident that there is a strong preference of oleic and linoleic acids for the 2-position and of the saturated acids for the 1- and 3-positions as is the case with most natural triglyceride mixtures (12).

The fourth and possibly the most interesting question of specificity, is whether estolide radicals are preferentially attached to the 2-position or to the 1- and 3-positions of glycerol. This can be determined for the four oils of Table III and fractions 1 and 2 (the mono- and diestolide triglyceride fractions) of oil S4 in Table IV, and the proportions of estolide moieties calculated to be in the 2- and in the 1- and 3-positions are shown in Table V.

The data listed in Table V show that in all cases there is a considerable proportion of the estolide residues esterified to the 2-position of glycerol. They give no clear indication however, of a marked positional specificity of such compounds in combination with glycerol. Thus, the oil M2 shows an apparently clear preference of estolide residues for the 2-position but fraction 2 of oil S4 indicates an equally clear preference for the 1- and 3-positions. If we assume that the 1- and 3-positions of glycerol are equal and equivalent in the fatty acids with which they are combined, and this is by no means necessarily a valid assumption (13), then in the remaining four samples of Table V the estolide moieties have a slight preference for the 2-position of glycerol. It should be emphasized, however, that the analytical results of the fractions derived by lipase hydrolysis which are summarized in Tables III and IV are not necessarily strictly representative of the true compositions of the whole oils or fractions. This is because we have no information on the effect of these unique estolide residues on the course of pancreatic lipase hydrolysis, except that they do not totally or even markedly inhibit it. Also, in no case was the conversion to monoglycerides and free acids complete, some diglyceride and occassionally even some residual triglyceride remaining in the hydrolysate. We cannot assume that the hydrolysis is strictly random in the acids it will liberate from the primary hydroxyls of glycerol in such unusual triglycerides and the wonder is

TABLE V

Distribution of Estolide Moieties (moles %) in Ergot Triglycerides[a]

Oil	Total	In 2-position	In 1+3-position	In 1 or 3-position[b]
S4	53.6	18.7	34.9	17.5
S4, Fraction 1	33.2	15.6	17.6	8.8
S4, Fraction 2	67.8	19.8	48.0	24.0
M2	6.3	10.0	1.7	0.9
M3	27.4	13.9	17.4	8.7
M4	41.0	15.6	27.1	13.6

[a] Calculated from the data in Tables I-IV.
[b] Assuming that the 1- and 3-positions are equal (see text).

that the estolide residues are hydrolyzed at all. If estolide residues are less readily removed from glycerol by pancreatic lipase than normal fatty acids then in any incomplete hydrolysis of a mixture containing estolide triglycerides the results would indicate a preference of estolide residues for the 2-position. Considerably more work clearly must be done before this question of the positional specificity of estolide radicals on triglyceride could be unequivocally answered.

Finally, from the analytical results herein described, can we infer anything about the biosynthesis of these ricinoleate-containing triglycerides of ergot oils? It is not possible to draw any conclusions as to the biogenetic precursor of ricinoleic acid in ergot oils, whether oleic acid, as in the case of castor oil (14,15), or linoleic acid. Our biosynthetic studies with laballed fatty acids show that linoleic acid is the immediate precursor (4). Our analytical results, however, encourage some speculations as to the course of the biosynthesis of estolide glycerides by *Claviceps*. The fact that we were unable to find any evidence of the presence of the polyestolide compounds illustrated in Figure 4 in the oils we examined suggests three possibilities. These are firstly, that an esterase responsible for acylating the ricinoleate hydroxyl groups to form estolides has a specificity which allows any acid other than ricinoleic acid or a preformed estolide acid to be esterified to the ricinoleate hydroxyl; or, secondly, that the enzyme sites for ricinoleate production and acylation are in close juxtaposition in the cell so that ricinoleate once formed is immediately acylated and is not available to the general fatty acid pool; or, thirdly, that the production of ricinoleate and its acylation is from a precursor acid which is already in combination with glycerol as a triglyceride. The second and third possibilities could either involve hydration of linoleic acid and subsequent acylation of the hydroxyl group so formed or indeed, might involve the formation of a ricinoleic estolide molecule directly by addition of a fatty acid across the 12,13-double bond of linoleate, the proton adding to the 13-position and the cation to the 12-position. The absence of triglycerides containing free hydroxyl groups in our ergot oils might even favor this last, very tentative suggestion.

There are two other pieces of evidence in favor of the suggestion that the effective substrate is a triglyceride. Firstly, there is the considerable proportion of estolides present on the 2-position of glycerol, indeed possibly even a preference for that position. It seems unlikely that the enzyme responsible for acylating the 2-position, normally so specific in selecting the C_{18} unsaturated acids, would adapt its selectivity to include what is effectively a C_{34} or C_{36} acid. The nonsubstituted fatty acids attached to glycerol do show the normal pattern of oleate and linoleate predominating at the 2-position and the saturated acids at the outside positions showing that the normal specificity in triglycerides is adhered to. Secondly, there is the finding that in oils with a range of ricinoleate contents the relative amounts of mono-, di- and triestolide triglycerides are apparently fairly similar, as shown in Figure 3. This would be rather hard to explain if the glycerides were synthesized from a general pool of fatty acids including either ricinoleic acid or estolide acids. However, if the synthesis of the ricinoleic acid and its acylation occurs from linoleic acid present in triglycerides then the statistical probability of, say, trilinolein being substituted twice or even three times before being released from the enzyme surface will be the same regardless of the total extent of ricinoleate production.

ACKNOWLEDGMENTS

P. G. Mantle of the Biochemistry Department, Imperial College, London supplied all the *Claviceps* samples used in this work.

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[Received Sept. 9, 1965]

Fatty Acid Distribution in the Bovine Pre- and Postpartum Testis[1]

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ABSTRACT

Testes from fetuses, calves, bulls and recently castrated animals were analyzed for total lipids, lecithin, cephalin, triglycerides, diglycerides, cholesteryl esters and cholesterol. Total lipids increase somewhat with age, but in the castrated animal the increase is more marked. Phospholipid content increases with age, but decreases in the castrated animal. Cholesterol decreases and triglyceride increases after birth and in the castrated animal. Polyunsaturated acids increase with age in all lipid classes. Eicosatrienoic acid is more abundant in fetal testicular lipids than in testes removed after birth. In the castrated testis there is a general decrease in the unsaturated fatty acids. Acids of the $\omega 6$ family are the predominant polyunsaturated acids and increase somewhat with age in all lipids. The $\omega 3$ family of polyunsaturated acids appears mostly toward the end of fetal life and increases after birth. Acids of the linoleate family reach approximately 25% of total acids in most lipid classes at maturity whereas the $\omega 3$ acids range from 1 to 9%.

INTRODUCTION

THE INVOLVEMENT OF certain polyunsaturated fatty acids (PUFA) in growth and development of the germinal epithelium of both male and female reproductive organs of the rat is well established (1–6). Thus far the correlation of fatty acid and lipid composition with development of reproductive organs has not been reported. Such studies are difficult using small experimental animals because of the size of the organs and the limiting amounts of lipids in immature organs. Because bovine testes are easily obtained and because the relationship of PUFA nutrition to reproductive function may be of economic importance, this species was chosen for such a study. The amounts and kinds of lipids of the testes and the fatty acid composition of the major lipid classes as a function of age of the animal, both in prepartum and postpartum life, are reported here.

[1] Presented at the American Dairy Science Association Meeting, Lexington, Ky., June 1965.

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MATERIALS AND METHODS

Testes from fetuses 4, 6 and 8.5 months old, from calves 1 and 4 months old and from adult animals 10 months of age were obtained at the time of slaughter at the local abattoir. The ages of the fetuses were determined by crown-rump measurements (7,8) and their physical appearances. The ages of immature and mature animals were obtained by estimates from their weight and body development at slaughter. Testes from 12-year-old bulls were obtained from breeding associations whose age records are exact. In addition, atrophied testes were obtained from two recently castrated animals. All of the animals were of the Holstein breed. Two samples of similar age were analyzed in each case and both values are given in the table, but are averaged in the figures. Testes were freed from surrounding tissue, weighed and frozen under saline solution, a procedure known to preserve polyunsaturated fatty acids in tissues for years.

Extraction and Chromatographic Separation of Lipids

After one week of storage the tissues were thawed, homogenized and extracted according to Folch et al. (9). The amount of total lipid was determined gravimetrically and expressed as percent of wet tissue weight. The content of neutral lipids and phospholipids was determined on an aliquot of each sample by silicic acid column chromatography (11). A glass column 40 cm × 2 cm was packed with 30 g of dried prewashed silicic acid and then preconditioned by washing with 5 × 100 ml portions of distilled chloroform. Approximately 50 mg of lipids were applied to the column. The neutral lipid fraction was eluted with 3 portions of 100 ml each of distilled chloroform and phospholipids with three portions of 100 ml each of distilled methanol. The solvent from each fraction was evaporated and the lipid fractions weighed. An aliquot of neutral lipid was fractionated by TLC using petroleum ether: diethyl ether:acetic acid (90:10:1) as solvent system and the two major components, triglyceride and free cholesterol, were estimated densitometrically (10). The chromatographic procedures had a total recovery of 90–95%.

Preparation of Fatty Acid Methyl Esters and GLC

Approximately 50 mg of total lipids were fractionated into phospholipids, triglycerides, diglycerides and cholesteryl esters by preparative thin-layer plates 0.5 mm thick, using petroleum ether:diethyl ether:acetic acid (90:10:1) as solvent system. The separated components were made visible by spraying with 0.2% dichlorofluorescein in methanol and marked under ultraviolet light (12), in correspondence to the authentic standards run simultaneously on the same plate. The identified compounds were scraped into a small glass column 10 × 100 mm and eluted with chloroform:methanol (90:10). Approximately 100 ml of solvent were used for 10 mg of lipids. The recovery was determined gravimetrically to be between 90–95%. Lecithin and cephalin were separated as pure fractions by diethylaminocellulose (DEAE) and silicic acid column chromatography (13). No attempt was made to isolate other phospholipids which constitute approximately 10% of the total. The purity of the fractions eluted from the column was ascertained on an aliquot by TLC using chloroform:methanol:water (65:25:4) as solvent system.

The eluted fractions from column chromatography and TLC were evaporated in a rotary evaporator until approximately 1 ml of liquid was left. This solution was then transferred to a tube and evaporated to dryness under nitrogen. The lipids were transesterified with 5% HCl-methanol, and the methyl esters were analyzed on a Beckman GC2 gas chromatograph with a hydrogen flame detector. An aluminum column, 6 ft × ¼ in. was packed with 20% ethylene glycol succinate with 2% phosphoric acid on Gas-Chrom P, 80–100 mesh. Helium was used as carrier gas at a flow rate of 80–90 ml/min and column temperature was 190–195C. The component esters were identified by comparison with known standards, and the components for which standards were not available were tentatively identified by calculated equivalent chain length (14), and corresponded to polyunsaturated acids whose structures have been determined (15). The data are reported as area percent of total fatty acids. Amounts less than 0.1% are expressed as trace.

The abbreviated nomenclature used indicates chain length, number of double bonds and position of the first double bond from the terminal methyl group. In PUFA, all double bonds are assumed to be *cis* and methylene-interrupted. Thus, 18:3ω3 is linolenate, 18:2 ω6 is linoleate and 18:1ω9 is oleate.

RESULTS AND DISCUSSION

Total lipid content, percent of neutral and phospholipids in the total lipids, and the content of free cholesterol and triglycerides in neutral lipids are presented in Table I. The total lipids of testes in the postpartum glands are higher than in the prepartum ones.

The percentage of phospholipids in the total lipid is approximately 35 in the fetal testes and 50 in testes removed after birth. The increase of phospholipids in the developing testes suggests a close association of this lipid component with the growth and development of the germinal epithelium. Boyd (16) reported an increase of 200–700% in phospholipid content of the ovaries of frogs during the production of ova. Studies of Siek and Newburgh (17) and Leslie and Davidson (18) on the increase in phospholipid content of chick brain during embryonic development bear close similarity to our observations in the growing testis.

Free cholesterol decreases from about 60% in fetal testes to about 30% in mature animals. This change in cholesterol concentration might be related to the synthesis of sex hormones in the testes of mature animals. The triglyceride

TABLE I

Content of Lipids in Testes as a Function of Age

	Prepartum			Postpartum				
	4 Months	6 Months	8.5 Months	1 Month	4 Months	10 Months	12 Years	Castrated
Total lipid (% Wet weight)	1.9–1.7	1.6–1.2	1.8–2.0	2.6–3.0	2.8–2.4	2.0–3.0	3.2–2.4	4.0–3.0
Phospholipid (% Total lipid)	38–32	40–36	34–38	53–47	58–50	52–48	60–50	34–30
Neutral lipid (% Total lipid)	69–55	57–63	66–58	50–46	47–39	44–48	46–40	67–63
Free cholesterol (% Neutral lipid)	64–56[a]	65–59	64–60	38–32	40–36	28–22	30–26	22–18
Triglyceride (% Neutral lipid)	5–3	3–2	4–3	8–6	9–5	13–9	12–10	36–28

[a] Averages of three measurements on each of two samples.

concentration follows a reverse pattern. It increases from 4% in 4 months fetal testis to 11% in the adult animal. Diglycerides and cholesteryl esters are only minor components, approximately 0.6 and 1.6% of total lipids, respectively (19), as reported in the literature. These lipids were therefore not measured quantitatively, and the samples were used for analytical gas chromatography.

The fatty acid composition of total lipids, phospholipids, triglyceride (TG), diglyceride (DG), cholesteryl esters (CE), lecithin (LEC) and cephalin (CEPH) were analyzed. Because the data are too numerous to present, only selected fatty acids showing relatively large and consistent differences in the pre- and postpartum age groups are shown in Figures 1 and 2. The complete tabular data are available upon request.

The major changes observed in the *triglyceride* fraction is the decrease of oleate (18:1 ω9) from 80% to about 25% during fetal life and an increase in 16:0. Linoleate (18:2ω6) and 22:5ω6 undergo a many-fold increase after birth. Arachidonate (20:4ω6) and docosatetraenoate (22:4ω6) follow similar courses of development, increasing from <0.1% to 3.2% and from 0 to 10.2%. Therefore, the acids of the linoleate family (ω6) are quantitatively important in this lipid fraction. Acids of the ω3 group do not appear in significant amounts in triglycerides at any age.

In the *diglyceride* fraction the total saturated fatty acids decrease as the bull ages. Linoleate increases twofold from the time the testes descend from the body cavity at a fetal age of 4 months until maturity. There is a further twofold increase from then to 12 years. Arachidonate and 22:5ω3 follow the same trend until the 10th month. Decreases were observed in the content of 16:1, 16:2, 18:0, 18:1 and 20:2 in diglycerides with increasing age. Acids of the ω3 family appear toward the end of the fetal period.

Diglycerides found in tissue lipid extracts might be artifacts caused by hydrolysis of triglycerides or phospholipids. This does not appear to be the origin of the diglycerides analyzed in this study, for the appearance and disappearance of individual fatty acids during development do not follow the same patterns in both diglycerides and triglycerides. For example, triglycerides contain no ω6 and ω3 acids during fetal life, whereas they appear in diglycerides. Although changes in fatty acid composition are parallel in diglycerides and

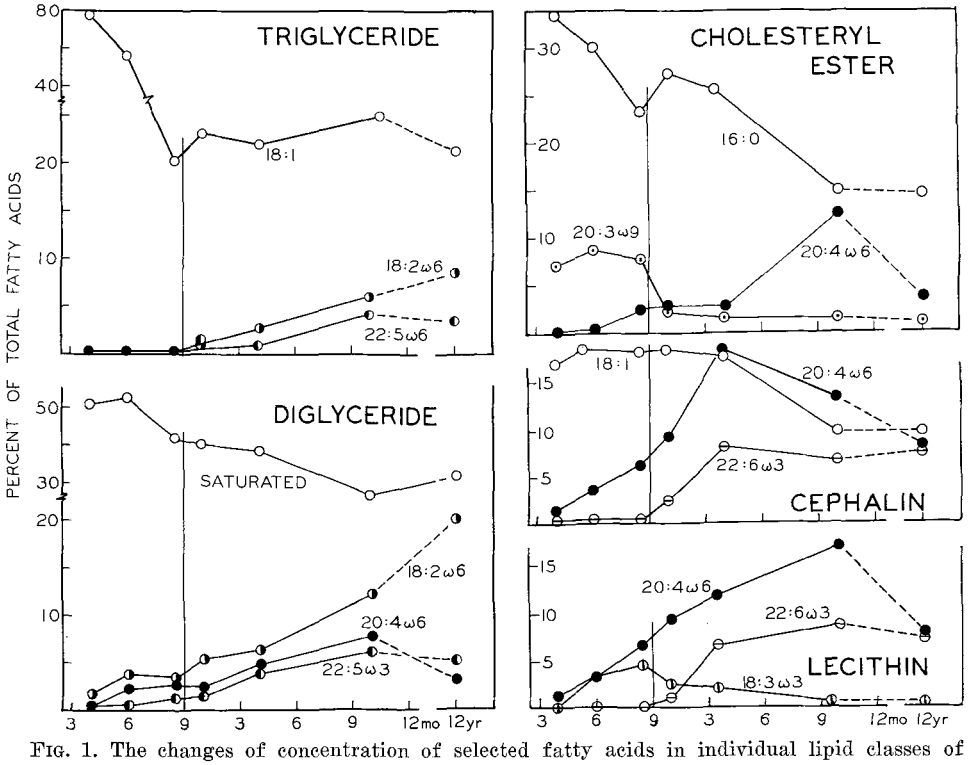

FIG. 1. The changes of concentration of selected fatty acids in individual lipid classes of testis lipids as a function of age. Parturition is indicated by a vertical line.

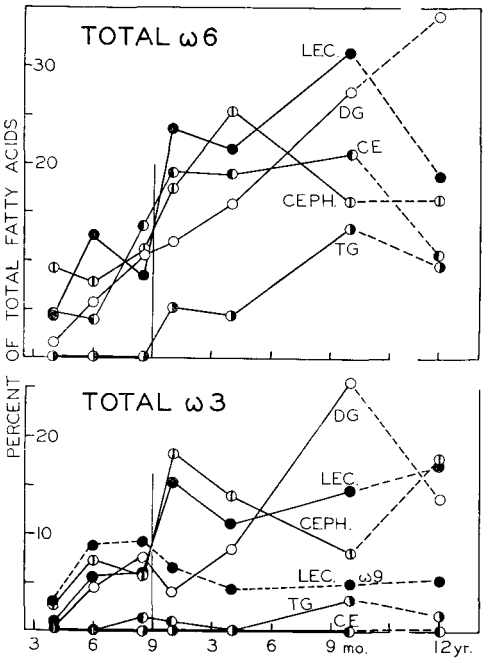

FIG. 2. The total ω6 and total ω3 fatty acids in individual lipid classes of testis lipids as a function of age. The total ω9 acid content of lecithin is included for comparison. Parturition is indicated by a vertical line.

cephalin in the prenatal stages of testis development, the changes are not parallel after one month postpartum. In a previous study 22:4ω6 was found in high concentration only in diglycerides and cholesteryl esters, suggesting that they were more related to each other than diglycerides to triglycerides or phospholipids (19). Thus, it appears that diglycerides are true components of testicular lipid, not artifacts.

In the *cholesteryl ester* fraction 16:0 decreases with age during both pre- and postnatal life. During fetal life 20:3ω9 remains rather constant and decreases after birth when dietary linoleate gives rise to 20:4ω6. Palmitate (16:0) and 16:2 tend to decrease during pre- and postnatal life, whereas 16:1, 18:1 and 18:2 increase. Acids of the ω3 group are absent in cholesteryl esters at all ages. Previously an increase of 20:3 ω9 in several tissues has been observed in EFA deficiency (20). The presence of this acid in fetal testes and its subsequent decrease upon maturity may reflect the relative deficiency of linoleate in that tissue during fetal life. The most striking change in the cholesteryl ester fraction occurs with arachidonic acid. This fatty acid increases from trace amounts in the fetal testes at 4 and 6 months to 12% of total fatty acids at maturity.

In the *cephalin* fraction (CEPH) arachidonate increased throughout prenatal life, and reached a maximum at 6 months postpartum, followed by a steady decrease until 12 years. 22:6ω3 is present only in traces in the fetal testes, but after birth there is a sharp increase. 18:3ω3 is present during fetal life but decreases after birth to only traces in mature animals, concurrent with the increases in 22:5 ω3 and 22:6ω3. Oleate decreases after puberty, and 14:0, 15:0, 16:1 and 18:2 also tend to decrease during the period studied.

In the *lecithin* fraction, as with cephalin, there is an increase of 20:4ω6 with age of the animal until maturity. In the 12-year-old animals the 20:4ω6 content was found to drop to approximately the prenatal level (8%). Linolenate is present prior to birth but decreases thereafter. 22:6ω3 is virtually absent during fetal life, and increases during postnatal life. The patterns of changes in the ω6 and ω3 metabolites are almost identical in the two phospholipid fractions. The content of 14:0, 15:0, 16:1, 16:2 and 18:0 decrease with age, whereas 16:0, 18:2ω6 and 20:3ω9 remained relatively constant.

The data suggest that from the time the testes descend from the body cavity at 4 months of fetal life the chain length and unsaturation of the fatty acids of all the lipid classes tend to increase with age. Most of the increase in the unsaturation comes after birth. Similar observations in the developing rat brain have been reported by Kishimoto et al. (21) for the fatty acids of the gangliosides and glycerophosphatides. Kirshman and Coniglio (22) found an increase in the concentration of polyunsaturated acids in the rat testicular tissue, particularly between the ages of 3 weeks and 3 months.

Figure 2 summarizes the principal changes in polyunsaturated acids of the ω6 and ω3 families of acids in the several lipid classes analyzed. Total ω6 fatty acids increase somewhat in all lipid classes during prenatal and postnatal life except in triglycerides, in which ω6 acids are absent until after birth. Most of the increase is after birth. With the exception of the diglyceride and cephalin fractions, the ω6 content of fatty acids in all lipid classes was less in 12-year-old animals than in 10-month-old bulls. In general, total ω3 acid contents of lecithin, cephalin and diglycerides increase somewhat during fetal life, and continue to increase thereafter. The content in cephalin and lecithin, however, shows a decrease

after one month postpartum. Measurable levels of ω3 acids appear only at the end of fetal life. The content of ω3 acids in triglycerides is very low until maturity is reached. The higher members of the ω9 family of fatty acids show an opposite trend, being higher in the fetal stage and declining as maturity is reached. This is exemplified in Figure 2 by the plot of ω9 fatty acids of lecithin, not including oleate.

Lipids and Fatty Acids of Testes of Castrated Animals

The testes were obtained from animals approximately 1 to 1½ months after castration. The current method of castration merely crushes the spermatic cord, and this leads to the atrophy of the testes. Thus, one can study the effects of degeneration of testes upon their lipid composition. Young bulls are castrated at approximately 6 months of age, and thus the data from these testes may be best compared with those of mature bulls at 10 months of age. In the castrate testes, total lipids amounted to 3.5 g/100 g wet tissue. Neutral lipids comprise 68% of the total lipids. These values are higher than found in normal testes (Table I). Triglycerides increase to about threefold more than in the mature bull, and free cholesterol decreases only slightly. This change may be the consequence of decreased phospholipid content in the degenerated testes.

The triglycerides and lecithin from the castrate testes contained approximately half as much 18:1ω9 as did 10-month-old bull testes. The content of oleate in the diglycerides tripled in the castrate testis, whereas the content in cephalin remained the same. The content of 20:3ω9 increased in triglycerides, diglycerides, lecithin and cephalin after castration, a change toward the composition seen early in fetal life. The total ω6 acids of diglyceride and lecithin were unaffected by castration, whereas this induced a marked decrease in triglycerides and cephalin. After castration the content of total ω3 acids increased in triglycerides and cephalins, whereas it decreased in diglycerides and lecithins.

The appearance of polyunsaturated fatty acids in testis lipids is slow during fetal life, and is most rapid in the first few months after birth. The predominant polyunsaturated acids in each lipid class are those of ω6 structure. In bulls 1 month of age and older the ω6 acids constitute about one fourth of the total fatty acids in lipids, whereas ω3 acids constitute 1 to 9%. The phospholipids containing high proportions of polyunsaturated acids are the principal lipids present in testis (19), and the ω6 acids can, therefore, be considered a significant structual component in the germinal epithelium.

The total unsaturation of the several lipid classes increases after birth. The double bond index, which indicates the average number of double bonds per fatty acid molecule ranges from 1.0 to 1.9 during fetal life in lecithin, cephalin, diglycerides and triglycerides. In postnatal life these values rise to 2.2 for the first three mentioned. However, the double bond index of triglycerides remained in the same range after birth. The degree of unsaturation influences the physical properties of lipids, and it thus appears that after birth, polyunsaturated acids accumulate in testis lipids giving them properties of increased fluidity.

ACKNOWLEDGMENT

Supported in part by Grant No. AM04524 and Research Fellowship Award No. 7-F2-AM-23, 963-01A1 from the National Institutes of Health.

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[Received Jan. 5, 1966]

Fractionation of Triglyceride Mixtures by Preparative Gas Chromatography[1]

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ABSTRACT

A semiautomatic system is described for gas-chromatographic separation and recovery of triglycerides of uniform molecular weight in milligram quantities. It employs an Aerograph Autoprep 700 (Wilkens Instrument and Research, Inc.) equipped with a stream splitter and a hydrogen flame ionization detector. The column is an aluminum or stainless steel tube (¼ in. O.D. × 2 ft) and contains silanized Chromosorb W (60–80 mesh) coated with 5% (w/w) JXR or SE-30. Five to ten milligrams of mixed triglyceride are injected at a time and the temperature is programmed exponentially from 150 to 350C. With split ratios of 1:5 to 1:10 collections of 20 to 50 mg of each peak can be made with some 10 to 20 injections.

INTRODUCTION

THE SEPARATION of natural triglycerides by gas-liquid chromatography (GLC) results in a segregation of the triglycerides on the basis of their molecular weight (1,2). Although each fraction contains only glycerides of the same carbon number these triglycerides usually contain an assortment of fatty acids esterified to glycerol. In order to identify the glycerides it is necessary to isolate the individual peaks and to determine their fatty acid compositions. The present report describes the separation and recovery of some synthetic and natural triglyceride mixtures in quantities large enough for rechromatography in the GLC apparatus and on thin-layer plates before and after enzymic and chemical transformations.

MATERIALS AND METHODS

Glycerides

The butter oil, its molecular distillates (3) and the coconut oil (4) used in this study have been described previously. The medium chain length (MCT) and long chain length (LCT) triglycerides were obtained from E. F. Drew and Company, Boonton, N.J. These triglycerides had been prepared by transesterification of fatty acid methyl esters of selected chain length with glycerol. The corn oil was Mazola and was purchased from a local market. Simple synthetic triglycerides of even carbon number and ranging from tricaproin to tristearin and triolein were obtained from the Applied Science Laboratories, Inc., State College, Pa.

Gas-Liquid Chromatography

An Aerograph Autoprep 700 (Wilkens Instrument and Research, Inc., Walnut Creek, Calif.) was modified by introducing a stream splitter and a hydrogen flame ionization detector. The flame detector was mounted on top of the thermal conductivity (TC) detector and was connected to the column through a post-column effluent splitter. The splitter (Fig. 1) consisted of two stainless steel tubes (⅛ in. and 1/16 in. O.D.) silver-soldered into the male Swagelock fitting (B) accepting the outlet end of the ¼ in. O.D. chromatography column (A). During chromatography the column effluent entered the splitter and was divided between the 1/16 in. bypass tube to the flame detector (C) and the ⅛ in. tube leading through

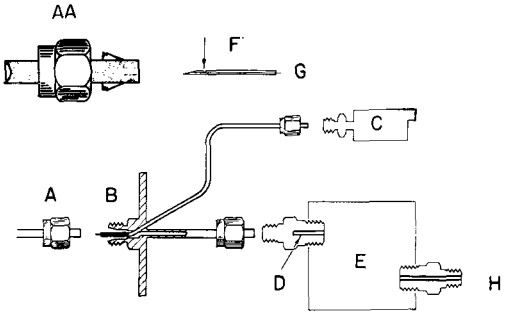

FIG. 1. Schematic representation of the post-column stream splitter showing its relation to column (A), flame detector (C), existing thermal conductivity detector (E) and the collector tube (H). AA, a magnified view of the outlet end of the packed column indicating the space inserted to accommodate the splitter needle (F). B, Swagelock fitting with 1/16 in. bypass tube containing notched needle (F) and restrictor wire (G). D, 1/16 in. restrictor in another Swagelock fitting. (Design and parts by Wilkens Instrument and Research, Inc.).

[1] Presented in part before the Canadian Committee on Fats and Oils, Ottawa, Ontario, Canada, October 1964.

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the thermal conductivity detector (E) to the collector (H). With a 1/16 in. O.D. outlet hole (D) in the Swagelock fitting accepting the ⅛ in. tube at the entrance to the TC detector, the approximate split ratio was 1:1 under the selected working conditions. By placing a 21 gauge needle (F) into the bypass tube at B the gas flow to the flame detector was restricted and the split ratio became 1:4.5 in favor of the collector. A further restriction resulting in a split ratio of about 1:10 was obtained by inserting a 0.007 in. diameter wire (G) into the needle. (A standard kit is now available from Wilkens Instrument and Research Inc., which converts the A-90-P or A-700 instruments to flame units).

The splitter needle actually sticks into the packing material in the column. In order to prevent the solid support from plugging the splitter needle, the column exit (AA) is prepared for entrance of the splitter by a preliminary penetration of the solid support by a 20-gauge needle.

The restrictor wire (G), about 2 in. long, is completely inserted in the needle.

For optimum sensitivity the flow to the flame tip was kept at about 25–30 ml/min requiring an overall flow of about 110–120 ml/min through column and about 90–100 ml/min to the collector (1:4 5 ratio). For a 1:10 split ratio, the flow through the column was set at about 300 ml/min. During the run, the detector oven housing, the splitter and the flame ionization detector were maintained at 325C. A special elbow heater (Wilkens Instrument and Research, Inc. accessory) was used to obtain a temperature of about 325 to 350C at the collector end of the tube. No special cooling devices were necessary for quantitative trapping of the eluted triglycerides in the collection vials partially filled with clean glass wool.

The columns used for the preparative runs were 2 ft × ¼ in. O.D. stainless steel or aluminum tubes. These were filled with hexamethylenedisilazane treated Chromosorb W (60–80 mesh) coated with 5% (w/w) SE-30 or JXR. The supports were supplied by Wilkens Instrument and Research, Inc., and the liquid phases by the Applied Science Laboratories, Inc. The analytical columns were similar except that 18 in. × ⅛ in. O.D. stainless steel tubes and 3% (w/w) JXR packings were used. All columns were first conditioned at 350C without flow for 2 hr then cooled to room temperature. They were conditioned under nitrogen at 325–350C until proportionally correct recoveries of the test triglyceride peaks were obtained.

Operation of the Preparative GLC System

The major steps involved in the operation of the preparative system used in this study consisted of the following: 1) adjusting gas flows and injector, detector and collector temperatures as described under the general conditions of operation; 2) selecting a Powerstat setting that will provide a suitable temperature gradient for the resolution at hand. (With starting temperatures of about 180C, a Powerstat setting of 65 to 70 gives temperature gradients that level off at about 320C permitting the separation of all the triglycerides from C_{28} to C_{54} in about 35 to 40 min); 3) allowing the column temperature to reach the upper limit and adjusting the cooling cycle timer to the number of minutes necessary to cool the column down to the starting temperature or slightly below it; 4) setting the precollection timer to ignore the solvent and any low boiling components emerging prior to the triglycerides; 5) injecting the sample into the flash evaporator or directly onto the column using a Hamilton syringe with either a 2 or a 6 in. needle, respectively, and recording the time and temperature of injection; 6) recording the temperature rise at regular time intervals and collecting peaks automatically by setting the peak signal activator switch on the recorder to the desired level. (As each peak appears on the recorder, the signal switch is tripped and the collector table is automatically advanced); 7) if necessary, using a postcollection timer to postpone the opening of the oven lid and the start of the cooling cycle until the remaining high molecular weight peaks have been vented or collected together in a separate collection bottle.

Thin-Layer Chromatography

The thin-layer chromatographic (TLC) examinations of the triglycerides before and after GLC and enzymic or chemical transformations were carried out using 20 × 20 cm plates coated with a 250 μ thick layer of Silica Gel G (Merck and Co.) (5). The prepared plates were reactivated after washing with methanol. The lipids were applied in spots containing about 0.1 to 0.5 mg of material per spot. The plates were developed with a mixture of petroleum ether and ethyl ether (120:80, v/v) to which 3 ml of formic acid was added. The developed plates were sprayed with a 0.2% solution of 2,7-dichlorofluorescein in ethanol. The lipid components were located by examining the plates under ultraviolet light. The system was capable of separating (in order

of increasing mobility) monoglycerides, diglycerides, free fatty acids, triglycerides, and hydrocarbons.

Infrared Spectrophotometry

The triglycerides (10 mg) recovered from the preparative gas chromatograph were dissolved in dry carbon tetrachloride (1 ml) and the solution examined in an infrared spectrophotometer in the range from 2.5 to 15 μ (Infracord 137, Perkin-Elmer Corporation) using sodium chloride cells with 0.5 mm light paths. These spectra were compared to those of the original triglycerides or their mixtures as well as to that of the bleed collected from the column.

Lipase Hydrolyses

The lipase hydrolyses of the recovered triglycerides were performed using the semimicro technique described by Luddy et al. (6) except that the digestions were carried out for 20 min at 37C on an ordinary laboratory shaker—water bath. Samples of recovered triglycerides, 10–25 mg, were digested with a pancreatin preparation called Steapsin (Nutritional Biochemical Corporation, Cleveland, Ohio), which had been successively extracted with cold acetone and ethyl ether prior to use. The product recovered from the enzymic digestion following acidification and petroleum ether extraction was diluted to ca. 1:10 with chloroform for TLC examination.

Esterifications

Transmethylations of the triglyceride mixtures were performed with 10% (w/w) sulfuric acid in methanol. The reagent was added in large excess (1–2 ml) to the recovered triglycerides (1–10 mg) and the reaction was completed in sealed tubes held at 80C for 16 hr. Transbutylations were performed similarly using a 10% (w/w) solution of sulfuric acid in dry n-butanol (7). Identical methods were used for the preparation of the esters from triglycerides recovered from the TLC plates together with the silica gel. The fatty acid esters were isolated by extraction with petroleum ether. The petroleum ether extracts were washed with distilled water and dried over sodium sulphate. Due to solvent evaporations the recoveries of the short chain fatty acid methyl esters were low. Nearly quantitative yields of short chain esters including the ester of butyric acid could, however, be obtained by preparing the butyl esters and avoiding solvent evaporations under a strong stream of nitrogen.

In order to detect the possible presence of free fatty acids in the triglyceride fraction recovered from the GLC, the recovered materials were allowed to react with freshly generated diazomethane (8). The fatty acids were analyzed in 5 ft × 1/8 in. O.D. stainless steel columns filled with SE-30 coated (5%, w/w) Chromosorb W (60–80 mesh) using temperature programming (7).

Quantitations

The weight proportions of the long chain triglycerides in the gas chromatograms were assessed by a direct comparison of their peak areas. The triglyceride proportions in mixtures of short and long chain triglycerides were obtained by reference to a standard response curve computed for simple triglycerides under the working conditions. The amounts and proportions of the individual triglyceride peaks recovered from preparative GLC were ascertained by rechromatography of the collected peaks in an analytical gas chromatograph together with a known amount of trioctanoin or tridecanoin.

The weight proportions of the short and long chain fatty acid esters were also obtained from response curves and were in good agreement with the proportions derived by multiplying the peak areas by the corresponding oxygen content of the ester and normalizing. Either the methyl or the butyl ester of heptadecanoic acid was used as an internal standard.

RESULTS AND DISCUSSION

Selection of Preparative Column

It had been shown earlier (2) that short narrow bore columns (18 in. × 1/8 in. O.D.) produce some of the most effective separations of natural triglyceride mixtures. Because of the thin film of the liquid phase, the capacity of these columns is small, rendering them impractical for preparative separations even with very large numbers of repeat injections. An increase in column diameter to 1/4 in. allows the separation of larger quantities of triglyceride (up to 0.1 mg per peak) which can be further raised by extending the column length to 5 ft. Since the longer columns require higher temperatures to complete the elutions (7), the advantages of the thin film coatings (1–2%, w/w) are largely lost and consideration can be given to shorter columns containing heavier coatings of liquid phase. The use of a 2 ft × 1/4 in. O.D. tube with a 5% liquid phase has been found to provide an acceptable compromise between column capacity

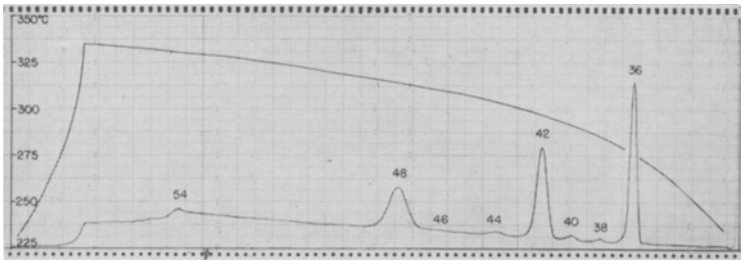

FIG. 2. Elution pattern recorded for a mixture of equal weight proportions of trilaurin (36), trimyristin (42), tripalmitin (48) and triolein (54) under conditions of preparative GLC. Column: 2 ft. × ¼ in. O.D. aluminum tube packed with 5% (w/w) JXR on silanized Chromosorb W (60–80 mesh). Injector: 325C, detector: 325C, collector: 350C. Temperature program as given in the figure. Nitrogen: 150 ml/min to the collector, 30 ml/min to the detector. 50 μl of 1% petroleum ether solution injected into the flash evaporator. The numbers identifying the triglyceride peaks refer to the number of C-atoms in the acyl groups.

and desirable operating temperatures without sacrificing the effectiveness of resolution. The recovery of the higher molecular weight triglycerides, however, is reduced and for quantitation appropriate calibrations must be used.

Recovery of Simple Triglycerides

Figure 2 shows the elution pattern recorded for a mixture of equal proportions of trilaurin, trimyristin, tripalmitin and triolein. As a result of the heavy coating of liquid phase (5% of support weight) and the small temperature gradient, the recovery of triolein is low. Similar low recoveries are experienced also with tristearin under these conditions. The proportions of the other triglycerides, however, are correct and correspond closely to those collected in the effluent, suggesting that the splitter system is satisfactory at least for the collection of widely spaced glyceride peaks. Twenty injections of 50 μl aliquots of a 5% solution of mixed triglyceride in chloroform yielded about 10 mg each of trilaurin, trimyristin and tripalmitin, but only trace (1 mg) of triolein. The purity of the collected peaks and their proportions were determined by rechromatography of the individual peaks in an analytical gas chromatograph together with a known amount of tridecanoin as an internal standard. Further tests of the preparative system with simple triglyceride mixtures indicated that the recoveries of the long chain glycerides varied greatly with the operating conditions, and that there may have been a preferential loss of the unsaturated C_{54} triglycerides.

Figure 3 indicates the approximate recoveries of various simple triglycerides from a preparative column with 5% liquid phase under optimum operating conditions. The low recoveries noted for triolein were not reflected in a general loss of all triglycerides containing unsaturated fatty acids. Mixed triglycerides containing oleic and linoleic acids in combination with medium chain length saturated fatty acids, could be readily recovered in apparently satisfactory condition. These triglycerides gave typical glyceride spectra in the infrared and could be completely digested by enzymic and chemical methods yielding the anticipated fatty acid compositions. The upper part of Figure 4 shows a representative repeat pattern recorded during the collection of the mixed C_{48} to C_{54} triglycerides of butterfat which were used for obtaining one of the infrared recordings. Everything past C_{46} was collected in the same tube. The fraction collector was indexed manually throughout the run. The clean front of the GLC elution pattern recorded at a

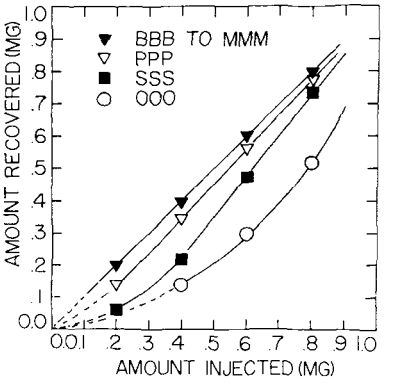

FIG. 3. Recoveries of simple triglycerides from a preparative GLC apparatus. The indicated quantities were injected directly onto the column as 1% solutions in chloroform. The triglycerides, tributyrin through trimyristin (BBB to MMM) were chromatographed under the conditions given in Figure 2, other triglycerides were recovered by programming the oven temperature from 250C to 350C.

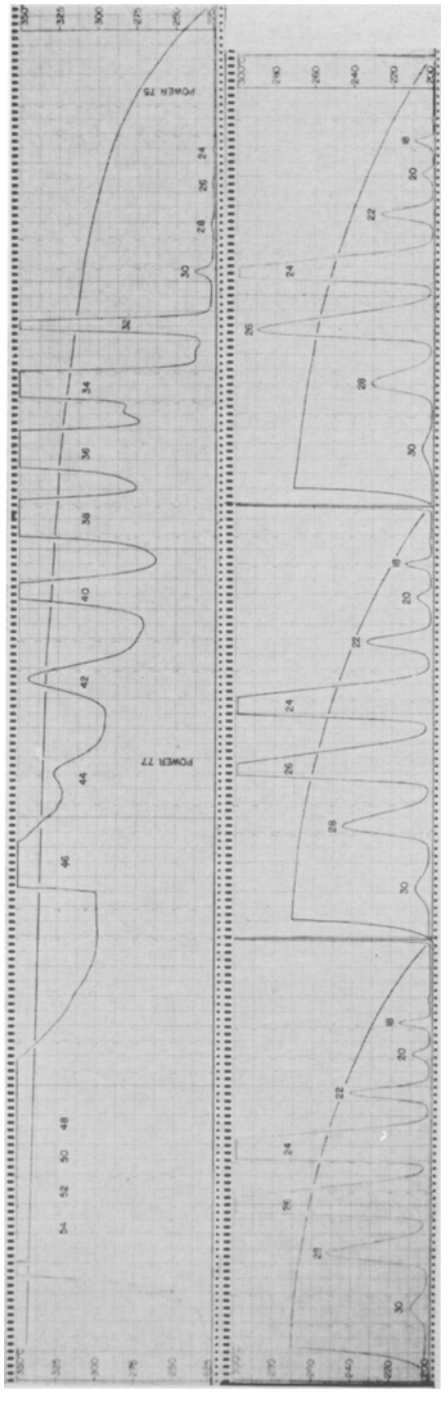

FIG. 4. Repeat patterns recorded during preparative GLC of triglycerides. Upper print: whole butter oil, 50 μl of 1% solution in petroleum ether; on-column injection. Temperature program as given in the figure. Other conditions as given in Figure 2. The large gap between the C_{46} and C_{48} peaks is due to a temporary levelling off in the temperature program resulting from manual operation. Lower print: MCT, 50 μl of a 10% petroleum ether solution injected into the flash evaporator. Injector 300C, detector 300C, collector 300C. Temperature program as given in the figure. Split ratio 1:10.

relatively low temperature indicates that the injection and volatilization of the samples was not accompanied by a fragmentation of the triglyceride molecules containing unsaturated fatty acids or by a liberation of free fatty acids.

All the collected triglyceride peaks were contaminated with small amounts of the liquid phase which was continually lost from the column. This silicone bleed could be separated from the triglycerides on thin-layer plates where the less polar polysiloxane fragments moved well ahead of the triglycerides. An examination of the recovered triglycerides in the infrared before and after the TLC purification indicated that the minor silicone contamination did not interfere with the recognition of the characteristic bands associated with the vibrations of the functional groups present in the triglyceride molecules (9).

The GLC and TLC examination of the recovered materials, before and after enzymic and chemical transformations, together with the infrared data confirm the earlier expressed belief (10) that the recovered fractions are homogeneous on the basis of molecular weight and represent undegraded triglycerides. It does not appear to have been recognized, however, that the collected glycerides are contaminated with column bleed.

Recovery of Complex Triglycerides

When working with natural triglyceride mixtures containing components differing by two carbons only, the timing of the rotation of the fraction collector becomes much more critical and a too early or too late indexing will result not only in incomplete recoveries of the desired peaks but also in cross-contamination. The difficulties arise from the relatively long distances that the triglyceride bands have to travel in separate tubes in order to reach the detector and collector simultaneously. Due to differences in the tube diameter, minor variations in flow rate bring about both changes in the split ratios and the times of travel in the empty tubes. Proper adjustment in the temperature of the collector and detector housing is the most important factor in the collection of pure components. Unless these variables are adequately controlled the recording of a good separation in the flame detector provides no assurance that the material collected will be pure.

The difficulties encountered are illustrated in an exaggerated way with the synthetic MCT sample, which was used for establishing the optimum operating conditions for the splitter

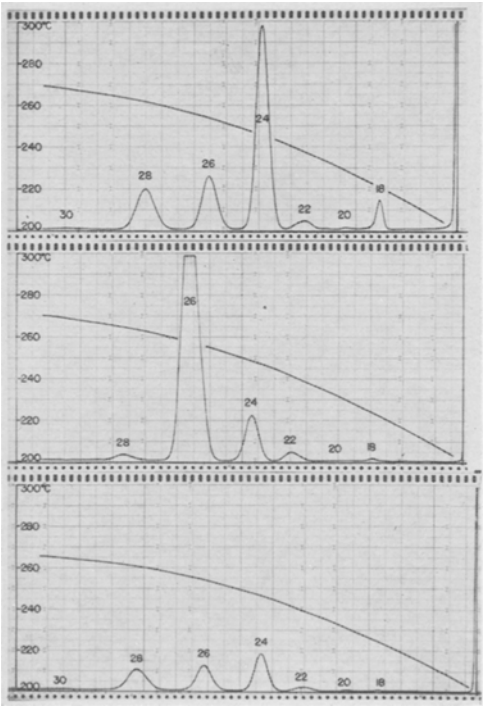

FIG. 5. GLC elution patterns recorded for triglyceride peaks collected under suboptimal conditions. Upper print: glycerides recovered with peak 24. Middle print: glycerides recovered with peak 26. Lower print: glycerides recovered with peak 28. Rechromatography conditions as given in Figure 4 for the MCT separations, except collector temperature 325C.

and the automatic collection system triggered by the recorder. The lower part of Figure 4 shows a repeat sequence of peaks recorded for a preparative run with the medium chain length triglycerides. Considering the low molecular weight of the material and the excellent resolution indicated by the flame detector, an effective collection of the resolved components would be anticipated. The composition of the recovered materials is shown in Figure 5. Each peak is seen to contain some of the preceding and some of the following peaks. The cross-contamination was due to a condensation of the effluent in the collector tube, the extent of which varied with the flow rates and detector temperatures. Although this condensation could be avoided by simply increasing the temperature of the collector tube, it did not necessarily provide for a quantitative collection of pure components, which also required correct indexing of the fraction collector. In order to collect fractions of uniform molecular weight it was necessary to maintain the collector tube about 25 to 30C above the detector and the latter about 25C above the column temperature at all times. It is believed that the maintenance of such temperature gradient is needed to overcome the condensing effects of any cold spots that may be present in the unevenly heated system.

Under the outlined conditions, the most complete collections are realized with the broader peaks. Closely spaced sharp spikes present

TABLE I

Analysis of a Synthetic Triglyceride Mixture (MCT)[a]

Distribution of triglycerides[b]

	Distribution types (mole %)						
Determination	18	20	22	24	26	28	30
Experimental	1.7	1.1	6.1	43.5	34.8	10.9	1.9
Random	0.01	0.6	8.6	46.7	34.3	9.0	0.8

Distribution of fatty acids[b]

	Acid types (mole %)					
	6:0		8:0		10:0	
Glyceride types	Experimental	Random	Experimental	Random	Experimental	Random
18	100	100				
20	66.6	66.6	33.3	33.3		
22	33.3	34.0	66.6	65.4	Trace	0.6
24	Trace	3.3	100	93.3	Trace	3.3
26	Trace	0.6	66.6	65.4	33.3	34.0
28			33.3	33.3	66.6	66.6
30					100	100

[a] The original glyceride sample contained the following fatty acids (as mole %): 6:0, 5.3; 8:0, 75.6; 10:0, 19.1. Other MCT samples also contained traces of 12:0 which resulted in the appearance of peak 32 and minor contributions to peaks 26, 28 and 30 in the GLC run.

[b] The recovery of the total fatty acid carbon was 100% (11).

problems as they may be easily missed or only partially recovered as a result of an incomplete correspondence in the arrival times at the two exit ports (detector and collector). Therefore the rather low temperature gradients obtained as the oven temperature asymptotically approaches a limiting value may be better suited for glyceride collection than the linear programs which in addition produce progressively closer spacing of the higher molecular weight components. Too flat peaks, however, cannot be reliably collected with this type of collector because any slight back pressure from the glass wool plugs in the collection bottles may result in accidental tripping of the activator switch and a further rotation of the turntable.

Using optimum conditions, quantitative collections of triglycerides of uniform molecular weight have been obtained for all components of the MCT sample. This mixture of synthetic triglycerides contained only C_6, C_8 and C_{10} fatty acids, which were shown (Table I) to have been nonrandomly incorporated into the triglyceride molecules. Quantitative collections have also been obtained for the major triglycerides of molecular distillates of butter (7) and coconut oil. With the latter fats the experimental information derived from preparative GLC can be greatly upgraded by collecting the triglycerides from material that has previously been segregated on the basis of unsaturation on TLC plates impregnated with silver nitrate. It is hoped to present these data at a later date.

ACKNOWLEDGMENTS

Earl Taft of Wilkens Instrument and Research, Inc., modified the Autoprep 700 and installed the post-column stream splitter and flame ionization detector. Applied Science Laboratories, Inc. donated liquid phases.

This work was supported by grants from the Special Dairy Industry Board, Chicago, Ill., the Ontario Heart Foundation, Toronto, Ontario, and the Medical Research Council of Canada.

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[Received Nov. 5, 1965]

A Comparative Study of the Phospholipids and Fatty Acids of Some Insects

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ABSTRACT

Phospholipids of 27 species of insects representing 6 orders and 20 families were examined by DEAE cellulose column chromatography to determine the choline/ethanolamine phosphoglyceride ratios, and by gas chromatography to determine the constituent fatty acids.

The phosphorus in the ethanolamine phosphoglycerides accounted for approximately 50% of the total lipid phosphorus in aphids (Homoptera) and in all but one family of Diptera (flies) examined while the phosphorus in the choline phosphoglycerides accounted for only about 25%. Ethanolamine and choline phosphoglycerides were present in approximately equal proportions in one family of Diptera and in the Coleoptera (beetles) examined. In the other insects examined choline phosphoglycerides predominated, ethanolamine phosphoglycerides comprising only about 25–30% of total lipid phosphorus as they do in most mammalian tissues.

Diptera in which ethanolamine phosphoglycerides were the major phosphatides were also characterized by high proportions of fatty acids less than 18 carbons long, particularly palmitoleic acid, in the neutral lipids. Aphids are characterized by a preponderance of 14-carbon fatty acids. The evidence suggests that predominance of ethanolamine phosphoglycerides is associated with a preponderance of shorter chain fatty acids in the neutral lipids.

Differences also exist between Diptera and other insects in the fatty acid compositions of different phosphatides, particularly with respect to the distribution of 18-carbon acids. The compositions observed in insects that contained large amounts of the choline phosphoglycerides are similar to those found in vertebrates. Similarities in fatty acid composition of the choline phosphoglycerides in such widely divergent organisms suggest that the fatty acids may play a greater role in phospholipid function than has heretofore been demonstrated.

[1] Contribution Number I.P.R.I. 74.

INTRODUCTION

THE RECENT OBSERVATION that the phospholipids of 3 species of the order Diptera (Insecta) contain more than 50% ethanolamine phosphoglycerides (EPG) and only about 25% choline phosphoglycerides (CPG) (1–3) contrasts sharply with findings in vertebrate animals where CPG generally represents 50% or more of the total phospholipid and EPG only about 25%. The further observation (4) that most Diptera are characterized by high levels (15+%) of palmitoleic (16:1) acid led to the suggestion (5) that an interrelationship might exist between EPG predominance and the high levels of palmitoleic acid.

A survey of phospholipid compositions and of fatty acid spectra within the neutral lipids and the main phospholipid classes of a broad group of insects was undertaken to attempt to determine if such a relationship exists, and to attempt to define it. The data obtained might also contribute to the larger problems of the significance of varying phospholipid composition and the specific variations in fatty acid composition between phospholipid classes (6). The results obtained form the substance of this communication.

PROCEDURES AND DATA

Procedures

Insects were obtained either from field collections or from laboratory reared stocks, usually as adults or mature larvae. The DEAE cellulose used was Cellex D (0.7 meq/g) obtained from BioRad Laboratories. All solvents were reagent grade and were redistilled before use.

Whole insects were extracted by grinding in a Waring blendor in 20 ml/g of chloroform-methanol 2:1. The extract was filtered and washed with dilute saline as recommended by Folch et al. (7). After evaporating the solvent under nitrogen the crude lipid was stored at −20C in chloroform solution until used.

Approximately 20 mg of this crude lipid was applied to a 2 × 20 cm column of DEAE cellulose in the acetate form that was prepared as recommended by Rouser et al. (8). Neutral lipids were eluted with chloroform and weighed. Polar lipids were then eluted with mixtures of chloroform and methanol and the effluent moni-

tored by phosphorus analysis (9). The identity of the fractions eluted from the column was checked by thin-layer chromatography (TLC) on Silica Gel G with chloroform:methanol:water (84:32:3).

Chloroform-methanol (9:1) eluted ninhydrin-negative material which on TLC had an R_f corresponding to authentic lecithin and sphingomyelin (the latter is not demonstrable in Diptera). This material is designated CPG. Chloroform-methanol (7:3) eluted material which gave a single ninhydrin-positive spot corresponding in R_f to authentic phosphatidyl ethanolamine. The remaining lipids were eluted with chloroform-methanol-ammonia (4:1 + 20 ml/liter conc NH_4OH) and were not further characterized except to show they were free of CPG or EPG. Replicate runs from the same initial crude lipid sample gave relative standard deviations (100 × standard deviation/mean) of 5.5% and 10.9% for the major (ca. 50%) and the minor (ca. 20%) components, respectively.

Transesterification of the fractions obtained by column chromatography was accomplished by the technique of Morgan et al. (10). The methyl esters obtained were chromatographed on a 5 ft × ⅛ in. stainless steel column packed with 12% diethylene glycol succinate on 100/110 mesh Anakrom AB at 190C in an Aerograph 204 equipped with a hydrogen flame detector. Fatty acid methyl esters were identified by comparison of their retention times with those of standards and by comparing logarithmic plots of retention ratios (11). For quantitation, areas under the peaks were measured either by Disc integrator or calculated from retention times height of peak (12). Quantitative results with National Heart Institute Fatty Acid Standard D agreed with the stated composition data with a relative error of less than 5% for major components (>10% of total mixture) and less than 10% for minor components (<10% of total mixture).

TABLE I

Phosphorus Content, as Per Cent of Applied Phosphorus, of Lipids Eluted from DEAE Columns

Insect		Stage[a]	CPG	EPG	Residue	P recovered	CPG/EPG
Diptera							
Musca domestica (Muscidae)		A	13.9	58.8	14.0	86.7	0.23
Euxesta notata (Otalidae)	(2)[b]	L	17.5	53.4	7.5	78.2	0.33
Nephrotoma sodalis (Tipulidae)	(2)	L	26.0	46.5	20.0	92.7	0.56
Strauzia longipennis (Tripetidae)	(2)	L	29.0	47.3	13.1	89.3	0.61
Hylemia antiqua (Muscidae)		A	17.4	52.9	7.2	78.2	0.34
Chironomus sp. (Chironomidae)		A	26.1	46.5	6.6	76.8	0.56
Phytophaga rigidae (Cecidomyiidae)		L	37.5	38.6	48.2	125.3	1.02
Rhabdophaga swainei (Cecidomyiidae)		L	48.1	44.2	9.2	101.5	1.08
Homoptera							
Anuraphis bakeri (Aphididae)		All	27.2	40.8	10.4	78.5	0.66
Prociphilus tesselatus (Aphididae)		All	29.7	49.1	7.2	86.0	0.60
Schizolachnus pini-radiatae (Aphididae)		All	33.7	54.3	6.2	94.2	0.62
Aphrophora parallela (Cercopidae)		NA	46.0	28.7	16.5	91.2	1.60
Lepidoptera							
Archips cerasivoranus (Tortricidae)	(2)	L	45.9	29.5	2.5	77.9	1.55
Paleacrita vernata (Geometridae)	(3)	L	48.7	25.8	16.5	87.5	1.89
Erannis tiliaria (Geometridae)	(2)	L	45.4	32.3	11.1	89.3	1.40
Hyphantria cunea (Arctiidae)		L	40.0	31.8	4.9	76.5	1.26
Datana integerrima (Notodontidae)		L	37.6	33.4	9.1	80.0	1.16
Hymenoptera							
Neodiprion sertifer (Diprionidae)	(4)	L	49.2	26.1	3.1	78.4	1.88
Monoctenus juniperinus (Diprionidae)		L	52.1	28.2	10.7	90.6	1.85
Pikonema alaskensis (Tenthredinidae)	(3)	L	47.8	30.3	11.6	89.7	1.58
Arge pectoralis (Tenthredinidae)		L	41.6	29.2	13.1	85.2	1.43
Coleoptera							
Pissodes strobi (Curculionidae)	(2)	P	36.0	36.0	3.4	79.0	1.00
Chrysomela crotchi (Chrysomelidae)		L	39.8	38.2	9.0	87.0	1.04
Altica ambiens alni (Chrysomelidae)		L	34.3	37.0	17.4	89.0	0.94
Tenebrio molitor (Tenebrionidae)		L	39.8	41.7	4.9	85.1	0.95
Orthoptera							
Diapheromera femorata (Phasmatidae)		A	57.4	35.5	7.0	85.0	1.62

[a] A = adults, P = pupae, L = larvae, N = nymphs.
[b] Bracketed numbers indicate replicate analyses, data then are means.

Data

The phosphorus content of the fractions eluted from DEAE cellulose are recorded in Table I and the CPG/EPG ratio calculated. In 6 of the 7 families of Diptera examined the CPG/EPG ratio was less than 0.7 indicating that EPG is the major component in the phospholipids and that this phenomenon occurs quite generally in Diptera. The Cecidomyiidae however have CPG/EPG ratios approaching 1.0 thus differing widely from the other Diptera examined.

The 3 species of the family Aphididae (Homoptera) also show ratios less than 0.7. The related cercopid bug, however, has a ratio of 1.60, or almost twice as much CPG as EPG. All the other insects examined had CPG/EPG ratios of 1.0 or higher, and thus resemble vertebrate tissues in phospholipid composition.

The results of the fatty acid analyses of the fractions obtained by column chromatography are presented in Tables II, III, IV and V. The neutral lipids (Table II) of all Diptera except the Cecidomyiidae contain very high proportions of palmitoleic (16:1) acid and more than 50% of the fatty acids are less than 18 carbons long in these Diptera. In the Cecidomyiidae (Diptera), as in the Lepidoptera, Hymenoptera, Coleoptera and Orthoptera, only about 30% or less of the fatty acids are less than 18 carbons long. In the Aphididae (Homoptera) more than 80% and in the related Cercopidae only 12% of the fatty acids are less than 18 carbons long. Thus in those species in which more EPG than CPG is present, and only in those species, more than 50% of the fatty acids in the neutral lipids are less than 18 carbons long.

Bromination of a number of neutral and phospholipid methyl ester samples indicates that where major proportions of 18:3 and 20:0 are found, 18:3 is the primary component with only low levels of 20:0 present.

The high levels of palmitoleic acid (16:1) found in the neutral lipids of most Diptera are found also in the CPG fractions of these insects (Table III), with generally lower but still significant levels in the EPG (Table IV) and Residue fractions (Table V). However, in

TABLE II

Fatty Acid Composition of the Neutral Lipid Fraction of Some Insect Fats as Weight Per Cent of Total Fatty Acid in Fraction[a]

	<12	12:0	14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3 20:0	>20
Diptera											
Musca domestica			6.8	2.4	22.8	24.0	4.5	29.3	3.3	1.3	2.4
Euxesta notata			4.9	4.3	23.7	19.4	3.6	20.7	18.3	4.6	
Nephrotoma sodalis		1.0	1.2	5.5	22.7	19.6	9.3	13.6	9.7	14.8[b]	
Strauzia longipennis			1.2	1.0	20.2	40.1	1.3	22.6	9.5		
Hylemia antiqua			2.4	1.1	19.1	39.4	1.9	23.6	8.1	1.2	
Chironomus sp.			2.2		26.6	21.2	3.4	30.2	9.3	3.1	1.8
Phytophaga rigidae		2.2	1.4		13.5	2.4	6.8	21.2	39.6	4.3	
Rhabdophaga swainei			1.4	1.2	11.3	8.2	2.6	43.7	27.8	3.2	
Homoptera											
Anuraphis bakeri	5.2	16.4	47.5		11.1	1.7	3.1	3.8	11.2		
Prociphilus tesselatus	8.6	17.3	55.4	4.8	5.5	1.0	1.3	4.3	1.7		
Schizolachnus pini-radiatae	6.4	10.9	60.2		13.2		1.8	3.5	2.0		1.2
Aphrophora parallela					10.2	1.2	10.6	45.1	26.3	1.3	
Lepidoptera											
Archips cerasivoranus			6.9		27.5	4.8	2.3	29.0	6.2	27.2	
Paleacrita vernata					24.6	1.1	3.0	34.4	8.0	28.4	
Datana integerrima			2.9		19.4	7.9	2.0	22.8	5.2	40.0	
Hymenoptera											
Neodiprion sertifer			2.0		12.9	2.0	4.5	37.5	15.7	17.5	1.0
Pikonema alaskensis			1.4		21.5	6.2	4.2	39.9	8.2	16.2[b]	1.4
Arge pectoralis					14.2	1.8	4.2	36.6	6.9	36.3[b]	
Monoctenus juniperinus			1.1		15.5	1.9	2.5	45.2	6.9	23.8	2.5
Coleoptera											
Pissodes strobi					17.4	10.9	2.3	49.6	14.0	3.9	
Chrysomela crotchi		4.2			24.2	1.5	4.7	31.1	5.5	29.4	
Tenebrio molitor			1.5		23.6	4.5	1.4	44.7	24.1	1.5	
Altica ambiens alni			1.4		22.6	1.6	7.3	23.3	7.6	36.1[b]	
Orthoptera											
Diapheromera femorata			1.8		18.1	6.8	8.9	42.2	5.3	17.0	

[a] Only those acids comprising more than 1% of the mixture are recorded.
[b] Shown by bromination to be primarily unsaturated.

TABLE III

Fatty Acid Composition of the CPG Fraction of Some Insect Fats as Weight Per Cent of Total Fatty Acid in the Fraction[a]

	<14	14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3 20:0	>20
Diptera										
Musca domestica		3.1		21.4	29.6	4.2	26.6	7.3	7.5	
Euxesta notata		4.8	2.9	23.2	31.8	2.4	22.0	7.2	5.7	
Nephrotoma sodalis		2.2	1.3	20.0	13.1	5.4	26.2	15.0	12.9	
Strauzia longipennis		2.0		20.9	21.1	5.5	24.3	17.5	4.9	
Hylemia antiqua		1.2		16.5	31.4	2.0	17.5	11.3	3.8	10.0
Phytophaga rigidae		1.1		10.5	1.7	5.5	24.3	17.5	4.9	14.6
Rhabdophaga swainei		3.2		13.8	5.2	8.9	32.9	29.7	4.1	
Homoptera										
Anuraphis bakeri	3.8	9.4		5.4	5.8	2.6	19.2	25.3	8.2	18.2
Prociphilus tesselatus	1.2	8.5	1.0	4.1	4.9	3.9	20.4	42.5	5.0	7.8
Schizolachnus pini-radiatae		8.3	6.7	5.1	5.3	5.1	20.0	38.8	11.5	5.6
Aphrophora parallela		3.1		7.8	2.2	9.7	15.5	56.7	1.6	3.3
Lepidoptera										
Archips cerasivoranus		3.3		21.4	5.6	9.1	19.0	14.2	27.3[b]	
Paleacrita vernata	1.0			15.0		6.9	13.4	19.1	43.7[b]	
Erannis tiliaria		3.5		15.4	1.4	7.1	7.6	26.1	38.9	
Hymenoptera										
Neodiprion sertifer		2.3		9.7		11.0	21.7	28.6	17.2[b]	10.2
Pikonema alaskensis		1.3		11.6	3.8	8.0	22.3	21.5	22.4[b]	10.3
Arge pectoralis	1.6	1.1		7.3	4.3	12.9	19.6	16.5	37.0	
Monoctenus juniperinus		3.2		11.4	2.8	10.0	23.5	18.3	22.0[b]	11.6
Coleoptera										
Pissodes strobi		1.9		16.0	6.8	9.4	29.0	28.7	5.9	1.9
Chrysomela crotchi		2.2		17.8		8.2	22.3	12.1	37.5	
Tenebrio molitor		3.1		15.5	2.4	8.7	21.4	48.9		
Altica ambiens alni		7.8	2.2	18.1	4.7	9.8	20.8	14.3	15.1	8.0
Orthoptera										
Diapheromera femorata		1.5		14.2	3.3	11.0	29.3	13.3	26.8	

[a,b] as in Table II.

the Aphididae the phospholipid fatty acid compositions do not reflect the neutral lipid pattern at all but resemble those of Lepidoptera or Hymenoptera.

Some other differences between the phospholipid fatty acid patterns in Diptera and other insects can also be discerned. Stearic acid (18:0) levels are consistently higher in the phospholipids than in the neutral lipids and higher in EPG than in CPG except in Diptera where no consistent differences between classes can be detected. Oleic acid (18:1) levels tend to be higher in the EPG fraction than in the neutral fraction of Diptera but are always higher in the neutral lipids of the other taxa. Such comparisons of neutral and phospholipid fractions were not meaningful in Aphididae because of the unusual neutral lipid composition. In Diptera linoleic acid (18:2) tends to be higher in the EPG than in CPG; in the other species examined linoleic acid tends to be higher in the CPG.

More generally, palmitic acid (16:0) levels are higher in the neutral lipids than in any phospholipid fraction, and linoleic acid is always higher in the phospholipids than in the neutral lipid fraction. A component with a retention time corresponding to hexadecadienoic acid (16:2) appears to be more prominent in the EPG (Table IV) and Residue (Table V) fractions and a component with a retention time corresponding to heptadecanoic acid (17:0) appears to be concentrated in the EPG fraction. The 18:3:20:0 combination was not concentrated in any particular fraction, but high levels were found in most taxa except in the Diptera and Aphididae examined. In CPG, there was usually a higher level of fatty acids longer than 20 carbons than there was in EPG.

DISCUSSION

That more EPG than CPG is found in the phospholipids of some Diptera had previously been established (1–3), as had the presence of large proportions of palmitoleic acid in the neutral lipids of many Diptera (2,4,13). In the present study we have shown that most, but not all, Diptera examined exhibit these characteristics together. When EPG is not the major phosphatide only low levels of palmito-

leic acid are found, as in the Cecidomyiidae. EPG is also the major phosphatide in the Aphididae which are unrelated to the Diptera but which in this and other studies (14) are shown to contain very high proportions of myristic (14:0) acid. It appears therefore that the occurrence in the neutral lipids of 50% or more of the fatty acids with chain lengths less than 18 carbons long is associated with the predominance of EPG, rather than as a characteristic of a particular phylogenetic grouping. Studies of lipids from other classes of organisms will be required to establish whether this is a general phenomenon. The physiological significance of such a relationship is obscure.

In the Diptera the phospholipid fatty acids reflect to some degree the pattern found in the neutral lipids. In addition, the fatty acid compositions of the various phospholipid classes differ from those found in insects and vertebrates where CPG is the major phospholipid, and may represent evolutionary responses to the high levels of EPG in these insects. Such responses are not, however, apparent in Aphididae and thus probably represent only one of many possible compensatory mechanisms.

The patterns of fatty acids in the phospholipids of non-Diptera resemble those cited by Ansell and Hawthorne (6) for phospholipids from mammals in that EPG contains more stearic acid than does lecithin which in turn contains more than do the glycerides (neutral lipid fraction). Glycerides, however, contain relatively more oleic and palmitic acids than do the phospholipids. These findings support the view that within broad groups (e.g., the animal kingdom) fundamental mechanisms are, generally, similar and that the fatty acid composition of phospholipids is of fundamental importance to function. It follows from this that the unique fatty acid composition of the phospholipids in Diptera is related to the high levels of EPG observed.

The effect of altered membrane phospholipid composition on the various membrane-bound or -mediated functions of the cell is still subject to considerable argument and speculation. Chapman (15) has indicated that the liquidity of EPG is considerably lower than CPG for purely steric reasons without invoking any charge or electronic effects. Bangham et al.

TABLE IV

Fatty Acid Composition of the EPG Fraction of Some Insect Fats as Weight Per Cent of Total Fatty Acid in the Fraction[a]

	<14	14:0	14:1	16:0	16:1	16:2	17:0	18:0	18:1	18:2	18:3 20:0	>20
Diptera												
Musca domestica		3.7	1.3	14.2	29.4	1.8		2.1	30.0	10.4	2.7	10.9
Euxesta notata		2.1		24.2	13.4			4.6	30.7	21.5	3.4	
Nephrotoma sodalis		4.7		23.9	10.2	4.4		4.8	20.9	17.2	11.1	
Strauzia longipennis		1.9		19.4	8.8		1.2	8.2	25.8	24.8	10.4	
Hylemia antiqua				17.6	31.5			1.4	29.2	13.7	5.0	
Chironomus sp.	3.4	2.7	1.2	20.2	13.5	1.7	4.8	17.2	27.6	7.0	3.0	
Phytophaga rigidae				15.6	2.0	1.8	2.5	13.3	9.3	51.8	5.7	
Homoptera												
Prociphilus tesselatus	8.8	4.2	1.9	6.3	4.8			11.5	18.0	27.4	5.5	9.6
Schizolachnus pini-radiatae		3.8	5.0	3.7				19.3	18.5	36.0	10.6	2.1
Aphrophora parallela		2.8	1.3	7.4	2.9	3.4		18.0	11.0	50.5	3.7	
Lepidoptera												
Archips cerasivoranus	2.1	4.1		14.6	3.2	3.7		10.2	20.1	12.2	31.7	
Paleacrita vernata				21.0	4.5	1.4	1.2	15.4	34.9	3.9	7.8	5.8
Erannis tiliaria		1.0		8.4		3.1		6.5	18.0	23.1	38.8	
Datana integerrima		2.0		11.0	2.0	5.1	1.2	9.3	20.1	11.3	37.3[b]	
Hymenoptera												
Neodiprion sertifer				7.4	2.0	2.5		11.5	21.4	19.9	18.7	18.5
Pikonema alaskensis		1.8		8.7	3.6	4.4		11.5	18.8	18.8	22.1[b]	9.6
Arge pectoralis	4.1	7.4		5.6	2.1	1.0		11.6	19.5	13.6	33.6	1.8
Monoctenus juniperinus				8.1	1.6	4.4		13.4	20.3	20.3	27.3	
Coleoptera												
Pissodes strobi		1.0		19.8	3.2			7.5	31.2	28.1	9.2	
Chrysomela crotchi		4.0		20.8	3.2		1.2	10.8	28.3	9.5	21.8	
Tenebrio molitor	4.1	3.1	1.0	12.2	1.1	1.1	1.3	13.0	20.3	38.6	6.2	
Altica ambiens alni	3.0	3.4	1.4	13.3			3.0	7.5	23.9	17.5	29.4	1.8
Orthoptera												
Diapheromera femorata		1.1		7.3	1.7	1.5		16.1	24.8	15.2	30.8	

[a], [b] as in Table II.

TABLE V

Fatty Acid Composition of the Residue Fraction of Some Insect Fats as Weight Per Cent of Total Fatty Acid in the Fraction[a]

	<14	14:0	14:1	16:0	16:1	16:2	18:0	18:1	18:2	18:3 20:0	>20
Diptera											
Musca domestica	4.1	2.6	1.2	26.2	15.1		10.3	19.6	6.2	3.1	9.0
Euxesta notata		2.4		15.6	4.9	1.7	8.9	21.9	19.1	24.9	
Nephrotoma sodalis	1.7	1.8	1.4	13.6	10.7	1.2	5.1	19.3	21.1	7.4	18.7
Strauzia longipennis	1.0	2.6	1.5	17.6	13.5		4.9	15.7	11.9	6.7	24.6
Hylemia antiqua		1.0		11.8	16.2		2.8	15.8	18.7	7.8	3.6
Chironomus sp.	2.9	2.9		15.2	19.3	3.0	6.8	23.9	12.7	6.5	4.2
Phytophaga rigidae		5.9		20.5	9.5		11.3	46.0	6.2		
Homoptera											
Prociphilus tesselatus	43.7	4.2	1.0	4.2	2.7		6.5	8.7	9.0	4.0	24.6
Schizolachnus pini-radiatae	7.6	11.0	0.9	9.3	5.5		10.0	29.3	24.0	6.0	
Aphrophora parallela				8.8	2.8	1.0	11.5	14.2	24.7	36.9	
Lepidoptera											
Paleacrita vernata		4.8		30.8	7.9		14.0	17.9	5.6	10.6	8.2
Erannis tiliaria		4.8		8.5	4.2	3.3	8.5	11.1	18.8	36.8	2.0
Datana integerrima	1.3	1.1	2.2	6.1	1.1	1.1	12.0	8.0	15.5	48.9	1.3
Hymenoptera											
Neodiprion sertifer		1.9		24.2	3.2		13.9	14.5	12.4	12.5	21.8
Pikonema alaskensis		2.5		21.1	5.1		13.9	28.3	15.6	13.2	1.7
Arge pectoralis		3.1	1.0	6.1	2.8	1.3	11.5	24.5	20.8	27.1	
Monoctenus juniperinus				16.9	8.4		8.4	19.4	18.0	32.7	
Coleoptera											
Chrysomela crotchi				18.9	6.8		8.3	23.7	17.0	25.1	
Tenebrio molitor	2.4	3.0		15.1	3.2	1.5	12.9	20.9	31.4	6.5	
Altica ambiens alni		3.4		12.3	4.9		11.2	14.6	31.0	22.8	
Orthoptera											
Diapheromera femorata	7.6	14.0		11.1	3.8	1.0	11.3	20.8	10.0	24.7	

[a] as in Table II.

(16,17) have shown that diffusion of univalent cations and anions out of discrete aqueous compartments bounded by a bimolecular layer of CPG is remarkably similar to the diffusion of such ions across biological membranes. The permeability of these layers is altered in a manner similar to biological membranes by biologically active steroids. The permeability to cations is also altered by increasing the negative surface charge of the membrane. Rojas and Tobias (18) have shown that at physiological pH both EPG and serine-phosphoglycerides are negatively charged whereas CPG is neutral or positively charged over a relatively wide pH range. There is thus good reason to postulate that the characteristics, both physical and physiological, of membranes in which the level of EPG is greater than CPG will be quite different from membranes in which CPG is the major constituent. Coupled with this are observations that the permeability of membranes may be altered by changing the fatty acid composition of the membrane phospholipids (19).

Some enzymes may require either a specific phospholipid class (20–22) or a specific mixture of phospholipids for optimal activity (23). Indeed, removal of phospholipid may even alter the substrate specificity of an enzyme (21,24). One can conclude therefore that Diptera, because of their EPG predominance and unique phospholipid fatty acid compositions, may provide the means by which phospholipid function and the role of fatty acids in phospholipids can profitably be studied. The aphids, with high proportions of EPG but "normal" fatty acid compositions, should also be useful in such studies.

One might also anticipate that the effect of biologically active compounds which act on the membrane will also be different in Diptera and aphids, since Bangham et al. (17) indicate that such actions are probably independent of polysaccharides, proteins, or active cell metabolism.

ACKNOWLEDGMENTS

Insects provided by personnel of the Insect Survey, Canada Department of Forestry, and of the Entomology Research Laboratory, Canada Department of Agriculture, Chatham, Ontario. Technical assistance provided by Mrs. S. Charrette.

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[Received Jan. 31, 1966]

Gas-Liquid Chromatography of Triglycerides from Erucic Acid Oils and Fish Oils[1]

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ABSTRACT

By critically selecting optimum operating conditions, quantitative gas-liquid chromatography of triglycerides has been extended to molecules containing substantial amounts of C_{20}, C_{22}, and C_{24} fatty acids. The triglycerides of four erucic acid oils (water cress, rapeseed, nasturtium, and *Lunaria annua*) and two fully hydrogenated fish oils (menhaden and tuna) have been quantitatively analyzed by this technique. The average fatty acid chain length calculated from the triglyceride composition of each oil agreed closely with that determined by GLC of its respective methyl esters. Several conclusions about the triglyceride composition of the fats analyzed are discussed.

INTRODUCTION

ALTHOUGH GAS-LIQUID CHROMATOGRAPHY (GLC) of triglycerides containing C_8 through C_{18} fatty acids is now used in many laboratories (1–9), erucic acid oils and fish oils have not been analyzed by this technique. Triglycerides containing long chain fatty acids usually show poor peak resolution and substantial losses during GLC.

An extensive investigation of the parameters affecting GLC of triglycerides (7) has shown that triglycerides with molecular weights as high as trierucin can be eluted when optimum operating conditions are employed. This paper reports a study of the quantitative GLC of C_{54} through C_{66} triglycerides and the analysis of erucic acid oils and fish oils by this technique.

EXPERIMENTAL

Materials

Trilaurin, tripalmitin, and tristearin of greater than 99% purity were purchased from Applied Science Laboratories, State College, Pa. Trielaidin, triarachidin, trierucin, and tribehenin of 99% purity were purchased from the Hormel Institute, Austin, Minn. GLC analyses of the individual triglycerides revealed traces of diglycerides and/or mixed-acid triglycerides in the triarachidin, tribehenin, and trierucin; and appropriate purity corrections were applied when using these three materials for calibration purposes.

Watercress (*Nasturtium officinale*), nasturtium (*Tropaeolum majus*), and *Lunaria annua* seeds were purchased from commercial suppliers. Rapeseed (*Brassica napus*) was obtained from the Saskatoon Wheat Pool, Saskatoon, Saskatchewan, Canada. Seeds from each species were ground and extracted with petroleum ether (30–60C bp) in a Soxhlet extractor for 4 hr. The entire seed was used for oil extraction except with *Lunaria annua* where the seed coat was removed prior to extraction. The oil contents of the seeds were: watercress, 12.0%; nasturtium, 5.6%; rapeseed, 40.8%; and *Lunaria annua,* 42.5%

Menhaden body oil (*Brevoortia tyrannus*) was obtained from a commercial source. A "little tuna" (*Euthynnus alletteratus*) weighing 4.06 kg was caught in the Gulf of Mexico 40 miles southeast of Freeport, Texas, on June 11, 1965, and was kept at −60C with dry ice until used 8 days later. After thawing, 1.82 g of lipid material was extracted from 173.0 g of wet muscle tissue using the chloroform/methanol extraction method of Bligh and Dyer (10). Each fish oil was fully hydrogenated by the method of Farquhar et al. (11) using distilled dioxane as a solvent in place of ethanol.

The triglycerides were isolated from each of the erucic acid oils and from the hydrogenated fish lipids by means of preparative thin-layer chromatography on 1.0 mm thick plates of Silica Gel G developed in 79/20/1 (v/v/v) petroleum ether/diethyl ether/acetic acid.

Methods

GLC of Fatty Acids. Methyl esters were prepared from the purified triglycerides using the standard H_2SO_4-methanolysis technique (8). The fatty acid composition was then determined by GLC analysis of the methyl esters on a 6 ft by 0.093 in. I.D. column containing 20% butanediol succinate polyester coated on acid-washed Chromosorb W as previously described (8). Peaks were identified by comparison with the elution times of known compounds and by comparison with the compositions reported by other workers for the

[1] Winner, AOCS Bond Award. Presented at the AOCS Meeting in Cincinnati, October 1965.

same oils (11–15). Branched chain acids in the hydrogenated fish oils were identified as those peaks which did not correspond to the elution times of a homologous series of straight chain methyl esters. Peak areas were determined by triangulation, and all fatty acid compositions are reported in mole percent.

GLC of Triglycerides. GLC analysis of triglycerides was carried out under the optimum conditions described by Litchfield, Harlow, and Reiser (7). An F&M 400 gas chromatograph equipped with a hydrogen flame detector and an automatic temperature programer was used. The 21 in. by 0.093 in. I.D. stainless steel column containing 3.0% JXR on 100/120 mesh Gas-Chrom Q was heated from 200 to 360C at 2–4C/min with a helium carrier gas flow rate of 100 ml/min. Under these conditions, 10–20 μg of triglyceride produced a full scale peak on a 1 mv recorder. In agreement with previous results (7), chromatograms showed no extraneous peaks which might indicate sample degradation in the flash heater. Sample peaks were identified by comparison with the retention times and elution temperatures of known compounds. Tristearin eluted at 290–300C and trierucin at 335–345C.

Quantitative response factors for the various carbon number triglycerides were determined from known composition mixtures of tripalmitin, tristearin, trielaidin, tri-11-eicosenoin, tribehenin, trilaurin, triarachidin, and trierucin. Trielaidin was used in the calibration mixtures since its detector response conveniently lies midway between triolein and trilinolein (7), the major C_{18} components of the erucic acid oils. The f_m values [molar calibration factors determined by the internal normalization technique (7)] of the simple triglycerides in the calibration mixtures were used to assign an average f_m value to each peak based on its estimated fatty acid composition. Typical f_m values for trilaurin, tristearin, and trierucin were 1.00, 0.71, and 0.71, respectively.

RESULTS AND DISCUSSION

The first step in the quantitative analysis of high molecular weight triglycerides was to determine the linearity of detector response to variations in sample size. Previous work has shown that some losses occur with triglyceride molecular weights above C_{42}, even under optimum operating conditions (7). Losses of tristearin were found to be proportional to the amount of sample injected, and detector response curves were linear with respect to sample size. Thus, losses could be accurately compensated for with calibration factors (7).

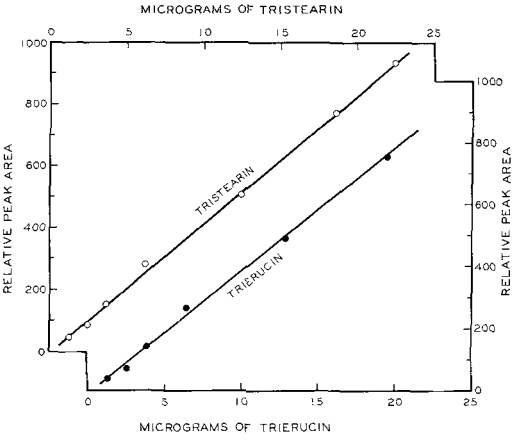

FIG. 1. Relationship between peak area and amount of sample injected for tristearin and trierucin. Operating conditions same as Figure 2 with oven temperature programed at 4C/min.

A similar check on the linearity of detector response was made with trierucin. These results and the comparative values for tristearin are shown in Figure 1. The plot of peak area vs. amount of triglyceride injected is linear over the 1–20 μg range in both cases. Below 1.0 μg, results were variable, and the curves may not be valid in this range. Theoretically, the trierucin curve should have a steeper slope than the tristearin curve, since trierucin contains a smaller percentage of oxygen than tristearin (16). In practice, however, the two plots were almost parallel, indicating that losses of trierucin were higher than those of tristearin. Using Ackman's calculation method for determining the theoretical relative responses of fatty acid esters in a hydrogen flame detector (16) and assuming that no trilaurin was lost during calibration runs, we estimated

TABLE I

Fatty Acid Composition of Erucic Acid Seed Oils (mole %)

Fatty acid	Watercress	Rapeseed	Nasturtium	*Lunaria annua*
14:0	0.1	0.1	....	....
16:0	10.7	4.0	0.8	1.3
16:1	0.3	0.3	0.1	0.1
18:0	2.5	1.4	0.1	tr
18:1	30.5	16.1	2.1	27.0
18:2	24.1	14.8	0.2	6.8
18:3	1.5	9.3	0.4	0.7
20:0	2.1	1.1	0.1	....
20:1	10.3	12.3	15.5	0.6
20:2	0.5	0.7	....	....
22:0	0.7	0.5	....	....
22:1	15.3	38.3	79.1	43.2
22:2	0.3	0.4	....	0.1
24:0	0.3	.	tr	....
24:1	0.6	0.6	1.6	20.2
Other	0.2	0.1	....	tr

TABLE II

Fatty Acid Composition of Fully Hydrogenated Fish Oils (mole %)

Fatty acid[a]	Menhaden body	Tuna muscle
12:0	0.2	0.1
13:0	....	0.2
14:0	10.5	4.7
Branched	0.2	0.1
15:0	0.7	1.1
Branched	0.1	0.1
16:0	37.3	31.8
Branched	0.1	2.4
17:0	1.2	2.0
Branched	0.1	0.3
18:0	21.5	21.2
19:0	0.4	0.4
20:0	14.8	9.2
21:0	0.6	0.4
22:0	11.3	25.0
23:0	0.1	....
24:0	0.9	1.0

[a] Listed in order of elution on butanediol succinate polyester GLC column.

that approximately 6% of the tristearin injected and 24% of the trierucin injected were lost during analysis. Under nonoptimum operating conditions, losses undoubtedly would have been much greater (7). Therefore, frequent calibration is essential for accurate results.

The fatty acid compositions of the triglycerides from the four erucic acid oils and the two hydrogenated fish oils were determined by GLC of their respective methyl esters on a polyester column (Tables I and II).

The triglycerides of each oil were separated according to carbon number using GLC on a 21-in. silicone column. The two fish oils were fully hydrogenated before triglyceride GLC to avoid possible thermal decomposition of the highly unsaturated fatty acids. The resulting chromatograms (Fig. 2) showed good peak resolution allowing accurate quantitation, except with the fish oil triglycerides. The presence of odd chain length and branched chain fatty acids in the two fish oils undoubtedly led to poorer resolution of triglyceride peaks. This effect was most noticeable with the tuna oil, which had 6.6% odd carbon number fatty acids, twice the amount found in the menhaden oil. The peak areas in each chromatogram were measured, and the carbon number distribution of each oil was quantitatively determined (Table III). The average fatty acid chain length was calculated from the triglyceride composition of each oil and also from the methyl ester GLC data using the method of Kuksis et al. (6). Comparison of the two values (Table IV) showed close agreement for all six fats, indicating that the carbon number distributions reported in Table III are close to the correct values.

Watercress seed oil contains 30.1% long chain acids ($C_{20}+C_{22}+C_{24}$). GLC results indicate that triglycerides containing both one (C_{56} and C_{58}) and two (C_{58}, C_{60} and C_{62}) long-chain acids are present in substantial amounts.

Rapeseed oil containing 39.2% C_{22} and 14.1% C_{20} fatty acids has no C_{66} (trierucin) and very little C_{64} (eicoseno-dierucin) present, indicating that no more than two long chain acids are usually present in any one triglyceride. The major peak in rapeseed oil, C_{62}, would correspond to a $C_{22}C_{18}C_{22}$ triglyceride such as oleo-

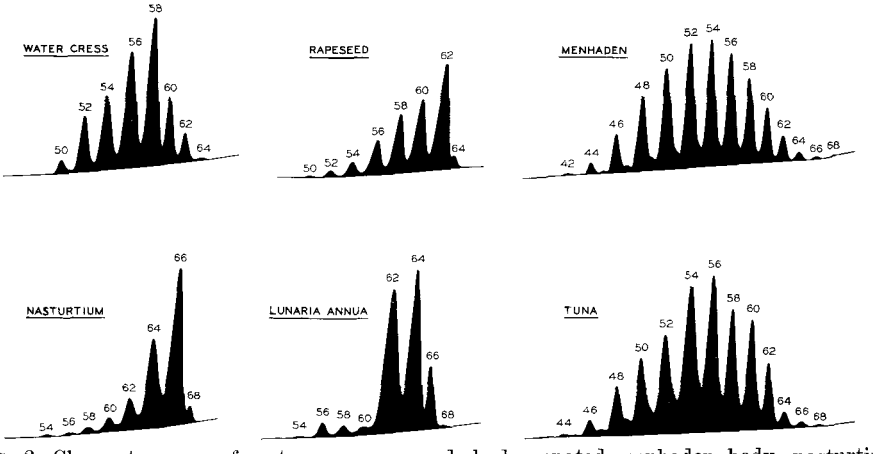

FIG. 2. Chromatograms of watercress, rapeseed, hydrogenated menhaden body, nasturtium, *Lunaria annua*, and hydrogenated tuna muscle oils. Operating conditions: F&M 400 gas chromatograph; 21 in. by 0.093 in. stainless steel column packed with 3.0% JXR on 100/120 mesh Gas-Chrom Q; column programed 200–360C at 2–4C/min. with 100 ml/min. He carrier gas; flash heater at 350C; detector base at 310–350C. Tallest peak in each chromatogram represents a 0.6–1.0 mv signal.

TABLE III

Triglyceride Composition of Erucic Acid and Fish Oils (mole %)

Carbon number	Water-cress	Rape-seed	Nastur-tium	*Lunaria annua*	Menhaden body	Tuna muscle
42	.....	.....	.....	.....	0.2	0.1
44	.....	.....	.....	.....	1.3	0.2
46	.....	.....	.....	.... .	4.6	1.6
48	.....	.....	.....	.....	9.9	5.1
50	2.2	0.5	.....	.....	14.3	8.7
52	10.3	1.8	.....	.....	17.8	13.0
54	16.4	4.1	0.4	0.2	17.4	20.8
56	26.7	10.0	0.6	2.2	14.5	19.5
58	30.7	19.5	1.4	1.7	10.2	13.0
60	9.6	24.9	3.4	1.6	6.1	10.8
62	3.8	38.8	9.4	44.1	2.6	5.4
64	0.3	0.4	31.6	39.9	0.8	1.3
66	.....	.....	52.0	9.9	0.2	0.4
68	.....	.....	1.2	0.4	0.1	0.1

dierucin or linoleo-dierucin. The second largest peak, C_{60}, could correspond to a $C_{20}C_{18}C_{22}$ triglyceride such as eicoseno-oleo-erucin or eicoseno-linoleo-erucin.

In nasturtium seed oil, the major triglyceride peak is C_{66}, undoubtedly mostly trierucin since the oil contains 79.1% erucic acid. The second largest peak, C_{64}, would be predominantly eicoseno-dierucin. The presence of C_{54} and C_{56} peaks is certainly unexpected, since nasturtium seed oil contains only 3.7% of fatty acids having less than 20 carbon atoms.

Lunaria annua seed oil contains 34.5% C_{18} and 63.5% C_{22} and C_{24} fatty acids. However, 93.9% of the triglycerides are either C_{62}, C_{64}, or C_{66}. To have such a composition, most of the triglycerides must contain one C_{18} acid and two long chain acids. Hence, the C_{62} peak would be mainly oleo-dierucin, the C_{64} peak mostly eruco-oleo-nervonin, and the C_{66} peak mostly oleo-dinervonin.

It is interesting to note the pattern in which increasing amounts of long chain fatty acids are incorporated into the triglycerides of erucic acid oils. Triglycerides containing three long chain fatty acids are only found in nasturtium seed oil, which contains 96.3% long chain acids and therefore must form considerable trierucin and eicosenodierucin. *Lunaria annua* and rapeseed contain 64.1% and 53.9% long chain acids, respectively, less than two-thirds the total; but no significant amounts of triglycerides containing three long chain acids are present in either oil. Watercress seed fat contains 30.1% long chain acids, less than one-third the total; and it definitely contains appreciable triglycerides with two long chain acids. If these oils are typical, it would appear that the enzymes synthesizing triglycerides in erucic acid oilseeds esterify only two long chain acids per triglyceride molecule. Only when the long chain acid content is above 66.7% are triglycerides with three long chain acids formed. On the other hand, when the long chain acid content is below 33.3%, there is no similar limitation to one long chain acid per triglyceride molecule.

TABLE IV

Average Fatty Acid Chain Length

Oil	Calculated from methyl ester data	Calculated from triglyceride data
Watercress	18.74	18.79
Rapeseed	19.80	19.85
Nasturtium	21.55	21.53
Lunaria annua	20.93	20.99
Menhaden body	17.61	17.78
Tuna muscle	18.34	18.37

Mattson and Volpenhein (17) and Grynberg and Szczepanska (15,18) have hydrolyzed numerous erucic acid oils with pancreatic lipase and have found that the long chain acids are mostly esterified at the 1- and 3-positions of the triglycerides. Such a distribution pattern would also limit long chain acids to two per triglyceride molecule, except above 66.7%. Hence, both GLC and lipase hydrolysis results independently point to the same distribution pattern in the triglycerides of erucic acid oils.

Menhaden oil and tuna oil triglycerides show very wide distributions of carbon numbers. Practically all possible di- and triacid triglycerides appear in the chromatograms. These results indicate that GLC will be helpful in the analysis of fish oil triglycerides whose exceedingly complex nature has heretofore resisted quantitative fractionation by chromatographic techniques. Quantitative results are reported here for only the even carbon number triglycerides, which represent the majority of triglycerides present in each oil. However, odd carbon number triglycerides are undoubtedly present, as indicated both by the fatty acid compositions

and the appearance of small peaks between the C_{42}, C_{44}, C_{46}, and C_{48} peaks. Complete analysis should, of course, include all the odd carbon number peaks; but this must await the availability of higher resolution columns for GLC analysis of triglycerides.

The successful quantitative GLC analysis of triglycerides containing C_{20}, C_{22}, and C_{24} fatty acids now extends the usefulness of this technique to all the molecular weights occurring in natural fats. However, no single method can completely resolve the complex mixtures of triglycerides in most natural fats. GLC is of greatest value when it is used as the final step in a series of consecutive fractionation techniques (8).

ACKNOWLEDGMENTS

Helpful technical assistance by Earline Miller and Carol Litchfield. Menhaden oil supplied by Bernadette Stevenson, rapeseed oil by the Saskatoon Wheat Pool. Taxonomic advice on fish by R. J. Baldauf. This work was supported in part by grants from the National Institutes of Health (AM 06011–04) and the Corn Products Institute of Nutrition.

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[Received Nov. 15, 1965]

Preparation of Sulfate Esters

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ABSTRACT

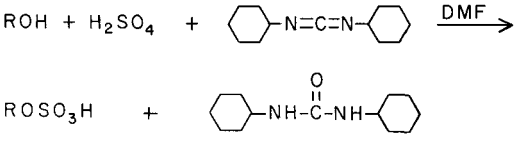
This communication reports a new method for the synthesis of sulfate esters, in good yield, under mild conditions. Sulfuric acid reacts with an alcohol and dicyclohexylcarbodiimide in a polar solvent to produce sulfate esters.

INTRODUCTION

DRAYER AND LIEBERMAN (1) have reported the isolation of cholesterol sulfate from human blood and gallstones. Lieberman et al. (2,3) have demonstrated steroid sulfates to be biosynthetic intermediates in vitro and in vivo and to have a relationship to some cancers. Haines (4), and Mumma and Gahagan (5) have reported the isolation and characterization of several naturally occurring sulfate esters. Because of the increased recognition of the biological importance of sulfate esters, it has become desirable to synthesize these esters and to study their properties.

Chlorosulfonic acid (6,7), sulfuric acid (8), and pyridine-sulfur trioxide (2,9) are reagents commonly used to produce sulfate esters. Many molecules of biological interest are not stable in the presence of these reagents and, therefore, a milder method of preparing sulfate esters was sought.

It was found that sulfuric acid reacts with an alcohol and dicyclohexylcarbodiimide (DCC) in a polar solvent such as dimethylformamide (DMF) to produce sulfate esters. This new reaction gives good yields under mild conditions, has practically no side reactions and the products are easily isolated.



Although carbodiimides have been used routinely for the synthesis of carboxylic acid esters, amides, and pyrophosphates, they have not been used to synthesize sulfate esters.

PROCEDURES AND DATA

Sulfate esters of saturated and unsaturated alcohols, sterols, and triterpenes have been prepared. The experimental conditions for four preparations are given; namely, the potassium salts of decyl, octadecyl, cholesteryl and testosterone sulfate. Each ester had the characteristic infrared sulfate ester absorptions at 1250–1200 cm^{-1}, 1081–1062 cm^{-1}, and 838–757 cm^{-1} (10). All reactions were followed radiochemically using trace amounts of $^{35}SO_4^=$ and each reaction step was analyzed by thin-layer chromatography. This procedure indicated that the best yields (70% or better, based upon the alcohol) were obtained when DCC and sulfuric acid were used in excess. The molar ratios commonly used were as follows: 1:1–2:5, alcohol:sulfuric acid:DCC.

Potassium Octadecyl Sulfate

Octadecanol, 1.55 mmole, dissolved in 4 ml of DMF, was added to 8.43 mmole of DCC dissolved in 2.5 ml of DMF in a 50 ml Erlenmeyer flask. Sulfuric acid, 3.20 mmole, dissolved in 2 ml of DMF containing 0.2 μc $^{35}SO_4^=$ was added dropwise to the alcohol-DCC mixture. Reaction immediately occurred as evidenced by the formation of the relatively insoluble dicyclohexylurea. After 1 hr at room temperature the reaction mixture was filtered to remove the insoluble dicyclohexylurea and the filtrate was neutralized with 1 N potassium hydroxide. Thirty milliliters of water was added to the filtrate and this solution was immediately transferred to a 250 ml separatory funnel and extracted with 50 ml of n-butyl alcohol. The water layer was re-extracted with 30 ml of n-butyl alcohol. The n-butyl alcohol layers were combined, washed with 40 ml of water and then the n-butyl alcohol was removed under vacuum at room temperature. The crude potassium octadecyl sulfate was recrystallized from absolute ethanol with a yield of 74%, mp 236–238C.

Analysis for $C_{18}H_{37}SO_4K$
Calculated: C, 55.69%; H, 9.60%; S, 8.25%
Found: C, 56.06%; H, 9.65%; S, 8.04%

Potassium Cholesteryl Sulfate

Cholesterol, 5.05 mmole, dissolved in 3.5 ml of DMF, was added to 24.4 mmole of DCC dissolved in 3 ml of DMF in a 50 ml Erlenmeyer flask. Sulfuric acid, 5.5 mmole, dissolved in 1 ml of DMF containing 0.2 μc $^{35}SO_4^=$, was added dropwise to the ice-cooled alcohol-DCC mixture. After thirty minutes the reaction mix-

ture was filtered. The precipitate was washed with 5 ml of DMF. The combined filtrates were neutralized with potassium hydroxide, and this solution was transferred to a 250 ml separatory funnel. One hundred milliliters of water and chloroform were added to the funnel and shaken. The insoluble cholesteryl sulfate remained at the interface between the two layers. The chloroform and water layers were removed and the insoluble sulfate ester was washed again with these same two solvents. The interfacial layer was filtered yielding crude potassium cholesteryl sulfate which was recrystallized from ethanol with a resulting yield of 74%, mp 247–248C.

Analysis for $C_{27}H_{45}SO_4K \cdot H_2O$

Calculated: C, 62.00%; H, 9.07%; S, 6.14%
Found: C, 62.43%; H, 9.24%; S, 6.21%

Potassium Decyl Sulfate

Decanol, 1.58 mmole, dissolved in 2.5 ml of DMF, was added to 7.90 mmole of DCC dissolved in 2.5 ml of DMF in an ice-cooled 50-ml flask. Sulfuric acid, 1.97 mmole, dissolved in 1.5 ml of cooled DMF containing 0.1 μc of $^{35}SO_4^=$, was added dropwise to the alcohol-DCC mixture. After thirty minutes the cooled solution was filtered, and the filtrate neutralized with 1 N potassium hydroxide. An additional 20 ml of water were added to the filtrate, stirred and the solution filtered. The crystals, additional dicyclohexylurea, were washed with 20 ml of warm water in small portions. The potassium decyl sulfate was water soluble and remained in the filtrate. The combined filtrates were transferred to a 250 ml separatory funnel, and 40 ml of n-butyl alcohol were added with mild swirling. The water layer was again extracted with 30 ml of n-butyl alcohol. The n-butyl alcohol was removed under vacuum at room temperature. The remaining crude potassium decyl sulfate was recrystallized from ethanol with a yield of 72%, mp 236–237C.

Analysis for $C_{10}H_{21}SO_4K$

Calculated: C, 43.43%; H, 7.66%; S, 11.60%
Found: C, 43.74%; H, 7.84%; S, 11.66%

Potassium Testosterone Sulfate

Testosterone, 0.90 mmole, dissolved in 2 ml of DMF, was added to 3.6 mmole of DCC dissolved in 2.5 ml of DMF in an ice-cooled, 50-ml flask. To this was added dropwise 1.1 mmole of sulfuric acid dissolved in 1.0 ml of cooled DMF containing 0.2 μc of $^{35}SO_4^=$. The following experimental procedure was the same as in the preparation of potassium decyl sulfate. The potassium testosterone sulfate also was water soluble and was isolated easily. The crude potassium testosterone sulfate was washed with hot ethanol. The product did not give a sharp melting point and was amorphous. The yield was 71%.

Analysis for $C_{19}H_{27}SO_4K$

Calculated: C, 53.72%; H, 6.89%; S, 7.55%
Found: C, 53.79%; H, 7.09%; S, 7.05%

DISCUSSION

The order of addition of the reactants is very important. Sulfuric acid will react directly with DCC. However, if an alcohol is present when the sulfuric acid is added to the DCC solution, the formation of the sulfate ester is preferred. At room temperature the reaction proceeds very rapidly and is complete within a few minutes, but a considerable amount of heat is produced and side reactions can occur. If the reaction temperature is kept below 25C, approximately 30 min are required for completion of the reaction, and no major side reactions take place. Therefore, in the afore mentioned procedures, the sulfuric acid was introduced dropwise, in dilute solution, to the cooled mixture of alcohol and DCC. The progress of the reaction was followed easily by the formation of the relatively insoluble dicyclohexylurea.

Although a number of methods were investigated, the best procedure for the isolation of the product seemed to be the extraction of the sulfate ester from an aqueous solution with n-butyl alcohol. The n-butyl alcohol can be removed readily under reduced pressure at room temperature leaving the desired sulfate ester. If the temperature is not kept low, side reactions occur and the solvent becomes sulfated. All the sulfate esters prepared were soluble in n-butyl alcohol and could be extracted almost quantitatively from the aqueous layer.

This method of synthesis appears to be of a general nature and well suited for the synthesis of sulfate esters of biochemical and detergent interest. It is particularly well adapted for radiolabeled syntheses because $^{35}SO_4^=$ can be used directly without prior preparation of chlorosulfonic acid or pyridine-sulfur trioxide. Also, the desired product can be isolated readily by thin-layer chromatography directly from the reaction mixture. Further studies are being conducted on the mechanism and versatility of this reaction.

ACKNOWLEDGMENTS

This work was supported in part by Grant AM08481 from the Institute for Arthritis and Metabolic Diseases of the Public Health Service.

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[Received Feb. 17, 1966]

A Comparison of Acyltransferase Activities in vitro with the Distribution of Fatty Acids in Lecithins and Triglycerides in Vivo

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ABSTRACT

The location and configuration of a double bond in a fatty acid influences the rate of its acyltransferase-catalyzed esterification to form lecithin and its distribution in vivo between the primary and secondary positions of triglycerides and lecithins.

Saturated acids of shorter chain length are transferred at rates similar to the long chain unsaturated acids.

The positional distributions of acids in the diglyceride units of liver triglycerides appear to be similar to that found in the lecithins.

Acyltransferase activities measured in vitro have a considerable predictive value in terms of the ultimate distribution of fatty acids in glycerolipids in vivo.

INTRODUCTION

MUCH OF THE RECENT WORK with acyltransferases supports the general concept that their activity may be important in controlling the fatty acid composition of tissue phosphoglycerides. The extent to which other reactions may control this composition is not clear, and it is evident that a series of enzymatic factors exist, all of which may exert some effect. Attempts to show selective esterifications in forming phosphatidic acid (1) were not as successful as those involving lecithin formation (2). Thus, the current evidence suggests that the characteristic distribution of fatty acids between the 1- and 2-positions may arise in part from a redistribution of acids after the nitrogenous base has been attached to the molecule. Further work has emphasized that the classification of fatty acids as either saturated or unsaturated is an unsatisfactory oversimplification (3) when describing their metabolic properties. Rather, each fatty acid should be regarded as having a characteristic metabolic fate.

This concept led us to consider the fate of unnatural fatty acids as well as a series of natural acids while testing the selectivity of the acyltransferases. A series of *cis* and *trans* isomers of octadecadienoate was used since three of the four acids are not encountered in normal metabolic processes. A comparison of the distribution of these acids in vivo with the relative esterification rates in vitro indicates the degree to which acyltransferase specificities could control the distribution of a fatty acid. Our results show that the enzymatic activities measured in vitro have a considerable value in predicting the ultimate distribution of fatty acids in glycerolipids in vivo.

EXPERIMENTAL

Synthesis of Acyl-CoA Derivatives

The procedure described by Seubert (4) was used to synthesize several saturated long-chain acyl-CoA esters. The derivatives of the polyunsaturated acids, however, are unstable in dilute aqueous solutions and the modifications described below provided high yields of thiol esters that were 100% active in the acyltransferase systems.

All solvents used in the following procedure contained 0.1–0.5% 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (Santoquin, ethoxyquin) as an antioxidant.[1] Tetrahydrofuran was distilled from $LiAlH_4$ immediately before use and all aqueous solutions were freshly prepared using water saturated with Santoquin. The reaction was carried out in a 50 ml Erlenmeyer flask and was stirred continously with a magnetic stirrer.

Coenzyme A (100 mg) was dissolved in 30 ml of aqueous tetrahydrofuran (50%), and the pH was adjusted to 7.5–8.0 with 1 *N* NaOH. The mercaptan content was estimated by removing 0.1 ml of the reaction mixture and adding it to a solution of 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (DTN) in phosphate buffer (pH 7.4) (5).

After the pH and mercaptan content of the solution were determined, 100 mg of the acylchloride was added and the pH readjusted to 7.5–8.0 with dropwise addition of NaOH. The mercaptan content was checked, and the addition of acylchloride and NaOH was continued until no mercaptan could be detected. At this point the reaction mixture was allowed to stand for 5 min and then 1 g of stearic acid was added to facilitate isolation of the less crystalline, unsaturated acyl-CoA esters in the subsequent steps.

[1] Antioxidant recommended by R. T. Holman and supplied by the Monsanto Co.

The reaction mixture was placed in a 100 mg round flask, chilled on ice, and 10 ml of 10% $HClO_4$ was added. The tetrahydrofuran was removed using a rotary evaporator, and the white precipitate was collected on a Buchner funnel while avoiding excessive exposure to 0_2. The solid was transferred to centrifuge tubes and washed thoroughly with ether to remove fatty acids. The residue of thiol ester was dissolved in 8 ml of cold water, the pH adjusted to 5.5 with a few drops of $NaHCO_3$, and the insoluble material (cellulose fibers, etc.) removed by centrifugation. The CoA thiol ester was reprecipitated by adding 2 ml of 10% $HClO_4$, collected by centrifugation, and washed with ether as before.

The yield was generally 60 to 70 μmoles of acyl-CoA per 100 μmoles of coenzyme A used as starting material. Santoquin interferes with the usual 232/260 assay for acyl-CoA (4), but the phosphorus content was measured (6) and compared to the extent of coenzyme A released (5) with acyltransferase systems to indicate that the material was 100% reactive (2).

Enzymatic Studies

The acyltransferase rates were determined spectrophotometrically under conditions of maximal velocity using DTN to measure CoA release as described earlier (2). The isomeric acylglycerol phosphorylcholine substrates were prepared by selective hydrolyses of lecithin and plasmalogen (3). Preparations of the 2-acyl-isomer generally required higher levels to saturate the enzyme than did the 1-acyl-isomer. The specific activities reported in this paper are the averages of three separate experiments with rat liver microsomes. The rates have been corrected for the small amount of hydrolase activity (+ 1.8 *mμmoles/min/mg protein*) noted in the controls. In addition, all reaction mixtures containing the 2-acyl-GPC[2] exhibited a very slow and consistent decrease in absorbance regardless of initial enzyme or acyl-CoA levels. The rate of decrease was proportional to the amount of the 2-acyl-GPC added and appeared to be due to traces of acetone-soluble compounds that reacted slowly with the yellow-colored arylmercaptan. Under the conditions used, the two corrections required for these reaction mixtures cancelled each other.

Feeding Experiments

In these studies, male rats of the Sprague-Dawley strain were made deficient in essential fatty acids by maintaining them from weaning to 4 months of age on a fat-free diet as described in previous work from this laboratory (8). Groups of a minimum of 5 animals each were then fed (as a supplement to the basic fat-free diet) 5% by weight of either *cis*-9, *cis*-12, *cis*-9,*trans*-12- or *trans*-9,*trans*-12-isomers of methyl linoleate for 18 to 20 days. The animals were killed, the livers excised, quick frozen on dry ice and stored at −20C. The lipid fraction was recovered by extraction with chloroform-methanol as previously described (8). The triglycerides were isolated by a combination of DEAE-cellulose and thin-layer chromatography (TLC) and the lecithins were isolated by a combination of DEAE-cellulose and ammonium silicate chromatography (9). The purity of the lecithins was confirmed by thin-layer chromatography and infrared analysis.

Positional Analyses of the Lecithins and Triglycerides

The lecithins were hydrolyzed with *Ophiogaphus hannah* (Ross Allen Reptile Institute, Silver Springs, Florida) phospholipase A, using conditions described by Robertson and Lands (10). After the hydrolysis was complete, as determined by periodic analysis with TLC, the products were isolated by preparative TLC. The fatty acid and 1-acyl-GPC fractions were separated by TLC on plates coated with chloroform-extracted Silica Gel G (A. G. Merck, Darmstad, Germany) using chloroform/methanol/water (70/30/3). The spots were detected by spraying the plate with 2′,7′-dichlorofluorescein and viewing it under ultraviolet light. The spots were scraped into test tubes with constricted necks for sealing. About 3 ml of methanol containing 6% (W/W) of dry HCl was added to each tube. The tubes were then flushed with nitrogen, sealed with a torch and heated in a boiling water bath to convert the sample to methyl esters.

The methyl esters were recovered and analyzed by gas-liquid chromatography using a F & M Model 609 hydrogen flame ionization gas chromatograph equipped with a 7 ft × ¼ in. column packed with ethylene glycol succinate polyester phase (EGSS-X, Applied Science Laboratories) on Chromosorb W, 100–200 mesh at 185C. The fatty acid composition was calculated directly from the proportionalities of the peak areas and expressed as weight percent. The conditions for linearity of detector response were determined with stan-

[2] The stereospecific assignment described by Hirschmann (7) is used to indicate the stereochemistry of the glycerides described in this paper. Glycerol-3-phosphorylcholine will be abbreviated as GPC.

dard mixtures of methyl esters obtained from The Hormel Institute.

Under the conditions employed, the geometric isomers of linoleate could not be separated from each other. However, since the animals were starved of lipid for four months the triglycerides and lecithins were virtually devoid of the naturally-occurring isomer of linoleic acid. Thus, 18:2 analyzed in each experiment may be considered to consist of the isomer of linoleic acid that was fed. That dietary *trans,trans* linoleic acid (linoelaidic acid) was deposited in the lipids of rats was demonstrated by a direct determination in a previous study (8).

The acids liberated by phospholipase A represent those originally esterified at the 2-position and the acyl-GPC content indicates the composition at the 1-position. The ratio of the percentage content of an acid at the 2- and 1-positions was then calculated. This ratio and the total percentage content of the acid in the total lecithins provide a fairly detailed description of the distribution of fatty acids in lecithins using only two values for each acid.

The distribution of the fatty acids in the 2-position of triglycerides was determined with pancreatic lipase under the conditions described by Mattson and Volpenhein (11) scaled down for use on a sample of about one-tenth the size employed by these workers. In this method the hydrolysis was stopped at about 50% completion. The monoglycerides obtained as products of the reaction were isolated from the free acids, di- and triglycerides by TLC. The fatty acid composition of the 2-monoglycerides was determined by forming methyl esters as described above. Since this lipase method does not allow distinction between the composition of the 1- and 3-positions, a ratio representing the distribution between the 2- and the 1-position was calculated using the average value for the primary positions. Thus the estimated 2/1 ratio (secondary/primary positions) for a given acid in the triglycerides is as follows.

$$\frac{2 \times (\%\text{ content in monoglycerides})}{3 \times (\%\text{ content in total triglycerides}) - (\%\text{ content in monoglycerides})}$$

In this way the distribution and composition of fatty acids in the triglycerides can be described using only two values similar to those used for the lecithins described above.

RESULTS

The results in Table I show that the acyltransferases can discriminate between the normal *cis* isomers and the unnatural *trans* forms. The *trans*-9,*trans*-12-isomer was esterified rapidly at the 1-position. When a *cis* configuration was present (in the *trans*-9,*cis*-12-isomer) the rate of esterification at the 1-position of 2-acyl-GPC was considerably diminished. When the *cis* configuration was in the 9-position, it interfered even more seriously with that acylation. The effect of configuration was not as marked on the rates of reaction at the 2-position although the *trans* dienoates reacted significantly less rapidly than the *cis* isomers. For each fatty acid considered in Table I, the enzymatic specific activities determined give different ratios for the rates of esterification at the 2- and 1-positions. In the case of the dienoates, the ratios extend over a 60-fold range from the all-*cis* to the all-*trans* isomers.

TABLE I

Acyltransferase Specificities of Rat Liver Microsomes

The reaction mixtures contained 25 mμmoles of acyl-CoA, 100 mμmoles of 1-acyl or 200 mμmoles of 2-acyl-GPC, and 0.25 mg of rat liver microsomal protein (treated with DFP (2)) in a final volume of 1.0 ml (see Experimental section).

Acyl-CoA[a]	Position acylated		2/1 Ratio
	No. 2	No. 1	
	mμmoles/min/mg protein		
18:0	1.7	19.6	0.087
16:0	3.5	20.3	0.17
14:0	4.3	7.6	0.56
12:0	6.5	7.0	0.93
16:1-c9	6.0	0.4	16.2
18:1-c9	12.4	3.9	3.2
18:1-t9	10.3	23.3	0.44
18:2-c9,c12	23.1	1.8	12.9
18:2-c9,t12	9.1	2.0	4.6
18:2-t9,c12	13.7	13.8	1.0
18:2-t9,t12	6.1	28.4	0.21
18:3-all c	14.6	1.8	8.0
20:4-all c	19.6	2.3	8.5

[a] The number notations indicate the chain length and number of double bonds, with the configuration and position indicated after the hyphen (e.g., elaidate is 18:1-t9).

The effect of decreasing chain length in the naturally-occurring saturated acids was a decrease in the rate of esterification at the 1-position with an increased rate of reaction at the 2-position. This is in agreement with earlier results (3) which indicated that some saturated acids of shorter chain length are handled like the unsaturated long chain acids. The reactivity found for 16:1 at the 1-position was particularly low, making the 2/1 ratio markedly higher than, for example, that found for 18:1. The low activity was checked many times but it consistently remained lower than that for any other acid tested. If the activity for reaction at the 1-position were approxi-

mately 2 mμmoles/min/mg protein, the 2/1 ratio would be 3, and thus closely resemble the results with 18:1.

The relative pattern of activities for the various acids in Table I has also been noted in this laboratory with the microsomal fraction of livers from rats that had been subjected to a variety of diets deficient in fatty acids such as starvation, fat-free and long-term essential fatty acid deficiency. The only noticeable difference from the values reported here was an elevated specific activity (per mg protein) for all acids tested with the enzymes prepared from rats with a severe essential fatty acid deficiency. However, in this case, the relative pattern of activities still remained the same. The observed distribution of octadecadienoate between the secondary and primary positions differed according to the isomer which was fed (Table II). The *cis,cis*-isomer was found predominantly at the 2-position, the *trans,trans* at the 1-position, and the *cis,trans*-isomer gave values intermediate between those two. Although the gas chromatographic method used to measure the acids in this work did not distinguish between the different geometrical isomers, the earlier results of Privett and Blank (8) indicate that isomers other than that added to the diet were not present to any appreciable extent. The calculated 2/1 ratios for the in vivo distribution of acids in the lecithins cover a wide range of values which is in interesting agreement with that found for the in vitro acyltransferase rates.

The positional distribution ratio for the *trans,trans*-isomer was very similar for both the lecithins and triglycerides. The distribution ratios for palmitate in vivo (given in Table II) were very similar to those found for the in vitro acylation rates, and were similar in both the lecithins and triglycerides. This result was not generally noted for those acids which are predominantly found at the 2-position (ratios greater than 1). Octadecenoate, for instance, gave values from 1.2 to 2.1 for the triglycerides and 0.8 to 2.5 for the lecithins. When the diet contained linoleate as a supplement, the resultant 18:1 content of the liver lecithin (see Table III) was only 9%, and a low value (0.8) (see Table II) was obtained for the 2/1 distribution of 18:1 in the lecithins. In these animals, arachidonate accounted for 33% of the total acids and was located almost exclusively at the 2-position. When the diet contained low levels of *cis* double bonds (fat free, *cis,trans,* or *trans,trans*) the content of monoenoic acids in the tissue lipids rose, particularly that of 16:1. In these cases the rise in monoenoate content occurred primarily at the 2-position, whereas the arachidonate content fell, and the 2/1 distribution ratio for the monoenoates in vivo approached values closer to the in vitro results.

DISCUSSION

In enzymatic studies with rat liver microsomes, the *trans* double bond seems similar to a saturated carbon chain in that the *cis*-9,*trans*-12-dienoate most closely resembles the *cis*-9-monoeneoate in acylation rates, whereas the *trans*-9,*trans*-12-dienoate most closely resembles the *trans*-9-monoeneoate. Also, esterification at the 2-position does not seem to be as sensitive to configurational differences as that at the 1-position which is in agreement with earlier results (3). The latter position is only slowly esterified by acids containing a *cis*-9 configuration. Additional comparisons of these acids suggest that at the 1-position the most linear *trans* configuration is slightly preferred even to the saturated chain. The ability of the *cis*-9 configuration to diminish this reaction rate more effectively than the *cis*-12 raises an interesting question of the effect of other positional isomers. The Δ9-desaturase system in rat

TABLE II

Positional Distribution of Fatty Acids in Rat Liver Lipids[a]

Dietary supplement	Fraction analyzed	2/1 Ratio				
		16:0	18:0	16:1	18:1	18:2[b]
Fat free	Lecithin	0.03	0.0	1.1	2.0	....
	Triglyceride	0.17	0.05	0.8	2.1	....
t9,t12	Lecithin	0.16	0.10	2.0	2.5	0.6
	Triglyceride	0.27	0.25	1.3	1.8	0.5
c9,t12	Lecithin	0.13	0.05	2.3	2.3	2.5
	Triglyceride	0.12	.....	1.1	1.8	1.4
c9,c12	Lecithin	0.14	0.11	0.7	0.8	5.5
	Triglyceride	0.15	0.5	1.0	1.6	4.9

[a] Within the limits of detection in these experiments the eicosapolyenoates, 20:3 and 20:4, were located at the 2 position.

[b] The values in this column differ from those in the others, in that each pair represents a different acid which was fed.

TABLE III

Composition of Rat Liver Lecithins and Triglycerides

Diet	Lipid fraction	18:0	16:0	16:1	18:1	18:2[a]	18:3[a]	20:3[a]	20:4[a]
Fat free	Lecithin	15.6	24.1	8.5	29.1	1.7	0.5	14.9	2.2
	Triglyceride	2.1	31.6	13.5	51.5	.....	....	.....	.....
t9,t12	Lecithin	11.6	18.5	10.2	35.7	13.3	3.9	5.0	1.4
	Triglyceride	3.3	28.6	13.2	49.2	5.7	....	.....	.....
c9,t12	Lecithin	15.7	21.9	5.0	29.3	8.7	....	7.4	12.0
	Triglyceride	1.5	27.1	10.3	55.0	5.3	0.8	.....	.....
c9,c12	Lecithin	19.2	20.9	1.5	9.2	14.6	....	1.5	33.0
	Triglyceride	2.4	29.4	7.7	53.5	5.4	....	.....	.....

[a] Different geometrical isomers are present depending upon the dietary acid.

liver is known to produce other positional isomers through chain lengthening or shortening reactions. This process is recognized to be the source of vaccenate (12), and may be responsible for 8,11-octadecadienoate and 4,7,10,13-eicosatetraenoate in the liver. The different acyltransferase specificities found in this paper suggest that future studies could show differences in acyltransferase activity for the different positional isomers (13) of the unsaturated acids.

The ratio of the enzyme-catalyzed rates of esterification at the 2- and 1-positions has been presented as a characteristic number for each acid. This ratio would predict the relative distribution in vivo of a fatty acid between the two positions of lecithin if the acyltransferases in the tissue were saturated with acyl-CoA and operating under the maximal conditions used for the in vitro experiments. This may often be a real situation since the reported levels of acyl-CoA in liver are at least 5–10 times higher than the K_m observed in our enzymatic studies (2). However, it would also be useful to note that these data suggest that competitive effects could occur with a mixture of acids. For instance a mixture of the *trans*-9,*cis*-12- and *trans*-9,*trans*-12-acids would give most of the *trans*-9, *cis*-12-isomer at the 2-position even though that acid has equal rates of esterification at both positions in a system where competition is not involved (Table I). Differences between the actual in vivo distributions and the enzymatic 2/1 ratios given in this paper could be due to such competitive effects. The *trans,trans* supplemented diet, for instance, produced lipids that contained mostly palmitate, stearate and oleate. Thus the octadecadienoate had only oleate as a significant competitor at the 2-position, whereas at the 1-position it competed with palmitate and stearate which both go well into that position. In such a situation, the 2/1 ratio could be expected to be shifted to a value (0.6) slightly higher than that noted in the noncompeting spectrophotometric assay system (0.21). Probably the most important additional factor altering the in vivo distribution in these experiments is the widely varying content of eicosapolyenoic acids in the lecithins as a result of the unusual diets. These acids account for about 60 to 70, 30 to 40, and 10 to 15%, of the acids at the 2-position of the phospholipid from animals of the *cis*-9, *cis*-12, the *cis*-9,*trans*-12, and *trans*-9,*trans*-12 supplemented diets, respectively. It should be emphasized that the enzymatic studies used washed rat liver microsomes in hope that the collection of acyltransferases in this preparation would be representative of the acylating activity of the intact liver. The general agreement between the in vitro and in vivo results suggests that the enzymes studied may be the significant part of those enzymes controlling fatty acid distributions. Furthermore, the observed retention of the relative specific activities of these enzymes under the variety of dietary conditions mentioned earlier suggests that they are a stable characteristic of the normal liver cell. In addition to these results with rat liver, a very close similarity in the observed acyltransferase 2/1 ratios to the distributions found in vivo has also been recognized recently for pig liver lipids (14).

In the case of those acids which have 2/1 distribution ratios greater than 1, there is relatively little of the acid at the 1-position of lecithin. Therefore when the 1,2-diglyceride unit is converted metabolically to triglyceride, incorporating such an acid into the 3-position would cause a marked percentage increase in its average content at the primary (1- + 3-) positions. This increase would then give a lower calculated 2/1 ratio for that acid in the triglycerides than in the choline-phosphoglycerides. On the other hand, the acids which are located predominantly at the primary position of lecithins would be predicted to exhibit less difference in the calculated 2/1 ratios for the two different lipids. These considerations may be significant in interpreting the differences in the 2/1 ratios seen in Table II.

At this time we cannot be certain whether the esterification at the 3-position is random, less specific than the acyl-CoA:phospholipid acyltransferases, or specific for unsaturated acids. More careful experiments which differentiate between the 1- and 3-positions of a triglyceride should help distinguish between these possibilities. One such experimental method has been described by Brockerhoff (15) who has recently noted that asymmetrical fats have an excess of palmitate at the 1-position with more oleate at the 3-position (16). Further results of this type will be particularly useful in determining the selectivity of the enzymes catalyzing the esterification of the 3-position. These results will also allow a more precise comparison of the distributions of acids between the 1- and 2-positions of the triglycerides for comparison with those found in lecithins.

At the present, we can already see a considerable similarity in the positional distribution of acids in the diglyceride units of liver lecithins and triglycerides. It is thus somewhat ironic that the discovery of acyltransferases began with the recognition that *the diglyceride unit of phospholipids* is metabolically different in some respect from that of the triglycerides (17). This conclusion was based on different rates of isotopic tracer incorporation, and it led to the present concept of the acyl-CoA:phospholipid acyltransferases incorporating fatty acids into lecithins without involving the diglycerides or triglycerides as intermediates. This metabolic difference has again been shown in the recent studies of stearic acid incorporation reported by Elofson (18). Comparison of the diglyceride radioactivity with that of the triglycerides and phosphoglycerides led Elofson to the conclusion that in vivo the absolute majority of labeled stearate in glycerolipids is incorporated via the acyl-CoA:phospholipid acyltransferase pathway. Thus the results with isotopic tracers indicate the differences in metabolic turnover rates of the ester bonds whereas the results of the positional analyses for each acid indicate that the 1,2-diglyceride units of lecithins and triglycerides are similar and may slowly equilibrate with each other. Such processes would allow the specificities of the acyl-CoA:acyl-GPC acyltransferases to be observed in both lecithins and triglycerides.

ACKNOWLEDGMENT

Supported in part by grants from the United States Public Health Service, AM-05310 and AM-04942.

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[Received Jan. 10, 1966]

Quantitative Recovery of Short Chain Free Fatty Acids after Gas Chromatography

DURING STUDIES of lipid metabolism in lung tissue, we found it desirable to analyze the radiopurity of short-chain fatty acids for substrates. For this purpose, we developed a simple, inexpensive collection system for attachment to a gas chromatograph that made it possible to recover chromatographed samples quantitatively for further analyses. The technique was equally useful for isolating components from a fatty acid mixture for subsequent characterization by independent analytical techniques, e.g. infrared spectroscopy. The short-chain fatty acids separated with this method have been notoriously difficult to handle due to their volatility. However, using this collection system with columns containing polar stationary phases and nonvolatile acidic components (1–3) we could analyze and collect the free acids easily.

Hexanoic, heptanoic, octanoic, nonanoic, and decanoic acids were purchased from Distillation Products, Inc. Octanoic acid was redistilled before use. C^{14}-labeled octanoic acid was purchased as sodium octanoate from New England Nuclear Corp. The fatty acids were separated on a 5 ft. × ¼ in. stainless steel column containing 15% (w/w) diethylene glycol succinate polyester (DEGS) and 3% (w/w) phosphoric acid on 100–120 mesh Gas-Chrom Q. Column temperature was 140C. The instrument used was a Barber-Colman Model 5000 dual column gas chromatograph equipped with thermal conductivity detector.

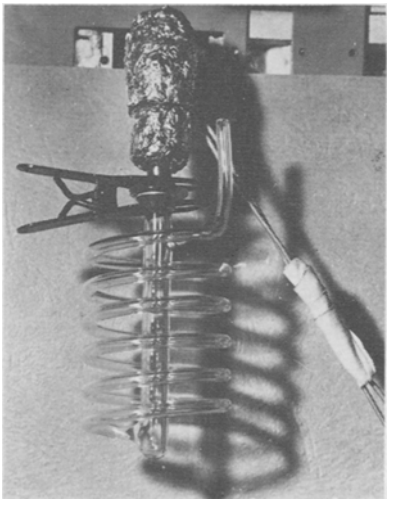

FIG. 1. Collection system. The 12 cm stainless steel connecting tube, ¼ in. I.D., fitted with a size 12/1 stainless steel ball joint, [Kontes Glass Co., Vineland, N. J., K-67450] is wrapped with a Briskeat flexible heating tape, ⅜ in. × 12 in., held with metal foil. The temperature within the ball-joint was 200C. The glass condensing vessel, made up with a female joint to match, is held in place by a spring clip. The height of the vessel from joint to bottom is 10 cm.

The sampling tube emerging from the detector was shortened and fastened to a Swagelok reducing union (⅛ in. × ¼ in.) which was placed on the inside wall of the detector oven. A stainless steel tube 12 cm long (¼ in. I.D.) with ball joint was attached to the Swagelok fitting, and was bent 90° so that the opening of the ball joint faced downward. A flexible heating tape (Briskeat ⅜ in. × 12 in.) was wrapped around the tube and covered with foil as shown in Figure 1. Heating the sampling tube was essential to good recovery. The glass collecting vessel was constructed so that the sample could be removed quantitatively by means of a capillary pipette introduced into the bottom of the vessel. The vessels were fastened to the sampling tube with a spring clip.

Sampling procedure: In advance, retention times of desired components were determined under conditions employed; one had in readiness the same number of collection vessels as samples were required. These vessels were chilled in an acetone/dry ice bath. Injection into the GLC column was then made and the recorder was observed. When the peak of the desired component appeared on the recorder, a collection vessel, kept immersed in the acetone/dry ice mixture, was attached to the sampling tube and was removed after the peak was complete. Times of attachment and removal for capturing a single component are shown by the dotted lines in Figure 2. A time lapse of several seconds was necessary under the conditions we employed for the sample to pass from the detector to the sampling tube. The neck of the vessel and the condenser were washed down carefully with a few milliliters of hexane immediately after removal from the apparatus to prevent loss of volatile sample.

Liquid scintillators used were 0.4% (w/v) 2,5 diphenyloxazole and 0.02% (w/v) 1,4-[2- (5-phenyloxazolyl)] benzene in toluene. The counting efficiency was 48%. Radioassay

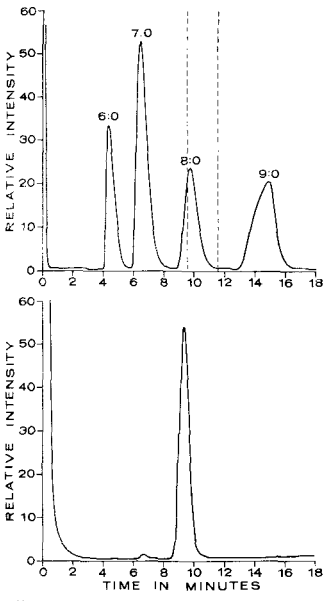

FIG. 2. Comparison of gas chromatograms of a mixture of fatty acids (upper curve) and of the collected sample of octanoic acid (lower curve). The dotted lines indicate times of attachment and removal of collection vessel. For conditions, see text.

was carried out using a Tri-Carb Scintillation Spectrometer (Packard Instruments, La Grange, Ill.).

Recovery was measured by injecting 10 μl of 0.1% C^{14}-labeled octanoic acid in hexane solution into the chromatograph, while the first collection vessel was already in place. All samples emerging for a period of 30 min. were collected in three fractions. The previously determined retention time of unlabeled octanoic acid was 12 min. Radioactivity was concentrated in the sample that coincided with the peak having the retention time of octanoic acid. Table I summarizes the results of two typical experiments. The recoveries of C^{14} in the octanoic acid peak were 90% and 102%.

It was easy to remove a single component from a mixture of short-chain fatty acids with this technique. First, separate samples of the desired acids were chromatographed to determine their retention times under the conditions employed. Following this, hexanoic, heptanoic, octanoic, and nonanoic acids (17 μl fatty acid mixture in 17 μl hexane) were injected. The desired fatty acid was collected after its peak appeared on the recorder and the collection vessel was removed before the following peak was anticipated, as indicated by the dotted lines in Figure 2. Rechromatography of the collected fatty acid showed a virtually pure sample, cf. lower curve, Figure 2.

TABLE I
Recovery of 1-^{14}C-Octanoic Acid after Gas-Liquid Chromatography

Collection time of sample after injection	Trial 1	Trial 2
min	cpm	cpm
0– 9	0	0
9–14	234	248
14–30	9	11
Total recovered	243	259
Percentage of injected sample recovered	94	107

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ACKNOWLEDGMENT

Work supported by USPHS Research Grant AM 06968.

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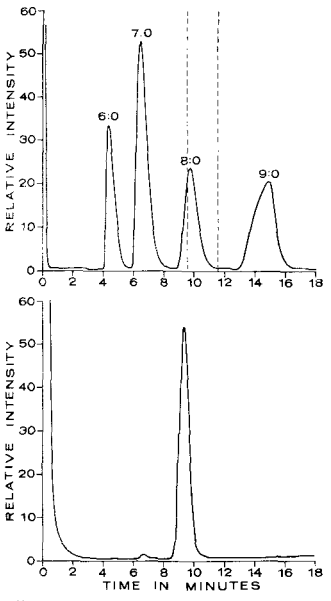
[Received March 7, 1966]

Quantitative Separation of Methyl 9-Hydroxystearate from Methyl 13-Hydroxystearate by Column Chromatography on Silica Gel

IN HIS REVIEW, Radin (1) cites methods for separation of hydroxy fatty acids; however, no mention is made of the quantitative column separation of different isomers of the saturated hydroxy fatty acids of the same chain length. Recently, Morris (2) described the migration behavior of a large number of saturated C_{18} isomeric hydroxy fatty acids on thin-layer chromatograms. This communication reports the quantitative separation of methyl 13-hydroxystearate from methyl 9-hydroxystearate by preparative column chromatography.



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Methyl 9-hydroxystearate was obtained by hydrogenation of methyl dimorphocolate (3) and purification on a silica gel column. Methyl 13-hydroxystearate was obtained by a procedure to be published shortly. Identity and purity of these compounds were established by mass spectrometry and were verified by comparing melting point, gas-liquid chromatography, thin-layer chromatography, and infrared spectroscopy characteristics with known pure isomers. The mass spectra of the isomeric methyl hydroxystearates give a characteristic fragmentation pattern by cleaving the carbon-carbon bonds on each side of the carbon atom to which the hydroxy group is attached. This fragmentation pattern was found for known pure methyl 8-, 9-, 10-, 12- and 13-monohydroxystearates and is consistent with the interpretation of the methyl 8- and 10-monohydroxystearates as given by Ryhage and Stenhagen (4).

The silicic acid used was the highly purified Adsorbosil powder for column chromatography (Applied Science Laboratories, Inc.) of mesh 100/140. (The pH of the powder when mixed with distilled water should not be below 4.) The glass column, 0.6 cm I.D. and 35 cm long, was equipped with a Teflon stopcock at the bottom to control flow. The solvent was redistilled *n*-hexane (bp 69C) and anhydrous absolute diethyl ether.

Adsorbosil (3.5 g) was slurried with 15 ml of solvent mixture (hexane-ether 98:2) and poured into the column with tapping to a height of about 13 cm. The rate of flow was then adjusted to 30 ml/hr and at least 10 ml of the same solvent was allowed to flow through the column until the liquid level dropped to the top of the column. A sample was then introduced. The size of the samples applied ranged from 30 to 150 mg methyl esters of fatty acids. If methyl esters of normal saturated fatty acids (e.g., methyl stearate) were present, the sample was applied in hexane-ether (98:2); when only methyl esters of hydroxy fatty acids were present, hexane-ether (90:10) was used. The eluate was collected in 2 ml fractions, the solvent evaporated under vacuum, the residue weighted, and a weight curve plotted.

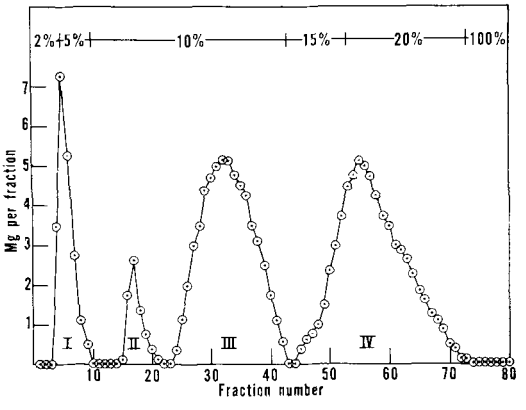

FIG. 1. Quantitative column separation of methyl stearate (I), methyl keto stearate (II), methyl 13-hydroxystearate (III) and methyl 9-hydroxystearate (IV) on Adsorbosil. A sample containing 150 mg was applied and collected in 80 fractions of 2 ml effluent each (98.5% recovery). Percentage refers to concentration of diethyl ether in *n*-hexane.

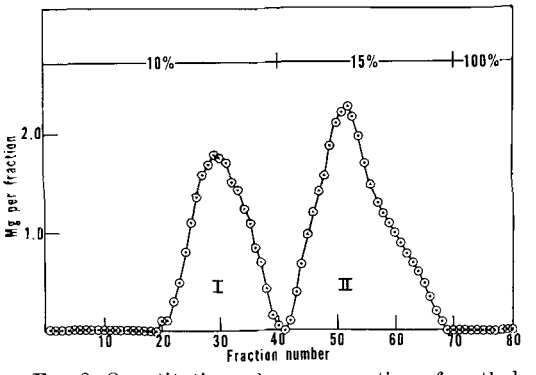

FIG. 2. Quantitative column separation of methyl 13-hydroxystearate (I) from methyl 9-hydroxystearate (II) on Adsorbosil. A sample containing 55 mg was applied on the column and 98.0% recovered. Percentage refers to concentration of diethyl ether in *n*-hexane.

The degree of separation obtained corresponding to the solvent system used is shown in Figures 1 and 2. More than 99% of the methyl 9-hydroxystearate was recovered with 100% purity, and more than 97% of the methyl 13-hydroxystearate, with greater than 95% purity. Each one of the isomers was rechromatographed by itself on the column and each gave a single peak. The identity of each peak was reconfirmed by its characteristic mass spectrum. A known weight of the isomers was then mixed together and refractionated on the column into the two known isomers, as described.

This procedure gives a fast quantitative separation of useful quantities of two hydroxy fatty acids. By varying the composition of the solvent system, a more complex mixture of hydroxy fatty acids could probably be separated.

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ACKNOWLEDGMENT

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[Received Feb. 16, 1966]

Cis-2-Octenoic Acid Administration and Essential Fatty Acid Synthesis

BRENNER ET AL. (1) were not able to confirm, in rats, the phenomenon of elongation of *cis*-2-octenoic acid to linoleic acid reported by Murty et al. (2,3) to take place in laying hens. In view of this conflicting response by the two classes of animals, and the differences in experimental conditions, it was thought desirable to repeat the study with rats under the same conditions used with laying hens.

Eight male weanling albino rats were reared on a fat-free diet (Nutritional Biochemicals Co., Cleveland, Ohio) for six weeks. All of the animals had access to the fat-free ration during the test period.

On the day of the test the animals were divided into 2 groups of 4 rats each. The controls were given hourly intraperitoneal injections of 62.5 μc of 1-C^{14}-sodium acetate for 4 hr. The animals in the experimental group, in addition to the acetate, were given individually 2 ml methyl *cis*-2-octenoate by stomach tube. Both groups were sacrificed at the end of the fourth hour and the liver, adipose tissue, and other viscera removed.

Total lipids were extracted with chloroform-methanol (2:1) and the methyl esters prepared by refluxing the lipids for 4 hr in 2% sulfuric acid in absolute methanol.

Preparative gas-liquid chromatography (GLC) (4.9 m × 6.25 mm O.D.; 30% SE-30 on 30–60 mesh firebrick) was used to collect liver methyl esters of the acids of carbon length 14, 16, 18 and 20 and viscera esters of carbon length 18. Preparative thin-layer chromatography (TLC) with silver nitrate impregnated Adsorbosil-1 (Applied Science Laboratories, State College, Pa.) was used to isolate liver fractions containing palmitic, palmitoleic, stearic, 18:1 and 18:2 acids and viscera 18:2 acids.

The 18:2 fraction from the viscera fat was oxidized with periodate-permanganate and methyl azelaate obtained by preparative GLC of the reaction products. The amount of methyl azelaate was determined by adding a known weight of methyl pimelate and comparing peak areas on an analytical chromatogram.

All methyl esters were mixed with 20 ml *scintillation solution* (4 g 2,5-diphenyloxazole and 100 mg 1,4-di(2,5-phenyloxazolebenzene) in one liter toluene at room temperature) and radioactivity determined by liquid scintillation spectrometry (Packard Instrument Co., La-Grange, Ill.).

We could find no evidence in the liver for the conversion of *cis*-2-octenoic acid by addition of acetate-1-C^{14} to any of the acids isolated. As seen in Table I, the effect of the octenoate was that of inhibition of fatty acid synthesis. Octenoic acid thus appears to differ from octanoic acid which Reiser et al. (4) observed to stimulate liver fatty acid synthesis of palmitic acid, possibly by elongation. Although the experimental conditions differed in the two studies, the contradictions in results cannot be explained entirely on these differences. Instead, it would seem that octenoate and octanoate are handled differently by animal tissues. Thus, while short chain saturated acids stimulate

TABLE I

Radioactivity of Liver and Adipose Tissue Lipids

Acid	Radioactivity	
	Control dpm/mg	Test dpm/mg
Adipose tissue fatty acids	1,199[a]	430
Liver fatty acids		
Composite	32,309	10,537
Individual		
14:0 + 14:1[b]	27,613[a]	7,310
16:0	25,107	12,766
16:1	4,740	3,246
18:0	14,577	8,461
18:1	16,641	5,938
18:2	2,268[c]	1,443
20:3 + 20:4[b]	4,776	3,035

[a] Each figure is the average radioactivity from 4 rats.
[b] 14:0 and 14:1, 20:3 and 20:4 were not separated, but were counted as a mixture.
[c] 18:2 acids were pooled to obtain enough material to determine specific activity.

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All methyl esters were mixed with 20 ml *scintillation solution* (4 g 2,5-diphenyloxazole and 100 mg 1,4-di(2,5-phenyloxazolebenzene) in one liter toluene at room temperature) and radioactivity determined by liquid scintillation spectrometry (Packard Instrument Co., La-Grange, Ill.).

We could find no evidence in the liver for the conversion of *cis*-2-octenoic acid by addition of acetate-1-C^{14} to any of the acids isolated. As seen in Table I, the effect of the octenoate was that of inhibition of fatty acid synthesis. Octenoic acid thus appears to differ from octanoic acid which Reiser et al. (4) observed to stimulate liver fatty acid synthesis of palmitic acid, possibly by elongation. Although the experimental conditions differed in the two studies, the contradictions in results cannot be explained entirely on these differences. Instead, it would seem that octenoate and octanoate are handled differently by animal tissues. Thus, while short chain saturated acids stimulate

TABLE I

Radioactivity of Liver and Adipose Tissue Lipids

Acid	Radioactivity	
	Control dpm/mg	Test dpm/mg
Adipose tissue fatty acids	1,199[a]	430
Liver fatty acids		
Composite	32,309	10,537
Individual		
14:0 + 14:1[b]	27,613[a]	7,310
16:0	25,107	12,766
16:1	4,740	3,246
18:0	14,577	8,461
18:1	16,641	5,938
18:2	2,268[c]	1,443
20:3 + 20:4[b]	4,776	3,035

[a] Each figure is the average radioactivity from 4 rats.
[b] 14:0 and 14:1, 20:3 and 20:4 were not separated, but were counted as a mixture.
[c] 18:2 acids were pooled to obtain enough material to determine specific activity.

fatty acid synthesis, their unsaturated counterparts hinder synthesis and are actually toxic.

Since azelaic acid could arise only from oxidation of linoleic acid (Δ9,12) in a mixture of 18:2 acids, the amount of azelaic acid radioactivity was taken as a measure of the amount of linoleic acid synthesis. The small levels of radioactivity observed in the azelaic acid (Table 2) of both control and test animals are in agreement with others who have found low levels of activity in linoleic acid from labeled acetate treated animals. However, this activity is very low compared to that of the other fatty acids (Table 1). It is, therefore, clear that no *cis*-2-octenoic acid was elongated to linoleic acid and the pathway proposed by Murty et al. (2) in hens is evidently inoperative in rats.

TABLE II

Specific Radioactivity of Azelaic Acid[a]

Animal	Test counts dpm/mg	Control counts dpm/mg
1	56	84
2	97	64
3	66	22
4	37	52

[a] Isolated as a product of viscera 18:2 acid oxidation.

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ACKNOWLEDGMENT

This study was supported in part by a grant from the National Institutes of Health (AM 06011).

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[Received Feb. 28, 1966]

Acyl Migration in the Conversion of Lecithin to 1,2-Diglyceride Acetates by Acetolysis

RECENTLY, RENKONEN (1,2) described the conversion of phosphatides to corresponding 1,2-diglyceride acetates to enable the application of triglyceride methods of structural analysis to these compounds. The conversion of phosphatides to the corresponding diglyceride acetates may be carried out by acetolysis (1–3) or via hydrolysis by phospholipase C (4,5) followed by acetylation in the usual manner. The acetolysis method gives high yields of 1,2-diglyceride acetates from lecithins as shown by Renkonen (1) but a shifting of fatty acids from their original positions in the lecithin molecule occurs and this reaction should not be incorporated in procedures for the determination of the positional arrangement of the fatty acids in these compounds. As an example of the type of shifting that occurs, the fatty acid composition of the fatty acids in the 2-position in the 1,2-diglycerides acetates obtained by the two procedures are compared in Table I on a sample of purified egg lecithin. The venom of *Ophiogaphus hannah* was used as the source of the phospholipase A for the assay of the fatty acids in the 2-position according to the procedure described by Robertson and Lands (6). The acetolysis reaction was carried out as described by Renkonen (1). Enzymatic conversion of lecithin to 1,2-diglycerides was carried out essentially as described by MacFarlane et al. (4) with *Clostridium perfringens*. Acetylation of the 1,2-diglycerides was carried out with pyridine-acetic anhydride. Pancreatic lipase hydrolysis of the 1,2-diglyceride acetates was carried out essentially as described by Mattson et al. (7) but using TLC for the isolation of the 2-monoglycerides.

TABLE I

Distribution of Fatty Acids in the 2-Position of Egg Lecithin and the 1,2-Diglyceride Acetates Prepared Therefrom

	Original lecithin (via phospholipase A)	1,2-diglyceride acetates (prepared via phospholipase C—acetylation)	1-2-diglyceride acetates (prepared by acetolysis)
16:0	0.9	1.1	28.4
16:1	0.7	0.7	1.7
18:0	....	0.1	12.3
18:1	55.1	56.5	33.4
18:2	34.8	33.3	17.7
20:4	8.5	8.3	2.9

Since phospholipase A specifically hydrolyzes the fatty acids in the β-position of lecithins and pancreatic lipase specifically hydrolyzes the fatty acids in the 1 and 3 positions of triglycerides, the fatty acid composition of the fatty acids liberated by phospholipase A from lecithins should be identical with that of the

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2-monoglycerides obtained as a product of the pancreatic lipase hydrolysis of the corresponding 1,2-diglyceride acetates.

Table I shows that the agreement in these data is near perfect when phospholipase C is employed for hydrolysis but that there is considerable disparity in the values when acetolysis was used.

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[Received Nov. 17, 1965]

Isolation of Methyl *cis*-15-Octadecenoate by Chromatography on a Silver-Treated Macroreticular Exchange Resin

PREVIOUSLY (1) WE REPORTED that *cis* and *trans* monoene fatty methyl esters can be separated by chromatography on a silver nitrate-treated macroreticular cation-exchange resin. Some fractionation of methyl *cis*-15-octadecenoate from the *cis*-9 and *cis*-12 isomers was noted. Now we find this fractionation great enough so that the *cis*-15 isomer can be isolated in approximately 25% yield by repeated passage of fractions rich in the *cis*-15 isomer through such chromatographic columns.

The starting material is a mixture of methyl *cis*-9-, -12-, -15-octadecenoates prepared by hydrazine reduction of linolenic acid followed by isolation of the monoene fraction by countercurrent distribution of methyl esters (4).

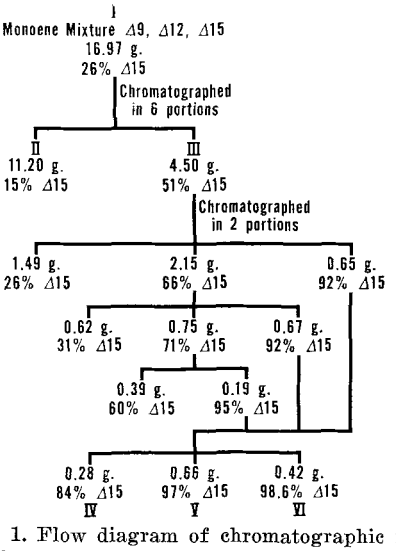

FIG. 1. Flow diagram of chromatographic isolation of methyl *cis*-15 octadecenoate.

The chromatographic procedure is similar to that previously described except that a larger column (approximately 215 cm × 2 cm I.D.) was used with a flow rate of 2 ml/min. A flow diagram outlining a typical series of chromatographic separations is shown in Figure

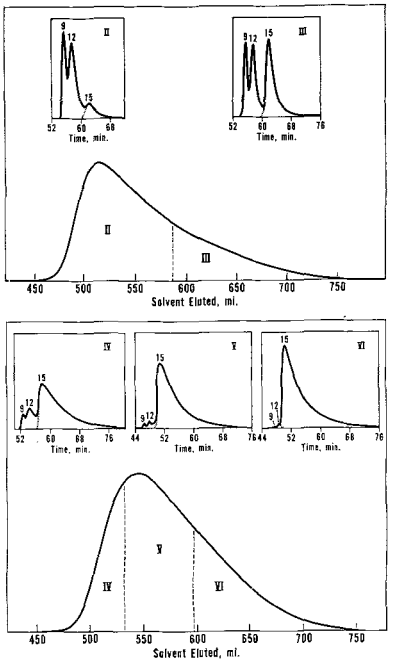

FIG. 2. Representative differential refractometric curves and gas chromatographic curves of fractions for separations in Figure 1. Top curve is the separation of original monoene mixture I to yield fractions II and III. Bottom curve is the final separation to yield fractions IV, V and VI.

2-monoglycerides obtained as a product of the pancreatic lipase hydrolysis of the corresponding 1,2-diglyceride acetates.

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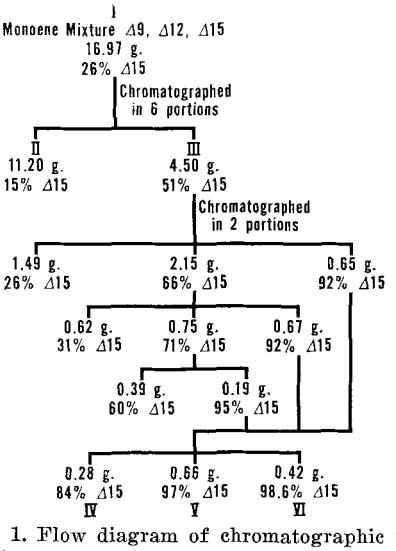

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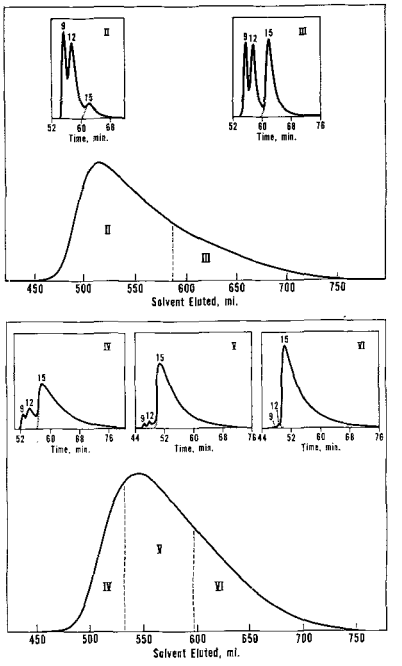

FIG. 2. Representative differential refractometric curves and gas chromatographic curves of fractions for separations in Figure 1. Top curve is the separation of original monoene mixture I to yield fractions II and III. Bottom curve is the final separation to yield fractions IV, V and VI.

1. Since the *cis*-15 monoene does not form a separate peak but only a *cis*-15 monoene-enriched tail on the main monoene peak, the cutting of fractions must remain a matter of experience and judgment. As a guide to the operation, Figure 2 presents refractometric curves of the first and last chromatographic separations from Figure 1. Fractions were analyzed by capillary gas chromatography on a 200 ft × 0.01 in. Apiezon L column to determine the *cis*-15 contents shown in Figure 1. The GLC curves of the fractions are also included in Figure 2.

Fraction VI was distilled under vacuum from a small short-path alembic flask. Physical properties of the distilled methyl esters were melting point 10C, N_{30}^{D} 1.4476 and density at 30C 0.8724 g/ml. Acids prepared from these esters melted at 40 to 41C. Equivalent chain lengths for the esters (2), measured on 200 ft × 0.01 in. capillary columns, including values for *cis*-9-octadecenoate (methyl oleate) for comparison, are listed:

	Apiezon L	DEGS
Methyl *cis*-15-octadecenoate	17.93	18.75
Methyl *cis*-9-octadecenoate	17.61	18.39

Comparison of the nuclear magnetic resonance spectrum of this compound with those of methyl oleate and methyl linolenate (3) shows that in common with linolenate the terminal methyl proton signal is shifted slightly downfield by the β-olefinic double bond.

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ACKNOWLEDGMENT

We are indebted to Miss Patricia Jonas and Lewis Coulson for much of the experimental work and to C. A. Glass for the nuclear magnetic resonance spectra.

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[Received March 7, 1966]

Lipid Synthesis in Peripheral Nerve from Alloxan Diabetic Rats[1]

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ABSTRACT

Decreased conduction velocity in the peripheral nerves of rats is noted after induction of diabetes. The slowing of nerve conduction is accompanied by a decrease in the in vitro incorporation of radioactive precursors into some of the myelin lipids isolated from nerve segments. Cerebroside synthesis is more depressed than that of any other fraction. A change in the type of cerebrosides synthesized is seen with a pronounced decrease in the rate of incorporation of saturated fatty acids.

INTRODUCTION

A NUMBER OF METABOLIC changes take place in experimental diabetes whether induced with alloxan or through pancreatectomy. Simultaneously with these changes the function of the peripheral nervous system is affected (1), and one functional alteration is shown schematically in Figure 1. The upper half represents a normal rat, the lower a diabetic one. Electrical stimulation is applied simultaneously to the sciatic nerves from normal and diabetic animals. The progression of the recorded impulse in the diseased nerve lags behind that of the corresponding impulse on the normal side. This is indicated by the relative positions of the symbols at time intervals labelled 1, 2, and 3. Such a slowing of nerve conduction velocity is also seen in human diabetes (2).

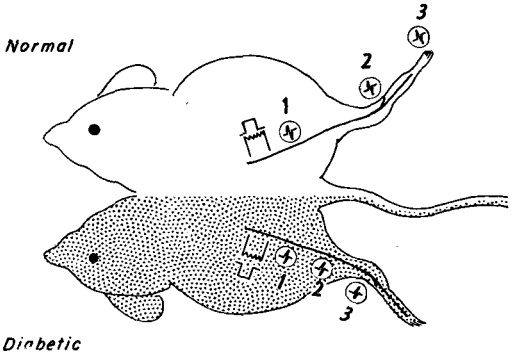

FIG. 1. Conduction of electrically evoked impulses in sciatic nerves from normal (upper half) and diabetic (lower half) rats. The distance a volley of impulses has traveled at time intervals 1, 2, and 3 is given by the location of the numbered symbol.

In order to investigate the reasons for the slower conduction, single nerve fibers were isolated from the sciatic nerve of diabetic rats and compared with normal nerve fibers (3). It was found that in the process of passage from node to node the impulse "leaked" out through each internode, thus delaying the excitation process. The "leakage" was due to a 70% decrease in the transverse resistance of the nerve fiber from the diabetic rat with no apparent change in the capacitance. Such a decrease in resistance could conceivably be due to alterations in myelin sheath composition. A study was undertaken of the composition and the lipid metabolism of nerve segments from alloxan diabetic rats to see whether any metabolic parallel to the pathophysiologic phenomenon described could be found.

MATERIALS

Stearoyl-1-^{14} and oleoyl-1-^{14}C-CoA were synthesized from the radioactive acids by the method described by Goldman and Vagelos (4). The acids were purchased from Nuclear-Chicago as were the sodium acetate-1-^{14}C and the galactose-1-^{14}C. Psychosine was prepared from crude cerebrin (Nutritional Biochemicals Corp.) by the procedure of Carter and Fujino (5).

The rats were of the Sprague-Dawley strain from Holtzman Company. Alloxan was obtained from Distillation Products Industries, and was prepared and injected in citrate buffer as described by Klebanoff and Greenbaum (6). The induced diabetic state was evaluated weekly and animals selected on the basis of the criteria previously given (1).

METHODS

Experimental

Nerves were quickly removed from the stunned animals and incubated in a Dubnoff shaker in Krebs Ringer-bicarbonate buffer at 37.4C for 2–4 hr unless otherwise indicated. The gas phase was usually 95% O_2 + CO_2; in a few experiments room atmosphere was used. Radioactive precursors were added after 30 minutes of preincubation.

[1] Winner, AOCS Bond Award. Presented at the AOCS Meeting, Cincinnati, October 1965.

Extraction of Lipids

Immediately after incubation the nerves were placed in a mortar and ground with dry ice. The pulverized material was transferred quantitatively and extracted according to Folch, Lees and Stanley (7).

Fractionation of Lipids

The peripheral nerve extracts were fractionated on silicic acid columns prepared according to Hirsch and Ahrens (8) and using their stepwise elution scheme with hexane, diethylether and methanol.

Cerebrosides were analyzed according to Kishimoto and Radin (9), i.e. after extraction and drying of the lipids the cerebrosides were freed from cholesterol and phospholipids on Florisil columns and purified via mild alkaline saponification and passage through ion-exchange resins. Methyl esters of fatty acids were obtained from the cerebrosides after acid methanolysis and esterification with dimethoxy propane (10). Normal and hydroxy esters were obtained and were further separated into saturated and unsaturated fractions after formation of the mercuric acetate addition products.

Cerebrosides were quantitated with a modified anthrone method (11).

Separation by TLC

Thin-layer chromatography was performed with the use of Adsorbosil as the adsorbent and a benzene-ethyl acetate (2:1) solvent system followed by chloroform-methanol-water (65:25:4). The components were identified by comparison with migration rates of standard compounds (Applied Science Laboratories, Inc.) and by staining with ninhydrin, Dragendorff and the ammonium molybdate-perchloric acid reagents as outlined by Wager, Hörhammer and Wolff (12). Lyso compounds were tentatively identified from their reactions with rhodamine B (0.05% in ethanol) under normal and UV light (12) and 2,4-dinitrophenylhydrazine, 0.5% in 2N. HCl was used for plasmalogens (13). Unstained zones were scraped from the plates, eluted with the appropriate solvent and used for radioassay.

Gas Chromatography

Gas-liquid chromatography was used for the analysis of fatty acid methyl esters. A RESCO 600 series gas chromatograph equipped with a strontium-90 detector was used. The columns were 6 ft × ¼ in. O.D. stainless steel. Packings used for fatty acid methyl ester analysis were: a) 20% diethyleneglycol succinate polyester coated on Chromosorb W, 80/100 mesh; and b) 5% SE-30 on Gas-Chrom RZ, 60/80 mesh. Argon flow was 80-100 ml per minute; the detector temperature was 240C and the column temperature varied.

The peaks were identified by comparison with standard mixtures and from a semilogarithmic plot of retention times versus chain length.

Radioactivity Determinations

Radioactivity was determined with a Packard Tri-carb Scintillation Spectrometer, using the scintillation mixture described by Bray (14). The counting efficiency was approximately 50%. Quenching was explored systematically by internal-standard technique.

RESULTS AND DISCUSSION

The results of incubation of excised peripheral nerve segments from diabetic and normal animals are shown in Table I. The rate of synthesis of triglycerides, 3-β-sterols, and the combined phospholipid and glycolipid fractions from acetate-1-^{14}C is decreased in the nerve fragments from the diabetic rats, as compared to the normal. The triglyceride fraction in peripheral nerve is probably chiefly in the connective tissue (15), varies greatly between animals (16), and is less likely to contribute to the excitatory process except for minor changes in external resistance. The depression in the synthesis of digitonin-precipitable sterols involves mostly cholesterol; other 3-β-sterols probably occur in relatively minor amounts. Changes in nerve sterol metabolism and content produced with inhibitors of cholesterol synthesis did not affect the nerve conduction velocity (17). The change in glycolipids and phospholipids appeared most promising, and the incorporation of acetate into these lipid subfractions from the normal and diabetic nerve

TABLE I

Total Radioactivity in Lipids from Sciatic Nerves of Normal and Diabetic Rats After Three Hours Incubation with Acetate-1-^{14}C

Lipids	Counts/min/g fresh tissue ± S.D. Normal	Diabetic
Triglycerides	14500 ± 1400 (21)	6300 ± 850 (18)
3-β-sterols	2400 ± 450 (21)	1480 ± 270 (18)
Phospholipids + glycolipids	7300 ± 850 (20)	3700 ± 420 (18)

Incubation mixture: Krebs-Ringer bicarbonate 2.0 ml; glucose, 9×10^{-2}M; potassium acetate, 1×10^{-3}M. Acetate-1-^{14}C, 8×10^{-6}M.
Atmosphere: 95% O_2 + 5% CO_2.
Time: 3 hr at 37C.
Number of animals given in parentheses.

TABLE II

Typical Distribution of Counts After Thin-Layer Chromatography of Sciatic Nerve Lipids. Precursor: Acetate-1-^{14}C

Lipid	Counts/min/g fresh tissue	
	Normal	Diabetic
Plasmalogen	1250	930
CEREBROSIDES	3200	660
Phosphatidyl ethanolamine	20	40
Cerebroside sulfatides	300	250
Phosphatidyl choline	2100	910
Lysophosphatidyl ethanolamine	600	700
Sphingomyelin	860	750
Lysophosphatidyl choline	40	140
Gangliosides	130	—[a]

[a] Cannot be distinguished from background Incubation mixture: See Table I. TLC on Silica Gel G developed with chloroform/methanol/water: 65/25/4.

was determined. The lipids were separated on thin-layer chromatograms. Individual lipids were eluted and counted as shown in Table II. Although decreased rates of synthesis were noted in several fractions, the cerebrosides were studied first because of their significance as myelin lipids (18).

The decreased synthesis of cerebrosides presumably could reflect a decreased synthesis of the whole or part of the cerebroside molecule. It seemed appropriate to make an in vivo study of the cerebroside synthesis in young animals in which the rate would be at an optimum (19). Diabetes was induced at the age of 8 days. At 16 days these animals and normal animals were injected intraperitoneally with either galactose-1-^{14}C or stearoyl-1-^{14}C-CoA (Table III). The amount of radioactivity incorporated into the cerebrosides from the two different precursors was measured. There were larger amounts of labelled stearoyl-CoA in the control nerve than in the nerve from the diabetic animal 12 hr after injection. No such difference was noted when radioactive galactose was used as precursor. The major difference in cerebroside synthesis between the normal and

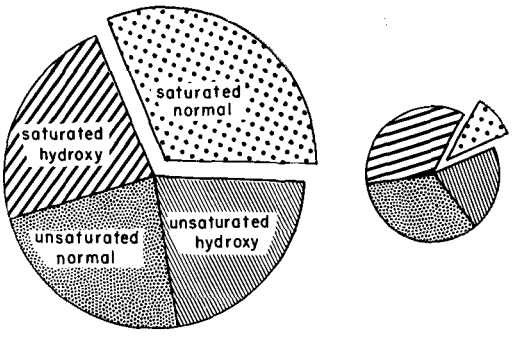

FIG. 2. Rate of synthesis of cerebroside fatty acids isolated from normal and diabetic sciatic nerves. The areas of the pie charts represent the rate of synthesis of total fatty acids; the segments, the rate of synthesis of the four groups of fatty acids: normal saturated, unsaturated and saturated and unsaturated hydroxy fatty acids. The decrease in the saturated, normal fatty acid fraction is emphasized.

the diabetic animal could be attributed to a decrease in the rate of incorporation of fatty acids.

The cerebrosides in nerves from normal and diabetic rats were isolated after incubation with acetate-1-^{14}C in vitro, and the fatty acids were separated according to Kishimoto and Radin (9). The rate of incorporation into all the fatty acids, as well as the amount of acetate taken up in each of four fractions was measured. The area of each circle in Figure 2 reflects the rate of synthesis in the nerves from the two kinds of rats. The segment representing rate of uptake into saturated, normal fatty acids is relatively much smaller in the diabetic animal.

One possible explanation would be an inability on the part of the enzyme systems in the diabetic organism to incorporate saturated acids into cerebrosides. Support for this interpretation is presented in Table IV. The

TABLE III

Incorporation in Vivo of Galactose-1-^{14}C and Stearoyl 1-^{14}C-CoA into Cerebrosides from Sciatic Nerves. Rats, 16 Days Old. Diabetic Group Alloxanized on 8th Day

Time after injection Hours	Galactose-1-^{14}C		Stearoyl-1-^{14}C-CoA	
	Normal counts/min/μ mole	Diabetic counts/min/μ mole	Normal counts/min/μ mole	Diabetic counts/min/μ mole
3	20	43	365	495
6	92	67	882	574
12	243	194	2045	690
24	63	100	1640	600

Intraperitoneal injection of galactose-1-^{14}C (1.6 mC/mM) 50 μC per kg. of body weight, or stearoyl-1-^{14}C-CoA (1.1 mC/mM) 50 μC per kg of body weight.

TABLE IV

Conversion of Acyl-1-^{14}C-CoA to Cerebrosides

Acceptor	Acyl-CoA	Radioactivity of the recovered cerebrosides in counts/min	
		Normal	Diabetic
Psychosine	Stearoyl-1-^{14}C-CoA	1465	14
Psychosine	Oleoyl-1-^{14}C-CoA	1300	1630

Incubation Mixture: Stearoyl-1-^{14}C-CoA 0.2μ mole = 0.22 μC or Oleoyl-1-^{14}C-CoA 0.2μ mole = 0.20 μC; Tween-20 1 mg; ATP 4 μ mole; Psychosine 1 mg; 500 mg. of sciatic nerve tissue was homogenized in Krebs-Ringer phosphate buffer and centrifuged twice at 800 g. The supernatant was made up to 2 ml with buffer and used.

system outlined by Brady (20) was used with cell-free extracts from either of the two kinds of sciatic nerves and psychosine as an acceptor. Stearoyl-1-^{14}C-CoA or oleoyl-1-^{14}C-CoA was the radioactive precursor. Only negligible amounts of the CoA ester of the saturated acid were incorporated into cerebrosides in this system when the extract was prepared from a diabetic rat's sciatic nerve. The uptake of unsaturated acyl-CoA was the same in both preparations.

One theoretical interpretation of the described changes is diagrammed in Figure 3. Psychosine reacts with three out of four classes of fatty acids; however, a block of some type interferes with the formation of saturated cerebrosides. Whether or not this block involves a psychosine acyl transferase is unclear since attempts to purify this enzyme have failed.

It remains to be determined if the change described in our experimental model of diabetic neuropathy is related to and sufficient to explain the alteration of the physiological properties of the nerve. Erwin and Block (21) have pointed out that the length and the degree of unsaturation of the fatty acids influence the properties of lipid molecules.

A biochemical parallel has recently been described in patients with Pelizaeus-Merzbacher's disease (22). Analysis of brain tissues from autopsy material revealed diminished concentration of cerebrosides (and sulfatides) associated with a deficit of long chain, unsaturated fatty acids.

The importance of such shifts in fatty acid composition must be weighed against the more generalized effect of the diminution in acetic

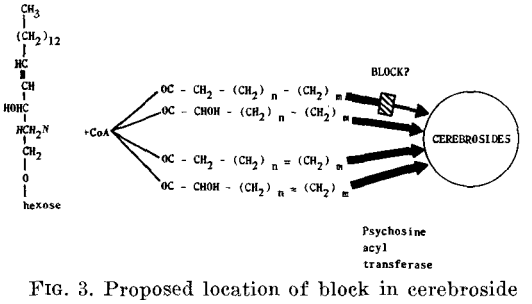

FIG. 3. Proposed location of block in cerebroside synthesis from psychosine and acyl-CoA esters.

thiokinase activity described by Adams and Field (23) and the possible osmotic effect of the now well-recognized accumulation of sorbitol and fructose in nerves from diabetic animals (24,25).

ACKNOWLEDGMENTS

This work was supported by USPHS Grant NB-05204 from the National Institute for Neurological Diseases and Blindness.

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[Received July 30, 1965]

The Synthesis of ^{14}C- and ^{3}H-Labeled Glycerol Ethers

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ABSTRACT

The racemic ^{14}C- and ^{3}H-labeled alpha and beta derivatives of octadecyl glycerol ether (batyl alcohol) and of hexadecyl glycerol ether (chimyl alcohol) of high specific activity were synthesized by treating the appropriate alkyl halides with a large excess of the potassium salts of isopropylidene or benzylidene glycerol. By use of the trifluoroacetic anhydride esterification procedure, the labeled diesters of alpha and beta octadecyl and hexadecyl glycerol ethers were prepared. The labeled monoesters of beta octadecyl and of beta hexadecyl glycerol ethers were isolated from the reaction mixtures by silicic acid column chromatography.

INTRODUCTION

THE ALKOXY GLYCEROL ethers make up a nonsaponifiable fraction found in both neutral and phospholipid extracts. The majority of the alpha alkoxy glycerol ethers found in nature consist of alkyl ether chains of hexadecyl (16:0—chimyl alcohol), octadecyl (18:0—batyl alcohol), and octadecenyl (18:1—selachyl alcohol) carbon units (1).

Because of our interest in studying the metabolism of the ether bond, it became necessary to synthesize a number of radioactive glycerol ethers. Various types of reactions have been used for the organic synthesis of both labeled and nonlabeled glycerol ethers (2–9).

This report describes the preparation of ^{14}C and ^{3}H-labeled glycerol ethers and their ester derivatives having high radiopurity and high specific activities.

RESULTS AND DISCUSSION

We found that the best method for preparing the labeled glycerol ethers was from the potassium salt of either isopropylidene glycerol or 1,3 benzylidene glycerol and the alkyl halides. Good yields of the labeled glycerol ethers were obtained using a modification of the procedure of Baylis and co-workers (2) (a large excess of the potassium salt and a longer reaction time).

In the initial synthesis of DL-α-1-^{14}C-batyl alcohol, the alkyl halide used was octadecyl-1-^{14}C-iodide. Subsequently, it was found that the alkyl bromides yielded fewer breakdown products, easier purification steps, and higher yields.

A summary of the labeled free glycerol ethers synthesized and their physical constants is given in Table I.

To determine the radiopurity of each labeled preparation, the compound was analyzed by thin-layer chromatography (TLC) in at least three different solvent systems. The TLC plates were then scanned at various sensitivities using a Packard Model 7200 Radio Chromatograph Scanner, or by preparing zonal scans (10).

Using the procedure of Stegerhoek and Verkade (4), we prepared dipalmitoyl-α-batyl alcohol which melted at 57–58C after two recrystallization from acetone at 0C. Thin-layer chromatography and silicic acid column chromatography were used to resolve two components from the mixture. TLC [hexane: diethyl ether: acetic acid (90:10:1 v/v/v)] revealed a major component with an R_f-0.90 and a trace component with an R_f-0.13. On silicic acid columns the major component (about 70%–80%) was eluted with benzene and concluded to be dipalmitoyl-α-batyl alcohol based on a melting point of 61C–62C, no infrared hydroxyl absorption, and 2.07 equivalents of ester (11) per molecule. The dipalmitoyl-α-batyl alcohol showed a decrease in melting point over a period of about four to five months (mp 61C–62C down to 57C–58C and finally to 53C–54C). Stegerhoek and co-workers (4) had mentioned that these compounds showed polymorphic change; our data confirm their findings. A separate paper from this laboratory will discuss in detail the infrared spectra and polymorphism of glycerol ether derivatives.

The chloroform eluate (mp 69C–70C) from the above mixture had 0.95 equivalents of ester (11) per molecule, and infrared analysis showed an absorption peak at 3550 cm^{-1} (indicating the presence of an hydroxy group). The second component was concluded to be monopalmitoyl-a-batyl alcohol. Carbon and hydrogen analysis for the dipalmitoyl-α-batyl alcohol and the monopalmitoyl-α-batyl alcohol were in agreement with the calculated values. The melting

[1] Work done in partial fulfillment for PhD Degree, Department of Biochemistry, University of North Carolina.

TABLE I

Glycerol Ethers

Compound	Melting point °C	Mixed melting point 1:1 (α:β) °C	Yield %	Radio purity %	Specific activity mc/mM	Analyses				Infrared absorption bands cm^{-1}
						Calculated		Found		
						%C	%H	%C	%H	
DL-α-l-^{14}C-batyl alcohol	70.5–71.5 [a]	66.5–67.5	44.0	99–100	^{14}C–0.88	73.20	12.87	73.10	12.71	OH—3580–3540 OH—1050–1040 CH_2—O—CH_2—1105–1085
DL-2-^{3}H-α-l-^{14}C-chimyl alcohol	63–64 [b]	58–59	72.2	100	^{14}C–1.49 ^{3}H–1.30	72.10	12.74	72.30	12.59	OH—3600–3570 OH—1055–1020 CH_2—O—CH_2—1105–1095
DL-β-l-^{14}C-batyl alcohol	70.5–71.5 [c]	66.5–67.5	43.0	99	^{14}C–0.74	73.20	12.87	73.10	12.89	OH—3570–3560 OH—1060–1030 CH_2—O—CH_2—1090–1080
DL-β-l-^{14}C-chimyl alcohol	62.5–63.5 [d]	58–59	49.5	100	^{14}C–0.75	72.10	12.74	72.44	12.71	OH—3605–3580 OH—1055–1010 CH_2—O—CH_2—1105–1095

[a] Ref. 2 mp 71–72C; [b] Ref. 2, mp 62–63C; [c] Ref. 3, mp 71C; [d] Ref. 2, mp 63C.

point of the monopalmitoyl-α-batyl alcohol (chloroform eluate mp 60C–70C) corresponded with that of the beta ester isomer reported by Stegerhoek and co-workers (4) (mp 69.5C–70C). Further infrared analysis demonstrated that the monopalmitoyl-α-batyl alcohol fraction is the β-palmitoyl ester of α-batyl alcohol.

We were unsuccessful in preparing microscale quantities of the diesters of the glycerol ethers from the acid chloride (prepared from the fatty acid and thionyl chloride). An overall yield of only 15%–20% could be obtained. More efficient esterification occurred by heating the fatty acid and trifluoroacetic anhydride (0.25 mM scale) in chloroform under anhydrous conditions. The mixed anhydride (12) formed was then esterified (72% yield) with the glycerol ether.

The trifluoroacetic anhydride method of esterification was used to prepare a number of labeled esters of glycerol ethers. A list of these compounds and their physical constants is given in Table II.

The distribution of radioactivity and the chromatogram of a typical labeled glycerol ether diester preparation after column purification is shown in Figure 1. Prior to column purification α-l-β-l-^{14}C-dipalmitoyl-α′-batyl alcohol, a trace of palmitic-l-^{14}C-acid, β-l-^{14}C-monopalmitoyl-α′-batyl alcohol, α-l-^{14}C-monopalmitoyl-α′-batyl alcohol, and a trace of unidentified material (other than free glycerol ether) were found to be present in the mixture. Attempts to separate the alpha and beta monoesters by preparative TLC on a milligram scale were not successful.

EXPERIMENTAL

Materials

Most of the solvents were Baker Analyzed quality. The commercial xylene was dried, redistilled, and stored over sodium before use. Spectrograde chloroform with silica gel preservative was obtained from Matheson Coleman and Bell and spectrograde hexane was purchased from Fisher Scientific Company. The unlabeled purified palmitic and stearic acids (purity 99% or greater) were obtained from the Hormel Institute, University of Minnesota. D-batyl and D-chimyl alcohols were obtained from Western Chemical Industries Limited, La Pointe Pier, Vancouver, Canada. The isopropylidene glycerol was obtained from the Aldrich Chemical Company and distilled.

The hexadecyl and octadecyl bromides and the trifluoroacetic anhydride were Eastman white label chemicals. Gas-liquid chromatography (GLC) on 12% ethylene glycol succinate

TABLE II

Esters of Glycerol Ethers

Compound	Melting point °C	Mixed melting point 1:1 (α:β) °C	Yield %	Radio purity %	Specific activity mc/mM	Analyses				Infrared absorption bands cm^{-1}
						Calculated		Found		
						%C	%H	%C	%H	
α-l-β-l-^{14}C-Dipalmitoyl-α'-Batyl alcohol	53–54 [a]	.....	69.0	99–100	^{14}C–3.08	77.50	12.76	77.30	12.69	OH Absent O ‖ —C— — 1735—1725 H_2—O—CH_2—1105—1095
α-l-β-l-^{14}C-Dipalmitoyl-α'-Chimyl alcohol	51.5–52.5	50.5–57.0	72.5	100	^{14}C–3.03	77.21	12.71	77.39	12.69	OH Absent O ‖ —C— — 1735—1715 CH_2—O—CH_2—1110
α-l-α'-l-^{14}C-Distearoyl-β-Batyl alcohol	63.5–64.0	.....	47.5	100	^{14}C–1.42	78.02	12.87	78.05	12.76	OH Absent O ‖ —C— — 1730—1720 CH_2—O—CH_2—1110
α-l-^{14}C-Monostearoyl-β-Batyl alcohol	58–59 [b]	.....	34.4	95	^{14}C–0.91	76.66	12.87	76.67	13.03	*OH—3595—3560* OH—1050—1035 O ‖ —C— — 1720 CH_2—O—CH_2—1110—1085
α-l-α'-l-^{14}C-Dipalmitoyl-β-Chimyl alcohol	57.5–58.0	50.5–57.0	51.7	100	^{14}C–3.29	77.21	12.71	76.96	12.66	OH Absent O ‖ —C— — 1720—1710 CH_2—O—CH_2—1105—1085
α-l-^{14}C-Monopalmitoyl-β-Chimyl alcohol	46.5–47.0	.....	42.5	98	^{14}C–0.77	75.75	12.71	75.67	12.76	OH—3600—3590 OH—1040—1030 O ‖ —C— — 1720 CH_2—O—CH_2—1105—1095

[a] Ref. 14, mp 53.5–54C; [b] Ref. 3, mp 57–59C.

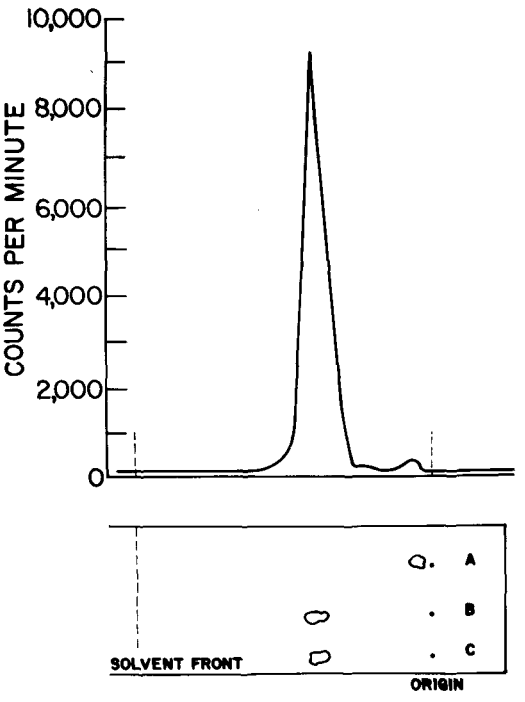

FIG. 1. A radiochromatogram scan and tracing of a chromatogram of purified α-1-β-1-^{14}C-dipalmitoyl-α'-batyl alcohol. The solvent system used was hexane: diethyl ether: acetic acid (95.5:1 v/v/v). *A*, synthetic unlabeled β-monopalmitoyl-α-batyl alcohol (mp 69C–70C). *B*, labeled dipalmitoyl-α-batyl alcohol. *C*, synthetic unlabeled dipalmitoyl-α-batyl alcohol.

polyester at 190C demonstrated that the hexadecyl bromide was 97% pure and that the octadecyl bromide was 94% pure.

The 1,3 benzylidene glycerol (mp 83C–84C) were prepared by the method of Piantadosi and co-workers (13) and the alkyl iodides by the procedure of Stegerhoek and co-workers (4).

Labeled halides, isopropylidene 2-^{3}H-glycerol, and the labeled fatty acids were purchased from New England Nuclear Corporation, Boston, Massachusetts. Unless otherwise stated, all of the labeled intermediates used for the syntheses were 99%–100% pure as determined by GLC and/or TLC.

Chromatography

Silicic Acid Columns. The silicic acid was washed with distilled water to remove fine particles. After filtration and drying at 110C–120C for 48 hr, 10 g of the silicic acid was used to prepare columns (20 × 200 mm) havin good separation properties and a fast flow rate (2–3 ml/min).

Thin-Layer (TLC). TLC plates were prepared with Silica Gel G, as described by Mangold (14). All of the labeled compounds in Table I and II had R_f values identical to the corresponding unlabeled synthetic compound.

Specific Activity Determinations

The specific activities were determined by measuring the radioactivity of a known quantity of the labeled compounds with a liquid scintillation spectrophotometer (Packard Tricarb Model 314EX) and were corrected for quenching by use of internal standards (10). The discriminator ratio method (15) was used for calculation of the radioactivity of each isotope in the double-labeled compound. The efficiency of the ^{14}C counting was 58–61%; for the simultaneous counting of ^{3}H and ^{14}C, the efficiency was 18% and 33%, respectively.

Synthesis

All of the synthetic procedures were carried out two or more times with unlabeled materials in order to standardize the yield and purity before the actual labeled materials were used. The melting points were taken with a Fisher Johns melting point apparatus and have been corrected. The carbon and hydrogen analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Michigan. The infrared spectra were obtained with a Perkin Elmer 421 recording infrared spectrophotometer.

DL- *-l-^{14}C-Batyl Alcohol* [l-(octadecyloxy-l'-^{14}C)-2,3-propanediol].

Using a 30 ml reaction filter flask equipped with magnetic stirrer, reflux condensor, and $CaCl_2$-P_2O_5 drying tube, 168 mg (4.30 mg atoms) of potassium metal in 5 ml of dry xylene was heated at 100C for 15 min with rapid stirring until the potassium was finely dispersed. Next 507 mg (3.84 mM) of 1,2 isopropylidene glycerol in 5 ml of dry xylene was added slowly to the potassium. The mixture was then heated at 145C–150C for 2 hr under dry conditions until all of the potassium had reacted. Finally 428 mg (1.12 mM) of octadecyl-l-^{14}C-iodide (1.00 mc) in 7 ml of xylene was added quantitatively and the reactants refluxed at 145C–150C for 72 hr. The cooled mixture was filtered to remove the potassium iodide and the filtrate collected in a 100 ml round bottom flask. After thoroughly washing the precipitate with spectrograde chloroform, the solvent of the combined filtrate was removed under reduced pressure.

The oil (360 mg) was hydrolyzed using ethanol: water: concentrated hydrochloric acid (75: 25:6 v/v/v) (3). The precipitate was then fil-

tered, washed thoroughly with distilled water, and dried in vacuo over P_2O_5.

The yellow precipitate (320 mg) was purified by silicic acid column chromatography. The material was dissolved in about 5 ml of warm benzene and was applied to a 10 g silicic acid column. Three fractions were eluted and collected with the following solvents: fraction I—150 ml benzene; fraction II—150 ml chloroform:benzene (72:25 v/v); and fraction III—150 ml chloroform:methanol (2:1 v/v). The results of a typical experiment were as follows: fraction I—138.0 mg (halide and hydrocarbon breakdown products); fraction II—4.5 mg (traces of fraction I); and fraction III—171.1 mg (glycerol ether—44.0% yield). The total recovery from the column was 99–101%.

The labeled α-batyl alcohol was recrystallized from two 5 ml-portions of spectrograde hexane at 0C. The purified glycerol ether was stored in spectrograde chloroform at −20C. The physical constants for the glycerol ethers are given in Table I.

DL-*2-^{3}H-α-l-^{14}C-Chimyl Alcohol* [l-(hexadecyloxy-1′-^{14}C)·2,3-(2-^{3}H)-propanediol].

The same procedure as described for the synthesis of α-batyl alcohol was used with the following exceptions: 18.75 mg (0.14 mM) of isopropylidene-2-^{3}H-glycerol (5.00 mc) and 488 mg (3.70 mM) of unlabeled isopropylidene glycerol, and 320 mg (1.05 mM) of hexadecyl-l-^{14}C-bromide (2.00 mc). The doubly labeled chimyl alcohol was obtained in a 72.2% yield.

DL-*β-l-^{14}C-Batyl Alcohol* [2-(octadecyloxy-l′-^{14}C)-1,3-propanediol].

The same procedure as described for the preparation of α-batyl alcohol was used with the following exceptions: 688 mg (3.82 mM) of 1,3-benzylidene glycerol (mp 83C–84C), 215 mg (0.65 mM) of octadecyl-l-^{14}C-bromide (1.00 mc), and 118 mg (0.35 mM) of unlabeled octadecyl bromide. After hydrolysis the precipitate was washed with 20–30 ml 50% ethanol. After purification by column chromatography, fraction III (143.2 mg—43.0% yield) was recrystallized twice from hexane as described above.

DL-*β-l-^{14}C-Chimyl Alcohol* [2-(hexadecyloxy-l′-^{14}C)-1,3-propanediol].

The same procedure as described for the preparation of β-batyl alcohol was used with the following exceptions: 160 mg (0.52 mM) of hexadecyl-l-^{14}C-bromide (1.00 mc) and 145 mg (0.48 mM) of unlabeled hexadecyl bromide. A 49.5% yield of labeled β-chimyl alcohol was obtained.

α-l-β-l-^{14}C-Dipalmitoly-α′-Batyl Alcohol [1,2-(dihexadecanoate-l′-^{14}C)-3-(octadecyloxy) propane].

The following reactants were placed in a 10 ml reaction filter flask equipped with magnetic stirrer, reflux condensor, and $CaCl_2$-P_2O_5 drying tube: 25.6 mg (0.10 mM) of palmitic-l-^{14}C-acid (1.00 mc), 102.4 mg (0.40 mM) of unlabeled palmitic acid, 0.10 ml (0.71 mM) of trifluoroacetic anhydride and 0.30 ml of spectrograde chloroform. The flask was heated under dry conditions with stirring for 2 hr at 45C–50C. After 2 hr 86.0 mg (0.25 mM) of unlabled D-batyl alcohol (mp 70C–71C) in 1.70 ml of spectrograde chloroform was added. The reaction was continued at 45C–50C for 20 hr. The reaction mixture was cooled and the chloroform was removed by a steam of nitrogen. The semisolid was stirred with three 3-ml portions of 20% Na_2CO_3, then 5 ml of distilled water, next with three 3-ml portions of 0.5N H_2SO_4, and finally with five 4-ml portions of distilled water. The white precipitate was filtered, dissolved in 5 ml of spectrograde chloroform, and then stirred with 5.0 g of Amberlite IR-45 ion exchange resin for about 15 min. The resin was filtered and the filtrate was collected in a 30 ml filter flask. The resin was thoroughly washed with chloroform to remove all traces of the glycerol ether diester. The combined filtrates were stirred with 5.0 g of Amberlite 1R-45 resin. After filtration the solvent was removed under reduced pressure at 40C. This material was dried in vacuo over P_2O_5. The mixture (196.3 mg) contained the labeled diester, traces of fatty acid, and the monoesters.

The precipitate (196.3 mg) was dissolved in warm hexane and applied to a 10 g silicic acid column. Four fractions were eluted and collected with the following solvents: fraction I—150 ml of hexane; fraction II—160 ml of hexane:benzene (80:20 v/v); fraction III—150 ml of benzene; and fraction IV—100 ml of diethyl ether. Fractions I and II contained less than 10 mg of nonpolar contaminates that may have been formed from the fatty acid. Fraction III contained 144.0 mg of the labeled diester (69.0% yield) and fraction IV (43.5 mg) contained the remaining more polar constituents. The total recovery from the column was 99%–100%. After recrystallization from two 5-ml portions of acetone at 0C, 140.4 mg of the labeled glycerol ether diester in spectrograde chloroform was stored at −20C. Data obtained for the labeled esters of the glycerol ethers are summarized in Table II.

α-l-β-l-^{14}C-Dipalmitoyl-α′-Chimyl Alcohol [1,2-(dihexadecanoate-l′-^{14}C)-3-(hexadecyloxy)-propane].

The same procedure as described for the synthesis of dipalmitoyl-α-batyl alcohol was used

with the following exceptions: 10.9 mg (0.04 mM) of palmitic-1-^{14}C-acid (1.00 mc), 117.1 mg (0.46 mM) of unlabeled palmitic acid, and 79.0 mg (0.25 mM) of D-chimyl alcohol (mp 62C–63C). A 72.5% yield (143.5 mg) of the labeled diester was obtained.

α-l-á-l-^{14}C-Distearoyl-β-Batyl Alcohol [1,3-(dioctadecanoate-1′-^{14}C)-2-(octadecyloxy) propane].

The same procedure as described above for the α-ether diester synthesis was used with the following exceptions: 5.3 mg (0.02 mM) of stearic-1-^{14}C-acid (1.00 mc), 135.7 mg (0.48 mM) of unlabeled DL-β-batyl alcohol (mp 70.5–71.5C). Fractions I, II, and III were collected as previously described; in addition fraction IV—150 ml of hexane:diethyl ether (90:10 v/v), fraction V—150 ml of hexane:diethyl ether (80:20 v/v), and fraction VI—100 ml of diethyl ether were eluted and collected. Fraction III contained 104.6 mg (47.5% yield) of the labeled diester; fraction IV—(33.6 mg) contained traces of the diester, acid, and monoester; fraction V—(53.6 mg) contained the labeled monoester; and fraction VI—(10.2 mg) contained the major portion of a very polar contaminant with traces of the monoester.

α-l-^{14}C-Monostearoyl-β-Batyl Alcohol [1-(monooctadecanoate-1′-^{14}C)-2-(octadecyloxy-3 propanol].

Fraction V—53.6 mg (34.4% yield) isolated from the labeled distearoyl β batyl alcohol preparation was recrystallized twice from 1-ml portions of acetone at 0C.

α-l-α′-l-^{14}C-Dipalmitoyl-β-Chimyl Alcohol [1,3-(dihexadecanoate-1′-^{14}C)-2-(hexadecyloxy)-propane].

The same procedure as described for synthesis of distearoyl β-batyl alcohol was used with the following exceptions: 27.5 mg (0.11 mM) of palmitic-1-^{14}C-acid (1.00 mc), 100.5 mg (0.39 mM) of unlabeled palmitic acid, and 79.0 mg (0.25 mM) of unlabeled DL-β-chimyl alcohol (mp 62.5C–63.5C). A 51.7% yield of the labled diester was obtained.

α-l-^{14}C-Monopalmitoyl-β-Chimyl Alcohol, 1-(monohexadecanoate-1′-^{14}C)-2-(hexadecyloxy)-3-propanol.

Fraction V (59.2 mg, 42.5% yield) from the dipalmitoyl-β-chimyl alcohol preparation was twice recrystallized at 0C from acetone.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Research Grants GM 12562-01 and GM 12562-02 from the National Institute of General Medical Sciences, National Institutes of Health.

Predoctoral trainee supported by Public Health Service Training Grant 5 TI-GM-404-04 from the National Institute of General Medical Sciences, National Institutes of Health.

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[Received March 28, 1966]

Pyrolysis Chromatography of Lipids. I. Mass Spectrometric Identification of Pyrolysis Products of Hydrocarbons [1]

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ABSTRACT

The products of pyrolysis at 600C of normal paraffins C_{10}–C_{18}, 2-methyl octadecane, 4-methyl octadecane, 6-methyl octadecane, cyclohexyl decane, cyclopentyl decane, 2,2,4,4,6,8,8-heptamethyl nonane, pristane and phytane were studied by means of a pyrolysis gas chromatograph directly coupled to a mass spectrometer. n-Paraffins yield a homologous series of n-olefins. Branched paraffins yield two homologous series, one of n-olefins and one of branched olefins. The n-olefin corresponding to the position of the branch is not formed. Interpretation of pyrolograms is similar in principle to the interpretation of mass spectra.

INTRODUCTION

SINCE THE WORK of Davison, Wragg and Slaney (1) in 1954, pyrolysis chromatography of synthetic polymers and high melting compounds (2) has become a routine method for their identification. The thermal fragmentation of a polymer causes fission at the weak bonds in the molecule and results in a complex cracking pattern which may be used as an empirical "fingerprint" to identify the parent compound. Recently pyrolysis gas chromatography has also been applied to the identification of volatile compounds, such as hydrocarbons (3), alcohols (4) and terpene acetates (5,6), employing infrared and ultraviolet spectroscopy as additional means of identification. Liddicoet and Smithson (7) have very recently applied pyrolysis chromatography to the qualitative identification of surfactants in the empirical fashion.

Our intent is to identify the products of pyrolysis of lipids with more certainty by employing a pyrolysis chromatograph directly coupled to a mass spectrometer. The combination of retention time data with molecular weight and mass spectra serves to identify more fully a pyrolysis fragment than either observation alone. This procedure should ultimately lead to the determination of unknown lipid structures by pyrolysis chromatography. Hydrocarbons were chosen for the initial exploratory study because their pyrolysis products would be simpler to interpret than what might be expected from more complex lipids.

MATERIALS AND METHODS

The hydrocarbons used in this study were found to be more than 98% pure by gas chromatographic analysis. Normal paraffins and cyclopentyl decane and cyclohexyl decane were obtained from the American Petroleum Institute. Pristane and isomeric methyl octadecanes were received from N. A. Sørensen of the Norwegian Technical University, Trondheim. Phytane was obtained from L. I. Smith of this University and 2,2,4,4,6,8,8-heptamethyl nonane from Aldrich Chemical Co.

Pyrolysis of the hydrocarbons was accomplished by injection of the neat sample into a Pyrex glass chamber 2 × 90 mm, electrically heated so that the exit temperature was 600C. The pyrolysis unit, shown in Figure 1, replaced the inlet port of an otherwise conventional Perkin Elmer model 800 gas chromatograph. As the temperature of pyrolysis was increased, the yield of products was increased and above 780C the pyrolysis was complete, since no original sample emerged from the gas chromatograph. However, above 600C the pattern of pyrolysis products became more complex by the formation of secondary and rearrangement reaction products. At a flow rate of 30 ml/min through the pyrolyzer, the residence time in the heated zone was 0.6 seconds. The sample size varied from 0.5 to 1.0 μl, and the yield of pyrolysis products varied from 50 to 80%, decreasing with increased sample size.

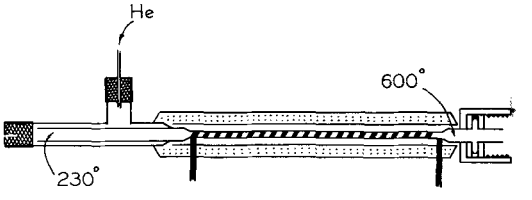

FIG. 1. Pyrolysis chamber constructed of Pyrex glass and substituted for the inlet port of a conventional gas chromatograph.

[1] Presented at the Symposium on "Mass Spectrometry of Lipids," AOCS Cincinnati, October 1965.

The effluent from the column was admitted to a Biemann helium separator (8) consisting of an internal sintered glass tube 8 × 190 mm and a concentric glass jacket evacuated to 1×10^{-3} Torr by a vacuum pump. The separator was connected by a heated 1 mm I.D. heavy wall glass tubing to the inlet pipe of a Hitachi Perkin-Elmer RMU6D mass spectrometer. Both the inlet and the exit of the helium separator were constricted by capillaries, the latter being of such a diameter that it passed 235 ml argon/min at a pressure differential of 15 psi. With the helium pressure on the GLC column at 18 psi, the flow through the column was 30 ml/min measured under atmospheric pressure. When the column was connected through the helium separator to the mass spectrometer, the analyzer pressure was in the range of 5×10^{-6} to 1×10^{-5} Torr, compared with a potential vacuum of 3×10^{-7} Torr when the column was removed and the inlet to the helium separator was plugged. Under the conditions of operation the ratio of the intensities of the helium peaks to the base peaks of the mass spectra of average chromatographic components varied from 1 to 10.

The gas chromatographic column used to separate the products of pyrolysis was a ⅛ in. × 6 ft aluminum tubing packed with 20% Apiezon L on Gas-Chrom P 100–120 mesh. The first 8 in. of the column were packed with uncoated support to avoid exposing the Apiezon L to helium at 600C as it emerged from the pyrolyzer. The final 6 in. of the column were packed with uncoated support to absorb the bleed from the column. The usual temperature program was from 40 to 240 C at 6C/min. At this rate the C_{18} compounds appeared before the end of the temperature program. Preliminary studies of the pyrolysis and the GLC separation of products from each substance were performed on an identical column in an F & M model 810 gas chromatograph, and the best conditions found were duplicated with the gas chromatograph mass spectrometer hookup.

The mass spectra of emerging substances were scanned at 80 eV at a rate of 9 seconds for m/e 1 to 300. The electron multiplier was operated at 1.5 to 3 kv and an attenuation of 100 (sensitivity 10). The ionization chamber was held at 230C. The exit slit of the source was 0.02 mm and the collector slit 0.3 mm. Spectra were recorded with a Honeywell 1508 Visicorder. For the rapid scan a 10 μf filter was employed across the input signal to the Visicorder to minimize noise. The source was equipped with a total ion monitor electrode, the signal from which was amplified by a Keathley Picoammeter and displayed on a strip chart recorder. Thus the total ion monitor served as the detector for the gas chromatograph and no time lag between GLC response and mass spectra existed. A representative pyrolysis chromatogram run with the gas chromatograph mass spectrometer hookup is shown in Figure 2. The broadened portions of the curve indicate the times during which mass spectra were recorded. The gas chromatographic curve was the response from the total ion monitor displayed on a strip chart recorder.

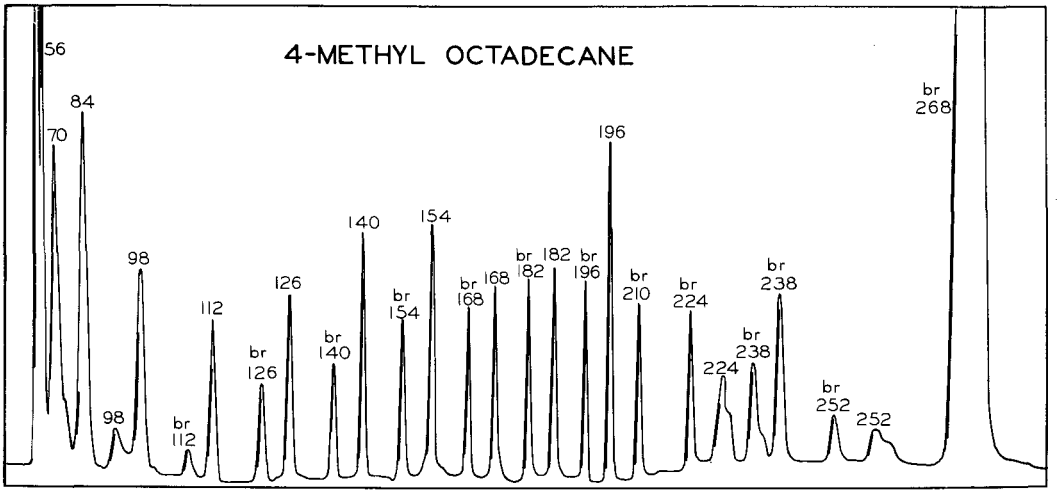

FIG. 2. Tracing from the total ion monitor during the pyrolysis-gas chromatography of 4-methyl octadecane. The broadened portions of the tracing represent those intervals during which mass spectra were recorded. Abscissa: time; ordinate: intensity of response of the total ion monitor.

Data are presented in the form of "pyrolograms," bar graphs in which the abundance of each component of the chromatogram is plotted against the equivalent chain length, ECL (9), calculated from normal paraffin standards. The proportion of each component to the total products of pyrolysis is shown by the height of the bar, as calculated from area percent of the pyrolysis chromatogram measured in the F & M instrument. Fragments of 4 carbons or less were present in roughly the same proportions as were the longer hydrocarbons, but were not separable by the conditions employed. Their yield was influenced by temperature variations more than the yields of the longer chain compounds. Since the consideration of these short chain compounds was unnecessary for the interpretation of structures of the parent substances, they were deemed of no interest and were not included in the total pyrolysis products in the above calculations. The excess unpyrolyzed starting material is depicted by a final bar in the pyrogram, and the height of this bar has no quantitative meaning. The ECL values were all calculated from data gathered on the Apiezon L column in the F & M instrument, and are presented on a scale based on even-chain normal paraffins as standards.

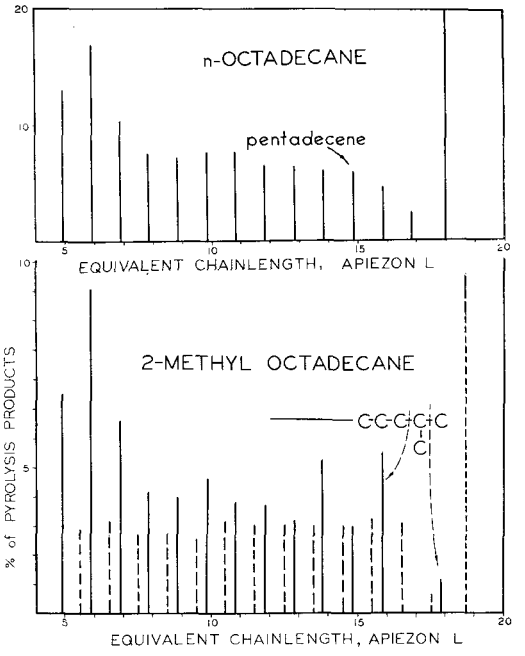

FIG. 3. Pyrolograms of n-octadecane and 2-methyl octadecane. Dashed bars represent branched substances.

RESULTS AND DISCUSSION

n-Octadecane is pyrolyzed to produce the entire series of n-olefins from C_{17} down to C_5 (Fig. 3). Quite comparable results were obtained with the pyrolysis of n-decane, n-dodecane, n-tetradecane and n-hexadecane. In all instances, the pyrosis of n-paraffins yielded the complete series of n-olefins containing numbers of carbon atoms equal to or less than the n-paraffin pyrolyzed. The identity of the products was deduced from their ECL values and verified in each instance by interpretation of their mass spectra, which revealed the expected molecular weights and the characteristics of n-olefins (10). However, the mass spectra do not permit location of the double bonds. The olefins from the scission of saturated hydrocarbons are largely 1-olefins (3).

Isomeric methyl octadecanes (11) were found to yield two series of homologs of olefins, one normal and one branched. The normal series extends as far as the number *n* of carbons in the longest branch of the molecule. The next member, corresponding to the carbon bearing the methyl group, is present only in traces. The member, n + 2, is again present representing a straight chain compound formed by the loss of the second largest branch. Subsequent members of the n-olefin series are absent except for the final one formed by the loss of the methyl group. In the case of 2-methyl octadecane (Fig. 3), scission of the bond between carbons 1 and 2 or between carbon 2 and the methyl carbon yields heptadecene. However, n-hexadecene is not produced from 2-methyl octadecane because both the bonds mentioned above must be broken to produce it, indicating that scission of two bonds is unlikely. Scission of the bond between carbons 2 and 3 yields pentadecene, and scission of subsequent bonds yields all the shorter chain n-olefins. Each possible scission in the straight chain end of the molecule produces a branched olefin, and the presence of all members of this series and the normal series with 6 or more carbon atoms was verified by mass spectrometry. Each branched olefin had a mass spectrum typical of a 2-methyl hydrocarbon and each had the molecular weight and typical spectrum of an olefin. Although it is contrary to Geneva nomenclature, pyrolysis products of branched hydrocarbons will be referred to in respect to the position of the branch rather than that of the double bond because the position of the double bond is not known, whereas, the reference to the original branch position clearly points out the relationship of a pyrolysis product to its parent compound.

4-Methyl octadecane (Fig. 2 and 4) produces all n-olefins up through C_{14}. n-Pentadecene is not produced by pyrolysis because two bonds are not simultaneously broken, but n-hexadecene is formed by breaking the bond between carbons 3 and 4. n-Octadecene is formed by loss of the methyl group. The smallest branched olefin which may be produced by scission of one bond has 6 carbon atoms. The smallest branched olefin identified was 3-methyl hexene, presumably formed by scission of the bond between carbons 6 and 7 of the right end of the molecule as drawn in Figure 4. Scission of each successive bond beyond 6–7 should produce a 4-methyl olefin in a complete series up to 4-methyl heptadecene. In addition, if bond 2–3 is broken a 2-methyl hexadecene should be formed. All the anticipated compounds were demonstrated in the pyrologram and were verified by mass spectrometry.

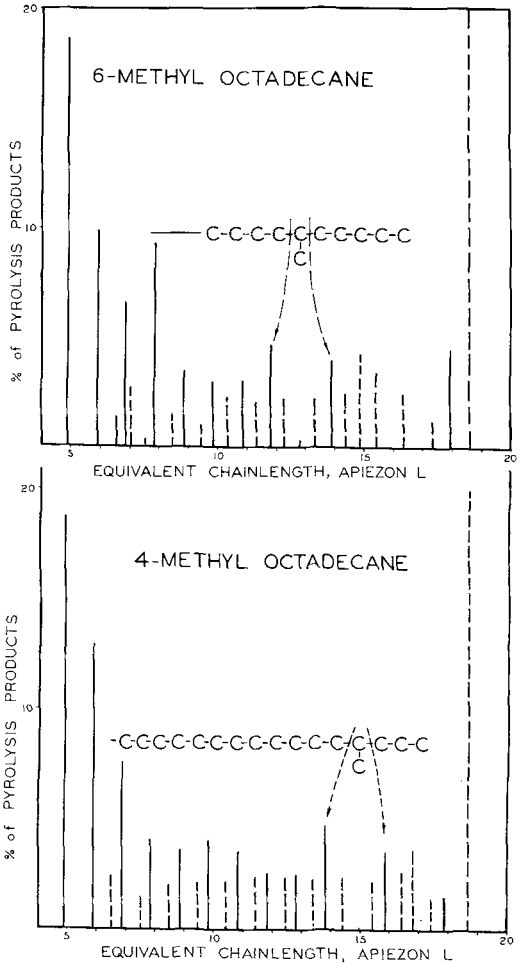

FIG. 4. Pyrolograms of 4-methyl octadecane and 6-methyl octadecane. Dashed bars represent the branched products.

6-Methyl octadecane yielded an interrupted series of n-olefins which is interpreted in the same manner as above. Scission of bonds in the longest branch of the molecule yield n-olefins whose maximum chain length is 12 carbons. Scission at bond 5–6 produces n-tetradecene. Loss of the methyl group yields octadecene. Scission at the 6–7 bond yields 2-heptene. Branched olefins formed by breaking a single bond should have a minimum of 8 carbon atoms and should exist in an unbroken series up to 6-methyl heptadecene. All these compounds appear in the pyrogram and were verified by their mass spectra. In addition, a branched pentadecene appeared with ECL value of 14.85. Its mass spectrum suggests that it is 3-methyl tetradecene, formation of which would require a rearrangement from the anticipated 2-methyl tetradecene.

Cyclohexyl decane should yield normal olefin fragments up through C_{10} and the whole range of cyclohexyl olefins less than 16 carbon atoms. The pyrogram shown in Figure 5 agrees with this prediction. Unfortunately in this experiment the early members of the series lower than C_7 did not separate well and their structures could not be confirmed by their mass spectra. However, the ECL values determined in the F & M instrument indicated the assignments shown in the pyrogram. In addition to the expected components, a small amount of n-undecene was detected, indicating some rupture of the cyclohexyl ring.

Cyclopentyl decane was also subjected to pyrolysis, but no mass spectra were measured on its products. However, the pyrolysis pattern was quite parallel to that found with cyclohexyl decane. The entire series of cyclopentyl olefins up to C_{15} were observed. n-Olefins up through C_{10} plus a small amount of C_{12} were found exactly analogous to the pyrolysis of of cyclohexyl decane.

2,2,4,4,6,8,8-Heptamethyl nonane pyrolyzed to yield principal fragments corresponding to rupture of bonds adjacent to methyl branches. Thus fragmentation at bonds 2–3 and 7–8 yielded isobutene as 50% of the pyrolysis products (Fig. 5). Loss of one of the nine methyl groups yielded multibranched fragments having 15 carbon atoms, mol wt 210. Scission at bond 3–4 yields a C_{11} fragment of mol wt 154. Breaking bond 4–5 yields two C_8 fragments, both of mol wt 112. Rupture of bond 5–6 yields C_7 and C_9 fragments of mol wt 98 and 126. Scission at bond 6–7 yields C_{11} and C_5 fragments of mol wt 154 and 70. In any of

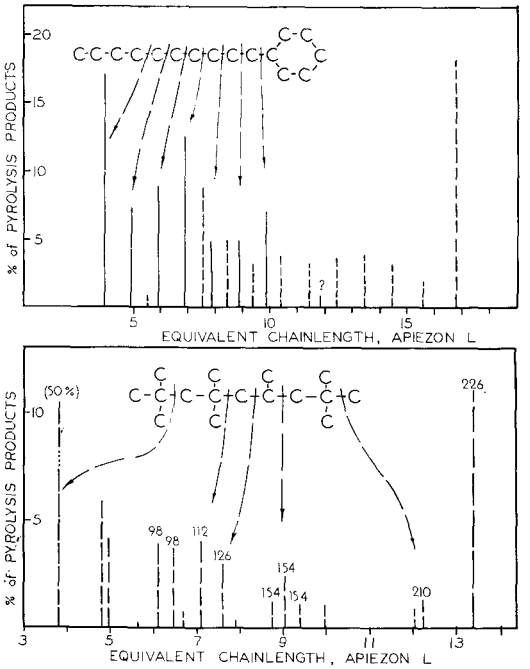

FIG. 5. Pyrolograms of cyclohexyl decane and 2,2,4,4,6,8,8-heptamethyl nonane. The numerals by each bar are the molecular weights measured by spectrometry.

these scissions, the locus of the double bond formed may be in more than one place, giving rise to isomers. Such multiplicity of fragments of equal mass was observed in the major products of fragmentation and may also explain some of the less abundant unidentified products. Perhaps the most revealing aspects of the pyrologram are the absence of principal fragments of mass 196 and 182 indicating the two methyl groups on either carbons 2 or 8, and the occurrence of minor fragments of mass 140, indicating the methyl group on carbon 6. The absence of a major fragment of mass 84 suggests the difficulty of forming a fragment of 6 carbons from either end of the molecule, thus indicating branching on carbons 4 and 6.

Pristane, or 2,6,10,14-tetramethyl pentadecane, is one of the naturally-occurring polymethyl hydrocarbons (12) and it has a symmetrical molecule. Cleavage of any of the four terminal methyl groups from the molecule should produce 2,6,10-trimethyl pentadecene. Because loss of two methyl groups from one end of the molecule is unlikely, 2,6,10-trimethyl tetradecene should not occur. Scission of the bonds between carbons 2 and 3 or carbons 13 and 14 should produce 2,6,10-trimethyl tridecene. Removal of 4 carbons and 5 carbons from one end of the molecule should yield 2,6,10-trimethyl dodecene and 2,6,10-trimethyl undecene, respectively. Rupture of the bond between carbons 5 and 6 (or 10 and 11) should yield two isomers of 2,6-dimethyl undecene. Formation of a 2,6-dimethyl decene should not occur. Dimethyl olefins of 10, 9 and 8 carbon atoms, but not of 7 carbon atoms, should be formed. In the main, the pyrologram of pristane agreed with these predictions (Fig. 6). However, two C_7 and two C_8 branched olefins were observed as well as some C_{12} branched olefin. However, the absence of C_{17} olefin, the near absence of C_{12} olefin, and the proportionately small amount of C_7 olefin indicate the positions of methyl groups in positions 2, 6 and 10, analogous to the behavior of monomethyl octadecanes and 2,2,4,4,6,8,8-heptamethyl nonane. Our observations on the structure of pristane agree with the formula proposed by Sørensen and Sørensen (12).

Phytane, or 2,6,10,14-tetramethyl hexadecane, is formed by the reduction of phytol. Phytane has one carbon atom more than pristane, giving it a terminal ethyl group. This asymmetry of the structure makes possible the formation of a continuous series of fragments, and all frag-

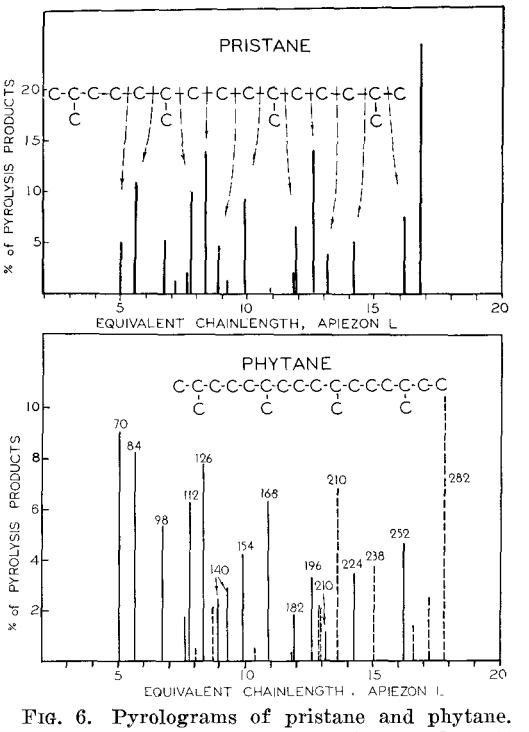

FIG. 6. Pyrologrames of pristane and phytane. Solid bars are products from pristane. Dashed bars are products unique for phytane. Numerals indicate molecular weights measured by mass spectrometry.

ments from C_5 through C_{19} were observed and measured (Fig. 6). The molecular weights given beside each fragment in the chart were verified by mass spectral measurement. In Figure 6 those fragments shown by solid bars correspond to those formed from pristane, for one end of the phytane molecule is identical to that of pristane. The dashed bars indicate those fragments not found with pristane and thus assignable to the right end of the molecule as shown in the chart. Considering the latter, the fragment of mol wt 238 represents the loss of the isopropyl group from phytane. Loss of isopropyl from pristane yields a fragment of 224. Loss of the isobutyl fragment from pristane leaves a mass of 224. Rupture of bonds 4–5 or 13–14 could yield C_{15} fragments of 210, both of which were observed and measured. Scission at bond 5–6 yields C_6 and C_{14} fragments of mol wt 84 and 196. Scission at bond 6–7 yields C_{12} and C_8 fragments of 168 and 112. Fragmentation at 9–10 yields two C_9 fragments of 126, and at 10–11 masses of 182 and 98 (C_{13} and C_7). Thus the principal predictable fragments from as complex a molecule as phytane were observed and confirmed to be present among its pyrolysis products.

DISCUSSION

This investigation of pyrolysis has concentrated on the effects of branching upon the products produced, and has demonstrated that with aliphatic branched compounds the structures can be readily deduced from the pyrolograms. To a considerable degree, the interpretation of pyrolograms is similar to the interpretation of mass spectra. In the pyrologram one deals with complete molecules and in mass spectra with ions of fragments, but in both the interpretations involve scissions of carbon to carbon bonds. The dissociation energies of carbon-carbon bonds in a straight chain are in the order of 75–80 kcal per mol, whereas those of bonds to a carbon bearing a branch are 70 kcal per mol (13). Hence, bonds adjacent to branches in molecules are more easily ruptured whether the energy of activation is thermal or bombardment by electrons. In the mass spectrometer, the conditions are such that molecules are widely separated under high vacuum, and molecules highly energized by electron bombardment at 80 eV cannot dissipate that energy by collision with other molecules. Therefore, they fragment one or more times each, and in all possible ways. In the pyrolysis, the much lower energy level imparted to the molecules is sufficient to disrupt only the weak bonds, and the energy is rapidly dissipated to surrounding helium molecules. The radicals produced by scission of carbon-carbon bonds expel hydrogen atoms to yield olefins. The hydrogen atoms may react with each other or with the column packing. Evidence for the presence of hydrogen gas was seen in the mass spectra of the resolved products which emerged first from the column.

If the pyrolysis is conducted near 600C the only products produced from paraffins are olefins. No evidence was seen for the products of disproportionation, dienes and saturated hydrocarbons. Hence, the pattern or spectrum of substances produced from a hydrocarbon molecule is kept as simple as possible—two olefin molecules from each possible mode of scission of the paraffin molecule. Thus, from a 20 carbon compound one deals with a maximum of 38 possible fragments, some of which may be duplicates. However, in a mass spectrometer, the number of fragments measurable is much greater. The yield of a pyrolysis product generally decreases with increase in molecular weight of the product; that is, the yield of butene from octadecane is greater than the yield of tetradecene. However, the yields of the higher fragmentation products are sufficient to allow their measurement and identification. The decrease in yield of fragments with increasing mass per charge in a high energy mass spectrum is much more drastic, suggesting that secondard fragmentation in pyrolysis occurs to a much lesser degree than in the mass spectrometer. The location of the branch in the molecule is deduced by the low proportion or absence of members of the homologous series of products corresponding to the position of the branch. The adjacent members of the series are usually present in higher proportions, indicating the relative ease with which the bonds to a carbon bearing a branch can be broken. This reasoning is analogous to the interpretation of mass spectra of branched compounds.

Aside from simplicity in interpretation of results, pyrolysis chromatography offers the advantage of simple equipment. All that is required is a gas chromatograph and a pyrolysis unit which can be fabricated in most laboratories from Pyrex glass. The gas chromatograph should preferably be capable of temperature programming if high molecular weight substances are to be examined.

Examination of substances by pyrolysis chromatography coupled to a mass spectrometer yields a more positive identification of the fragments, for their molecular weights can be determined precisely, and often their structures can be deduced, identifying substructures of

the parent substance. This technique should extend the molecular range which can be admitted to a mass spectrometer. Complex molecules which have insufficient vapor pressure for examination by mass spectrometer can be converted to more volatile pyrolysis products which yet retain some of the features of the original structure. In some mass spectrometers pyrolysis can be performed on high molecular weight samples and the spectra obtained are those of mixtures. However, with pyrolysis-chromatography-mass spectrometry, the mass spectra are measured upon individual components and should be easier to interpret.

The present study has attempted to reduce the empiricism of pyrolysis chromatography by identifying the products of pyrolysis via mass spectrometry. With the information so gained, the products of pyrolysis of hydrocarbons can now be predicted, and their structures may be deduced with more certainty than was possible from previous "fingerprint" patterns.

ACKNOWLEDGMENT

Supported in part by NIH Grant HE 03559.

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[Received Jan. 12, 1966]

Effect of Diet Handling on Nutritional Studies with Used Frying Fats [1]

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ABSTRACT

A four-week experiment to study the significance of careful diet handling was carried out with weanling rats fed purified rations containing 15% of various fats. Fresh soybean oil was the fat in the control diet and the other fats, which had been used to prepare food by a commercial-type deep-frying operation, were soybean oil, partially hydrogenated soybean oil with iodine value (I.V.) 70, partially hydrogenated soybean oil with I.V. 108, and cottonseed oil. A purified diet was fed ad libitum.

Treatment of the dietary groups in regard to preparation and handling of the rations proved to be highly significant. That is, as opposed to weekly mixing and twice weekly feeding of the diets, daily preparation and feeding along with the use of antioxidants and refrigeration of the ingredients resulted in a much superior growth rate and a higher efficiency of feed conversion. Since this very significant response became apparent in less than four weeks, the importance of careful handling to minimize secondary effects within the diet must be emphasized. The fresh soybean oil control, and all of the used frying fats gave similar results.

INTRODUCTION

THERE IS ALWAYS A QUESTION in nutritional experiments whether a particular component of the diet being studied is compatible with other ingredients. Also, once the diet is prepared, is special care needed to prevent or reduce deterioration? It was felt that in order to do meaningful experiments with fats which had been exposed to heat, such as in the preparation of fried foods, an investigation of diet handling conditions was desirable.

Literature reports have indicated vitamin destruction due to oxidized fat. Among these, Barnes et al. (1) found that a biotin deficiency was caused by the oxidation of biotin synthesized in the intestine, when rancid fat was included in the diet. Holman (2) reported the destruction of vitamin A in the presence of oxidized fat; before 10% of linoleate carrier was oxidized, virtually all of the vitamin was gone. Witting and co-workers (3) stated that dietary riboflavin and pyridoxine levels were factors in the nutritional behavior of thermally oxidized fats. Excess riboflavin seemed to be of special value. Therefore, it is possible that much of the effect in feeding studies where depressed growth has been seen may have resulted from improper handling of the diets, with consequent oxidation reactions of the fats resulting in products which can lead to subsequent damage to other dietary nutrients.

Based on these and other observations of deterioration with laboratory-heated fats, it has been implied by some investigators that similar changes may occur in fats used for commercial deep-frying of foods. However, Keane et al. (4) found no deleterious effects from commercially-used frying oils, and stressed the value of a nutritionally adequate diet.

I recognize that fats can be damaged by heating and oxidation, and that under extreme conditions these changes may result in impaired growth when the abused fats are fed at high levels to animals. In most studies reported, however, the conditions of laboratory heating were much more drastic than usage conditions adopted in commercial practice, and extrapolation of such results is unwarranted. Commercial frying conditions may vary greatly from laboratory heating depending on the operation. Variables include the quantities and type of foodstuffs and moisture, as well as deviations in turnover rate and agitation during the heating process.

Poling et al. (5) studied the nutritional value of fats after use in commercial deep-fat frying. No mention was made of how the diets were handled from the standpoint of frequency of mixing, and storage conditions. They showed that unsaturated fats, which had been damaged in the laboratory by long heating at high temperatures under oxidative conditions, reduced energy values and caused increases in liver weights. In contrast to the laboratory heated fats, they found that the majority of the commercially used fat samples showed no impairment in nutritional values. Slight increases in liver weights were observed.

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Many papers in this field do not define the feeding conditions other than to list the diet ingredients, and to indicate ad libitum feeding (6–10). Therefore, there is a lack of information on how important actual feeding techniques are in nutritional studies with used fats. In view of this, a rat experiment with careful diet handling and special feeding techniques was undertaken to study their effects on growth, feed efficiency and fat utilization. The fats were prepared by a controlled commercial-type deep-frying process typical of the industry.

EXPERIMENTAL

Fats Studied

To prepare each of the four used fats for the feeding study, a Toastmaster Fryer was charged to capacity with 15 lb of fat, heated and thermostatically controlled at 182C. There were 10 fryings per day for 5 consecutive 8-hr days to give a total of 50 fryings during 40 hr of heating. Each frying consisted of 1.5 lb of French-fry potatoes (6 min) followed by 1.5 lb of breaded shrimp (3 min) and then 0.85 lb of onion rings (2 min). The kettle was allowed to recover to the 182C temperature each time before the new food was fried. Approximately 3 lb of fresh fat was added to the fryer daily to maintain the charge.

Fleischman et al. (11) stressed the point that poorly-controlled heating during frying can cause much more extensive chemical changes in fat than occurs during carefully controlled commercial frying.

All of the following experimental fats were refrigerated until they were mixed into the rations: 1) fresh soybean oil; 2) used soybean oil, I.V. 130 and 117; 3) used soybean oil, I.V. 108 and 101; 4) used soybean oil, I.V. 70 and 74; 5) used cottonseed oil, I.V. 107 and 100. The iodine values were obtained before and after the fats were used for frying. Sample 1 was not heated, and samples 3 and 4 were partially hydrogenated.

Feeding Method

Weanling male rats of the Holtzman strain were housed in individual cages with screen-wire floors in a room maintained at 74 ± 2F and 50 ± 5% relative humidity. They all were given Purina Laboratory Chow pellets for three days. At this time the 150 animals were distributed randomly into 10 groups of 15 animals each on the basis of litter and body weight. The experimental diets shown in Table I were fed ad libitum for 4 weeks. Distilled water was also provided ad libitum. Soybean oil was used as the carrier for the fat-soluble vitamins, and as such supplied an adequate level of essential fatty acid.

TABLE I

Composition of Diets

Ingredients	Per cent by weight
Casein	27.0
Sucrose	44.0
Vitamin mixture in sucrose [a]	5.0
Vitamin mixture in soybean oil [b]	2.0
Salt mixture U.S.P. XIV [c]	4.0
Cellu Flour	3.0
Fat	15.0

[a] Furnished the following in mg per 100 g of ration: 0.3 menadione, 0.4 thiamine, 0.5 riboflavin, 2.0 niacin, 2.0 calcium pantothenate, 0.4 pyridoxine, 0.025 biotin, 300 choline chloride, 200 inositol, 10 ascorbic acid, and 10 paraaminobenzoic acid.

[b] Furnished the following per 100 g of ration: 1,200 I.U. vitamin A, 1,200 I.U. vitamin D_2, and 14.7 I.U. vitamin E as D-α-tocopheryl acetate.

[c] Nutritional Biochemicals Corporation, Cleveland, Ohio.

Each of the five fats was studied under two different conditions of diet handling as follows:

A. Diets were prepared weekly, not refrigerated, and fed only twice a week.

B. Diets were prepared and fed daily. In this case, enough basal diet, minus fat, to last a week was mixed and refrigerated. Fat-soluble vitamins in soybean oil as well as the experimental fats were added to the basal during the daily preparation. The fats for these diets were protected with 70 ppm of added antioxidant (BHA and BHT).

Data Obtained

During the four-week experiment, records of body weight gain and feed consumption were kept. For the last ten days feces were collected from ten rats from each dietary group. Total fecal fatty acids were determined by the method of Hoagland and Snider (12). The values for fat absorbability were calculated on the basis of total fatty acid in both the diet and feces. Caloric efficiency data were calculated.

Most data were analyzed statistically including analysis of variance and the Tukey "d test" for minimum significant difference as described by Scheffe (13). For any two means to be significantly different, the variance between the two must exceed the minimum significant difference value given.

At the end of the feeding study the rats were killed, autopsied, and inspected for gross pathology. The livers were removed and weighed.

RESULTS AND DISCUSSION

The values for feed consumption (Table II) on an individual dietary group basis did not

TABLE II

Feed Consumption, Weight Gain, Grams of Gain Per Gram of Feed, Fat Absorbability and Caloric Efficiency[a]

Dietary fat[b]	Average values[c]									
	Feed eaten[d]		Weight gain[e]		Grams of gain per gram of feed[f]		Fat absorbed[g]		Calories per gram gain[h]	
	A	B	A	B	A	B	A	B	A	B
	g	g	g	g	g	g	%	%		
SBO (control)	361.3	358.9	177.6	192.6	.49	.54	94.4	94.9	11.9	9.6
Used SBO (I.V. 130)	357.5	377.5	179.9	205.0	.50	.54	92.3	92.7	10.9	9.2
Used SBO (I.V. 108)	356.7	376.4	183.1	198.2	.51	.53	90.2	91.5	10.7	9.5
Used SBO (I.V. 70)	357.2	369.7	173.3	196.1	.49	.53	88.0	89.0	11.1	9.2
Used CSO (I.V. 107)	367.7	372.4	180.9	198.3	.49	.53	87.9	90.3	11.4	9.4
Average for treatments	360.1	371.0	179.0	198.0	.50	.53	90.6	91.7	11.2	9.4

[a] For statistical evaluation $P \leq 0.05$.
[b] SBO = soybean oil and CSO = cottonseed oil.
[c] A = diets prepared weekly and fed twice a week. B = diets prepared and fed daily.
[d] Differences are not statistically significant.
[e] Differences are significant when they exceed the d value of 15.2.
[f] Differences are significant when they exceed the d value of .03.
[g] Differences are significant when they exceed the d valu eof 1.7.
[h] Calories eaten (minus those in unabsorbed fat) which were needed for each gram of weight gain. Differences are significant when they exceed the d value of 1.6.

vary significantly by statistical analysis, either as a result of the type of dietary fat or condition of handling. However, whenever used fats were fed the animals tended to eat more with daily feeding (category B).

Weight gain results are also in Table II. Within a feeding category, A (weekly) or B (daily), all fats performed alike. However, there was a much greater gain in weight by the animals fed daily, as seen in column B. In this respect, the combined treatment effect, indicated by "average for treatments" was highly significant by statistical analysis. Figure 1 is presented to illustrate the percentage increase in the rate of weight gain due to special handling and daily feeding. The points were plotted at the end of each week, and are representative of the gain during that week. By the slope of the curve it is evident that by far the greatest benefit was recorded during the fourth week. Because of the definite trend as the experiment progressed, from a longer study one should expect a greater effect than this 30% increase in weight due to special handling of the diets.

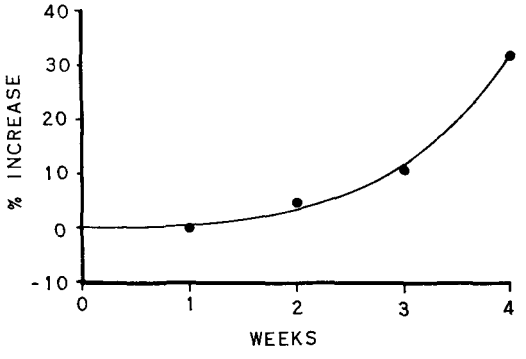

FIG. 1. Increase in rate of weight gain per week due to daily preparation and feeding of the diet, as opposed to weekly preparation and twice-weekly feeding [(Gain of B × 100/Gain of A) − 100].

In general, on an individual fat basis (Table II) efficiency was improved significantly by daily feeding, and the over-all effect of the treatment was statistically significant. Within each diet-handling category (A or B) there was no difference as a result of feeding the various fats.

The fresh soybean oil was absorbed better than any of the used fats (Table II). Furthermore, among the used frying fats values for the last two samples (soybean oil I.V. 70, and cottonseed oil I.V. 107) were especially low compared with the absorbability values for the fresh soybean oil. Dietary calories required per gram of body weight gain were calculated with allowance for the absorbability of each fat. These values in Table II show clearly an over-all economy in growth response from careful diet handling and daily feeding (category B).

No difference in liver weights due to the type of fat was noted (Table III). Nevertheless, daily preparation of the diet (B) as opposed to weekly preparation (A) resulted in distinctly heavier livers for all rats fed the used fats. These values reflected the heavier body weight found for the groups fed daily. Conversion of the data to the basis of grams organ weight per kilogram body weight eliminated any difference. This is shown by the average for treatments. At gross autopsy no abnormalities were observed in any of the rats.

There was no evidence that the used fats

TABLE III

Liver Weights

Dietary fat[a]	Average values[b]			
	Weight per animal		Grams/ kilogram body weight	
	A	B	A	B
	g	g		
SBO (control)	13.7	13.6	56.2	53.4
Used SBO (I.V. 130)	13.0	15.0	55.9	56.9
Used SBO (I.V. 108)	14.1	15.0	57.3	56.5
Used SBO (I.V. 70)	13.1	15.2	55.1	58.6
Used CSO (I.V. 107)	13.9	14.7	57.9	56.7
Average for treatments	13.6	14.7	56.5	56.4

[a] SBO = soybean oil and CSO = cottonseed oil.
[b] A = diets prepared weekly and fed twice a week. B = diets prepared and fed daily.

consumed in these diets were toxic to the animals. This is in confirmation of results reported by others (4,5,14–16) with fats used under practical conditions such as restaurant frying. On the other hand, used frying fat in my rat diet depressed growth and lowered the efficiency of feed conversion unless special handling conditions were employed to protect the ration against oxidation effects. Lea (17) has emphasized that since fat oxidation has a destructive effect on vitamins (A, E, K, C and several of the B group) oxidized fat toxicity is likely to be complicated by vitamin deficiency or increased vitamin requirement. With the inferior results from a weekly mixing and twice-weekly feeding regimen in the current study, vitamin deficiencies in the animals were suspect. Indeed, preliminary tests in these laboratories have shown that several of the B vitamins including biotin, riboflavin, calcium pantothenate, pyridoxine and inositol are involved.

Results from feeding experiments with rations high in readily oxidizable components, such as certain fats, may be misleading unless special care is taken to protect the dietary ingredients from secondary effects due to oxidation. The length of time the ration remains in the feeder (even just a few days) can be critical.

ACKNOWLEDGMENTS

Doyle Johnson prepared the used frying fats, and Granville A. Nolen provided technical assistance.

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[Received Jan. 7, 1966]

Phospholipase A Properties of Several Snake Venom Preparations[1]

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ABSTRACT

The hydrolytic properties of the venoms of seven species of snakes, *Crotalus adamanteus, Ancistrodon contortrix, Naja naja, Bothrops atrox, Ophiophagus hannah, Crotalus atrox* and *Vipera russeli,* were studied with purified lecithins and mixtures of lecithins of known fatty acid and class composition as substrates.

The relative rates of hydrolysis of the fatty acids by the above venoms were studied by analysis of the products of the reaction at intervals during the course of the reaction. Of the seven venoms studied, that of *Ophiophagus hannah* was the only one which did not give some degree of preferential rate of hydrolysis of individual fatty acids.

In general, saturated fatty acids were liberated faster than unsaturated fatty acids; differences in the rates of the hydrolysis of individual saturated and unsaturated fatty acids were also observed. Individual classes of lecithin were also hydrolyzed at different rates. For the determination of the distribution of the fatty acids between the α- and β-position of lecithin, the reaction should be carried to completion. If the reaction requires a prolonged time to go to completion, it should be carried out under nitrogen to prevent autoxidation.

INTRODUCTION

VARIOUS FACTORS that influence the hydrolysis of lecithins by phospholipase A, which specifically hydrolyzes the fatty acid in the β-position (1–3), have been studied extensively (4,5). However, there are a number of aspects of the reaction which have not been clearly defined. Quantitative conversion of lecithin to fatty acids and lysolecithin is not generally attained and there is some evidence that different fatty acids may be hydrolyzed at different rates. In regard to the latter, it has been observed that hydrogenated lecithin is generally more difficult to hydrolyze than unsaturated lecithins (4). Moore and Williams (6) developed a method for the determination of the lecithin classes on the basis that they are hydrolyzed at different rates. The above factors are very important for the quantitative determination of the distribution of fatty acids between the α- and β-positions of lecithin via phospholipase A hydrolysis and are the subject of this investigation.

MATERIALS AND METHODS

The venoms of the following species of snakes were obtained from the Ross Allen Reptile Institute, Silver Springs, Florida: *Crotalus adamanteus, Ancistrodon contortrix, Naja naja, Bothrops atrox, Ophiophagus hannah, Crotalus atrox* and *Vipera russeli.*

Egg lecithin was prepared from fresh hen eggs, soybean lecithin from a commercial sample obtained from Nutritional Biochemicals Corporation, and safflower lecithin from a crude preparation supplied to us by John Kneeland of the Pacific Vegetable Oil Company. The lecithins were purified by DEAE cellulose and ammonium silicate column chromatography as described by Rouser et al. (7). The neutral lipids were separated first by fractionation of the crude preparations in 2 gram batches on a 3.0 × 37 cm column of DEAE cellulose by elution with chloroform. After the neutral lipids were separated, the lecithin was eluted with a 9:1 (v/v) ratio of chloroform:methanol. The lecithin preparations obtained from the DEAE column were purified by passing them through an ammonium silicate column (3.0 × 40 cm). Materials of lower polarity than lecithin were eluted first with chloroform:methanol (4:1, v/v) containing 0.5% water. The lecithin was then eluted by increasing the amount of water in this solvent to 1.5%. The composition of the eluate was analyzed by TLC. All fractions containing pure lecithin were combined. No impurities could be detected in the final preparations by two-dimensional TLC (8) or infrared spectral analysis which was used to confirm the identity of each product.

Samples of fully hydrogenated lecithins were prepared by catalytic hydrogenation of samples of the isolated lecithins in a mixture of ethyl ether:alcohol (3:7) with a platinum catalyst at room temperature and 50 lb pressure. The final products were purified by the silicic acid-silicate water chromatographic technique described by Rouser (7). This technique provided the most effective means for removal of the catalyst.

[1] Presented at the AOCS Meeting, Cincinnati, October 1965.

Completeness of the hydrogenation was determined by GLC analysis of the constituent fatty acids. No unsaturated fatty acids could be detected in any of the final products.

Phospholipase A hydrolysis was carried out under the conditions described by Robertson and Lands (9) with several modifications in the work-up of the products of the reaction. The reaction was carried out on 5 to 50 mg of lecithin dissolved in 5 ml of ethyl ether. To this solution was added 100 μl of snake venom solution which consisted of 5 mg of venom and 0.4 μmoles of $CaCl_2$ per ml of tris buffer at pH 7.5. The reaction was usually carried out in a 10 ml volumetric flask at 20C in an inert atmosphere (by purging the solutions with nitrogen) and with vigorous stirring by means of a magnetic stirrer.

At predetermined times the reaction was stopped by the addition of about 10 ml of methanol to the reaction mixture in a 50 ml Erlenmeyer flask. Approximately 5 g of anhydrous sodium sulfate was added to this solution maintained under an atmosphere of nitrogen. After 30 min the solution was decanted and the sodium sulfate was washed twice with 20 ml portions of distilled chloroform. The solutions were combined, reduced to a volume of about 0.5 ml and applied to a 20 × 20 cm chromatoplate coated with chloroform-extracted Silica Gel G. The products of the reaction, lysolecithin, fatty acids and unreacted lecithin were separated with chloroform:methanol:water (70:30:3) and scraped from the chromatoplate into ampules (test tubes with a constricted neck for sealing) containing a small known amount (1 to 5 mg) of methyl pentadecanoate which served as an internal standard in the subsequent analysis. Esterification was carried out with dry methanolic HCl (6%, W/W). The sealed ampules containing about 3 ml of this reagent were heated in a boiling water bath for 2 hr after flushing them with nitrogen. The crude methyl esters were recovered by extraction with ethyl ether:petroleum ether (1:1) by the usual method and analyzed by gas-liquid chromatography (GLC). The percent hydrolysis for the reaction was determined by comparing the percent of internal standard (methyl pentadecanoate) to the total fatty acid composition of each fraction.

The GLC of methyl esters was carried out with an F&M Model 600 flame ionization instrument equipped with a 7 ft × ¼ in. column packed with 15% ethylene glycol succinate polyester on Chromosorb W at 185C. The fatty acid composition was determined directly from the proportionalities of the peak areas.

RESULTS AND DISCUSSION

The importance of conducting the hydrolysis of lecithins with phospholipase A in an inert atmosphere is indicated by a TLC analysis of the products of the reaction shown in Figure 1. When the reaction was carried out in the presence of atmospheric oxygen, artifacts were formed which could be detected by TLC. One such substance had an R_f of about 0.9 (Fig. 1); polar material was also formed which was difficult to separate from lysolecithin. Artifacts apparently arise from autoxidation because they may also be detected in the chemical hydrolysis of lecithin if the reaction is not carried out in an inert atmosphere. The amount of the artifacts may be small with highly active venoms, but when an hour or more is required to complete the hydrolysis, it should be conducted under an atmosphere of nitrogen to prevent autoxidation.

Analysis of the products at partial and complete hydrolysis of various samples of lecithin with the seven different venoms (Table I) showed that the composition of the fatty acids released in the reaction varied considerably with the degree of hydrolysis with all venoms except

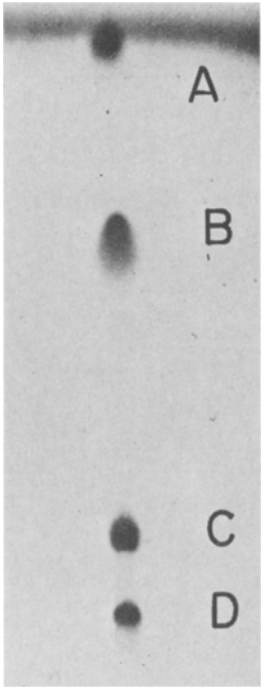

FIG. 1. Thin-layer chromatography of the products of the partial hydrolysis of lecithin with the venom of *Crotalus adamanteus* in the presence of air: *A*, indicates artifact; *B*, fatty acid; *C*, unhydrolyzed lecithin; *D*, lysolecithin.

TABLE I

Hydrolysis of Lecithins
(Composition of Liberated Fatty Acids)

Venom	*Naja naja*		*Bothrops atrox*		*Ophiophagus hannah*		*Crotalus atrox*		*Vipera russeli*		*Crotalus adamanteus*		*Ancistrodon contortrix*	
Lecithin	48.9% HE in safflower		44.0% HE in safflower		37.4% HE in safflower		37.4% HE in safflower		34.4% HE in soybean		47.8% HE in soybean		37.9% HE in soybean	
% Hydrolysis	48.0	100	21.8	100	37.1	100	30.0	100	32.0	100	43.4	100	24.7	100
16:0	5.6	3.2	4.6	2.4	2.7	2.5	2.8	2.3	2.4	1.2	3.2	2.3	3.1	1.5
18:0	64.4	48.8	62.7	41.0	35.6	31.2	38.3	31.7	48.6	27.9	58.4	43.5	60.9	30.1
18:1	4.9	8.7	3.8	7.2	7.7	8.9	7.9	8.0	6.6	8.6	5.2	6.6	4.4	8.2
18:2	20.5	36.0	23.9	45.8	51.3	54.3	47.5	54.6	34.4	54.8	26.9	40.3	23.0	50.9
20:0	4.6	3.3	5.0	3.6	2.7	3.1	3.5	3.4	4.3	2.5	4.3	3.7	5.9	4.4
18:3	...								3.6	5.0	2.0	3.6	2.6	4.9

HE = hydrogenated egg lecithin

Ophiophagus hannah. In the early stages of the hydrolysis the proportions of the saturated fatty acids in the liberated acids were generally highest, and that of the unsaturated fatty acids the lowest. As the reaction proceeded the concentration of the unsaturated fatty acids increased. The general pattern is shown more clearly in Table II by analyses made at several stages in the hydrolysis. The results with *Ophiophagus hannah* are also shown in Table II for comparison. Moore and Williams (6) made a similar observation on the analysis of the composition of the fatty acids released at several stages in the hydrolysis of egg lecithin by *Crotalus adamanteus*. These workers attributed the increase in the saturated fatty acids to a selective hydrolysis of the α-unsaturated-β-saturated class on the assumption that the α-saturated-β-saturated class was not attacked in the early stages of the hydrolysis. In the above experiments (Tables I and II) mixtures of hydrogenated and natural lecithins were employed in order to have a sample that contained an appreciable amount of the disaturated type. In accordance with the observation of Long and Penney (4), hydrogenated lecithin alone was hydrolyzed very slowly. When hydrogenated lecithin was mixed with a natural lecithin as in the above experiments, the rate of hydrolysis was not decreased, however, and these mixtures were hydrolyzed at about the same rate as the natural lecithins.

Since the concentration of the fatty acids may influence the rate at which they are hydrolyzed, the specific reaction rate of the hydrolysis of the major fatty acids was determined

TABLE II

Composition of Liberated Fatty Acids

Bothrops atrox (44.0% hydrogenated egg in safflower)

% Hydrolysis	16:0	18:0	18:1	18:2	20:0
6.0	5.4	61.5	4.4	23.5	5.2
21.8	4.6	62.7	3.8	23.9	5.0
85.5	3.1	50.3	6.5	35.6	4.5
95.2	2.6	42.4	6.2	45.2	3.6
Complete	2.4	41.0	7.2	45.8	3.6

Crotalus adamanteus (47.8% hydrogenated egg in soybean)

% Hydrolysis	16:0	18:0	18:1	18:2	20:0	18:3
20.2	4.2	52.6	5.1	31.9	4.4	1.7
43.4	3.2	58.4	5.2	26.9	4.3	2.0
56.4	3.2	54.7	5.2	30.4	4.2	2.3
89.6	2.3	40.9	6.6	42.9	3.9	3.5
Complete	2.3	43.5	6.6	40.3	3.7	3.6

Vipera russeli (34.4% hydrogenated egg in soybean)

% Hydrolysis	16:0	18:0	18:1	18:2	20:0	18:3
32.0	2.4	48.6	6.6	34.4	4.3	3.6
59.1	1.9	36.3	7.7	45.9	3.3	4.8
77.6	1.7	35.7	8.3	46.8	3.2	4.4
Complete	1.2	27.9	8.6	54.8	2.5	5.0

Ophiophagus hannah (37.4% hydrogenated egg in safflower)

% Hydrolysis	16:0	18:0	18:1	18:2	20:0
6.2	3.7	30.7	9.1	54.1	2.4
18.4	3.2	33.1	7.9	52.9	2.9
37.1	2.7	35.6	7.7	51.3	2.7
81.9	2.2	32.7	8.3	53.6	3.2
Complete	2.5	31.2	8.9	54.3	3.1

from the plot of the extent of hydrolysis vs. the log of the concentration of each of the fatty acids. This plot conformed to the kinetics of a first order reaction and the specific rate was determined from the slopes of these relationships (Table III). The results showed that with these samples of lecithins the saturated fatty acids were liberated faster than the unsaturated fatty acids and in the order of 16:0 > 18:0 > 20:0. The rates of the hydrolysis of the different unsaturated fatty acids were about the same in these experiments. In some experiments the relative rates of hydrolysis of the unsaturated fatty acids appeared to follow the degree of unsaturation, but the differences were not great enough to be considered significant. Although this pattern of rates of hydrolysis of the individual fatty acids was common to all the venoms in Table I, except for *Ophiophagus hannah,* judging from the comparison of the changes in the fatty acid composition at various stages in the hydrolysis, different patterns may be expected from phospholipase A enzymes from different sources and, perhaps, with different lecithin substrates. It is plausible that the major pathway for the synthesis of lecithins of particular structure involves specific phospholipase A enzymes acting in concert with specific acyl transferase enzymes in accordance with mechanism proposed by Lands and Hart (10). Accordingly, one may expect to find phospholipase A enzymes having little or no specificity such as that in the venom of *Ophiophagus hannah,* as well as those which exhibit specific properties.

Since the venom of *Ophiophagus hannah* does not exhibit a preferential rate of hydrolysis of the individual fatty acids and it is highly active, the fastest acting of those tested, it is ideally suited for the analytical determination of the distribution of the fatty acids between the α- and β-positions of the lecithins.

In order to illustrate the error that may be encountered in the determination of the distribution of the fatty acids between the α- and β-position of lecithins when the reaction is not carried to completion with venoms which exhibit a preferential rate of hydrolysis of the individual fatty acids, the results of the hydrolysis of samples of lecithin with *Crotalus adamanteus* at 43% and complete hydrolysis is shown in Table IV. In these experiments the distribution of the fatty acids in the α- and β-positions was determined independently as follows:

$$\%\ \text{in}\ \beta\text{-position} = \frac{100 \times \%\ \text{in liberated acids}}{2 \times \%\ \text{in original lecithin}}$$

$$\%\ \text{in}\ \alpha\text{-position} = \frac{100 \times \%\ \text{in lysolecithin}}{2 \times \%\ \text{in original lecithin}}$$

The results in Table IV show that at 43% hydrolysis an appreciable error is encountered in the determination of the percent of distribution of the fatty acids between the α- and β-positions with *Crotalus adamanteus,* as evidenced by the discrepancy in calculated values for the percent distributed between the two positions which should add up to 100. Furthermore, the error from results based on the analysis of the liberated fatty acids is in the opposite direction to that determined on the basis of the fatty acid composition of lysolecithin. In contrast to the venom of *Crotalus adamanteus,* that of *Ophiophagus hannah* gave values that agreed close to 100% recovery within limits of the experimental error at all stages in the hydrolysis (Table IV).

Since Moore and Williams (6) proposed that differences in the rates of the hydrolysis

TABLE III

Specific Reaction Rates of the Hydrolysis of Fatty Acids

Fatty acid	34.4% Hydrogenated egg in soybean	44.0% Hydrogenated egg in safflower
	Vipera russeli	*Bothrops atrox*
16:0	1.20	1.20
18:0	1.14	1.08
20:0	1.13	1.04
18:3	.65	....
18:2	.55	.58
18:1	.63	.57

TABLE IV

Determination of the Percent Distribution of Fatty Acid Between α- and β-Positions

	Crotalus adamanteus (47.8% hydrogenated egg in soybean)					
	43.4% Hydrolysis			100% Hydrolysis		
	β-pos	α-pos	$\alpha+\beta$-pos	β-pos	α-pos	$\alpha+\beta$-pos
16:0	7.8	122.7	130.5	5.2	94.6	99.8
18:0	95.7	38.5	134.2	68.4	31.7	100.1
18:1	36:6	30.5	67.1	53.2	47.6	100.8
18:2	37.9	27.7	65.6	59.4	40.4	99.8
20:0	82.7	.....	82.7	77.1	22.9	100.0
18:3	26.3	28.9	55.2	50.0	50.0	100.0
	Ophiopagus hannah (37.4% hydrogenated egg in safflower)					
	37.1% Hydrolysis			100% Hydrolysis		
16:0	6.9	93.9	100.8	5.4	94.7	100.1
18:0	69.5	31.0	100.5	65.5	34.2	99.7
18:1	47.0	54.8	101.8	60.1	41.2	101.3
18:2	57.4	41.9	99.3	62.1	38.1	100.2
20:0	77.8	19.4	97.2	70.5	27.3	97.8

of the fatty acids may be related to differences in the rates of hydrolysis of the classes of lecithin, we determined, in one experiment, the relative amounts of the different classes in the unreacted lecithin remaining at various stages of the hydrolysis. Should any differences exist in the class composition of the unreacted lecithin in such an experiment, it would indicate that the classes were being hydrolyzed at different rates. The results (Table V) showed that the class composition was different at the various stages in the reaction when the venom of *Crotalus adamanteus* which hydrolyzed the fatty acids at different rates was used. Thus the different classes were hydrolyzed at different rates in accordance with the observations of Moore and Williams (6).

However, in contrast to the results obtained by Moore and Williams (6), the results in Table V showed that the disaturated class appeared to be hydrolyzed the fastest. The hydrolysis of this class was so slow in Moore and Williams' experiments which were carried out on egg lecithin, they presumed that no significant amount of hydrolysis of this class occurred in the early stage of the reaction and based their method for the determination of class composition on this premise. Since egg lecithin contains on a very small amount of the disaturated class (SS), it appears that the difference in our results probably can be explained on the basis of a concentration effect (the sample used in our experiment contained 48.6% of the disaturated class). However, as pointed out by Moore and Williams, many experiments would have to be made on pure lecithin classes to define the precise kinetics of the reaction. Also, since the rate of hydrolysis of the disaturated class is much faster in mixtures than alone, the kinetics of the hydrolysis of a mixture of classes as found in natural lecithin would be exceedingly complex. Regardless, it appears that since the individual fatty acids may be hydrolyzed at different rates and the rate of the reaction is related to concentration, it is doubtful that there is any simple relationship between the rate of hydrolysis of the individual fatty acids and the lecithin classes that can be used for the quantification of the latter.

TABLE V

Analysis of Unreacted Lecithin at Various Degrees of Hydrolysis with *Crotalus adamanteus* (47.8% hydrogenated egg in soybean lecithin)

Type		% Hydrolysis		
α	β	zero	43.4	89.6
S	S	48.6	19.0	31.4
U	S	0.8	2.0	0.7
S	U	17.0	67.4	53.1
U	U	33.6	11.6	14.8

S and U = saturated and unsaturated fatty acids, respectively.

ACKNOWLEDGMENT

Supported in part by USPHS Grant HE 05735 from the National Institutes of Health.

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[Received Jan. 31, 1966]

Stereochemistry of α-Parinaric Acid from *Impatiens edgeworthii* Seed Oil[1]

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ABSTRACT

α-Parinaric acid is a major constituent fatty acid (48%) from *Impatiens edgeworthii* Hook F. seed oil. Partial hydrazine reduction of the tetraene gave products which permit defining the stereochemistry of α-parinaric acid. Its structure is *cis*-9, *trans*-11, *trans*-13, *cis*-15-octadecatetraenoic acid. The tetraene readily reacts with maleic anhydride to give the Diels-Alder product with no *trans*-unsaturation. The formation of this adduct is contrary to previous reports.

INTRODUCTION

FIRST REPORTED in 1933 to be a constituent of the seed oil *Parinarium laurinum* of the Rosaceae family (1), α-parinaric acid was initially considered a geometrical isomer of eleostearic acid. Two years later Farmer and Sunderland (2) designated it as a 9,11,13,15-octadecatetraenoic acid. Since then, numerous members of the genus *Impatiens* of the Balsaminaceae family have been reported to contain α-parinaric acid (3,4).

During the more than 30 years that α-parinaric acid has been known, efforts to learn its stereochemistry have been frustrated (5,6). Both Riley (5) and Kaufmann and Sud (6) failed to obtain Diels-Alder adducts by reaction with maleic anhydride. Kaufmann and Sud concluded that *trans*-double bonds could not be adjacent.

Impatiens edgeworthii Hook F. seed oil, not previously studied, has 48% of α-parinaric acid. This communication defines the stereochemistry of α-parinaric acid isolated from *I. edgeworthii* seed oil.

PROCEDURES AND DATA

General Methods

Gas-liquid chromatographic (GLC) analyses were carried out with a Burrell Kromatog K-5, and the retention values were treated as described by Miwa et al. (7). Free acids were identified by comparing retention times of observed peaks with those of components of a standard mixture. Quantities are reported as area percent. Infrared spectra were determined on carbon disulfide solutions with an Infracord Model 337 spectrophotometer; ultraviolet spectra, on isooctane solutions with a Beckman DK-2A far ultraviolet spectrophotometer; and melting points, on a Fisher-Johns block. Extractions and most reactions were performed in a nitrogen atmosphere either in the dark or in flasks wrapped with aluminum foil. Most products were maintained as petroleum ether solutions during storage or transfer manipulations.

Preparation of Mixed Methyl Esters

I. edgeworthii seed was flaked in a roller mill, and the resulting flakes were immediately immersed in pentane-hexane (bp 33–57C). The oil was removed by extraction in a Soxhlet apparatus.

I. edgeworthii seed oil (13.7 g) in ca. 2 liters of pentane-hexane was stirred 20 hr with 100 ml of 2 *N* ethanolic potassium hydroxide at room temperature. Water (100 ml) and ethyl ether (600 ml) were added, and the mixture was chilled in an ice bath. Free acid liberated by dropwise addition of *N* hydrochloric acid during nitrogen agitation was taken up in the organic phase. The aqueous phase was further extracted with two 250-ml portions of ethyl ether. The combined ethereal solution was washed with water and then dried over sodium sulfate. Almost all the solvent was removed at room temperature under a stream of nitrogen. The fatty acid concentrate was esterified by reaction with diazomethane (8).

GLC analyses gave the relative amounts of esters with retention characteristics like those of common esters, and ultraviolet analyses provided the amount of tetraene present. Table I shows the composition of the total ester mixture based on GLC and ultraviolet analyses.

Isolation of Methyl α-Parinarate

The mixture of esters was fractionated by countercurrent distribution (CCD) in a 200-tube Craig-Post apparatus with the solvent system hexane-acetonitrile. The esters were divided among the first 10 tubes of the apparatus, and 10 ml of upper phase was added

[1] Honorable Mention, AOCS Bond Award. Presented at the AOCS meeting, Cincinnati, Ohio, October 1965.
[2] No. Utiliz. Res. Dev. Div., ARS, USDA.

TABLE I

Composition of Water-Insoluble Acids from *Impatiens edgeworthii* Seed Oil

Type of acid	Ester, %	Type of acid	Ester, %
14:0	trace	18:2	6
16:0	5	18:3	26
18:0	3	18:4 conj.	48[a]
18:1	11		

[a] Conjugated tetraene was determined by ultraviolet analyses by using λ max 302.5 mμ $E^{1\%}_{1\,cm}$ 2560 equal 100%.

at each transfer stage. After 190 transfers, upper phase was decanted into a fraction collector combining 4 transfers per fraction until 990 transfers had been made. Solvent was removed, in vacuo, from selected fractions to give the weight distribution plot shown in Figure 1.

Samples were taken at either side of the expected position of the tetraene peak to locate the peak and still conserve and preserve sample. With the exception of 870 through 873, transfers 634 through 906 were combined and stored in the dark as a 0.2% hexane solution at −18C. A sample taken after 6 weeks' storage had infrared and ultraviolet spectra like that of the initial preparation.

Spectral Data

Methyl Ester. The infrared spectrum of the tetraene in carbon disulfide solution has characteristic absorption at the 10–11 μ region (10.08 S, 10.30 W, 10.53 M, 10.74 W, and 10.88 μW). The ultraviolet spectrum of the tetraene has maxima at 278.5, 289.5, 302.5, and 317 mμ with $E^{1\%}_{1\,cm}$ = 870; 1,690; 2,560; and 2,240, respectively.

Impatiens Oil. The ultraviolet spectrum of *I. edgeworthii* seed oil has maxima at 280, 290, 303.5, and 318 mμ with $E^{1\%}_{1\,cm}$ = 410; 800; 1,230; and 1,100, respectively. The infrared spectrum of the oil in carbon disulfide has absorbance at 10.09 S, 10.32 W, and 10.53 M, and 10.75 μW.

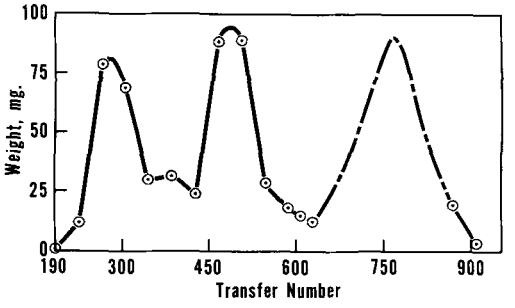

FIG. 1. Countercurrent distribution of methyl esters from *Impatiens edgeworthii* seed oil. Dashed portion of the plot is an estimate based on recovery.

Hydrogenation Studies

Impatiens Oil. A portion of the oil (0.179 g) was reduced as an ethanol solution in the presence of a platinum catalyst. A portion of the saturated oil was transesterified in 3 ml of methanol containing 2% of sulfuric acid. The esters were isolated in the usual manner, and GLC analyses showed 4.8% of methyl palmitate and 95.2% of methyl stearate. Some of the saturated oil was saponified; then the solvent was removed from the alkaline solution, and the remaining solid was chilled, covered with ethyl ether, and acidified with hydrochloric acid. Acetic acid was found in the ethereal solution by GLC analyses.

Methyl α-Parinarate. Pure methyl α-parinarate (0.094 g) was hydrogenated in ethanol with a platinum catalyst. The ester absorbed 3.9 mole equivalents of hydrogen to yield a white solid melting at 34.5–36.0C. GLC analyses indicated 97+% of methyl stearate and about 2% of methyl "oleate."

Oxidation of α-Parinarate

A portion (0.41 g) of the tetraene was oxidized with permanganate-periodate according to the method of von Rudloff (9). The resulting cleavage products were extracted with six 150-ml portions of ethyl ether. The combined ether extract was dried over sodium sulfate. Solvent was removed in vacuo at 0 C. A free acid sample was shown by GLC analysis to contain propionic and acetic acid (4:1). The acetic acid is probably the result of over oxidation. The remainder of the cleavage products was esterified with diazomethane. Solvent was removed in vacuo to leave 0.313 g of esters. GLC analyses indicated 90% of dimethyl azelate, 2% of dimethyl suberate, and lesser amounts of other products of shorter retention times.

Partial Hydrazine Reduction

A portion of the methyl α-parinarate (2.09 g) was reduced with hydrazine as previously described for the reduction of α-eleostearic acid (10). Aliquots were removed at predetermined time intervals to evaluate the progress of the reduction (Fig. 2). For ultraviolet determinations, arbitrarily selected ε-values were used for calculating conjugated diene (28,300), conjugated triene (47,000), and conjugated tetraene (74,400). Since the tetraene gives rise to a

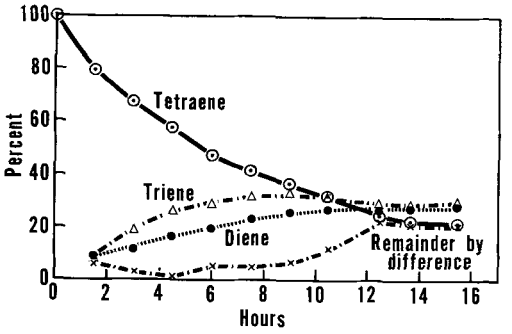

FIG. 2. Hydrazine reduction of *Impatiens edgeworthii* tetraene as estimated by ultraviolet analyses.

peak at about 279 mμ which overlaps the triene peak at 270 mμ, an empirical correction was applied in the determination for conjugated triene. The correction factor, optical density apparent triene—0.18 X optical density of the tetraene peak at 317 mμ = optical density of corrected triene, was deduced by determining the absorbance at 270 mμ for various concentrations of pure tetraene and relating the absorbance changes at 270 mμ to the absorbance of the secondary peak for tetraene at 317 mμ.

Fractionation of Hydrazine-Reduced Products

Methyl esters (1.926 g) of the hydrazine reduction product obtained after 15.5 hr were fractionated by CCD as described previously. The esters were added to tube O with 10 ml of hexane and 40 ml of acetonitrile. At each transfer, 10 ml of equilibrated hexane was added to tube O. After 200 transfers the upper phase was decanted into the fraction collector combining 2 transfers per fraction. Solvent was evaporated from selected fractions, and the

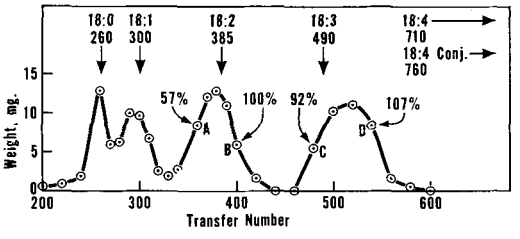

FIG. 3. Countercurrent distribution of a hydrazine-reduction product mixture from *Impatiens edgeworthii tetraene*. The numerals across the top indicate the position of expected maxima for nonconjugated fatty acid methyl esters. Fractions A, B, C, and D refer to selected fractions on which detailed spectral data were obtained. The percentages are approximate amounts of conjugation as determined by ultraviolet spectroscopy.

TABLE II

Spectral Data on Selected Countercurrent Distribution Fractions from Hydrazine-Reduced Tetraene[a]

Sample designation		Major chromaphore		
Fraction	Transfer No.	Infrared	Ultraviolet max.	ε
A	360	*trans,trans*	231.0	18,800
B	400	*cis,trans*	231.5	27,400
C	480	*cis,trans,trans*	268.5	43,000
D	540	*cis,trans,trans*	268.5	50,100

[a] See Fig. 3.

residues were weighed to give the data shown in Figure 3. Spectral analyses of selected fractions are summarized in Table II.

Maleic Anhydride Adduct of Methyl α-Parinarate

Preparation. The tetraene (0.057 g) was refluxed 2 hr with 5 ml of benzene and 0.049 g of maleic anhydride. Solvent was removed in vacuo. The product was dissolved in ethyl ether, washed with four 50-ml portions of water, and dried over sodium sulfate. Ether was evaporated in vacuo to give 0.067 g of product. Absorption bands at 5.39 and 5.64 μ (anhydride carbonyl) showed the presence of adduct, and ultraviolet spectral analysis showed about 32% of tetraene. After trituration with pentane-hexane, 0.027 g of insoluble purified adduct was obtained. The infrared spectrum of the purified adduct showed no trans-unsaturation. Adduct formed in a similar manner darkened while being dried in an Abderhalden apparatus. Elemental analyses of the product gave values about 2% low with respect to calculated carbon. Additional adduct was prepared, and the product was hydrogenated with a platinum catalyst. The saturated adduct was isolated from hydrogenated starting materials by chromatography on a silica column.

Anal. Calcd. for $C_{28}H_{38}O_5$: C, 70.01; H, 9.71. Found: C, 70.38; H, 9.68.

Oxidation. The tetraene Diels-Alder product was ozonized in methanol essentially by the procedure of Ackman et al. (11). The bulk of the formic acid solvent from the peracid cleavage of the ozonides was removed by distillation through a fractionating column. GLC analyses of the free acids showed propionic and formic acids. Methyl esters of the cleavage products gave GLC peaks corresponding to dimethyl azelate (90%), dimethyl suberate (5%), and lesser amounts of products with shorter retention times.

trans, trans-Diene Concentrate

Isolation. Transfers 372 through 379 and 382 through 389 from CCD of hydrazine-

reduction products (Fig. 3) were combined to give 0.104 g. As evidenced by infrared spectroscopy, a *trans,trans*-conjugated diene fraction (0.016 g) was isolated by crystallization from acetone at −70C.

Cleavage. The *trans,trans*-conjugated diene was oxidized with permanganate-periodate in 30% *t*-butanol (9). The fragments obtained correspond to those expected from cleavage of the mixture shown in Table III.

cis, trans-Diene Concentrate

Isolation. Transfers 392 through 399 from CCD of hydrazine-reduction products (Fig. 3) were combined to give 0.037 g of a mixture of dienes. The fraction had an infrared spectrum like that of a mixture of *cis,trans*- and *trans, trans*-conjugated dienes. The residue was reacted 3 hr with maleic anhydride as above to yield 0.044 g. A portion of the reaction product (0.034 g) was fractionated on a silica gel column with hexane-ethyl ether (49:1). A nonadducted fraction (0.010 g) was obtained, λ max 233 mμ, ϵ = 25,200. The infrared spectrum of the nonadduct indicated *cis,trans*-conjugated diene with ca. 10% of *trans,trans*-conjugated diene (12).

Cleavage. The *cis,trans*-conjugated diene was oxidized with permanganate-periodate as previously described. The cleavage fragments correspond to those expected from oxidation of the mixture shown in Table III.

Preparation of α-Parinaric Acid

A portion of the methyl α-parinarate hexane solution (30 ml) was stirred overnight at room temperature with 5-ml of 2*N* ethanolic potassium hydroxide. The saponification mixture was worked up as described for the saponification of *Impatiens oil.* Solvent was removed in vacuo to yield 0.072 g of α-parinaric acid. Its infrared spectrum at the 10–11 μ region is like that of the methyl ester, except the bands at 10.53, 10.74, and 10.88 μ are superimposed on the broad medium intensity OH deformation (out of plane) band from the carboxyl group (13). The absorbance of the band at 10.08 μ gave K = 1.44.

Attempts to obtain the melting point of α-parinaric acid between microscope cover slides emphasize the reactivity of this acid. When heating α-parinaric acid at our usual rate, ca. 2 to 3C per min, softening occurred at 60C. As the temperature continued to rise, the appearance of the sample changed to indicate apparent resolidification. At about 130C, the sample became bright yellow, but it darkened at higher temperatures. A fresh sample, placed on the melting point block at 55C and rapidly heated, melted at 65–70C. Upon standing overnight under nitrogen purge, α-parinaric acid gave a hard lump with no appreciable solubility in carbon disulfide. Even at a high rate of heating, this hard material failed to melt at temperatures up to 200C. The infrared spectrum of the tetraene artifact as a Nujol mull had little absorption in the 10–11 μ region.

DISCUSSION

Pure conjugated tetraene from *Impatiens edgeworthii* seed oil was isolated as its methyl ester by CCD. It readily absorbed 4 mole equivalents of hydrogen to give methyl stearate; thus, a normal C-18 skeleton was indicated. Oxidative cleavage of the ester gave fragments indicating that the unsaturation is in the 9,11,13,15-positions. Comparing absorbance for the band at near 10.1 μ (K = 1.44) with the absorbance values summarized in Table IV (14) indicates the presence of 2 *trans*-double bonds in a conjugated system. To determine the configuration of each double bond, the tetraene was partially reduced with hydrazine. The resulting mixture was resolved according to unsaturation by CCD. Spectral analyses of selected fractions, summarized in Table II, indicate that the two *trans*-double bonds are adjacent. This finding is contrary to the report by Kaufmann and Sud (6) on their studies with parinaric acid from other *Impatiens* species.

Kaufmann and Sud proposed that α-parinaric acid has one of the three possible structures in

TABLE III

Compositions of Dienes from Hydrazine Reduction[a]

Isomer	*trans,trans* %	*cis,trans* %
9,11	12	39
9,13	....	4
9,15	....	5
11,13	71	14
11,15	....	1
13,15	16	35

[a] Determined by GLC analyses of oxidative cleavage fragments.

TABLE IV

Infrared Absorbance Values of Known Conjugated Acids

Ester	Double bond configuration	K[a]
Punicate	*cis,trans,cis*	0.52
Diene	*cis,trans*	0.495
α-Eleostearate	*cis,trans,trans*	1.68
Diene	*trans,trans*	1.43
β-Eleostearate	*trans,trans,trans*	2.39

[a] Values determined for absorbance maxima at near 10.1 μ (14).

which no two *trans*-double bonds could be adjacent. The spectral evidence from the present study provides for two structural patterns; i.e., *trans,trans,cis,cis* or *cis,trans,trans,cis* for configuration of the double bonds. The conjugated trienes, which could result from partial reduction of these two possible structures, can be easily identified by infrared spectroscopy. The spectrum of a *cis,cis,trans*-conjugated triene isomer has been reported (15), and Tolberg et al. (14) have published the spectrum of a *cis,trans,trans*-conjugated triene. We have evidence for only the *cis,trans,trans-* (*trans, trans,cis-*) conjugated triene (Table II); therefore, provided hydrazine reduced the double bond in a manner analogous to α-eleostearic acid (10), the parent tetraene has the *cis,trans, trans,cis*-configuration of double bonds. This structure is further supported by data in Table III obtained by cleaving concentrates of *trans, trans-* and *cis,trans*-conjugated dienes. Conclusive proof of configuration was obtained by preparing the maleic anhydride adduct of the tetraene. Other authors (5,6) have reported attempts to prepare maleic anhydride adducts from α-parinaric acid, but they were not successful. The infrared spectrum of the Diels-Alder product showed no *trans*-unsaturation. Cleavage fragments from oxidation of the adduct were those expected for a product formed by adduction across the two central double bonds. Therefore, α-parinaric acid is *cis*-9, *trans*-11,*trans*-13,*cis*-15-octadecatetraenoic acid.

The hydrazine reduction of the tetraene proceeds essentially as it did with α-eleostearic acid (10). The bulk of the products were conjugated; however, evidence was obtained for the presence of some nonconjugated products.

The presence of acetic acid as a triglyceride constituent in *I. edgeworthii* seed oil is in accord with studies of seed oils from other *Impatiens* species (4). The infrared spectrum of α-parinaric acid from the current study is similar to the spectrum reported by Kaufmann and Sud (6) for impatienic acid. This acid was reported to isomerize to α-parinaric acid on standing overnight. This transformation was evidenced by spectral analyses and melting point data. Our attempts to repeat this isomerization phenomenon resulted in complete loss of α-parinaric acid during the overnight storage to give an intractable solid. The infrared and ultraviolet spectra of methyl α-parinarate were unchanged when stored in solvent and protected from light.

In recent publications Takagi (16) described studies with the tetraene from *Parinarium laurinum* of the Rosaceae family. Using the hydrazine approach, he obtained results similar to ours. Therefore, the acid from both *P. laurinum* and *I. edgeworthii* seed oils is identical. These findings are in accord with the theory recently advanced by Gunstone (17) for the possible biosynthesis pathway to conjugated polyenes.

ACKNOWLEDGMENTS

Gas-liquid chromatographic analyses by J. W. Hagemann. Microanalyses by Mrs. Clara McGrew. Translation of reference 16 by Dr. T. K. Miwa.

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Keto Fatty Acids from *Cuspidaria pterocarpa* Seed Oil[1]

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ABSTRACT

The seed oil of *Cuspidaria pterocarpa* contains three keto fatty acids with unusually long carbon chains: 15-oxo-*cis*-18-tetracosenoic (5.4%), 17-oxo-*cis*-20-hexacosenoic (13.4%), and 19-oxo-*cis*-22-octacosenoic (3.3%) acids. These acids were isolated by countercurrent distribution of the corresponding methyl esters. Their structures were established by oxidative degradation, by reduction to known compounds, and by nuclear magnetic resonance and infrared spectra.

INTRODUCTION

FATTY ACIDS CONTAINING ketone functions are rarities in natural lipids of plant origin. Apparently, the only ones whose complete structures have been recorded in the literature previously are 4-oxo-*cis*-9,*trans*-11,*trans*-13-octadecatrienoic (α-licanic) and 9-oxo-*trans*-10, *trans*-12-octadecadienoic acids. The former is a major constituent of Brazilian oiticica oil (1). The latter of these two was found recently as a minor constituent of *Dimorphotheca* seed oil (2).

Preliminary investigation of seed oil of *Cuspidaria pterocarpa* (Cham.) DC. (family Bignoniaceae) suggested the presence of three keto fatty acids. The present paper describes the isolation and structural elucidation of these keto acids. The structure of one has been reported in a preliminary communication (3).

EXPERIMENTAL PROCEDURES AND RESULTS

General Methods

Esterifications and transesterifications were carried out as follows, except where otherwise 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 30C–60C). Combined extracts were dried over sodium sulfate and evaporated in vacuo.

Gas-liquid chromatographic (GLC) analyses were carried out exactly as described by Miwa and co-workers (4), including the use of apparatus and detector described.

Thin-layer chromatography (TLC) was performed on plates coated with Silica Gel G (according to Stahl) with the solvent system hexane-diethyl ether-acetic acid (80:20:1).

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 A-60 spectrometer on 1% deuteriochloroform solutions.

Melting points were determined with a Fisher-Johns block and are uncorrected.

Preparation of Mixed Methyl Esters

Coarsely ground seeds of *Cuspidaria pterocarpa* (Cham.) DC. (13.87 g) were extracted overnight in a Soxhlet apparatus with petroleum ether (bp 30C–60C). Upon evaporation of solvent, 7.87 g of oil was obtained. Its IR spectrum showed a double carbonyl peak having a strong maximum at 1740 cm^{-1} (ester) and a weaker one at 1720 cm^{-1} (ketone).

A 3.21 g portion of the oil was converted to a mixture of methyl esters by transesterification; a yield of 3.12 g of methyl esters was obtained. Their IR spectrum was very similar to that of the oil in the carbonyl region. The esters had the following composition (expressed as wt %): C_{16}, 2.6; C_{18}, 68.3; C_{20}, 5.2; C_{22}, 0.9; C_{24} keto, 5.4; C_{26} keto, 13.4; and C_{28} keto, 3.3. It became apparent from the amount of C_{28} keto ester isolated subsequently that the value obtained by GLC for this compound was considerably too low. Consequently, the weight percentages indicated here for the various components have been corrected on the basis of estimates of the amounts of keto esters in fractions obtained by countercurrent distribution.

In order to determine whether acid-catalyzed transesterification appreciably altered the composition of *Cuspidaria* oil, methyl esters also were prepared by two other procedures: *Cuspidaria* oil was saponified by stirring 24 hr with 1 *N* ethanolic potassium hydroxide at ambient temperature under a nitrogen atmosphere. A portion of the free acids thus obtained was esterified with diazomethane. Another portion was esterified with 1% methanolic sulfuric acid. The three ester preparations were essentially identical as judged by TLC, GLC, and IR. Only 1.4% of unsaponifi-

[1] Presented at the AOCS Meeting, Los Angeles, April 1966.

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

ables was found in the oil. Consequently, the acid-catalyzed transesterification method was chosen for large-scale preparation of methyl esters from *Cuspidaria* oil because of its simplicity.

Countercurrent Distribution of Methyl Esters

Countercurrent distribution (CCD) of *Cuspidaria* mixed methyl esters was carried out with an acetonitrile-hexane system by the general procedure of Scholfield et al. (5). An 11.01 g portion of the mixed esters was divided among the first 6 tubes of a 200-tube Craig-Post apparatus. Ten milliliters of upper phase and 40 ml of lower phase were used throughout the distribution. After the 200 fundamental transfers had been completed, upper phases were decanted into a fraction collector; four transfers were combined in each tube. Apparently the entire starting sample emerged from the apparatus by the time 600 transfers had been applied. The three keto esters formed three discrete peaks, satisfactorily resolved from one another. Unfortunately, however, these three coincided with peaks formed by esters of common fatty acids that were present in larger amounts—oleate, linoleate, and linolenate. Results of GLC analyses of esters from selected CCD tubes are given in Table I. Concentrates of the individual keto esters suitable for isolation of more highly purified compounds for characterization were obtained by combining contents of appropriate tubes.

TABLE I

Composition of CCD Fractions
(Results estimated by GLC and expressed as area percent)

Tube number[a]	Transfers[b]	% Keto esters[c]	% Non-Keto esters[d]
20	280	0.7 C_{28}	11.2 C_{16}, 14.2 C_{18}S, 38.5 C_{20}, others
25	300	9.6 C_{28}	13.6 C_{16}S, 72.2 C_{18} (Mostly C_{18}I), 3.7 C_{20}
30	320	16.7 C_{28}, 1.0 C_{26}	16.3 C_{16}S, 73.9 C_{18} (Mostly C_{18}I), 3.2 C_{20}
35	340	13.4 C_{28}, 18.9 C_{26}	54.8 C_{18}I, 31.4 C_{18}II
40	360	44.0 C_{26}	55.3 C_{18}II
50	400	32.4 C_{26}	66.4 C_{18}II
55	420	30.8 C_{26}, 2.1 C_{24}	66.0 C_{18}II
60	440	12.2 C_{26}, 31.8 C_{24}	51.7 C_{18}II + C_{18}III
65	460	0.3 C_{26}, 44.9 C_{24}	54.3 C_{18}III
70	480	36.9 C_{24}	62.8 C_{18}III
80	520	22.8 C_{24}	77.3 C_{18}III
90	560	16.5 C_{24}	83.0 C_{18}III

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

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

[c] The indicated percentages are subject to error, especially in the case of the C_{28} keto ester (see text).

[d] Where indicated the symbols have the following significance: S = Saturated; I = one double bond; II = two double bonds; III = three double bonds.

Isolation and Preliminary Characterization of Keto Esters

The individual keto esters were isolated by low-temperature crystallization. The hexane solutions of mixed esters that resulted from combining CCD fractions were reduced considerably in volume and then stored at −18C for 3 days. The resulting mixtures were then filtered with conventional Büchner funnels at the same temperature; collected products were washed with a small amount of cold solvent.

C_{26}-Keto Ester (Ib). Low-temperature crystallization of material from CCD tubes 41–54 afforded 1.12 g of C_{26}-keto ester (Ib), mp 41.5–43.5C. When 0.049 g of this was recrystallized from methanol, 0.018 g was provided, mp 44–45C. Ib had strong IR maxima at 1720 and 1745 cm^{-1}. With platinum oxide catalyst, Ib consumed 1.1 moles of hydrogen in ethanol solution. The NMR spectrum of Ib is summarized in Table II and Figure 1.

Anal. Calcd. for $C_{27}H_{50}O_3$ (422.67): C, 76.7; H, 11.9. Found: C, 76.6; H, 12.0.

C_{24}-Keto Ester (Ia). Low-temperature crystallization of esters from CCD tubes 66–79 (Table I) provided 0.349 g of Ia, mp 35–37.5C. Recrystallization of 0.080 g of this product from aqueous methanol afforded 0.061 g of Ia, mp 40.0–40.5C. Its NMR spectrum is summarized in Table II.

Anal. Calcd. for $C_{25}H_{46}O_3$ (394.62): C, 76.1; H, 11.8. Found: C, 75.6; H, 11.5.

C_{28}-Keto Ester (Ic). An 0.214 g portion of Ic was obtained by low-temperature crystallization of esters from CCD tubes 21–29 (Table I), mp 47C–49C. Recrystallization of 0.035 g of this product from methanol provided 0.033 g of Ic having mp 50.0–51.5C. Its NMR spectrum is summarized in Table II.

Anal. Calcd. for $C_{29}H_{54}O_3$ (450.72): C, 77.3; H, 12.1. Found: C, 76.8; H, 11.9.

Sodium Borohydride Reduction of C_{26}-Keto Ester

An 0.200 g portion of Ib was dissolved in 30 ml of methanol; a solution of 0.6 g of sodium

TABLE II

NMR Spectra of *Cuspidaria* Keto Esters

Assignment	τ-Value ppm	Number of protons Ia	Ib	Ic
CH_3, terminal	9.12	3	3	3
CH_2, shielded	8.73	28	32	36
CH_2, *a* to carbonyl or double bond	7.62	10	10	10
OCH_3	6.33	3	3	3
Olefinic H	4.63	2	2	2
Total protons		46	50	54

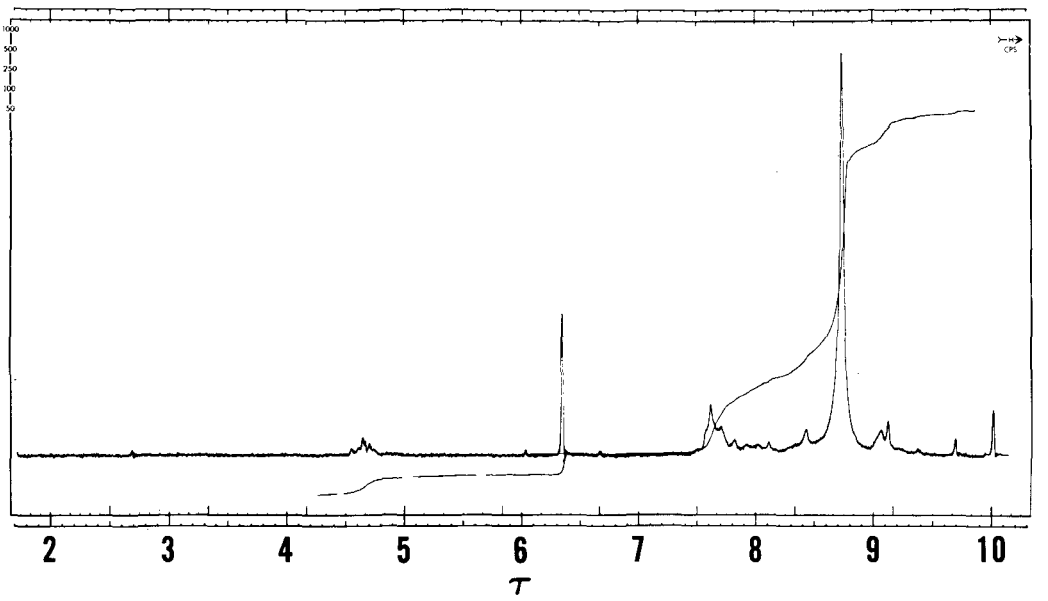

FIG. 1. NMR spectrum of C_{26}-keto ester (Ib).

borohydride in 20 ml of methanol was added. After the initial vigorous reaction had subsided, the mixture was refluxed 4.5 hr under a nitrogen atmosphere, acidified with hydrochloric acid and then extracted repeatedly with petroleum ether (bp 30–60C). Combined extracts were dried over sodium sulfate, and then evaporated. The resulting residue was reesterified since IR indicated some hydrolysis of the carbomethoxy grouping. The crude product, 0.211 g, had mp 36–42C. Two recrystallizations from aqueous methanol afforded 0.018 g of ester IIb, mp 46–47C. The IR spectrum of IIb showed maxima at 1740 cm^{-1} (ester) and 3635 cm^{-1} (OH).

Anal. Calcd. for $C_{27}H_{52}O_3$ (424.69): C, 76.4; H, 12.3. Found: C, 76.5; H, 12.5.

Reductive Deoxygenation of C_{26}-Keto Ester

An 0.106 g portion of Ib was hydrogenated at ambient temperature in 20 ml of methanol with platinum oxide catalyst. The saturated product, IIIb, was recrystallized twice from methanol; 0.039 g having mp 70.0–70.5C was obtained.

Anal. Calcd. for $C_{27}H_{52}O_3$ (424.69): C, 76.4; H, 12.3. Found: C, 76.7; H, 13.0.

Saturated keto ester IIIb was reduced with sodium borohydride essentially as was Ib. The reduction product was subjected to hydrogen iodide-phosphorus reduction by the general procedure of Meakins and Swindells (6). An 0.071 g portion of saturated hydroxy ester was refluxed 18 hr with 5 ml of hydriodic acid and 50 mg of red phosphorus. The mixture was diluted with water and extracted repeatedly with petroleum ether. Combined extracts, after having been dried with sodium sulfate, were evaporated. The oily residue was refluxed 4 hr with 0.20 g of granular zinc, 1 ml of hydrochloric acid, and 5 ml of methanol. The reduction product (IVb) was isolated by extraction with petroleum ether. Evaporation of combined extracts afforded 0.018 g having mp 57–59C. Two recrystallizations from methanol provided a sample of IVb having mp and mixed mp 61.5–62.0C (lit. mp 62C (7)). A mixture of ester IVb and an authentic specimen of methyl hexacosanoate was not resolved during GLC.

Permanganate-Periodate Oxidation of Keto Esters

Each of the three esters was oxidatively cleaved by von Rudloff's permanganate-periodate method, specifically the modification in which *t*-butyl alcohol is used as a cosolvent (8). Each yielded hexanoic acid as the only monobasic acid cleavage product, as determined by GLC.

An 0.126 g portion of Ib yielded 0.141 g of oxidation products. Two recrystallizations from benzene afforded 0.069 g of γ-keto acid Vb, mp 123–124C. Vb was obtained as a free acid because the strongly alkaline conditions during the workup hydrolyzed the ester grouping.

Anal. Calcd. for $C_{20}H_{36}O_5$ (356.49): C, 67.4; H, 10.2. Found C, 67.4; H, 10.3.

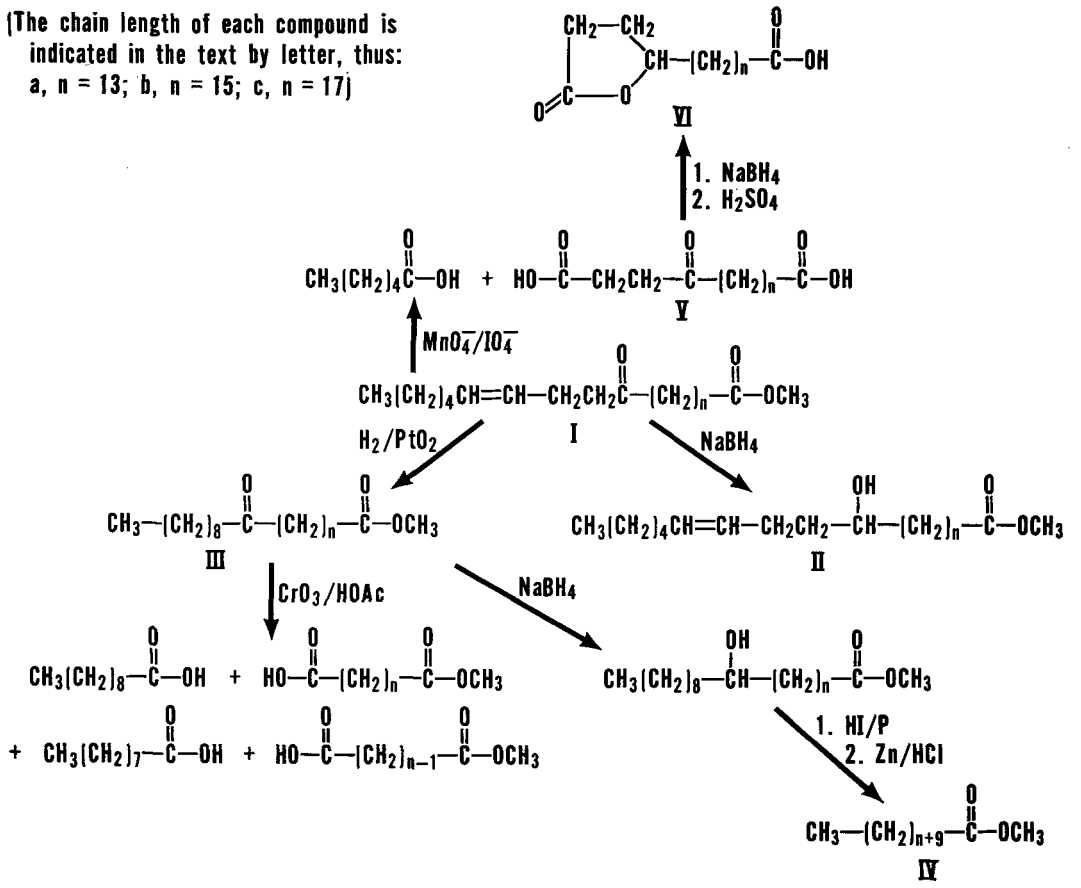

FIG. 2. Flow sheet and reaction scheme.

Vb was converted to a methyl ester for determination of IR spectrum; this showed maxima at 1720 (ketone) and 1745 cm^{-1} (ester).

An 0.137 g portion of Ia yielded 0.143 g of oxidation products. Two recrystallizations from benzene provided 0.073 g of 4-oxooctadecanedioic acid (Va), mp 122–123C. Elementary analyses of Va were not obtained owing to the accidental destruction of the analytical sample.

Oxidation of 0.088 g of C_{28}-keto ester provided 0.065 g of oxidation product. This product was recrystallized from benzene-hexane, and 0.034 g of 4-oxodocosanedioic acid (Vc), mp 119.5–121.0C, was obtained.

Anal. Calcd. for $C_{22}H_{40}O_5$ (384.54): C, 68.7; H, 10.5. Found: C, 68.2; H, 10.4.

Conversion of γ-Keto Acids to γ-Lactones

Each of the three γ-keto acids (Va, Vb, and Vc) was reduced with sodium borohydride in methanol. The reduction products were lactonized by treatment with aqueous mineral acid. The following example is typical of the procedure used:

Sodium borohydride (0.270 g) dissolved in 15 ml of methanol was added to 0.036 g of Vb dissolved in 10 ml of methanol. After the vigorous initial reaction subsided, the mixture was refluxed 2 hr, and then diluted with water, acidified with hydrochloric acid, and extracted with ethyl ether. The crude product thus isolated was refluxed 4 hr with 1% aqueous sulfuric acid. Extraction with ethyl ether and evaporation provided 0.032 g of 4-hydroxydocosanedioic acid lactone (VIb). Recrystallization from benzene-hexane provided 0.023 g of VIb, mp 95.0–95.5C. The IR spectrum of VIb showed maxima at 1720 cm^{-1} (carboxylic acid) and 1775 cm^{-1} (γ-lactone.)

Anal. Calcd. for $C_{20}H_{36}O_4$ (340.49): C, 70.5; H, 10.7. Found: C, 70.2; H, 10.6.

Chromium Trioxide Oxidation of Keto Esters

Each of the three keto esters—Ia, Ib, and Ic—was hydrogenated preparatively, and then

cleaved with chromium trioxide-acetic acid as described by Meakins and Swindells (6) with some modifications. The following will serve to illustrate:

Ester IIIb (0.028 g) was stirred 1 hr at ambient temperature with a solution of 0.35 g of chromium trioxide in 2.5 ml of acetic acid and 0.3 ml of water. Then the mixture was diluted with water and extracted with ethyl ether. The combined extracts were dried over sodium sulfate, and then distilled with a short Vigreux column to remove solvent. The residue was esterified and examined by GLC. C_9 and C_{10} monobasic esters were found together with C_{16} and C_{17} dibasic esters. A considerable number of other degradation products were also present; these appeared to be derived mainly from the dicarboxylic acids.

IIIa and IIIc likewise yielded nonanoate and decanoate as degradation products when treated in the manner just outlined. In addition, the C_{24} ester yielded C_{14} and C_{15} dicarboxylic acids, and the C_{28} ester afforded C_{18} and C_{19} dicarboxylic acids as cleavage fragments.

DISCUSSION

Ester Ib contains an unconjugated carbonyl group, since its IR spectrum shows a maximum at 1720 cm^{-1} in addition to the one due to the ester group (1745 cm^{-1}). The reduction of keto ester Ib to methyl hexacosanoate shows that Ib must possess a normal C_{26} carbon skeleton. The NMR spectrum of Ib is in full accord with this conclusion since it shows only one C-methyl group. Ib must contain one double bond, since it consumes one molar equivalent of hydrogen and yields two acidic fragments when oxidized with permanganate-periodate. The IR spectrum of Ib shows that this double bond is *cis*. Its NMR spectrum (Fig. 2) shows but two olefinic protons (4.65τ), and thus indicates but one double bond. The formation of hexanoic acid as one product of permanganate-periodate oxidation of Ib indicates that the double bond must be six carbons from the end of the chain—i.e. in the Δ^{20} position. The other fragment from the oxidative degradation, keto acid Vb, must be a γ-keto acid since it can be reduced to a γ-lactone. This observation indicates that the double bond and oxygen function of Ib must have a 1,4-relationship. Our inference is supported by the results of chromium trioxide oxidation of saturated ester IIIb, since both lines of evidence place the oxygen function at C-17. As expected, Ib underwent facile reduction to unsaturated hydroxy ester IIb in the presence of sodium borohydride. Thus Ib must be an ester of 17-oxo-*cis*-20-hexacosenoic acid.

The structural elucidation of the C_{24} and C_{28} keto acids (Ia and Ic) followed the same general scheme. Oxidative cleavages showed that each contains one double bond and that it has the same position relative to the keto group and the terminal methyl group as does the double bond in Ib. The NMR spectra of the three keto esters are very similar and indicate that all have only one C-methyl group and one olefinic bond. All three spectra are characterized by a signal at 7.62τ. According to Hopkins (9) this chemical shift is associated with methylene protons in the grouping $-CH=CH-CH_2CH_2-\overset{O}{\overset{\|}{C}}-$. The NMR spectra of the three differ only in the number of shielded methylene protons found (8.73τ). Thus Ia must be 15-oxo-*cis*-18-tetracosenoic acid, and Ic is 19-oxo-*cis*-22-octacosenoic acid.

The smooth oxidation of keto esters Ia, Ib and Ic by permanganate-periodate to afford hexanoic acid and γ-keto acids could not have been anticipated with certainty, since cases have been encountered in which the formally expected cleavage products of long-chain compounds have been destroyed by this reagent (10,11,12). von Rudloff recently reported that 2,5-hexanedione is oxidized appreciably on prolonged treatment with permanganate-periodate (13).

The chromium trioxide-acetic acid cleavage method appears to be less satisfactory for these C_{24} to C_{28} acids than for those in the C_{18} to C_{20} range. Undesired degradation products were more numerous and were formed in larger amounts than with shorter-chain acids.

The cause of the error in values for the C_{28} keto ester in GLC analyses of mixtures and of methyl esters is not known; it may be low detector response and/or nonquantitative elution from the column. Since GLC analyses were applied to CCD fractions, the values in Table I are unavoidably subject to some error. At present, we do not have a satisfactory method for determining the C_{28} keto ester in lipid mixtures.

The keto acids of *Cuspidaria pterocarpa* seed oil combine two features rarely found in lipids of higher plants—ketone functions together with chain lengths longer than C_{22}. Their biogenetic origin presents an interesting problem for speculation. The co-occurrence of these homologs, differing only in the number of methylene groups between the keto and carboxyl functions, suggests that the three have the same direct

precursor. However, either the double bond or the carbonyl function could be introduced after the carbon chain already had been elaborated. There is now evidence, incidentally, that oleic is the direct precursor of ricinoleic acid (14,15).

Seed oils of comparatively few species in the family Bignoniaceae have been studied. Some that have been examined are sources of unusual acids; many of these have conjugated unsaturation (16,17). No consistent pattern of fatty acid composition of seed oils is apparent in this family.

ACKNOWLEDGMENTS

GLC analyses by J. W. Hagemann and R. Kleiman; microanalyses by M. Rogers, B. Heaton, and L. P. Burgess; NMR spectra by L. W. Tjarks; computation of weight under CCD curves by W. F. Kwolek; seeds and botanical identification by Q. Jones and A. S. Barclay, USDA, Beltsville, Md.

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[April 5, 1966]

Isolation and Characterization of Glycerides in Human Hair Lipids by Thin-Layer and Gas Chromatography

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ABSTRACT

Techniques for the quantitative analysis of hair lipids using thin-layer chromatography (TLC) together with a proximate analysis of components in one sample deduced by these criteria are presented. Mono-, di- and triglycerides were separated by TLC using Silica Gel G as adsorbent. The chromatoplates were developed with 98% acetone + 2% petroleum ether. Glycerides moved with the solvent front. The requisite portions were scraped off the plates and extracted with acetone and ether. Further TLC, limiting the migration of triglycerides and diglycerides was afforded by use of 95% ethanol as solvent in one direction while monoglycerides moved with the solvent front. For the separation of monoglycerides, chloroform was used as solvent in a second direction. Reference standards and several mixtures were run simultaneously and the spots identified by charring with concentrated sulfuric acid containing dichromate. Additional checking was affected by IR spectra. For determination of glyceride composition, methyl esters of the component fatty acids were prepared by transesterification and submitted to gas chromatography. Comparison of the levels of each of the constituent fatty acids showed no remarkable differences between the three classes of glycerides in one hair lipid pool. Although certain discrepancies in the amounts of a few fatty acid components might be construed for one pool of lipids from hair of white full-headed men (WF-9A) in contrast to findings with two Negro pools, no unequivocal conclusions can be drawn presently.

The speed and versatility of thin-layer chromatography (TLC) and its ability to resolve compounds with minor differences in chemical structure, make it especially valuable as an analytical technique for the quantitative analysis of human sebum or hair lipids.

The complexity of the mixture of fatty acids in the human skin surface lipids has been known since the gas chromatographic investigation of James and Wheatley (1). Other studies also have substantiated the presence of straight and branched chain, odd- and even-carbon numbered as well as mono- and dienoic acids (2–7). A TLC method has also been described by Kaufmann and Viswanathan (8) for the separation of free cholesterol, esters and squalene of human sebum and scalp lipids. Bey (9) applied column, TLC and gas chromatographic methods to lipids removed from soiled or worn clothes. For general properties of sebum, the reader is referred to the reviews of Rothman (10) and Montagna et al. (11).

In the present investigation, methods were selected for the separation of the major components from adult human male sebum or hair lipids directly by TLC, greater emphasis being directed to the isolation of the glycerides and analysis of their component fatty acids by gas chromatography. A comparison was then made of the glyceride contents and of the distribution of fatty acids in lipids pooled according to race and scalp condition.

MATERIALS AND METHODS

Lipid Samples and Reagents

Hair cuttings were carefully collected and pooled from adult Negro-full-headed and balding (CF and CB) and white full-headed males (WF) who abstained from the use of hair dressings. These were extracted with redistilled petroleum ether (b 30–60C) according to the procedures presented earlier (12,13).

All reagents were of high or analytical grade. Solvents were redistilled, and several, especially acetone, were purified by passage through a column of alumina. Silica Gel G was used in TLC and all requisite solvents were checked for any discolored fronts. In this conjunction, Rouser et al. (14) showed that great care must be exercised in TLC of lipids, wide variations in their migration being encountered depending on amount and type of gel preparation and on the methods of activation and cooling plates. The cleansing of the plates is also of prime importance (15).

TLC Procedures

Method 1. Plates measuring 20 × 20 × 0.4 cm were coated with Silica Gel G at a thickness

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of 0.25 mm (16). The plates were dried at 25C for 16 hr, heated in an oven at 110C for 30 min and stored over anhydrous silica gel. The lipid sample in ether was introduced in volumes of 5–10 μl on the starting line 2 cm from the edge of the plate and development carried out at 20C in a chamber (17) saturated with 98% acetone + 2% petroleum ether. The solvent was allowed to ascend 15 cm from the starting point. Staining of spots was affected by charring with concentrated sulfuric acid-dichromate mixture. In this way, four spots resulted as shown in Figure 1 for Pool CF-9A.

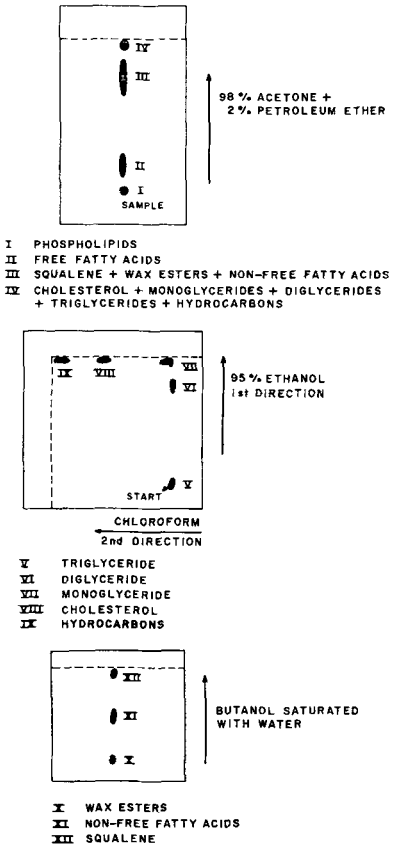

FIG. 1. *Upper-* TLC of human hair lipids employing silica gel G and developed with 98% acetone + 2% petroleum ether (Method 1). The spots were visualized by charring with sulfuric acid-dichromate mixture. *Middle-* Resolution of Fraction (IV) into five subfractions (V-IX, inclusive) by two-dimensional TLC. The plate was developed with 95% ethanol in the first direction and chloroform in the second. *Lower-* Separation of Fraction (III) by TLC using butanol saturated with water as developing medium and leading to three spots as designated, squalene occurring in Fraction (XII).

With the charred spots as reference, the respective portions were carefully scraped from the plates and the four adsorbent pools stored at 0C until further processed. Each was repeatedly eluted with suitable solvents, filtered by centrifugation or through sintered glass and concentrated at 25–30C or lower. In order to minimize atmospheric oxidation and deterioration, a nitrogen atmosphere was employed and generally, complete removal of solvent was avoided until analysis. This was especially true of Fraction I, or the phospholipids; the solvent mixture in this case contained chloroform: methanol (1:1 by volume). Fractions II and III were extracted from the gel with ether and acetone as well as ether, respectively; for spot IV, acetone was employed.

The components of Fraction IV from the above method were further resolved by two-directional TLC as shown in Figure 1. The plates were first developed with 95% ethanol and in the second direction, chloroform comprised the medium. In this way, five spots (V to IX, inclusive) occurred and these were eluted with acetone and ether. For the separation of squalene, wax esters and non-free fatty acids, Fraction III from the initial TLC sep-

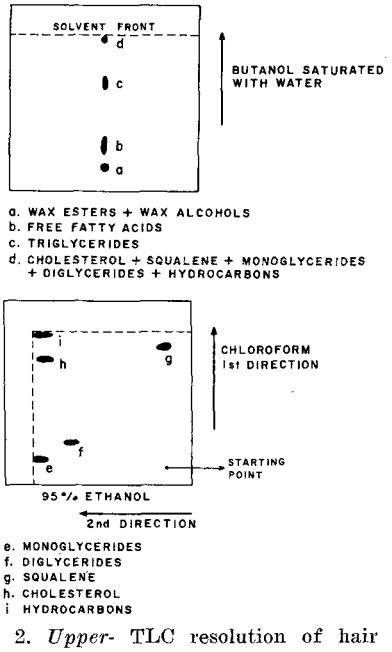

FIG. 2. *Upper-* TLC resolution of hair lipids into four fractions by Method 2, the plates being developed with water-saturated butanol. *Lower-* Further separation of Fraction (d) by two- dimensional TLC. The solvents were chloroform and 95% ethanol in the first and second directions, respectively.

aration was also resubmitted to chromatography and the plates developed in butanol saturated with water. Fractions X, XI and XII resulted on elution of the adsorbent portions with acetone and ether (Fig. 1).

Method 2. The above procedure was modified in that the chromatoplates were first developed with water-saturated butanol, four fractions being produced (a-d, inclusive; Fig. 2). The location of phospholipids was not unequivocal. Further resolution of Fraction (d) into five subfractions (e-i, inclusive) was afforded by two-dimensional TLC, chloroform being used in the first, and 95% ethanol as developing medium in the second direction. All fractions were exhaustively eluted with acetone and ether. Lipid samples weighing 2–2.5 g were processed employing Methods 1 and 2.

Transesterification of Glycerides

The glycerides were transesterified by refluxing with sodium methoxide in methanol for 30 min by the method of Luddy et al. (18). The mixture was cooled, the excess methoxide neutralized with 30% acetic acid and the methyl esters removed with hexane. The extract was washed three times with 10% ethanol and the hexane removed under nitrogen. Gas chromatographic analyses were performed directly on the ester mixtures and on aliquots hydrogenated in a Parr low pressure apparatus at 25C in the presence of Adams (platinum oxide) catalyst.

TABLE I

Proximate Analysis of Hair Lipids Pooled from White and Negro Men (C-W-9)

Component	Wt. %
Phospholipids	8.0
Free fatty acids	12.3
Triglycerides	24.1
Diglycerides	11.8
Monoglycerides	2.3
Cholesterol	2.6
Squalene	14.3
Wax esters	1.4
Hydrocarbons	5.6
Wax alcohols, non-free fatty acids and undetermined components	16.6

Gas Chromatography

All analyses were carried out on a Barber Colman model 10 gas chromatograph employing the radium ionization detector. The two U-shaped borosilicate columns measured 8 ft × ¼ in. and contained 15% diethylene glycol succinate and 5% SE-30 on Gas-Chrom P (60–80 mesh), respectively. The samples were dissolved in the least amount of ether and volumes of 5 μl injected. The column, injection and detector temperatures were 190C, 225C and 240C, respectively. The argon inlet pressure was adjusted to 20 lb to maintain an exit flow

TABLE II

Fatty Acid Distribution Among Mono-, Di- and Triglyceride Fractions Isolated by Thin-Layer Chromatography[a]

Relative carbon number[b]	Tentative fatty acid identification	Fatty acids (Area %)				
		Mono-glycerides	Di-glycerides	Tri-glycerides		
		CF-26A	CF-26A	CF-26A	CB-44A	WF-9A
8.0	n-Octanoic	1.6		0.2	5.1	1.2
9.0	n-Nonanoic		1.5	0.4	3.7	0.8
9.6	C_{10}-Branched		1.5			
10.0	n-Decanoic	0.8	1.5	0.4	1.2	1.0
10.6	C_{10}-Monounsaturated		1.4	0.3		0.7
11.0	n-Undecanoic	0.5	0.7	0.3		
11.5	C_{12}-Branched		1.6	0.3		0.9
12.0	n-Dodecanoic	1.9	2.2	1.6	1.3	2.5
12.4	C_{12}-Monounsaturated	0.6	0.8	0.3		
13.0	n-Tridecanoic	0.9	0.3	0.7		0.4
13.7	C_{14}-Branched	1.4	1.3	1.1		1.2
14.0	n-Tetradecanoic	9.5	10.2	10.4	10.5	8.6
14.5	C_{14}-Monounsaturated	5.0	6.1	5.9	2.8	4.6
14.8	C_{14}-Diunsaturated				0.7	
15.0	n-Pentadecanoic	7.9	7.2	9.4	8.7	4.0
15.5	C_{16}-Branched	1.4	2.7	3.3	3.5	4.8
16.0	n-Hexadecanoic	24.3	23.8	25.2	23.2	15.1
16.2	C_{16}-Monounsaturated	10.3	11.4	12.1	14.2	23.5
17.0	n-Heptadecanoic	3.4	2.4	2.8	2.4	3.6
17.4	C_{18}-Branched	2.8	2.6	1.6	2.4	3.9
18.0	n-Octadecanoic	8.8	7.7	9.6	6.7	3.6
18.3	C_{18}-Monounsaturated	11.6	12.2	11.1	13.0	18.3
20.0	n-Eicosanoic	7.3		2.8		

[a] For the source of adult male hair lipid pools, the codings C, W, F and B refer to Negro, white, full-headed and balding, respectively.

[b] Based on separation on DEGS column as described in the text.

TABLE III

Summary of Fatty Acid Distribution Among Mono-, Di- and Triglyceride Fractions of Several Hair Lipid Pools

Glyceride Fraction	Lipid Pool[a]	Relative fatty acid distribution (area %)			
		Total n-Saturated acids		Branched	
		Even	Odd	Chain	Unsaturated
Mono-	CF-26A	54.2	12.7	5.6	27.5
Di-	CF-26A	45.4	12.1	9.7	31.9
Tri-	CF-26A	50.2	13.6	6.3	29.7
Tri-	CB-44A	48.0	14.8	5.9	30.7
Tri-	WF-9A	32.0	8.8	10.8	47.1

[a] For the source of adult male hair lipid pools, the coding C, W, F, and B refer to Negro, white, full-headed, and balding, respectively.

rate of approximately 72 ml/min. Qualitative characterization of the peaks was based on relative carbon number (15) as compared to known mixtures supplied by Applied Science Laboratories, Inc. Peak areas were corrected for relative response as compared to the C_{16} (methyl palmitate) peak.

RESULTS AND DISCUSSION

The application of TLC-Method 1 is illustrated by the proximate analysis given in Table I for hair lipids pooled from adult male white and Negro subjects. Known reference mixtures, including glycerides from Distillation Products Industries, were chromatographed side by side with sebum in order to verify spot location. Each of the moieties of Table I was ascertained by direct gravimetric analysis. The amount of mono-, di- and triglycerides increased greatly in the order stated, the respective levels being 2.3, 11.8 and 24.1 wt %. Infrared spectroscopy was also used for preliminary identification of the glyceride fractions isolated by the two TLC methods described above.

The composition of fatty acid methyl esters obtained by transesterification of triglycerides from Negro balding and full-headed male hair lipids (CB-44A and CF-26A) and from full-headed white men (WF-9A) are presented in Table II. Acids below C_{14} were rather low in yield, totals of 11.3, 5.6 and 8.7% occurring in the CB, CF and WF pools, respectively; these were normal or straight chain except for two branched components (C_{10}-C_{12}) in both the CF and WF mixtures. The occurrence of the remaining fatty acid peaks was essentially the same for all three pools. Thus, although the n-C_{14} ranged 8.6–10.5% for the three, the levels of n-C_{16} and n-C_{18} were rather similar for both CB and CF (n-C_{18}: 23.2 and 25.2%; n-C_{18}: 6.7 and 9.6%) but in marked contrast to WF (n-C_{16}: 15.1%; n-C_{18}: 3.6%). The differences are further reflected in the data summarized in Table III. It will be noted that the unsaturated fatty acids range higher in the WF pool. In fact, the over-all yields of saturated and unsaturated C_{16} as well as C_{18} acids were virtually the same for the three pools, except that the unsaturated moieties occurred at higher levels in WF. Before unequivocal conclusions can be advanced, further triglyceride pools must be processed according to the categories and the data submitted to statistical analysis, studies of which are currently in progress. In this conjunction, it is known that the free fatty acid content of sebum or hair lipids can rise on storage due in large measure to hydrolytic cleavage. Such changes will obviously affect the ester and glyceride distribution.

The fatty acid findings on transesterification of the mono-, di and triglycerides of Pool CF-26A (Tables II and III) are worthy of comment. The percentage composition of the acids was nearly the same for each mixture except possibly for a rather minor decrease in the level of unsaturated acids of the monoglycerides. It should be mentioned that the results reported in the tables are relative quantities (corrected area percentages) identified in the gas chromatographic tracings and not necessarily the actual weight percentages. However, since each TLC fraction represented a purified glyceride cut and as no appreciable quantities of fatty acids less than C_8 or greater than C_{20} were found, the values reported are close approximations to the total weight percentage in each fraction.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service Grant, CA 06487, from the National Cancer Institute.

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[Received July 7, 1965]

An Ethanolamine Plasmalogen Artifact Formed by Acetone Extraction of Freeze-Dried Tissue [1]

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ABSTRACT

Extraction of freeze-dried tissues by acetone results in the in vitro production of an acetone derivative (imine) of the ethanolamine phosphatides. Some of the properties of the acetone imine of ethanolamine plasmalogen are discussed.

INTRODUCTION

THE CHROMATOGRAMS of our first extract of cat placenta revealed a large plasmalogen positive spot (*Im*) above the phosphatidyl ethanolamine (Fig. 1) which was subsequently isolated free of phosphatidyl ethanolamine from a silicic acid column. We were, however, unable to detect (*Im*) in chloroform-methanol extracts from 12 other cat placenta samples nor in similar extracts from human, dog or rat placenta.

Acetone extracts prepared from freeze-dried rat kidneys, for the purpose of chromatographically examining the neutral lipid fraction, revealed a high relative proportion of the (*Im*) plasmalogen as did the chloroform-methanol soluble fraction of these acetone extracted kidneys.

This communication is a report of our observations of this material (*Im*) which appears to be the imine derivative of ethanolamine plasmalogen-acetone.

PROCEDURE AND DATA

Extracts for chromatographic analysis, of the tissues listed below [2] were prepared from freeze-dried and from wet fresh tissue using 20 ml of acetone and/or chloroform:methanol per gram dry weight of tissue or the equivalent volume for the wet tissue assuming it to be 80% water. A chloroform soluble fraction was obtained from the chloroform:methanol extracted wet tissues by the procedure of Folch et al. (1).

Chromatograms of these various extracts were made on silicic acid impregnated filter paper

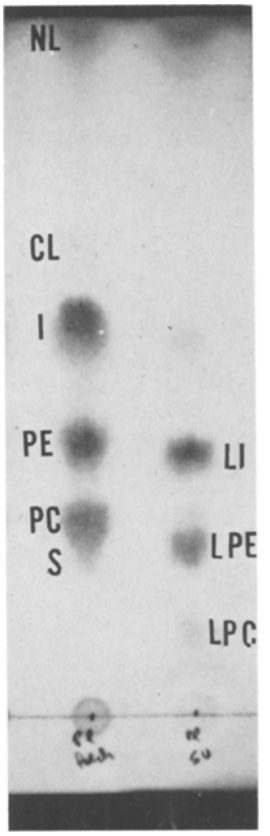

FIG. 1. Chromatographic analysis of the first cat placenta extract showing an unusually large proportion of the ethanolamine imine plasmalogen (I). Lecithinase A from snake venom (s.v.) hydrolyzed the imine as well as the natural glycerophosphatides with the production of the corresponding lysocompounds. Neutral lipid (NL), cardiolipin (CL), ethanolamine imine phosphatide (I), phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), and sphingomyelin (S) are clearly shown in the chromatograms at the left; LI, LPE, LPC, in the chromatograms at the right, are the corresponding lysophosphatides produced by the action of the snake venom. The chromatogram was developed in a solvent system consisting of 2, 6-dimethyl-4-heptanone: pyridine: methanol: water (150:75:10:12). The lipids were visualized by the plasmal reaction and counterstained with rhodamine 6G, only I, PE, LI and LPE were plasmal positive.

[1] This investigation was supported by USPHS Grants 5 RO1 AM 9088 and T1HE 5133.

[2] Kidney of rat, cat, dog, rabbit, guinea pig and snake; placenta of rat, cat, dog, rabbit and man; brain of mouse and guinea pig; erythrocytes of dog and man; ovary and uterus of rat; liver of rat and heart of dog and man.

(S & S 2043b), identification of the resolved lipids was accomplished by the aid of multiple spot-tests and authentic phosphatide samples run concurrently (2,3). The most useful spot-tests for the purposes of this report were the plasmal and ninhydrin reactions. Production of the lysophosphatides was accomplished, in situ, on the paper chromatograms using an aqueous snake venom preparation for the source of lecithinase A (*3*).

Acetone (1) and chloroform:methanol (2) extracts were dried over nitrogen at 40C and dissolved in chloroform for fractionation on silicic acid columns containing 2 g of silicic acid (G. Frederic Smith Co.). Elution of the neutral lipids and the various phosphatides was done by successive additions of chloroform, chloroform:methanol (95:5), and chloroform:methanol (90:10). Five-milliliter fractions were collected and examined by paper chromatography as above.

The paper chromatographic analysis revealed that the acetone extracts obtained from all of the freeze-dried tissues contained (*Im*) evident on the chromatogram as a spot above ethanolamine plasmalogen and below cardiolipin and which could not be demonstrated in any of the other extracts (Fig. 2). Spot-test analysis showed that (*Im*), unlike ethanolamine plasmalogen, was only ninhydrin positive after heating the sprayed chromatogram at 100C for 2 min (Fig. 3). The lecithinase A product of (*Im*) similarly showed the same ninhydrin characteristic (Fig. 3) in contrast to the rapid reaction of phosphatidyl ethanolamine and lysophosphatidyl ethanolamine (and their respective plasmalogen analogs) at room temperature. *Im* was eluted from the silicic acid columns by chloroform:methanol (95:5) coming off after cardiolipin and before phosphatidyl ethanolamine and could be obtained only from the various acetone extracts from freeze-dried tissues as described above. A good yield of (*Im*) could only be obtained following treatment with acetone for at least 18 hr and never from acetone extracts prepared from wet tissue. In every tissue the relative proportion of phosphatidyl ethanolmine and (*Im*) was similar, i.e., about 10:1 as shown in Figure 2.

We, at first, believed that (*Im*) was the monomethyl derivative of ethanolamine plasmalogen. Monomethyl phosphatidyl ethanolamine (a synthetic preparation of high purity was kindly supplied by Dr. Erich Baer) also required heating in order for the ninhydrin color reaction to occur: this possibility, however, was eliminated since the R_f of monomethyl phosphatidyl ethanolamine in the tetrahydrofuran solvent system was significantly less than phosphatidyl ethanolamine. Nevertheless the ninhydrin property did suggest that (*Im*) was an N-substituted analog of phosphatidyl ethanolamine. When a mixture of the (*Im*) and ethanolamine plasmalogen, obtained from a silicic acid column fractionation (similar to that shown in Fig. 3) was hydrolyzed by 1*N* HCl and water-soluble hydrolysis products examined by both a) a Technicon amino acid analyzer and b) chromatography of the dinitro-fluorobenzene derivatives only ethanolamine could be

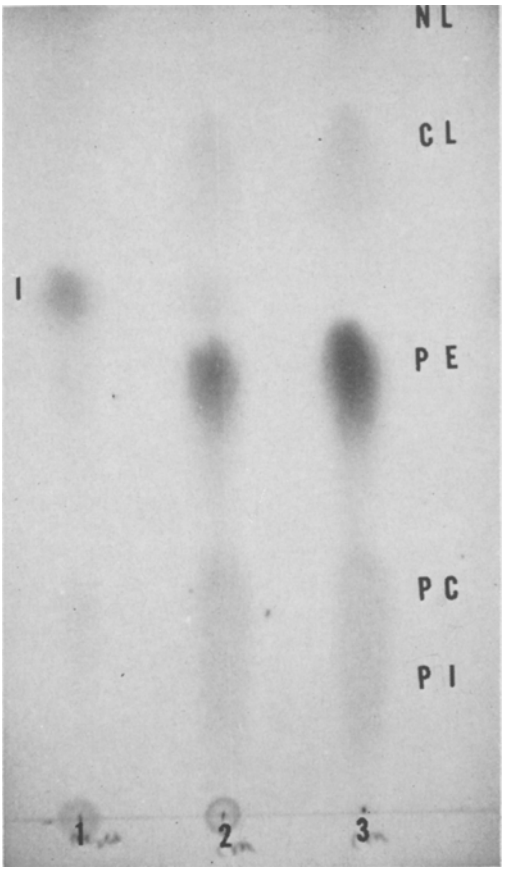

FIG. 2. This chromatogram was prepared from freeze-dried rat kidney extracted by acetone, *1*, followed by chloroform methanol, *2; 3* is a chloroform:methanol extract of freeze-dried rat kidney. Neutral lipid (NL) and imine (I) are the principal lipid components of the acetone extract. By comparison with the total lipid extract *3* there is a diminution in the amount of (PE) as imine to the acetone extract, (PI) is phosphoinositide. Developed in tetrahydrofuran: 2, 6-dimethyl-4-heptanone:water (150:20:14) and stained by the plasmal reaction and rhodamine 6G. Only I and PE were plasmal positive.

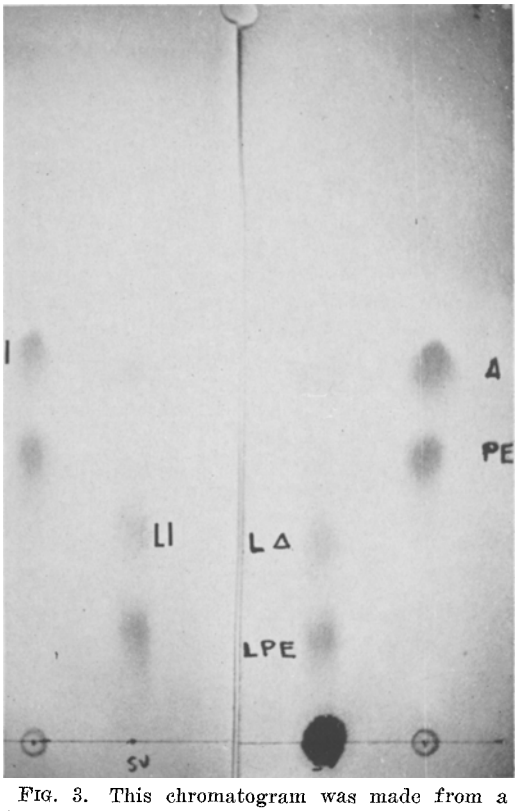

FIG. 3. This chromatogram was made from a chloroform: methanol (95:5) eluate obtained by silicic acid column chromatography of the acetone extract of freeze-dried dog kidney. After putting the four samples on the paper, lecithinase A *from snake venom* was applied at 2 and 3. The chromatogram was run in the tetrahydrofuran system as in Figure 2. The left half of the chromatogram was stained by the plasmal reaction and the right half by ninhydrin. The imine (I), ethanolamine plasmalogen (PE) and their corresponding lysocompounds (LI) and (LPE) were positive by the plasmal reaction. PE and LPE were positive for ninhydrin at room temperature; heat (100C for 2 min) was required to produce ninhydrin staining of the imine (Δ) and lyso imine (LΔ).

detected. It was therefore probable that through reaction with acetone we had converted a portion of the tissue ethanolamine plasmalogen (and presumably the phosphatidyl ethanolamine also) into an imine. The failure to demonstrate (Im) in extracts of wet tissue probably implies the necessity for anhydrous conditions since imine formation is a dehydration reaction and that under the conditions of our extractions the reaction equilibrium did not permit more than 10% imine formation. Indeed, addition of water to extracts containing the acetone derivative appears to force the reaction slowly to the left with the reappearance of ethanolamine plasmalogen.

Although the phosphatides are considered to be insoluble in acetone, it is apparent from the chromatograms that not only phosphatidyl ethanolamine, and especially the acetone derivative, are soluble in acetone but also a significant fraction of the phosphatidyl choline (cf Fig. 2). We found that (*Im*) is even soluble at −20C both in acetone and in ethanol. We expect that this is the explanation for the acetone soluble phosphatides of erythrocytes reported by Hanahan (*4*) where the predominant phosphatide is phosphatidyl ethanolamine. Presumably the first cat placenta extract had inadvertently been made with a chloroform: methanol mixture containing acetone. Tetrahydrofuran containing only 10% acetone is an effective lipid solvent and shows the usual proportion of the imine plasmalogen. Phosphatidyl ethanolamine and (*Im*) could be resolved on paper chromatograms by 2, 6-dimethyl-4 heptanone: pyridine: methanol: water (150:75:10:12) and by 2, 6-dimethyl-4 heptanone: methanol: dichloroethane: water (100:40:15:5) but not by 2, 6-dimethyl-4 heptanone: acetic acid: water (100:40:7).

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[Received March 14, 1966]

Quinones and Quinols as Inhibitors of Lipid Peroxidation

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ABSTRACT

The influence of biological quinonoid compounds upon oxidative polymerization of lipids has been compared with that of simple quinones and antioxidants. A new procedure for the accelerated production and measurement of oxidative polymerization was used for this comparison. The biological quinones were found to be relatively ineffective as retarders of oxidative polymerization. Heme-catalyzed lipid peroxidation, as measured by oxygen uptake, was inhibited by ubiquinone and ubiquinol, both having about one fourth of the antioxidant capacity of α-tocopherol. The peroxidation of mitochondrial lipid in vitro was inhibited by the presence of exogenous ubiquinone indicating that this compound may contribute towards the protection of the organelle in vivo.

INTRODUCTION

THE UBIQUINONES are widely distributed in nature and much interest has centered on their participation in biological oxidation-reduction reactions (1). In addition certain ubiquinones and their heterocyclic derivatives, ubichromenols and ubichromanols, have been found to alleviate the symptoms of vitamin E-deficiency in some species (2). However, little is known about the ability of these compounds to act as antioxidants, despite the well-known antioxidant properties of quinols and the extensive use of quinones in polymer technology as inhibitors of free radical polymerization of unsaturated monomers (3). The present study considers the effects of quinones and quinols on lipid peroxidation. In particular, the role that ubiquinone might play in contributing towards the ability of the mitochondrion to resist lipid peroxidation is examined.

PROCEDURES AND DATA

Oxidative Polymerization

Commercial grade linolenic acid (Mann Research Labs.) was polymerized by oxidation at elevated temperature. Seven milliliter aliquots of this oil, containing 10^{-2} M benzoquinone, hydroquinone, α-tocopherolquinone, ubiquinone-6, menadione, α-tocopherol dimer [prepared by the alkaline ferricyanide oxidation of α-tocopherol (4)], α-tocopherol and butylated hydroxytoluene (BHT), were polymerized within Ostwald-Cannon-Fenske viscosimeters (size 400) by heating these in a waterbath at 50C, 70C and 90C and passing oxygen through each viscosimeter at the rate of 500 ml/min. The increase in viscosity of each sample was measured at intervals over 4–6 hr and compared with the increase in viscosity of controls containing only linolenic acid. Under these conditions oxidative polymerization is a function of temperature and oxygen flow rate and both must be uniform for all samples. Table I compares the effect of these compounds on oxidative polymerization at the three temperatures used; 50C, 70C and 90C.

Manometric Measurements

The effect of ubiquinone-6 and ubiquinol-6 upon the heme-catalyzed peroxidation of methyl linolenate was studied by manometric measurement of oxygen uptake. The quinol was prepared from the quinone by reduction using sodium borohydride in ethanol, acidification with 1 N HCl and extraction into light petroleum ether. The reaction mixture, 1 ml per 6 ml Warburg flask, consisted of 10% methyl linolenate in ethanol containing 2×10^{-6} M hemoglobin and 10^{-2} M ubiquinone-6 or ubiquinol-6. In similar flasks 10^{-2} M α-tocopherol replaced ubiquinone-6. Controls contained no ubiquinone-6 or α-tocopherol. The reaction mixture was shaken at 37C in oxygen. Table II shows the effect of these compounds on lipid peroxidation as measured by oxygen uptake.

TABLE I

Some Quinones and Related Compounds as Inhibitors of the Oxidative Polymerization of Linolenic Acid

Test substance, at 10^{-2} M concentration	Increase in viscosity over 4 hr / Increase in viscosity of control over 4 hr × 100		
	50C	70C	90C
p-Benzoquinone	5	54	79
p-Hydroquinone	3	28	74
Tocopherolquinone	67	96	75
Ubiquinone-6	117	93	106
Menadione	104	116	87
α-Tocopherol dimer	4	40	75
α-Tocopherol	0	40	72
Butylated hydroxytoluene	0	0	45

TABLE II

Ubiquinone-6 and Ubiquinol-6 as Inhibitors of Heme-Catalyzed Lipid Peroxidation of Methyl Linolenate in Ethanol

Inhibitor	Oxygen uptake, μl O_2 (S.T.P.)/hr.
None	93
10^{-2} M Ubiquinone-6	22
10^{-2} M Ubiquinol-6	19
10^{-2} M α-Tocopherol	5

Mitochondrial Peroxidation

Mitochondria were prepared from livers of twenty 350 g male Sprague-Dawley rats by a modification of the technique of Schneider (5). The suspending medium was 0.25 M sucrose + 10^{-3} M EDTA, nuclei and cell debris were centrifuged down at 1,000 $\times$ g for 10 min, mitochondria were centrifuged down, resuspended and centrifuged again at 6,500 $\times$ g for 10 min. A second washing and the final suspension of the mitochondria were made with 0.175 M KCl + 0.02 M Tris buffer (pH 7.4 at 37C) to yield a preparation which contained 60 mg protein per milliliter. Aliquots of this mitochondrial suspension were prepared which contained 2.0, 1.0, 0.5 and 0.2 mg ubiquinone-6 per milliliter of mitochondria. Each peroxidizing system (volume 6.0 ml) contained 1.5 ml of a mitochondrial suspension of ubiquinone-6, 2.5 μmoles KCN and 250 μmoles Na succinate in KCl-Tris buffer. One set of controls contained no succinate so that the capacity of the mitochondria to reduce ubiquinone-6 was minimal, the other controls contained no ubiquinone-6. Each 6.0 ml reaction mixture was shaken for 7 hr at 37C in a 100 ml flask containing oxygen. At intervals 1.0 ml samples of each reaction mixture were withdrawn and lipid peroxidation was measured by the thiobarbituric acid (TBA) reaction. Under similar but anaerobic conditions where the suspending medium was 0.25 M sucrose + 0.05 M Tris, exogenous ubiquinone-6 was rapidly reduced by the mitochondria as shown by the disappearance of the 275 $m\mu$ ultraviolet absorption peak.

TBA Reaction

To each 1.0 ml sample was added 4.0 ml 5% trichloroacetic acid. After centrifugation, 3.0 ml of the supernatant and 1.0 ml of 1% TBA were heated together at 100C for 10 min. The resultant red color was measured at 532 $m\mu$. Figure 1 shows the influence of exogenous ubiquinone-6 upon the rate of mitochondrial peroxidation. The rates of lipid peroxidation were plotted as a function of the concentration of ubiquinone-6.

DISCUSSION

Oxidative Polymerization

The effects of certain quinones and related compounds on the rate of oxidative polymerization of linolenic acid at 70C are shown in Figure 1, and are compared at three different temperatures in Table I. The unsubstituted quinones, benzoquinone and hydroquinone, showed inhibition of oxidative polymerization consistent with their use as retarders of vinyl polymerizations. However, the substituted quinones, α-tocopherolquinone, ubiquinone-6 and menadione, did not appreciably affect the course of the polymerization. These data, and the known instability of substituted quinols, indicate that alkyl substituents stabilize the quinone ring and inhibit the formation of the semiquinone free radical. Steric hindrance by the relatively large alkyl groups of the biological quinones could render them ineffective as retarders of oxidative polymerization of lipids. The dimeric oxidation product of α-tocopherol retarded the reaction in a similar manner to that of α-tocopherol. This effect may be due to homolytic scission of the dimer to form free radical inhibitors, at these elevated temperatures. Differences in antioxidant activity are not the result of different rates of volatilization of the test compounds since the more volatile compounds of lower molecular weight showed higher antioxidant activity than the less volatile compounds with aliphatic side chains.

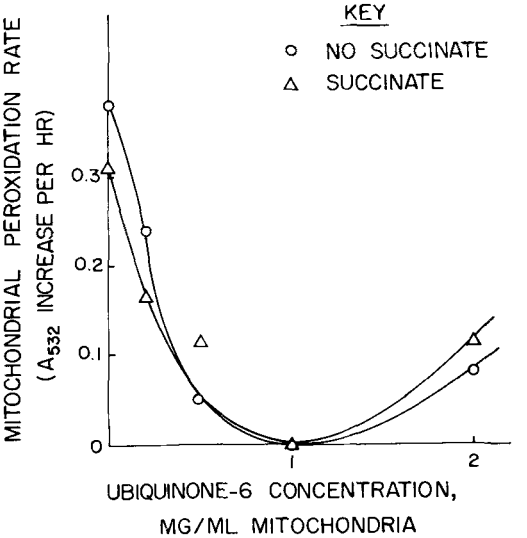

FIG. 1. Mitochondrial peroxidation as a function of the concentration of exogenous ubiquinone-6.

Lipid Peroxidation

Table II shows the effects of ubiquinone-6 and ubiquinol-6 on the heme-catalyzed peroxidation of methyl linolenate in ethanolic solution. Under these conditions ubiquinone-6 and ubiquinol-6 showed some antioxidant activity which was not apparent under the drastic conditions used for oxidative polymerization. The protection afforded by the quinone and quinol appear to be about one fourth of that given by α-tocopherol. These figures are of the same order as those reported by Lea and Kwietny (6) for relative antioxidant activities of α-tocopherol and ubiquinone derivatives in aqueous ester emulsions undergoing lipid peroxidation.

Similar studies by Kaufmann and Garloff (7) showed that ubiquinone-6 and ubiquinone-10 exhibited weak antioxidant activity when methyl linoleate was peroxidized in 2-ethoxyethanol. However these workers found that, in an aqueous emulsion of potassium linoleate in phosphate buffer pH 7.2, these ubiquinones have an antioxidant activity greater than that of α-tocopherol. The relatively lower activity of α-tocopherol in such a system may be due to the influence of heavy metal catalysts since the same workers found that the presence of EDTA would increase the antioxidant activity of α-tocopherol above that of the ubiquinones.

Mitochondrial Peroxidation

Mitochondria were peroxidized in the presence of different concentrations of exogenous ubiquinone-6 and the relative rates of peroxidation were measured by the TBA method. The possible influence of the electron transport-linked reduction of ubiquinone-6 on the stability of the mitochondrion was studied by carrying out the peroxidations both with and without succinate, a substance for the reduction of ubiquinone-6. Because the presence of an individual substrate has a marked effect on the rate of peroxidation it was not possible to substitute a competitive inhibitor, such as malonate, in the place of succinate, to block the reduction of ubiquinone-6.

As shown in Figure 1 lipid peroxidation decreased with increasing concentration of ubiquinone-6 up to the level of 1 mg ubiquinone-6 per milliliter of mitochondrial suspension, where lipid peroxidation was completely inhibited over the 7-hr period of the experiment. Above this level of ubiquinone-6, lipid peroxidation was again evident. Many antioxidants exert a prooxidant effect at high concentrations and such an effect may be the cause of increased peroxidation at high ubiquinone-6 levels. In the absence of succinate, lipid peroxidation reached a steady level after about 4 hr, but in the presence of succinate the rate of peroxidation continued to increase and did not reach a steady state until about 7 hr. Figure 1 illustrates the rate of lipid peroxidation as a function of the concentration of ubiquinone-6 and shows that the presence of succinate as a substrate for the mitochandrial reduction of ubiquinone-6 has little effect on the inhibition of peroxidation by the quinone. Though ubiquinol might be expected to afford more protection to the mitochondrion than ubiquinone, under these conditions no such protection was observed. The reason may be that, in this highly aerobic system, direct oxidation of ubiquinol by molecular oxygen proceeds much faster than the concomitant mitochondrial reduction of ubiquinone, preventing any significant accumulation of ubiquinol.

Tappel and Zalkin (8) showed that isolated mitochondria were susceptible to lipid peroxidation and that this could be prevented by the addition of α-tocopherol to the system. The antioxidant theory of vitamin E function postulates that the role of endogenous mitochondrial α-tocopherol is to stabilize the structural polyunsaturated lipid against oxidative damage. Though the primary role of ubiquinone is almost certainly that of a co-factor, undergoing oxidation-reduction reactions, these results indicate that the presence of the quinone in the mitochondrion at a concentration reported to be ten times that of α-tocopherol (9) could give the organelle significant protection against oxidative damage.

ACKNOWLEDGMENTS

This work was supported by Grant No. AM-09933 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.

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[Received June 6, 1966]

Altered Partition of Serum Cholesterol and Cholesteryl Ester in a Petroleum Ether-Ethanol-Water System After Incubation

DIFFERENTIAL PARTITION of free and esterified cholesterol between aqueous ethanol and petroleum ether has been used for quantitative analysis of serum lipids. Under the conditions of Galanos et al. (1) free cholesterol appears in the aqueous ethanol phase and esterified cholesterol in the petroleum ether phase. In contrast, we have observed conditions under which free cholesterol partitions into the petroleum ether phase while esterified cholesterol remains in the aqueous ethanol phase. A modification of the conditions results in both free and esterified cholesterol appearing in the petroleum ether phase. These observations serve as the basis for additional analytical procedures. The conditions are described below.

Acetone-ethanol 1:1 lipid extracts of human serum were prepared as described by Blankenhorn et al. (2). Extracts (10 ml) were dried, dissolved in 5 ml of absolute ethanol and incubated at 37C for 60 min. After incubation 5 ml of deionized water were added and the aqueous ethanol mixture was then extracted with 10 ml of petroleum ether (bp 60C–75C). All of the free cholesterol (determined by means of the Liebermann-Burchard reaction) was found to be present in the petroleum ether phase after a single partition; esterified cholesterol remained in the aqueous ethanol phase. When incubation was omitted both free and esterified cholesterol appeared in the petroleum ether phase in keeping with the partition coefficients of the pure substances in the solvent system.

Values for total free cholesterol (Table I) obtained by the solvent partition method showed close agreement with those obtained by the digitonide precipitation method of Sperry and Webb. When C^{14}-labeled cholesteryl palmitate and stearate were added to serum lipid extracts and incubated as described above, all of the label was found in the lower (aqueous ethanol) phase. On the other hand, after incubation of the pure cholesteryl esters alone, the label was found in the petroleum ether phase (Table II). These results were further confirmed by chromatography on silicic acid impregnated paper. Chromatograms were developed in hexane, stained with rhodamine 6G, and visualized under ultraviolet light. Cholesterol was detected in the upper phase and cholesteryl esters in the lower phase.

The optimal conditions for incubation (60 min, 37C) were determined in studies in which the incubation period was varied between 0 and 90 min with 15-min intervals and incubation temperatures from 0C to 100C (0, 20, 37, 50, 80, 100). Incubation in methanol rather than ethanol did not affect the distribution of free and esterified cholesterol.

Column chromatography was employed to demonstrate the effects of removal of protein and phospholipid. After removal of both protein and phospholipid by Florisil fractionation (2) both sterol and sterol ester partitioned into the petroleum ether phase. Total cholesterol (free and esterified) in the petroleum ether phase after one partition in 6 experiments averaged 138% of the control value (range 124%–150%). Elimination of protein from the reaction mixture by fractionation on DEAE-cellulose columns in accordance with the principles elaborated by Rouser et al. (3) resulted in a substantially higher value for total cholesterol in the upper phase (287% of control) after a single partition.

TABLE I

Comparative Analyses of Free Cholesterol Content of Human Serum[a]

Sample No.	Solvent partition, average	Digitonide precipitation, average
1	86 (85,87)	87 (86,88)
2	78 (79,76)	73 (74,72)
3	111 (110,112)	107 (109,104)
4	188 (187,188)	180 (181,179)
5	73 (73,73)	69 (73,65)
6	70 (71,69)	66 (66,66)

[a] Values given as mg/100 ml of serum. Individual values are enclosed within parentheses.

TABLE II

Distribution of Labeled Cholesteryl Esters in Aqueous Ethanol-Petroleum Ether

Sample	CS[a]–C^{14} counts/min	Per cent	CP[a]–C^{14} counts/min	Per cent
Labeled sterol added to serum extract:				
Upper phase	8270	1.9	9525	2.3
Lower phase	43900	98.1	409000	97.7
Labeled sterol added to solvent system:				
Upper phase	321400	97.5	408000	99.3
Lower phase	8350	2.5	3340	0.7

[a] Cholesteryl stearate-1-C^{14} and cholesteryl palmitate-1-C^{14} were obtained from the New England Nuclear Corp. Radioactivity was determined on 1 ml aliquots of the upper and lower phases.

Analysis of protein in the lower phase with the biuret reaction indicated the presence of 5 mg of protein in a volume of extract representing 1 ml of serum. This material, when brought to an initial pH of 10.0 and titrated electrometrically with 0.001 N HCl, yielded pK_a values of 8.2 and 5.2.

The experimental results suggest that, upon incubation in ethanol, a chemical complex is formed. The complex appears to consist of esterified cholesterol, phospholipid, and protein. This complex remains in the aqueous ethanol phase while free cholesterol is selectively and quantitatively distributed into the petroleum ether phase of the biphasic system. While the significance of the observation which we have reported here can only be speculative at this time, the similarity in composition of the chemical complex (protein 51%; phospholipid 26%; esterified cholesterol 24%) to Macheboeuf's lipoprotein (4) is striking. Since the partition after incubation is very reproducible, the procedure can be used for quantitative analysis. A series of 25 separate analyses carried out on the same serum sample gave a mean free cholesterol value of 61 mg/100 ml with a standard deviation of ± 3.3%.

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[Received March 28, 1966]

Optically Active Aceto-triglycerides of Oil from *Euonymus verrucosus* Seed[1]

THE PRESENCE OF acetic acid in seed oils of the Celastraceae was reported some years ago (1,2) with the suggestion that the acid was probably not part of the triglycerides. We have found that in *Euonymus verrucosus* Scop. the acetic acid occurs as a monoaceto-triglyceride. The acetoglycerides are optically active and this activity derives only from the asymmetry of the central carbon atom of the glycerol. Such measurable optical activity of unaltered natural triglycerides has not been reported previously.

Chromatography of the petroleum ether (30–60C) extract from ground seeds of *E. verrucosus* on a silicic acid column yields some 90% of the extract as a fraction (I) differing from common triglycerides in its response to infrared (IR) spectroscopy, thin-layer chromatography (TLC), and gas-liquid chromatography (GLC). (I) adsorbs IR more strongly at 7.3 and 8.1 μ than do common oils. The absorption at 7.3 μ is attributed to terminal methyl groups and that at 8.1 μ to short-chain esters; both are equal in intensity to the corresponding absorption of acetylated pure distearin.

Direct GLC of (I) in a 0.5 meter column of 3% JXR (3) showed only two components. The retention times corresponded with those of trilaurin and acetodistearin used as standards. The C_{36} component (excluding C of glycerol) can be attributed to a triglyceride containing C_2, C_{16}, and C_{18} acids and the C_{38} component, to one containing C_2 and two C_{18} acids. Mobility on TLC (silica gel G, impregnated with boric acid; hexane:ether, 70:30) agreed with that of synthetic α-acetodistearin (R_f = 0.67) and differed definitely from that of β-acetodistearin (R_f = 0.75).

A portion of the dried soaps from saponification of (I) was extracted with ethanol. GLC of the solution revealed only glycerol as a solute. Another portion of the soaps was acidified under ether. The only short-chain acid found on GLC of the ethereal solution was acetic acid.

GLC of esters prepared from (I) by acid-catalyzed methanolysis showed only esters of the common C_{16} and C_{18} fatty acids.

Quantitation of the nuclear magnetic resonance (NMR) spectrum of completely hydrogenated (I) provided additional confirmation of the proposed structure. Peaks were found at 9.1 τ (long-chain terminal methyl protons), 8.7 τ (methylene protons), 8.0 τ (acetate protons), 7.8 τ (protons alpha to the carboxyl

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(I) showed specific optical rotations in a Cary recording spectropolarimeter (hexane, 25°, c = 4) of −0.5° at the sodium D line, −0.8° at 450 mμ, and −1.9° at 350 mμ. Hydrogenation of the material did not alter the rotation.

On transesterification of the aceto-triglyceride in the spectropolarimetric cell with 5% HCl (anhydrous) in methanol:hexane (4:1), the specific rotation at 350 mμ decreased from +2.3° at 15 min to +1.8° at 45 min, +0.6° at 120 min, and 0.0° at 195 min. At the end of this time, TLC showed the presence of only methyl esters and glycerol.

The disappearance of optical activity with the elimination of the chiral center in the glycerol, in conjunction with the other data presented, precludes the presence of any other chiral centers previously reported in triglycerides optically active because of asymmetry in a fatty acid moiety.

Comparison with synthetic products of known configuration shows that the natural material is essentially all (*S*)-α-aceto-triglyceride.

This work will be described later in greater detail.

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ACKNOWLEDGMENTS

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[Received June 6, 1966]

The Hydrolysis of Long Chain Trisaturated Triglycerides by Pancreatic Lipase

AN EARLIER REPORT (1) from this laboratory described a rapid, semimicro technique which could be used for judging the purity of synthesized triglycerides. This technique was based on the action of pancreatic lipase at 40C. Therefore, its application to trisaturated glycerides was limited because lipase had decreased activity with higher melting solid substrates (2).

It seemed that the technique might be extended to the higher melting triglycerides by employing a low melting glyceride as carrier. The data in Table I show this to be the case. However, since highly pure fatty acid methyl esters were easier to obtain than pure triglycerides, it was found much more convenient to use the former for this purpose. The data indicate that methyl esters as well as triglycerides can be effective carriers for high melting substrates. Furthermore, no lessening in specificity of lipase for hydrolyzing terminal acids of triglycerides was observed. The use of methyl esters has the additional advantage that further analysis of monoglycerides isolated from hydrolysis products is not complicated by the presence of monoglycerides produced from the carrier.

TABLE I

Lipase Hydrolysis[a] of 1 Palmito-Distearin[b]

	Fatty acid composition, mol %			
	MG		FA	
Carrier	16:0	18:0	16:0	18:0
Triolein	2[c]	98[c]	49[c]	51[c]
Methyl oleate	1	99	48[c]	52[c]
Methyl pentadecanoate	2	98	50[d]	50[d]

[a] 5 min reaction time; other conditions as described (1). Ca 15 mg carrier, 35 mg triglyc. were used.
[b] Composition of PSS: 33.3 mol % 16:0, 66.7 mol % 18:0.
[c] 18:1 from carrier ignored in calc.
[d] 15:0 from carrier ignored in calc.

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Column Chromatography of Lipids Containing Hydroxy Fatty Acids

HYDROXYLATED COMPOUNDS, found as decomposition products in oxidized fats, may be absorbed and transported via the lymph. Their influence on the composition of lymph lipids has not been determined, and quantitative methods were needed for the separation of these compounds prior to such studies. In the present study, a method is presented for the separation of synthetic mixtures containing hydroxy fatty acids and their triglycerides as well as lymph lipids containing these compounds.

In order to achieve complete separation of lipids containing neutral lipids, hydroxylated lipids and phospholipids, two silicic acid columns were used. The first silicic acid column was prepared as follows: 100 mesh silicic acid (activated by heating at 110C overnight in an oven) was slurried with hexane and poured into the column and allowed to pack under gravity and a slight pressure of nitrogen to form an adsorbent column, 2.4 cm × 10.8 cm. Up to 350 mg of lipid sample may be added to the column before overloading occurs. The column was prewashed in reverse order with 100 ml each of the solvents used for elution. The level of the final wash was allowed to drop to the top of the silicic acid column, and the lipid sample dissolved in a minimum amount of redistilled hexane was pipetted onto the column.

The major lymph lipid classes were separated by stepwise elution according to the general scheme of Garton and Duncan (1) and Fillerup and Mead (2). After prior treatment with Amberlite IRA 400 to remove free fatty acids, cholesterol esters were eluted with 100 ml Skellysolve F, triglycerides with 300 ml 5% diethyl ethyl-Skellysolve F, cholesterol with 200 ml chloroform and phospholipids with 300 ml methanol containing 4% water. A flow rate of 1.6–2.0 ml per minute of solvent was maintained by a slight positive pressure of nitrogen on the column. Fractions were combined, evaporated to dryness in vacuo, weighed and stored at 0C under a layer of nitrogen. The homogeneity of each fraction was determined by thin-layer chromatography (TLC). Triglycerides composed of hydroxy fatty acids were removed from phospholipids by separation on a second silicic acid column as follows: Seventeen grams of activated silicic acid was added to a 100 ml burette column as a slurry in anhydrous methanol. The absorbent was allowed to pack under gravity and by a slight pressure of nitrogen; the column was washed with two 50 ml portions of anhydrous methanol and 5% methanol-chloroform. The sample was dissolved in a minimum amount of 5% methanol-chloroform and pipetted onto the column. Triricinolein was eluted with 300 ml 5% methanol-chloroform while the phospholipid fraction was eluted with 300 ml methanol containing 4% water. Each fraction was filtered, the solvent removed under vacuum, the sample weighed and its homogeneity determined using TLC. A solvent system composed of petroleum ether (40C–60C) diethyl ether-glacial acetic acid, 90:10:1, v/v/v, was employed for the separation of cholesterol esters, triglycerides and free fatty acids. Another solvent system composed of petroleum ether (40C–60C)-diethyl ether-glacial acetic acid, 70:30:2, v/v/v, proved effective for the complete separation of the more polar cholesterol, phospholipids, mono- and diglycerides, ricinoleic acid and triricinolein. The spots were visualized by spraying with 50% aqueous sulfuric acid solution saturated with potassium dichromate and heating at 150C for 30 minutes.

In the course of our studies concerning the adsorption of ricinoleic acid from the small intestine, it was found that lipid obtained from the lymph of rats fed ricinoleic acid or triricinolein, contained these materials in the form of free fatty acids, mono-, di,- and triglycerides. The presence of these compounds had an adverse effect on the routine separation of lipids using silicic acid chromatography and conventional solvent systems. Free ricinoleic acid exerted a strong polar effect on the absorption of other lipids. It could not be eluted as a definitive peak; it appeared to tail throughout the triglyceride, cholesterol, and phospholipid fractions. It was conveniently removed along with other free fatty acids by treatment of the lipid mixtures with Amberlite IRA-400 prior to silicic acid chromatography (3). Mono- and diglycerides of ricinoleic acid were eluted with the cholesterol fraction when chloroform was employed as the eluting solvent. Triricinolein was eluted along with phospholipids in both standard mixtures and in lymph lipids when methanol was employed as the eluting solvent. In order to separate the hydroxylated triglyceride from phospholipids, it was necessary to employ another silicic acid colmn. In this way,

triricinolein was separated from phospholipids and from the column with 5% methanol in chloroform. The phospholipids were then eluted with methanol containing 4% water. An illustration of the separations obtained from this column using synthetic lipid mixtures are illustrated in the Table. The separations achieved were clear-cut and recoveries were better than 98%.

TABLE I

Recovery of Triricinolein and Ricinoleic Acid on a Silicic Acid Column

	Charge, mg	Recovery, mg	Percent recovery, %
Lipid sample I			
Ricinoleic acid	9.5	10.0	105.2
Phospholipid [a]	15.7	15.4	98.1
Total	25.2	25.4	100.8
Lipid sample II			
Triricinolein	9.9	10.6	107.0
Phospholipid [a]	14.9	16.1	108.0
Total	24.8	26.7	107.8

[a] Synthetic dipalmitoyl lecithin.

No attempt was made to obtain further separation of cholesterol from the partial glycerides since cholesterol can be determined colorimetrically. The mono- and diglycerides may also be estimated by oxidative methods.

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[Received Feb. 11, 1966]

Investigation of the Glyceride Structure of *Cardamine impatiens* L. Seed Oil

THE FATTY ACID METHYL esters resulting from transesterification of *Cardamine impatiens* L. (Cruciferae) seed oil contain 17% of *erythro*-13,14-dihydroxydocosanoate, 6% of *erythro*-15,16-dihydroxytetracosanoate, and smaller amounts of the C_{18} and C_{20} dihydroxy acid methyl esters (1). The unusual composition of this oil prompted further investigation to determine whether the dihydroxy acids are actually triglyceride substituents and, if so, how they are attached to the glycerol moiety.

Thin-layer chromatographic (TLC) analysis of the seed oil on Silica Gel G plates with light petroleum (bp 30–60C) and diethyl ether (70:30, v/v) indicated two major components, one of which was normal triglyceride. The unusual triglyceride component (I) had an R_f intermediate between that of the castor oil triglyceride containing one free hydroxyl group per molecule and that of the triglyceride containing two free hydroxyl groups per molecule. Infrared analysis of a liquid film of the seed oil on NaCl plates showed an absorption band due to hydroxyl (3,470 cm^{-1}) and bands at 1,370, 1,235 and 1,022 cm^{-1} indicative of acetate groups. The positions of these bands are similar to those reported by Stodola et al. (2) for 8,9,13-triacetoxydocosanoic acid.

Component I was separated from the normal glycerides on a silica gel column with the same solvent as for the thin-layer separation. After the fractions having hydroxyl absorption in their infrared spectra were combined, they totalled about 40% of the weight of oil applied to the column. The combined material was shown 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). At least one of the hydroxyl groups of each vicinal diol grouping must be bound in some manner because the glyceride did not react with periodate; nor did it form an isopropylidene derivative. Component I was optically active, $[\alpha]_D^{27.5C}$ + 1.4° (C = 10, $CHCl_3$), but the dihydroxy acids resulting from hydrolysis of I appeared to be optically inactive (1).

The nuclear magnetic resonance (NMR) spectrum of I indicated the probable presence of an acetate group (signal at 7.93 τ, sharp singlet). This observation supported infrared evidence for acetate functions. Acetic acid was identified by GLC of the free acids obtained by saponification of I, but no reliable quantitative measure of acetate has been made. Phosphorus, nitrogen and sulfur were shown to be absent.

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The mixed methyl esters obtained by refluxing I with 1% H_2SO_4 in methanol for 3 hr were crystallized from a 15% (w/v) diethyl ether solution overnight at −18C. TLC analysis of the liquor and the crystals showed that nearly quantitative separation between ordinary methyl esters and dihydroxy methyl esters was achieved. The composition of each fraction was determined as previously described (1) and these results indicated that the glyceride contained approximately 2 moles of nonhydroxylated long-chain fatty acids per mole of dihydroxy fatty acid.

The hydroxyl-containing glyceride (I) was hydrolyzed with the glycerol ester hydrolase isolated from *Ricinus communis* (castorbean) seed. Only two major hydrolysis products (II, III) resulted (by ether extraction of the aqueous system) as evidenced by two spots on TLC analysis. One of these spots (II) proved to be the normal free acids (oleic, linoleic, etc.) that had been liberated. No dihydroxy acids were liberated and no starting material remained. This enzyme is known to be specific for glyceryl ester linkages (4). Under the conditions used here, it hydrolyzed soybean oil (used as a control) completely, but it did not hydrolyze methyl oleate or 9(10)-hydroxy-10(9)-propionoxystearic acid to any measurable extent.

The unidentified enzymic hydrolysis fragment (III) was isolated by column chromatography on silica gel and the infrared spectrum indicated that it contained both carboxyl and ester functions. After the free carboxyl groups were esterified by treatment with diazomethane, the product (IV) gave only one spot on TLC analysis. The infrared spectrum of this esterified product showed hydroxyl absorption and somewhat more intense acetate absorption than the spectrum of I. Acetate protons (7.93 τ) and methoxyl protons (6.33 τ) were shown to be present by NMR. Since there was no band at 5.76 τ due to glycerol α-carbon protons, the glycerol moiety was not present. The bands due to methoxyl and C-methyl protons had approximately the same area. The hydrolysis fragment (III) was readily transesterified with 1% H_2SO_4 in methanol to give a ratio of 1 mole of long-chain nonhydroxylated methyl ester to 1 mole of dihydroxy methyl ester.

Some structural feature of III apparently prevents complete liberation of dihydroxy acids when III is refluxed with N ethanolic KOH for 2 hr. A byproduct (V) that is the result of partial hydrolysis (contains no acetate groups) is obtained in about a 10% yield, while the remaining 90% of the hydrolysis mixture results from complete hydrolysis of III. Though seemingly resistant to alkaline hydrolysis, byproduct V can be completely hydrolyzed under acidic conditions. Treatment with 1% H_2SO_4 in methanol yields nonhydroxylated methyl esters and dihydroxy methyl esters in a 1:1 mole ratio.

Elemental analyses indicate that IV contains 18.4% oxygen (by difference). This high oxygen content, together with the alkaline hydrolysis results, the presence of acetate, and the NMR spectra, suggests that compound IV has structural features that cannot be completely accounted for in terms of ester linkages between ordinary fatty acids and long-chain dihydroxy acids. An equivalent number of methoxyl and C-methyl protons, as shown by the NMR spectrum of IV, is particularly surprising. If IV were comprised of a nonhydroxylated long-chain acid esterified with a hydroxyl group of a long-chain dihydroxy acid methyl ester, the number of C-methyl protons would be twice that of the methoxyl protons. The spectrum suggests, therefore, that a dicarboxylic acid with one of its carboxyl groups free is incorporated in III. Compound IV would then have the same number of C-methyl and methoxyl protons. At present, we have no evidence for a dicarboxylic acid fragment among the hydrolysis products.

Preliminary work on cleavage of I with pancreatic lipase gave inconclusive results. Further work was not attempted due to lack of seed. Structural work on this unusual seed lipid will be resumed when a sufficient supply of *Cardamine impatiens* seed becomes available.

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ACKNOWLEDGMENTS

Gas-liquid chromatographic analyses by J. W. Hagemann, NMR spectra by C. A. Glass, and seeds supplied by Q. Jones, USDA Crops Research Division, Beltsville, Maryland.

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[Received May 6, 1966]

Phosphonolipids. VII. Synthesis of Phosphonic Acid Analogues of Diether L-α-Lecithins

THE DISCOVERY of the existence in nature of complex lipids containing phosphonic acid instead of phosphoric acid has aroused considerable interest as to their biosynthesis and biological role. However, the isolation of phosphonolipids in the presence of phospholipids which they resemble closely in structure as well as physical and chemical properties offers considerable difficulties. To facilitate the isolation of phosphonolipids from natural sources, their structural identification and the elucidation of their biological role, the synthesis of phosphonic acid analogues of phospholipids thought to be most likely to occur in nature is being undertaken in this laboratory. We have already reported the synthesis of *phosphonic acid analogues* of L-α-cephalins (1), L-α-lecithins (2) and diether L-α-cephalins (3).

We wish now to make a preliminary report on the synthesis of *phosphonic acid-analogues* of diether L-α-lecithins (Formula A). These compounds with two identical alkyl radicals having 14, 16 or 18 carbon atoms per radical were obtained via the following series of intermediates: (I) Diethyl-2-bromoethylphosphonate ⟶ (II) 2-Bromoethylphosphonic acid monoanilinium salt (2) ⟶ (III) 2-Bromoethylphosphonic acid (2) ⟶ (IV) 2-Bromoethylphosphonic acid monochloride (2) ⟶ (Va) Di-O-octadecyl L-α-glyceryl-(2-bromo ethyl) phosphonate. Melting point, 42.5–43.5C, $[\alpha]_D^{25}$–2.8° in dry chloroform, (c, 10). *Anal.* Calcd. for $C_{41}H_{84}O_5PBr$ (768): C 64.12, H 11.03, P 4.03, Br 10.41. Found: C 64.06, H 10.93, P 4.02, Br 10.02.

(Vb) Di-O-hexadecyl L-α-glyceryl-(2-bromoethyl) phosphonate. Melting point 46.5–47.5C, $[\alpha]_D^{25}$–3.0° in chloroform (c, 9.5). *Anal.* Calcd. for $C_{37}H_{76}O_5PBr$ (711.9): C 62.42, H 10.76, P 4.35, Br 11.23. Found: C 62.59, H 10.76, P 4.42, Br 10.92.

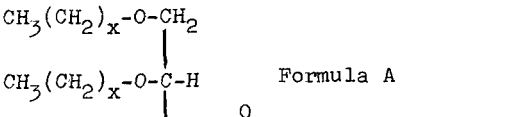
(Vc) Di-O-tetradecyl L-α-glyceryl-(2-bromoethyl) phosphonate. Melting point 47–48C, $[\alpha]_D^{25}$–3.2° in chloroform (c, 11). *Anal.* Calcd. for $C_{33}H_{68}O_5PBr$ (655.8): C 60.44, H 10.45, P 4.72, Br 12.19. Found: C 61.22, H 10.25, P 4.64, Br 11.96. ⟶ Compounds VIa -c were dried over phosphorus pentoxide at 25C and a pressure of 0.02 mm Hg.

(VIa) Di-O-actadecyl L-α-glyceryl-(2-trimethylammoniumethyl)-phosphonate. Melting point 190–192C, $[\alpha]_D^{27}$ + 3.5° in chloroform-methanol (3:2); c, 10, M_D + 26.7°. *Anal.* Calcd. for $C_{44}H_{94}O_6NP$ (764.2): C 69.15, H 12.40, N 1.83, P 4.05. Found: C 69.43, H 12.44, N (Kjeldahl) 1.70, N (Dumas) 1.95, P 4.14.

(VIb) Di-O-hexadecyl L-α-glyceryl-(2-trimethylammoniumethyl)-phosphonate. Melting point, 192–194C, $[\alpha]_D^{25}$ + 3.4° in chloroform-methanol (3:2); (c, 9), M_D + 24.1°. *Anal.* Calcd. for $C_{40}H_{86}O_6NP$ (708.1): C 67.85, H 12.24, N 1.98, P 4.37. Found: C 67.44, H 12.05, N (Kjeldahl) 1.92, N (Dumas) 1.94, P 4.35.

(VIc) Di-O-tetradecyl L-α-glyceryl-(2-trimethylammoniumethyl)-phosphonate. Melting point, 195–197C, $[\alpha]_D^{26}$ + 4.0° in chloroform-methanol (3:2); (c, 10), M_D + 26.1°. *Anal.* Calcd. for $C_{36}H_{78}O_6NP$ (652): C 66.32, H 12.06, N 2.15, P 4.75. Found: C 66.66, H 12.11, N (Kjeldahl) 1.96, N (Dumas) 2.08, P 4.83.

The homogeneity of compounds VIa-c (Fig. 1) was confirmed by one-dimensional thin-layer



Formula A

VIa X= 17, VIb X= 15, VIc X= 13

chromatography on silica gel H using two solvent-mixtures: chloroform-methanol-water (65:25:4), and chloroform-methanol-7M ammonium hydroxide (230:90:15). In each case a single spot was obtained. The R_f values of compounds VIa, b and c are practically identical, and are the same in both solvent mixtures, i.e. 0.21, 0.22 and 0.22, respectively.

Compounds VIa-c, on drying at 80C and a pressure of 0.02 mm Hg. lose the elements of one mole of water.

The melting points of compounds VIa, b and c were determined simultaneously, using capillary tubes and an electrically heated metal block. The temperature of the block was raised from room temperature to 160C at a rate of 12–13C/min, and from thereon at a rate of 4C/min. At about 180C, the compounds formed translucent masses. These coalesced suddenly

with the formation of a meniscus, at the temperatures reported as melting points.

ACKNOWLEDGMENT

Work supported by a grant (MT-684) from the Medical Research Council (Canada).

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[Received March 30, 1966]

An Improved Tank for Development of Preparative Thin-Layer Chromatograms

HIGH-RESOLUTION PREPARATIVE thin-layer chromatography (TLC) is often hampered by the low capacity of the adsorbent. Often overloading and poor resolution can be overcome by increasing the thickness of the adsorbent layer although heavy layers (> 1 to 2 mm) tend to slide from the plate when developed in the conventional ascending manner.

The new unit overcomes limitations of conventional preparative TLC by allowing development of high capacity layers up to 3 mm thick without adsorbent fracture; accordingly, development time is decreased.

The tank is constructed of plexiglass and is designed to accommodate a standard 8 × 8 in. TLC plate (Fig. 1). Developing solvent need be prepared in small quantities only because it is contained in a shallow stainless steel trough. The TLC plate is supported at the top by a narrow ledge so that it meets the solvent in the tray at an angle of 15° with the horizontal. The inner surface of the tank's hinged lid is covered with filter paper that is drawn taut and taped to the outside top surface. Development time is greatly reduced when the tank lid is lined with paper and cooled with dry ice. When wet with TLC solvent, this paper helps to maintain the saturated atmosphere necessary for rapid development. Cooling with dry ice, placed in a 200 mm petri dish resting on the lid during TLC development, establishes a thermal gradient which causes circulation of solvent vapors and keeps the tank atmosphere and filter paper saturated with solvent.

This tank has been used for all types of plates with a variety of solvents; it is unaffected by bromine atmospheres and polar solvent vapors; its plexiglass body is protected from liquid solvent attack by the stainless steel reservoir.

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[Received April 18, 1966]

FIG. 1. Preparative thin-layer chromatography tank that accommodates 8 × 8 in. plates and uses a minimum of solvent.

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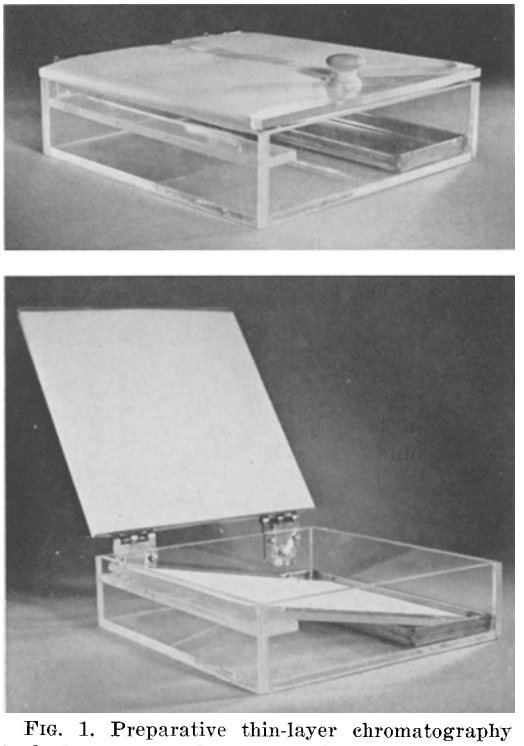

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[Received April 18, 1966]

Mechanism of Lipoxidase Reaction: Origin of the Oxygen Incorporated into Linoleate Hydroperoxide

LIPOXIDASE IS KNOWN to be specific for a *cis,cis*-1,4 pentadiene system such as linoleic acid, and this acid yields an optically active isomer (1); Hamberg and Samuelsson have suggested that lipoxidase attacks the ω-6 carbon atom specifically (2). They showed that during lipoxidase-catalyzed oxidation of linoleic acid 70% of 13-hydroperoxy-octadeca-9,11-dienoic acid is formed and only 30% of the 9-hydroperoxyoctadeca-10,12-dienoic acid. Following the development of an analytical procedure that enabled us to separate quantitatively methyl 13-hydroxystearate from methyl 9-hydroxystearate (3), we found an even higher specificity for lipoxidase, i.e., the exclusive formation of 13-hydroperoxyoctadeca-9,11-dienoic acid (4).

Although always assumed, but never demonstrated, the hypothesis is that the oxygen molecule incorporated into the hydroperoxide as a result of lipoxidase catalyzed oxidation comes from the gas phase. The source of the oxygen molecule in lipoxidase oxidation became suspect because of studies on hydrogenation of sorbic acid (5). During the homogeneous catalytic hydrogenation of this acid with pentacyanocobaltate, the hydrogen incorporated into the hydrogenated molecule originated in the H_2O molecule and not in the H_2 gas phase as predicted. Also, the complexity of the free radical reaction with lipoxidase and the presence of aqueous and gaseous sources for oxygen led us to question the source of the oxygen in the hydroperoxide.

Two experiments were designed to answer this question. The first, the incubation of lipoxidase with linoleic acid in a $H_2{}^{18}O$ buffer with $^{16}O_2$ in the gas phase; and the second, the same incubation but in normal $H_2{}^{16}O$ buffer with isotopic $^{18}O_2$ in the gas phase. The incubated products were methylated, reduced, and fractionated by column chromatography and the methyl hydroxystearate was analyzed by mass spectrometry. No isotopic ^{18}O was detected in the product of the first experiment that used $H_2{}^{18}O$ water, yet all the hydroxystearate obtained from the second experiment that used $^{18}O_2$ was labeled. These experiments demonstrate that lipoxidase does not catalyze oxygen exchange between the water and gas and that the oxygen incorporated into the hydroperoxide molecule by lipoxidase comes from the gas phase.

A detailed description of this investigation is being prepared for publication.

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[Received May 6, 1966]

LETTERS TO THE EDITOR

Comments on the Analysis of Some Triacid Triglyceride Mixtures

Sir: Brockerhoff has recently discussed the difficulty of resolving a mixture of triacid triglycerides (TG) with methods presently available (4). We wish to point out that in the special case where oleate is one of the acids and the other two are saturates, use of a lipase specific for oleate would give the additional information necessary to completely resolve the mixture. The microorganism, *Geotrichum candidum*, produces a lipase highly specific for oleic acid regardless of position (2,6), which appears suitable for this analysis.

Brockerhoff's stereospecific analysis (BSA) (3) will provide information of the type in Table I. A second analysis involving lipolysis of the triacid TG mixture with *G. candidum* lipase, isolation of the 1,2 saturated fatty acid diglycerides, reaction with phenylphosphoryl dichloride, and digestion with phospholipase A would give directly the proportions of one of the critical pairs (see Table II); namely, those that contain oleate in the 3-position (Hirschmann). Since BSA tells us the M% of each acid in each position, and the additional analysis with *G. candidum* lipase yields the ratios of one critical pair, the others can be calculated by difference.

For purposes of illustration, we have assumed that BSA gives values (for example Ia + Ib) listed in Table I and analysis two gives 66.6% 18:0 in the FFA. The isomer proportions (see Table II) can now be calculated with the aid of the following equations:

$$\text{Ia} = (18{:}0\%)\ (\text{Ia} + \text{Ib})$$
$$\text{Ib} = (16{:}0\%)\ (\text{Ia} + \text{Ib}) = (\text{Ia} + \text{Ib}) - (\text{Ia})$$
$$\text{IIa} = (\text{Ia} + \text{IIa}) - \text{Ia}$$
$$\text{IIb} = (\text{Ib} + \text{IIb}) - \text{Ib}$$
$$\text{IIIa} = (\text{Ia} + \text{IIIa}) - \text{Ia}$$
$$\text{IIIb} = (\text{Ib} + \text{IIIb}) - \text{Ib}$$

Since phospholipase A will also hydrolyze one of the positions of a β-phosphatide (7), a partially independent technique is available to determine the critical pair containing secondary oleate. Likewise, after exhaustive digestion of the α-phosphatides, pancreatic lipase (5) could be used to substantiate the ratios of the 1-position oleate critical pair.

While the properties of *G. candidum* lipase have not been thoroughly explored, it appears that the lipase can be highly specific for linoleic acid under certain conditions (1), thus triacid TG combinations containing linoleic acid should also be amenable to complete structure analysis. Theoretically, the procedure outlined above can be applied to triacid TG containing both oleic and linoleic acid in combinations with a saturated acid. In this case an additional step is necessary to separate from the products of *G. candidum* lipolysis, those diglycerides containing one double bond. At present, there is not enough information to allow speculation on the extension of the method to glycerides containing linolenic acid.

This procedure, then, for most natural fats—once the individual TG triacid combinations are isolated—would allow complete structure elucidation of all major TG's except the trisaturated TG's. This latter type awaits the discovery of lipases specific for certain fatty acids or a specific position such as the 2 or 3 position of a TG.

We should point out that the methods as detailed above requires the *G. candidum* lipase be capable of attacking the unsaturated linkage at either primary position. This aspect has not been investigated; however, we believe that stereospecificity is highly unlikely for a lipase which attacks both the primary and secondary positions of a TG. At any rate, if the enzyme were found to be stereospecific, the TG combinations discussed above could still be resolved, since the derivatives of at least two of the oleate critical pairs could be isolated after treatment with *G. candidum* lipase. We are currently investigating the properties of this lipase and

TABLE I[a]

Positional Distribution of an Oleate Triacid Triglyceride

Position	Fatty acid 16:0	18:0	18:1
	M%		
1	33.3	33.3	33.3
2	33.3	33.3	33.3
3	33.3	33.3	33.3

[a] Adapted from Brockerhoff (4).

TABLE II

Isomer Proportions of an Oleate Triacid Triglyceride Mixture

	I[a]		II[a]		III[a]	
	A	B	A	B	A	B
Position						
1	16:0	18:0	16:0	18:0	18:1	18:1
2	18:0	16:0	18:1	18:1	18:0	16:0
3	18:1	18:1	18:0	16:0	16:0	18:0
M%[b]	22.2	11.1	11.1	22.2	11.1	22.2

[a] Critical pairs of oleate triacid triglycerides.

[b] Calculated from FFA released by digestion with phospholipase A of 3-phosphatides prepared from products of *G. candidum* lipolysis (i.e., 66.6% 18:0, 33% 16:0).

hope to further establish the utility of this enzyme in TG structure investigations.

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ACKNOWLEDGMENT

Supported in part by Public Health Service Research Grant AM-02605-08 from the Institute of Arthritis and Metabolic Diseases.

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[Received May 13, 1966]

A Solved Problem of Triglyceride Analysis

Sir: A complete stereospecific analysis of triglyceride mixtures requires a considerable amount of data. However a recent letter in your March issue incorrectly states that "with our present methods this is still an impossible task." We wish to affirm that the difficulties can be overcome with perserverance and the methods now available.

The triglycerides that contain only one type of acid cause no difficulty. Fortunately the triglycerides that contain two different types of acid can often be resolved to a greater extent than the total number of alkene groups in the molecule would indicate (e.g., 18:0–18:0–18:2 is separable from 18:0–18:1–18:1 and 18:0–18:0–18:3 *from* 18:0–18:1–18:2 on $AgNO_3$-silicic acid plates). The triglyceride species with only two acids have only 3 possible isomers and are easily analyzed.

The triglycerides that contain three different acids require the most consideration in structure determination. They can be readily classed into three isomeric pairs which must then be further resolved.

Position	Triglycerides		
1	AB	AC	BC
2	BA	CA	CB
3	CC	BB	AA

The relative amounts of these triglycerides most probably reflects the availability of the different diglyceride precursors. With no steric selectivity, the esterification of C at position 3 would be expected to produce ABC and BAC in the same relative abundance as the pairs ABB and BAB or ABA and BAA are produced. The latter sets of triglycerides are isomeric pairs that are easily analyzed and could be used to suggest the ABC/BAC ratio.

A fully rigorous structure proof of the three-acid triglycerides requires only the following steps:

(a) Cleavage of the six-isomer mixture to 1,2- and 2,3-diglycerides by triglyceride lipase.

(b) Isolation and analysis of the 1,2-diacylglycerol-3-phosphates produced by diglyceride kinase action as described in Federation Proceedings 25, 521 (1966).

(c) Separation of the three isomeric pairs of phosphatidate derivatives according to their acid content.

AB	AC	BC
BA	CA	CB
PP	PP	PP
x	y	z

The relative amounts of x, y and z are indicated by the content at the 3 position of C, B and A respectively.

(d) Phospholipase A cleavage of each pair to determine the AB/BA, AC/CA and BC/CB ratios.

If the results of the unequivocal analysis show that the AB/BA ratio is constant in all the triglyceride species, future analyses could then use the values obtained from the more easily measured diacid triglycerides. We hope these comments will adequately indicate that the difficulties of triglyceride analysis can be overcome.

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[Received April 28, 1966]

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[Received April 28, 1966]

The Cholesterol Complex in the Myelin Membrane

Sir: The lipid composition of purified myelin obtained from different animal species and prepared by sucrose density gradient techniques has been reported by a number of laboratories. Although Finean (1) and, more recently, Vandenheuvel (2) have proposed that the myelin subunit is composed of glycerophosphatide-cholesterol and/or sphingolipid-cholesterol complexes, there remains a substantial fraction of noncholesterol lipid which is in excess of the cholesterol when calculated as a molar ratio. In this paper we will correlate turnover data published elsewhere with analyses of the lipid composition of myelin and propose the identification of those lipids which may be complexed with cholesterol.

We have recently determined the rate of turnover of each of the different myelin lipids in the rat and have found that inositol phosphatide, lecithin, and serine phosphatide, when labeled with acetate-1-C^{14}, appear to turn over somewhat more rapidly than the remaining lipids, ethanolamine phosphatide, sulfatide, cerebroside, sphingomyelin, and cholesterol. The lipids which are more active metabolically show a half-life ranging from 5 weeks to 4 months, while the half-lives of the more stable lipids vary from 8 to 14 months (3).

In his myelin model, Vandenheuvel (2) has proposed that the long chain fatty acids are interdigitated in a tightly packed arrangement, thus being subject to intermolecular forces which would provide maximum stability. In general, the results of our turnover rate experiments lend support to such a concept as the lipids containing the highest proportion of long chains, the sphingolipids, appear to show the longest half-lives. The shorter chain fatty acids, which are known to be found in such compounds as lecithin, have shorter half-lives. The ethanolamine phosphatide, however, which is one of the more stable lipids, also contains short chains.

TABLE I

Molar Ratios of Rat Brain Myelin Lipids

"Stable" Lipids	
Cholesterol	1.00
Galactolipids	0.60
Sphingomyelin	0.08
Ethanolamine phosphatide	0.38
Sum	1.06
"Labile" Lipids	
Serine phosphatide	0.17
Lecithin	0.21
Inositol	0.03
Sum	0.41

We have noted recently that in our analyses of the lipid composition of myelin, the molar sum of the galactolipid, the ethanolamine phosphatide and the sphingomyelin, which comprise the metabolically stable lipids, approximates the molar concentration of cholesterol. In Table I is shown the ratio of the sum of these components to cholesterol when the molar concentration of cholesterol is equal to 1.00. The same calculations applied to myelin lipid compositions of human, ox, guinea pig, rat, rabbit, mouse and monkey from other laboratories reveal similar correspondence.

We think it reasonable, therefore, that the sphingolipids and ethanolamine phosphatide may exist as cholesterol complexes in myelin, the association with cholesterol resulting in increased metabolic stability. Those lipids which have been found to be more labile may exist in the myelin membrane in an uncomplexed form and may function in a somewhat different manner.

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[Received March 17, 1966]

Chemical Composition of the Wax Secreted by a Scale Insect (*Ceroplastes pseudoceriferus* Green)

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ABSTRACT

The wax material in the secretion of a scale insect, *Ceroplastes pseudoceriferus* Green was analyzed chemically with special interest to the composition of higher fatty acids and higher alcohols. The wax consists of 34.2% fatty acids, 27.1% unsaponifiable matter and 29.5% resin acids. The fatty acids were found to be a complex mixture of 15 normal acids ranging from C_8 to C_{32}. Of these, octacosanoic, triacontanoic and dotriacontanoic acids comprise over 30% of the wax. Presence of relatively large amount of unsaturated fatty acids of the C_{18} series (2.8% of the wax) is of particular interest.

From the unsaponifiable fraction, only one saturated straight chain alcohol, hexacosanol, was detected (2.7% of the original wax). The other unsaponifiable matter was considered to be cyclic or branched carbon chain, and consisted of at least 12 to 20 compounds. The resin acid fraction was also found to be a complex mixture of at least 13 to 14 components.

INTRODUCTION

THE SCALE INSECT, *Ceroplastes pseudoceriferus* Green (Insecta; Hemiptera) has a waxy covering of large bulk, making up 60% to 70% of the total (insect and covering) weight, throughout its life history. This material consists of honeydew and wax, which are secreted from particular glands of the insect. The mode of secretion was briefly described (1). The amino acid and carbohydrate composition of the water soluble honeydew was analyzed and discussed previously (2,3). This paper is one of a series on the waxy secretion of this insect, and deals with the composition of the wax, with special reference to the composition of higher fatty acids and alcohols.

EXPERIMENTAL

Separation of Crude Wax

The overwintering female adults were collected from twigs of infested tea plants in Shizuoka-ken, Japan, from September to December. All the insects collected were stored in a deep freezer at −20C until used.

The procedure applied was virtually that described in the preceding paper (2). The insects with waxy covering were shaken lightly with chloroform to dissolve the external wax. The chloroform layer was thoroughly washed with water, and evaporated to dryness. The resulting light yellow wax material, referred to as crude wax hereafter, was stored in darkness over silica gel in vacuo until further treatment.

Fractionation of Crude Wax

All operations for removing organic solvents were done in vacuo at lower than 30C under nitrogen. To crude wax (27.2 g) dissolved in a small volume of chloroform was added a large excess of ethanol. The amount of 14.5 g of white precipitate, referred to as hard wax, was obtained by filtration.

The residue from the ethanolic filtrate was dissolved in petroleum ether (bp, 30C–60C), and extracted three times with 50% aqueous ethanol, and then three times with 2.5% KOH in 50% aqueous ethanol. The combined 50% ethanol extracts yielded 2.7 g of yellow solid referred to as fraction A. The combined alkaline ethanol extracts were acidified with sulfuric acid, and the acidic material extracted with petroleum ether. The amount of 4.6 g of yellow solid was obtained (fraction B). From the remaining petroleum ether solution after washing with alkaline ethanol, 3.1 g of yellow paste was obtained (soft wax).

Saponification of Hard and Soft Waxes

The procedures of Kariyone et al. (4) were followed for saponification and separation of the fractions. As the result of the saponification, 2.2 g of white solid saponifiable matter and 1.5 g of yellow paste unsaponifiable matter were obtained from 4.0 g of original hard wax, and 1.2 g of yellow solid saponifiable matter and 1.8 g of brown liquid unsaponifiable matter from 3.1 g of soft wax.

Separation of Straight Chain Alcohols as Urea Adduct

Unsaponifiable matter derived from the hard and soft wax was dissolved in benzene, and 6 to 7 volumes of urea were added. The mixture was refluxed for 15 minutes, cooled to room temperature with shaking, and stored in a refrigerator overnight. The isolated urea adduct

was treated with hot water to liberate the straight chain alcohols.

Infra-red Absorption Analyses

A Hitachi EPI-2 infrared spectrophotometer was used. All the solid samples were examined as KBr tablets, but the liquid samples as a thin film on an NaCl plate.

Gas-Liquid Chromatography

The apparatus used was a Shimadzu GC-2B gas chromatograph, equipped with a thermal conductivity detector. Stainless steel spiral columns, 1 meter long and copper spiral columns, 2 meters long with 4 mm diameter were used. The following four operating conditions were applied; I) 10% silicone SE-30 on 60 to 100 mesh Celite-545 at 250C, II) 30% high vacuum silicone grease on 60 to 100 mesh Celite-545 at 230C, III) 20% silicone DC-550 on 40 to 60 mesh Celite-545 at 190C, and IV) 15% polyethylene glycol adipate on 40 to 60 mesh Celite 545 at 190C. Helium was used as the carrier gas (70 ml/min), and the detector filament current was adjusted to 200 mA at the operating temperatures. All the samples of fatty acids were analyzed as their methylesters which were prepared with diazomethane in ether.

Identification of fatty acid methyl esters and alcohols was achieved by comparing their retention times with those of standard or by plotting the logarithm of the retention times versus carbon chain length.

RESULTS

Fractionation of Crude Wax

From the infrared analysis, it is concluded that the crude wax consists largely of esters (absorption maxima at 1750–1730, 1240 and 1170 cm^{-1}), with some free carboxyl groups (1710 cm^{-1}) and some double bonds (3080 −3000, 890 cm^{-1}).

The hard wax constitutes 53% of the crude wax. Infrared absorption spectra show peaks at 1730, 1240 and 1165 cm^{-1}, corresponding to esters; and a shoulder at 1710 cm^{-1} and a weak absorption at 885 cm^{-1} suggest the presence of a free carboxyl group and a double bond, respectively.

The soft wax constitutes about 11% of the crude wax and gives absorption maxima at 1730, 1240 and 1170 cm^{-1} indicative of esters, and at 1640 and 890 cm^{-1} indicating the presence of double bonds.

Fractions A and B amount to about 13% and 17% of the crude wax, respectively. The infrared absorption spectrum of fraction A shows maxima at 1690, 1300–1200 and 950 cm^{-1} indicative of free carboxyl group, and a shoulder at 1650 cm^{-1} with weak absorption at 890 cm^{-1} probably indicative of the presence of unsaturated compounds. The spectrum of fraction B is quite similar to that of fraction A, with the exception of weaker absorption at 3400 cm^{-1} than A. Fraction A may contain more hydroxyl radicals than fraction B.

Fatty Acids from Hard Wax and Soft Wax

The saponifiable matter from the hard wax constituted 30% of the crude wax. The infrared absorption spectrum indicated the presence of saturated higher fatty acids. According to gas chromatographic analyses under condition I, this sample was a mixture of three components, octacosanoic, triacontanoic and dotriacontanoic acids. Their relative amounts were calculated as follows: C_{28}—7.8%, C_{30}—72.7% and C_{32}—19.5%. Neither appreciable amount of homologues lower than octacosanoic acid nor of homologues higher than dotriacontanoic acid were found.

The saponifiable matter isolated from the soft wax comprised about 4.5% of the crude wax. Its infrared analysis suggested that this fraction contained some unsaturated compounds. Gas chromatography carried out under condition I gave three peaks corresponding to octacosanoic, triacontanoic and dotriacontanoic acids. Furthermore, chromatograms obtained under condition IV revealed the presence of relatively large amount of lower homologues. The saponifiable matter from the soft wax was a complex mixture of fatty acids ranging in carbon chain length from 8 to 32. The presence of a large amount of the C_{18} series unsaturated fatty acids is of particular interest. The overall results of analyses on saponifiable matter of the hard and soft waxes are given in Table I.

Alcohols from Hard Wax and Soft Wax

The unsaponifiable matter of the hard wax constituted 20% of the total. With urea treatment 1.45 g of unsaponifiable matter gave 0.19 g of a white solid from the urea adduct fraction and 1.11 g of yellow viscose liquid from the nonurea adduct fraction. The latter liquid is considered to be a mixture of cyclic and branched chain compounds. The infrared absorption spectrum of the white solid showed a feature characteristic of a saturated straight chain higher alcohol. Gas chromatographic analysis of the alcohol under condition II gave only one peak, corresponding to hexacosanol (C_{26}).

The unsaponifiable matter of the soft wax constituted 6.5% of the original crude wax.

TABLE I
Fatty Acid Composition of Saponifiable Matter of Hard Wax and Soft Wax

Fatty acid	Hard wax		Soft wax	
	% in acids of hard wax	% in crude wax	% in acids of soft wax	% in crude wax
8:0	—	—	+	+
10:0	—	—	+	+
11:0	—	—	+	+
12:0	—	—	+	+
13:0	—	—	+	+
14:0	—	—	+	+
15:0	—	—	+	+
16:0	—	—	+	+
18:0	—	—	1.7	0.1
18:1	—	—	7.4	0.3
18:2	—	—	36.4	1.6
18:3	—	—	21.1	0.9
28:0	7.8	2.3	5.6	0.3
30:0	72.7	21.4	21.1	1.2
32:0	19.5	5.7	6.5	0.4

+ : trace, — : no detectable amount.

No appreciable amount of any compound reacted with urea to form the urea adduct. Almost all components of the unsaponifiable matter of the soft wax are considered to be cyclic and branched chain compounds.

The infrared absorption spectra of nonurea adducts of the unsaponifiable matter of both the hard and soft waxes were similar; both exhibited spectra indicative of hydroxyl groups and double bonds. Gas chromatography carried out under conditions I, II and III showed that these two fractions are complex mixtures of from 12 to 20 compounds. Though several components were common in both fractions, it is noteworthy that the fraction isolated from the soft wax contains larger amounts of short carbon chain compounds than that from the hard wax.

Fraction A and Fraction B

Gas chromatographic analyses of these fractions under condition I did not result in any appreciable peak. After esterification with diazomethane, however, a number of peaks appeared under conditions I and II. Gas chromatographic patterns of esterified fraction A and fraction B were quite similar to each other. These two fractions consist mainly of compounds with free carboxyl groups reacting with diazomethane to form their methyl esters. No normal fatty acid was found at appreciable levels under condition IV. It is assumed that these fractions are mixture of resin acids.

DISCUSSION

The overall results of the present analyses are summarized in Table II. Further fractionation of the unsaponifiable cyclic and branched chain compounds and resin acids has not been conducted. Because of the amount of these fractions in the crude wax, however, isolation and identification of their components should be undertaken in the future.

TABLE II
Composition of the Crude Wax

Saponifiable matter	34.2%
Caprylic, capric, undecanoic, lauric, tridecanoic, myristic, pentadecanoic and palmitic acids	trace
Stearic acid	0.1
Oleic acid	0.3
Linoleic acid	1.6
Linolenic acid	0.9
Octacosanoic acid	2.6
Triacontanoic acid	22.6
Dotriacontanoic acid	6.1
Unsaponifiable matter	27.1
Hexacosanol	2.7
Cyclic and branched chain compounds	24.4
Resin acids	29.5

The hard wax consists of esters of straight chain acids (C_{28}, C_{30}, C_{32}) and straight chain (C_{26}), cyclic and branched chain alcohols, and the soft wax is a complex mixture of esters of acids ranging from C_8 to C_{32} with cyclic and branched chain alcohols. The unsaturated fatty acids of the C_{18} series were detected in the soft wax at a relatively high level. The occurrence of unsaturated compounds, dodecenoic and tetradecenoic (C_{14}) acids in the wax of a scale insect *Tachardina theae* was also reported by Kono and Maruyama (5).

According to Kono and Maruyama (6,7), the wax of nine Japanese species of scale insects contains C_{26} and C_{30} straight chain fatty acids and a C_{26} straight chain alcohol as their main components. The wax secreted by *Ceroplastes destructor* in Australia also consists of C_{26} and C_{28} straight chain saturated fatty acids and alcohol and their esters (8), with large amounts of C_{12} saturated fatty acid and C_{12} unsaturated alcohol (9).

Mukai et al. (10), working on lipids of *Icerya purchasi,* isolated straight chain alcohols of C_{27}, C_{26} and C_{24}. Chibnall and his co-workers (11,12) reported that the main components of waxes produced by some scale insects were straight chain fatty acids and alcohols with even numbers of carbon atoms ranging from 26 to 36. Bee wax of four species of bees also contain C_{26} to C_{36} straight chain fatty acids and alcohols, as well as large amount of paraffins (12).

There are some reports about the composition of cuticular wax of insects other than scale insects. In such insects as the mormon cricket *Anabrus simplex,* cockroach *Periplaneta americana,* silkmoth *Bombyx mori* and adult housefly *Musca domestica,* significant level of hydrocarbons occurred in the cuticular waxes (13–

16). On the other hand, the cuticular wax of the cattle tick *Boophilus microplus,* consists mainly of a mixture of saturated acids and alcohols of chain length of C_{30} (17).

Although the mechanism of biosynthesis of insect wax should be an interesting field of investigation, little work has been done with scale insects except for the schematic presentation by Chibnall and Piper (18). Piek (19), working on the biosynthesis of beeswax, speculated that the oenocytes synthesize the wax acids and hydrocarbons from acetate derived from the glycolysis in the fat body, but that fat body cells themselves synthesize the esters and their component acids and alcohols from acetate.

Comparing the relative amount of carbohydrates in the honeydew excreted by scale insects and that in aphids' honeydew, it is reasonable to assume that the higher fatty acids and higher alcohols in the wax are originated from excessive carbohydrates in the host plant sap on which the insect fed (3). The fatty acid fraction from the body lipid of this scale insect is a complex mixture of straight chain fatty acids ranging from C_7 to C_{20}, and the predominant components are the short chain acid (C_{10} and C_{12}) (20). There is no appreciable amount of alcohols or hydrocarbons higher than C_{20} in the body lipid (20). On the basis of these facts, it seems that the long chain fatty acids and alcohols in the wax are synthesized from short carbon skeletons at the time of secretion. On the other hand, it is doubtful that the resin acids are metabolic products of the scale insects. These acids may be derived from host plant sap and excreted without any change. Kono and Maruyama (21) determined the chemical structure of a resin acid isolated from the wax of *Ceroplastes rubens* and named it rubabietic acid. They also suggested that this resin acid was derived from the host plant sap.

ACKNOWLEDGMENTS

Shoziro Ishii, Kyoto University and Chisato Hirano, National Institute of Agricultural Sciences, Tokyo provided guidance and encouragement during the course of this study. L. I. Gilbert of Northwestern University, USA read the manuscript and J. Kanazawa and S. Goto, the Inspection Station advised gas-liquid chromatographic and infrared spectrometric analyses.

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[Received April 26, 1966]

The *trans*-3-Enoic Acids of *Grindelia oxylepis* Seed Oil

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ABSTRACT

trans-3-Hexadecenoic acid (14%) and the previously unreported *trans*-3-octadecenoic acid (2%) have been identified in seed oil of *Grindelia oxylepis* Greene, Compositae. Evidence was also found for the existence of other acids with *trans*-3 unsaturation.

INTRODUCTION

NUMEROUS SEED OILS from plants of the family Compositae show infrared absorption at 10.36 μ indicative of nonconjugated *trans* unsaturation (3). In *Calea urticaefolia* oil, a major component (35%) was identified as *trans*-3,*cis*-9,*cis*-12-octadecatrienoic acid (1). Gas-liquid chromatography (GLC) indicates the presence of this acid in most of the Compositae showing isolated *trans* unsaturation and, in many, indicates the co-occurrence of a component with equivalent chain lengths (ECL) (11) of 15.9 in an Apiezon L column (16) and 16.6 in a LAC-2-R 446 column. These chain lengths suggest that the component is an unusual hexadecenoic acid. Hopkins and Chisholm (7) reported the presence of *trans*-3-hexadecenoic acid in the seed oil of *Helenium bigelowii,* a member of the Compositae. Their report is the first published information about *trans*-3-hexadecenoic acid in a seed oil, but it has been found in other plant parts (2,13). The only other acid with *trans*-3 unsaturation recorded as present in a seed oil is the *trans*-3,*cis*-9,*cis*-12,*cis*-15-octadecatetraenoic acid from *Tecoma stans* (8).

trans-3-Hexadecenoic acid has now been identified in the seed oil of *Grindelia oxylepis* Greene, along with small amounts of the previously unreported *trans*-3-octadecenoic acid. Their isolation and identification are described in this paper.

Grindelia, a member of the family Compositae, is a genus of herbaceous or suffruticose annuals, biennials or perennials commonly referred to as "gumweeds" or "tarweeds" due to their characteristically resinous-viscid foliage. The genus contains some 57 species which occupy a greater part of western North America and much of South America and which are usually adapted to xeric, semidesert or desert environments (15).

Grindelia oxylepis Greene is an erect annual herb, 2–5.5 dm in height, occurring in moist places on plains, valleys and old fields in the Mexican States of Coahuila, Chihuahua, Durango and San Luis Potosí (14). The seed sample of this species was collected by USDA botanists from a population of wild plants growing in the mesquite-grassland near Parral, Chihuahua. Field observations of wild stands at the time of seed collection suggest that this species may have excellent potential as a new crop prospect if uses for its oil should develop.

METHODS

General

Analyses by GLC were carried out with a Burrell Kromatog K-5 as described earlier (10). Methyl esters were prepared by acid-catalyzed methanolysis.

Isolated *trans* unsaturation was determined essentially by AOCS method Cd 14–61 with a 1-mm KBr cell in a Perkin-Elmer Model 337 spectrophotometer.

A Varian A-60 spectrometer was used to measure nuclear magnetic resonance (NMR) in deutero-chloroform solution containing tetramethylsilane as internal standard.

Methyl esters and triglycerides were saponified by refluxing the sample in 1*N* ethanolic potassium hydroxide for 3 hr.

Melting points were determined with a Fisher-Johns melting point apparatus.

Permanganate-periodate oxidations were carried out according to the method of Lemieux and von Rudloff (9). The products were esterified and analyzed by GLC in both polar and nonpolar columns.

Hydrogenation was done in ethanol in a micro hydrogenator with a platinum oxide catalyst or with a micro vapor-phase hydrogenation accessory fitted to a Loenco GLC unit. Conditions for the latter procedure were as reported by Mounts and Dutton (12), and the full depth of the catalytic bed (2% palladium) was used to provide maximum hydrogenation.

Qualitative thin-layer chromatography (TLC) was performed on 20 × 20 cm plates spread with a 250 μ layer of Silica Gel G impregnated with 30% of silver nitrate. Adsorbent was removed from 2 cm on each side of the plate, glass strips 1 mm thick were placed on the cleared areas, and a 20 × 20 cm cover plate

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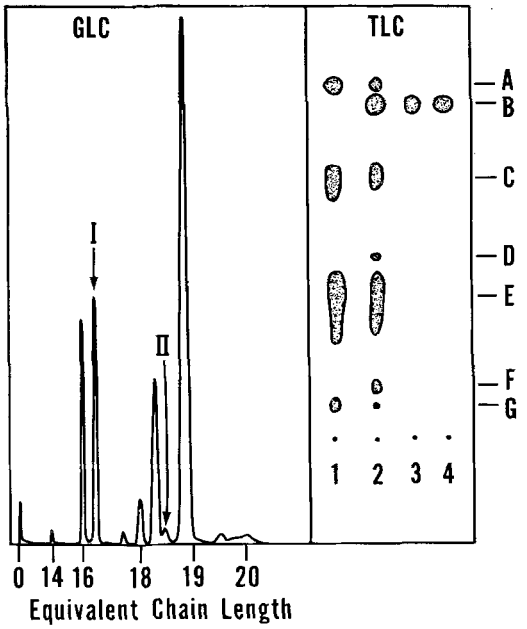


FIG. 1. Analysis of *Grindelia oxylepis* methyl esters by gas-liquid and thin-layer chromatography. GLC of methyl esters on a 10 ft × ⅛-in. column packed with 20% LAC-2-R 446. Component I is methyl *trans*-3-hexadecenoate; component II is methyl *trans*-3-octadecenoate. TLC sample 1 is soybean oil methyl ester: (1-A) saturates, (1-C) *cis* monoenes, (1-E) *cis* dienes, (1-G) *cis* trienes. Sample 2 is *Grindelia* methyl esters: (2-A) saturates, (2-B) *trans* monoenes, (2-C) *cis* monoenes, (2-D) presumed *trans* diene, (2-E) *cis* dienes, (2-F) *trans*-3, *cis*-9,*cis*-12-octadecatrienoate, (2-G) *cis* triene. Sample 3 is methyl *trans*-3-hexadecenoate. Sample 4 is methyl *trans*-3-octadecenoate.

was clamped on to make a "sandwich" chamber 1 mm deep (6). The chromatogram was developed with benzene in an 11 × 22 × 24 cm chamber. Spots were detected either by spraying with 0.2% dichlorofluorescein and observing under ultraviolet light or by charring with sulfuric acid-dichromate solution.

Fractionation of Methyl Esters

Fractionation of mixed methyl esters from *G. oxylepis* oil was accomplished by GLC, TLC and column chromatography in various combinations. In one preparation, the initial separation was made in an Aerograph A-700 "Autoprep" equipped with a 20 ft × ⅜ in. aluminum column packed with 30% SE-30 on 42/60 mesh Chromosorb P. Column temperature was 270C and the helium flow was 200 ml/min. Manual injection of twenty-three 100-μl samples yielded a total of 1.03 g of methyl esters, 0.01 g of C_{14}, 0.23 g of C_{16} and 0.79 g of C_{18}. The C_{14} fraction was contaminated with carryover of C_{18} fractions from previous injections and was not analyzed further.

The C_{16} fraction was separated into methyl palmitate and an unknown ester by TLC on 1-mm layers of Silica Gel G impregnated with 30% silver nitrate on 10 × 34 cm plates. Twenty-five 1-μl spots were placed on each plate and the chromatogram was developed with benzene in a 14 × 45 cm cylinder. Movement of the solvent front to 32 cm completely separated the two components, whereas chromatography on 20 × 20 cm plates did not. Bands containing the components were removed from the plates by the vacuum device of Goldrick and Hirsch (5).

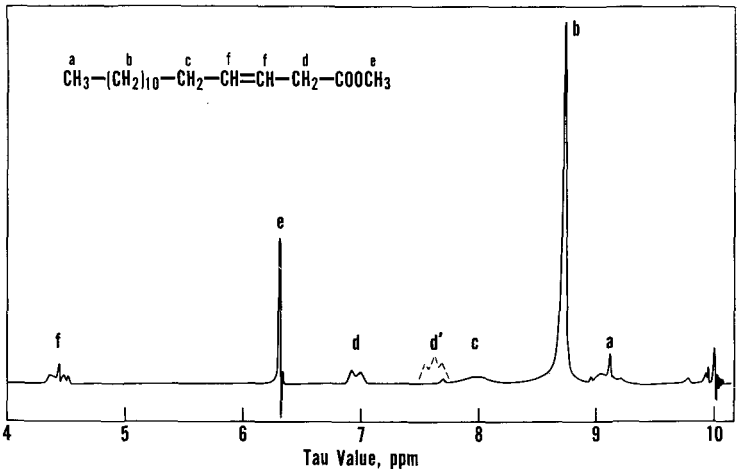
Components in the C_{18} fraction were separated by chromatography on a column packed with a macroreticular ion-exchange resin (Amberlyst XN1005) saturated with silver ions. Conditions were essentially as described by Emken et al. (4), except that the flow (0.6 ml/min) was under gravity and the effluent was collected in 2-ml fractions. Fractions were combined on the basis of their analysis by TLC on $AgNO_3$-Silica Gel G.

RESULTS AND DISCUSSION

The oil extracted from the ground "seed plus pericarp" of *Grindelia oxylepis* Greene by petroleum ether (30C–60C) amounted to 9% (dry basis). GLC of the methyl esters from the oil indicated the following composition (area percent): 16:0, 9%; 16:1^t, 14%; 18:0, 3%; 18.1%, 15%; 18:1^t, 2%; 18:2, 55%; 18:3 ^{t,c,c}, 1%; other, 1%. The separation by TLC in the presence of silver nitrate is shown in Figure 1 with the curve from GLC of the *Grindelia* esters. Analysis of the mixed esters by IR showed the presence of *trans* unsaturation equivalent to 18.6% if calculated as methyl elaidate.

Identification of trans-3-Hexadecenoic Acid

The unknown component constituting 58% of the C_{16} fraction obtained by preparative GLC was isolated by TLC. It contained *trans* unsaturation equivalent to 111.4% when calculated as methyl elaidate or 100.3% as C_{16} monoene. On the basis of 16:1, one mole of unknown methyl ester absorbed 0.96 moles of hydrogen. GLC of the hydrogenated ester on polar and nonpolar columns indicated the acid moiety to be palmitic acid. The free acid recovered after saponification melted at 59.5C–60.5C, alone and mixed with authentic palmitic acid. Analysis of the oxidative cleavage products of the C_{16}



Peak	Tau	Protons (Found)	Protons (Theory)
a	9.12	2.8	3
b	8.72	20.1	20
c	7.97	2.6	2
d	6.95	1.9	2
e	6.30	2.9	3
f	4.60	1.7	2

FIG. 2. Nuclear magnetic resonance spectrum of methyl *trans*-3-hexadecenoate. Peak d represents the protons α to the carboxyl group when 3,4 olefinic unsaturation is present. Peak d' is the pattern expected when these protons are shielded as in methyl stearate.

trans monoene by GLC showed only tridecanoic acid. Confirmation of the location of the double bond at the 3,4 position was given by the doublet at 6.95 *tau* in the NMR spectrum (Fig. 2). Elemental analysis of the ester agreed with that calculated for a 16:1 ester (Found: 12.0% H, 75.7% C; theory: 12.0% H, 76.0% C).

Thus the unknown acid is the *trans*-3-hexadecenoic acid.

Identification of trans-3-Octadecenoic Acid

Analysis by GLC of the monoene fraction, isolated after TLC of the mixed esters on a preparative plate containing silver nitrate, revealed a component with an ECL of 17.9 by the Apiezon L column and 18.6 by the LAC-2-R 446 column, exactly 2 units longer than the ECL's of the *trans*-3-hexadecenoate. Comparison of the peak area of the slower component with that of the 16:1 indicates the presence of 2% of *trans*-3-octadecenoic ester in the mixed esters of the original oil.

The C_{18} fraction (0.79 g) from the Autoprep was applied to a column of the macroreticular resin in the silver form. Saturated and *trans* monoenoic esters were eluted together and then partially separated by preparative TLC. The composition of the monoene fraction by GLC was: 16:0, 0.8%; *trans*-16:1, 3.9%; 18:0, 9.2%; *trans*-18:1, 86.2%. Infrared analysis indicated absorption equivalent to 84.5% of methyl elaidate. Partial hydrogenation in the GLC accessory (12) produced stearic acid as the major component. Some of the starting material remained unchanged, but some was isomerized to give a component with the ECL of methyl oleate. The *trans*-16:1 present in the mixture reacted in an analogous manner. Oxidation of the monoene fraction produced pentadecanoic acid as the major component as well as small amounts of tridecanoic, palmitic and stearic acids. The ratio of the pentadecanoic acid to the tridecanoic was the same as that of the *trans*-18:1 to the *trans*-16:1 before oxidation. As with the C_{16} fraction, confirmation of the 3,4-position of unsaturation was given by the doublet at 6.87 τ in the NMR analysis.

Tentative Identification of an Unusual Octadecadienoic Acid

In addition to the saturated and *trans*-monoene esters discussed above, four other fractions were collected from the silver-containing resin column: a small amount of rapidly moving unknown material, *cis* mono-

enoic esters, unknown esters and *cis,cis*-18:2 ester. Emken et al. reported that the *cis,cis* dienoic esters are retained in the column. Failure of our column to retain them may reflect unidentified differences in the preparation and conditioning of the column. The unknown esters were analyzed by GLC, TLC, IR, micro vapor-phase hydrogenation and permanganate-periodate oxidation. GLC showed no difference from linoleate on either the Apiezon L or LAC-2-R 446 columns. In TLC with $AgNO_3$ present, the unknown migrated between methyl oleate and linoleate (Fig. 1), the expected position for a diene with one *trans* bond. IR confirmed the presence of *trans* unsaturation. Partial vapor-phase hydrogenation gave methyl stearate, as the major component, and smaller amounts corresponding to *trans*-3-octadecenoate, "oleate" and the starting material. Oxidation yielded a major component, corresponding to azelaic acid, and a trace of hexanoic acid. During work-up of the oxidation product, solvent was removed on a steam bath, with a stream of nitrogen directed on the surface. Under such conditions methyl hexanoate, if present, would have been mostly lost by volatilization. The evidence suggests strongly that the unknown is the ester of *trans*-3,*cis*-12-octadecadienoic acid. The *cis* monoenes and *cis,cis* dienes were not investigated beyond demonstrating the absence of *trans* bonds.

Other trans Acids

Mixed methyl esters from the whole oil of *G. oxylepis* were fractionated on the silver-containing resin column, and analysis of the fraction containing saturated and *trans* monoenoic esters showed the major components to be the expected 16:0, *trans*-16:1, 18:0 and *trans*-18:1. Present also were minor components with ECL's of 13.9, 14.9 and 16.9 on the Apiezon L column and 14.6, 15.7 and 17.6 on the LAC-2-R 446 column. These ECL's suggest that these three components are the esters of *trans*-3-monoenoic acids containing 14, 15 and 17 carbon atoms.

The *trans*-3,*cis*-9,*cis*-12-octadecatrienoic acid is a minor component in oil of *G. oxylepis*. Its presence was indicated both by GLC and by TLC with silver nitrate (Fig. 1), but not by chromatography on the macroreticular resin column.

A report is being prepared on the composition of a large number of Compositae oils, many of which show evidence on GLC of the presence of *trans*-3 acids.

ACKNOWLEDGMENTS

T. L. Mounts performed the vapor-phase hydrogenation; E. A. Emken provided assistance with the macroreticular resin column; Quentin Jones and A. S. Barclay, New Crops Research Branch, ARS, USDA, identified and supplied *Grindelia oxylepis* seed and botanical descriptions of the genus and species.

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[Received May 3, 1966]

Low Temperature Direct Methylation of Lipids in Biological Materials[1,2]

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ABSTRACT

The procedure for low temperature methylation of fatty acids in lipids by sulfuric acid-methanol has been adapted to direct methylation of fatty acids of lipids in biological materials without prior extraction of the lipids. Successful application requires a solution or a suspension of fine particles of the lipid bearing material in ether. Concentrated sulfuric acid is added to the solution or suspension at low temperatures followed by addition of absolute methanol. The acid is neutralized by methanolic KOH and the esters extracted. Application of the method to prepare methyl esters of lipids in cream, blood serum, swine liver and kidney tissue, and cells of yeast on *Staphylococcus aureus* show that fatty acid composition based on this method compares with that determined by methylation of extracted lipids.

INTRODUCTION

THE ANALYSIS OF LIPIDS in biological tissues has posed many problems of extraction, hydrolysis, and preparation of derivatives suitable for their determination. The advent of gas-liquid chromatography (GLC) and the ready identification and quantification of fatty acids as their methyl esters has been a great step in improving lipid analysis. However, except for very few cases, no direct determination of the fatty acid composition of lipids in biological materials without prior extraction or separation of the lipids has been accomplished. Luddy et al. (4) accomplished this by basic interesterification with sodium methylate to form methyl esters of lipids of plasma, cells, liver and soybeans. They reported a 96% conversion of fatty acids in glycerides to methyl esters, a 92–93% conversion of fatty acids in cholesterol esters to methyl esters, and conversion of the fatty acids in phospholipids to methyl esters in amounts greater than afforded by saponification and subsequent esterification of the fatty acids. Basic hydrolysis and interesterification proceeds well with glycerides and phosphoglycerides. Shinowara and Brown (6) reported good yields of methyl esters after 36 hr methanolysis of phospholipids with 5–10% dry HCl as catalyst. It is well known that sphingolipids are highly resistant to basic but are readily subject to acid hydrolysis and therefore the fatty acid composition of such lipids would be expected to be incompletely accounted for by a method using base catalysis. The effect of strong base catalyzed interesterification on conjugation of double bonds has not been clearly elucidated and reported although Luddy et al. (4) noted only slight increases in ultraviolet absorptivity in the diene and triene regions over those found in the original samples. Jamieson and Reid have shown that no isomerization of linoleic acid occurs when oils containing linoleic acid are saponified at temperatures not exceeding 120C.

The low temperature sulfuric acid-methanol method of McGinnis and Dugan (5) for methylation of fatty acids in glycerides proved to be rapid, complete, and without any apparent effect on fatty acids in fats with various physical properties and composition. Since the conditions employed were such that they implied a possible ready access of reactants to lipids in complex systems it was therefore decided to explore the application of this method to analysis of the fatty acid composition of biological materials without prior extraction of the lipids.

MATERIALS AND METHODS

Materials used were as described in the earlier paper (5). Successful application has been made to analysis of the fatty acid composition of cream, blood plasma, liver and kidney tissue, dry yeast, and the cells of *S. aureus*.

Methylation of Fatty Acids in Cream

The amount of 1 ml cream was pipetted into a 125-ml Erlenmeyer flask. Twenty-five milliliters of ethyl were added. The contents of the flask were mixed by a magnetic stirrer while the temperature was lowered to −65C by means of a dry ice-acetone bath. Two milliliters of

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[1] Michigan Agricultural Experiment Station Publication No. 3803.

[2] Presented in part at the AOCS Meeting in Chicago, 1964.

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TABLE I

Fatty Acid Composition of Cream as Determined from Methyl Esters Prepared by Direct Methylation and by Methylation of a Lipid Extract

Fatty acid carbon No.	Whole cream		Lipid extract
	Ice bath[a]	Dry ice bath[b]	Dry ice bath
	%	%	%
6:0	1.8	1.5	1.9
8:0	1.6	1.5	1.6
10:0	3.3	3.3	3.2
11:0	.3	.4	.4
12:0	3.8	3.8	3.9
14:Br(?)	.1	.1	.1
14:0	12.0	12.1	12.1
14:1	1.8	1.7	1.8
15:0	1.1	1.0	1.1
16:Br(?)	.2	.2	.2
16:0	29.8	30.2	29.8
16:1	2.5	2.7	2.5
17:0	.7	.6	.8
18:Br(?)	.1	.2	.2
18:0	11.1	11.5	11.5
18:1	25.3	24.9	25.2
18:2	3.1	2.9	2.6
18:3	1.5	1.3	1.1

[a] Refers to methylation by methanol-sulfuric acid conducted at the temperature of an ice-water bath.

[b] Refers to methylation conducted at the temperature of a dry ice-acetone bath.

concentrated sulfuric acid were added from a microburette at a rate of 1 ml/min. The contents of the flask were blanketed with CO_2 and stirred for 10 min. Fifteen milliliters of methanol were added followed by 13 ml of 35% methanolic-KOH. The mixture was allowed to warm to room temperature before it was transferred to a 500 ml separatory funnel containing 75 ml deionized water. Another 75–100 ml of water and 20 ml of pentane were used to rinse the flask. The funnel was shaken and allowed to separate into a clear aqueous phase and a pentane-water emulsion. The emulsion was transferred to a 125-ml Erlenmeyer flask. The aqueous layer was extracted again with two 15-ml portions of pentane. The pentane emulsions were combined and rinsed with a 15% solution of NaCl until they were clear and then washed with two 10-ml portions of deionized water. The methyl ester preparation was concentrated by evaporation of the pentane, on a water bath, under nitrogen to a volume of 0.2 ml.

Methylation of Fatty Acids in Plasma

Ten milliliters of blood were taken from the bracial vein of a leghorn type laying hen using a heparinized syringe and centrifuged at 16,750 rpm at −2 to 0C for 15 min in a Serval RC-2 refrigerated centrifuge. The plasma was decanted into a 125 ml Erlenmeyer flask. The remainder of the procedure was as outlined above for cream.

Methylation of Fatty Acids in Kidney and Liver Tissues

Five grams of kidney or 2.5 g of liver were homogenized in a Virtis homogenizer for 5 min with 50 ml of ethyl ether. The ether-tissue mixture was decanted into a 125-ml flask. The residue was homogenized with an additional 25 ml of ether for 3 min. The ether mixtures were combined and methyl esters were prepared as outlined above for cream.

Lipids were extracted from tissues by the methods reported by Kuchmak and Dugan (3). The lipids after being freed of solvent were methylated by the method of McGinnis and Dugan (5). All of the ester preparations were spotted on micro-chromatoplates to check for completeness of esterification. The solvent and adsorbent system used was similar to that reported previously (5).

Studies with Microorganisms

S. aureus was grown in trypticase soy broth for 24 hr at 37C with continuous shaking in a water bath. The cells were harvested at the end of the incubation period, washed twice with normal saline and the packed cells so obtained were used for fatty acid analysis.

Fleischmann's active dry yeast packets were

TABLE II

Fatty Acid Composition of Chicken (Laying Hens) Blood Plasma as Determined from the Methyl Esters Prepared by Direct Methylation and by Methylation of a Lipid Extract

Fatty acid C. No.	Whole plasma				Lipid extract			
	Sample 1	Sample 2	Sample 3	Av.	Sample 1	Sample 2	Sample 3	Av.
	%	%	%	%	%	%	%	%
14:0	.3	.4	.3	.3	.3	.4	.3	.3
16:0	25.4	25.2	25.5	25.4	25.4	25.3	25.5	25.4
16:1	3.3	3.4	3.2	3.3	3.2	3.4	3.2	3.3
18:0	9.8	10.1	9.8	9.9	9.7	9.9	9.8	9.8
18:1	44.4	44.3	44.4	44.4	44.3	44.2	44.2	44.2
18:2	15.0	14.9	15.1	15.0	15.0	14.9	15.0	15.0
18:3	.2	.3	.3	.3	.4	.4	.4	.4
20:4	1.5	1.5	1.5	1.5	1.6	1.6	1.6	1.6

TABLE III

Fatty Acid Composition of Swine Liver as Determined from the Methyl Esters Prepared by Direct Methylation of the Lipids in the Tissue and by Methylation of a Lipid Extract

Fatty acid carbon No.	Whole liver			Lipid extract		
	Sample 1	Sample 2	Av.	Sample 1	Sample 2	Av.
	%	%	%	%	%	%
14:0	trace	trace	trace	trace	trace	trace
16:0	12.2	12.7	12.5	12.1	12.2	12.2
16:1	.4	.5	.5	.4	.6	.5
17:0	.4	.5	.5	.3	.4	.4
18:0	34.5	34.6	34.6	33.6	34.3	34.0
18:1	13.8	13.8	13.8	13.0	13.8	13.4
18:2	18.8	18.2	18.5	19.8	17.9	18.9
20:4	19.6	19.7	19.7	20.6	20.8	20.7

obtained from a local grocery shop and the contents were used as such.

Preparation of Methyl Esters of Cell Lipids

a) Extraction of lipids and methylation by the method of Hornstein et al. (1). Two grams of the cells were hydrolyzed for 2 hr with 25 ml of 6*N* HCl at 110C. The hydrolyzed mixture was allowed to cool to room temperature and 25 ml of deionized water were added. The lipids were then extracted with 40 ml of n-pentane. The process was repeated twice for a total of three extractions. The samples were dried over anhydrous sodium sulfate and the methyl esters were prepared using the method of Hornstein et al. (1).

b) Method for direct methylation of cell lipids. Two grams of the cells were homogenized with 40 ml of methyl ether in a Virtis homogenizer. The suspension was transferred to a 125 ml Erlenmeyer flask and cooled to −65C with constant stirring. Four milliliters of concentrated sulfuric acid were added slowly (1 ml/min) and the mixture stirred for 10 additional minutes. Fifteen milliliters of absolute methanol were added and this was followed by 26 ml of 35% methanolic KOH.

The remaining procedure was as reported above.

Gas Chromatography

An F and M Scientific Corp. Model 500 programmed temperature chromatographic unit with thermal detector was used with a 7 ft × 0.25 in. O.D. coiled column packed with 20% diethylene glycol succinate polyester and 1% phosphoric acid on 80/100 mesh acid-washed Chromosorb W. The helium flow rate was 75 ml/min. The detector temperature and injection port temperature were 250C. The column temperature was varied for the fat studied, and temperature programming from 125–210C was used for methyl esters from cream.

RESULTS

The data in Table I show the fatty acid composition of cream determined by GLC of the methyl esters prepared by the methods described here. A comparison has been made of methylation at ice bath vs dry ice bath temperatures and these values in turn were compared with those obtained by methylation of the lipids from a total lipid extract of cream. Thin-layer monitoring for completeness of esterification showed a faint triglyceride spot in the cream sample methylated at 0C. The fatty acid composition obtained for whole cream compared very well with that from the lipid extract and demonstrated that it was not necessary to first remove the lipids from cream in order to make methyl esters of the fatty acids comparable in composition to those from a lipid extract from cream.

Table II shows a comparison of the fatty acid composition of whole plasma from chicken blood with that for a lipid extract from the same blood. The results are quite comparable and the sample-to-sample reproducibility is clearly evident.

The data in Table III show how well the fatty acid composition of swine liver, as deter-

TABLE IV

Fatty Acid Composition of Swine Kidney as Determined from the Methyl Esters Prepared by Direct Methylation of the Lipids in the Kidney Tissue

Fatty acid carbon No.	Sample 1	Sample 1	Av.
	%	%	%
14:0	.6	.6	.6
16:0	23.8	24.6	24.2
16:1	1.2	1.6	1.4
17:0	.7	.7	.7
18:0	16.9	17.1	17.0
18:1	19.5	19.0	19.3
18:2	20.2	19.6	19.8
18:3	trace	trace	trace
20:1	.3	.3	.3
20:2	1.1	1.0	1.1
22:0	3.0	2.9	3.0
20:4	12.8	12.6	12.7

TABLE V

Fatty Acid Composition of Yeast Cells as Determined from Extract Methyl Esters and as Determined from Methyl Esters Prepared by the Direct Method

Fatty acid	Trial 1		Trial 2	
	Lipid extract	Cells	Lipid extract	Cells
	%	%	%	%
?	trace	trace	trace	trace
?	trace	trace	trace	trace
?	trace	trace	trace	trace
16:0	11.8	11.7	11.6	11.8
16:1	24.3	24.7	22.2	22.4
18:0	25.3	24.9	24.5	24.4
18:1	23.9	23.1	23.5	23.6
18:2	9.1	9.3	10.6	10.0
18:3 ?	5.6	5.8	8.0	8.0

mined from a direct methylation of the lipids, compares with that for a lipid extract from the same liver.

In Table IV is seen the fatty acid composition of lipids in two samples of swine kidney as determined by gas-liquid chromatographic analysis of methyl esters prepared by the method described here. No comparison was made with an extract of lipids from kidney tissue. Since the thin-layer chromatographic monitoring showed no triglyceride present in the esterified sample, it is confidently felt that the data shown represent properly the fatty acid composition of the kidney lipids.

The data in Table V show how the fatty acid composition of extracted yeast cell lipids compares with that determined on the whole cell by the direct methylation method employed here. The lipid extract from yeast and from *S. aureus* was methylated by the method of Hornstein et al. (1) as in the previous paper. This provided a comparison with a different method for methylation of the fatty acids as well as comparing methylation of lipid extract with that of lipid in the original system.

In Table VI is shown the fatty acid composition of a lipid extract from cells of *S. aureus* with that from whole cells as determined by GLC of the methyl esters of the fatty acids in the cell lipids.

TABLE VI

Fatty Acid Composition of *S. aureus* Cells as Determined from the Methyl Esters Prepared by Methylation of an Extract and from Methyl Esters Prepared from the Entire Cell by the Direct Method

Fatty acid	Sample 1[a]		Sample 2[a]	
	Extract[b]	Cell[c]	Extract[b]	Cell[c]
	%	%	%	%
?	trace	trace	trace	trace
?	5.1	5.6	1.1	1.5
14:0	43.1	42.8	37.6	38.2
?	4.9	4.9	2.6	1.9
16:0	4.4	4.6	8.5	7.8
16:1	12.1	13.4	14.6	14.8
?	trace	trace	trace	trace
18:0	12.3	13.1	17.0	16.5
18:1	2.4	1.6	4.0	4.4
?	trace	trace	trace	trace
18:3 ?	16.5	16.8	14.1	14.6

[a] The differences in the two trials are due to the difference in the two batches of cells grown at two different times.

[b] Methyl esters of extract lipids prepared by the method of Hornstein et al. (1).

[c] Methyl esters of lipids in whole cells prepared by the method of McGinnis and Dugan (5).

DISCUSSION

The direct methylation of fatty acids in lipids of biological materials can be accomplished by using the low temperature sulfuric acid-methanol method. Comparison of values for fatty acid composition of lipids in a variety of biological materials with those of a lipid extract from the same materials demonstrates the reproducibility of the method and completeness of reaction. A major factor in successful application of the method appears to lie in proper preparation of the sample prior to methylation. The apparent completeness of reaction with liver lipids, which are notably variant in lipid classes, indicates the applicability of the method to a variety of lipid classes.

This method, as any other method which involves water washing, encounters difficulties with the short chain acids since the methyl esters are appreciably water soluble. Consequently no values were given for butyric acid composition of cream. It is apparent from Table I that the observation made in the previous paper (5) that reaction at ice bath temperatures is almost equally effective with the reaction at the temperature of a dry ice-acetone bath is valid here. Many determinations of lipid composition of biological matter can be expeditiously performed by this technique.

ACKNOWLEDGMENT

This study was supported in part by National Institutes of Health Graduate Training Grant No. T1-ES-16.

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[Received April 17, 1966]

Lipoprotein Synthesis. I. Rat Plasma Lipoprotein Composition and Synthesis from Radioactive Precursors

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ABSTRACT

The in vivo synthesis of rat plasma lipoproteins was studied by the use of isotopic protein and lipid precursors. Labelled amino acids, palmitic acid and tripalmitin were administered by stomach tube and the radioactivity in the plasma lipoproteins was determined following preparative ultracentrifugal isolation at densities of 1.006, 1.019, 1.063 and 1.21 g/ml.

In response to triglyceride feeding, amino acid composition of the high density lipoprotein changed little, but in the low density lipoproteins proportionality in the amino acid pattern was changed as reflected by increases and decreases in certain amino acids.

Isotopic amino acids were not incorporated in proportion to the relative abundance with which they occurred in the lipoproteins. Triglyceride feeding markedly stimulated isotope utilization, especially in the low density fractions. Methionine, though only present in small amounts, was extensively utilized and it is suggested that this amino acid may play a significant role in the synthesis of lipoproteins, other than the role of a methyl donor for phosphatidylcholine.

INTRODUCTION

It has been proposed that a protein template or matrix determines the lipid composition of the plasma membrane and the local orientation of the lipids in the bimolecular leaflet of the plasma membrane (1,2). Our interest in the structure and in the interaction of proteins with lipids was markedly stimulated by observations of Mueller et al. (3,4), who found that a few, and apparently highly specific, protein fractions rendered reconstituted lipid bilayer membranes electrically excitable. It seemed reasonable to assume that the structure of the lipid bilayer had been altered (or ordered) by the protein in such a way as to render certain areas of it electrically excitable. Since the surface area of the reconstituted membranes was only a few mm^2 (thickness 60–90Å), local lipid-protein interaction in this system was not readily amenable to analytical experimentation. Model systems which would yield further information on the effects of proteins on lipid ordering and composition were considered. Lipoproteins, which are micellar aggregates of lipids covered by protein layers, present aspects that are quite analogous to those of the plasma membrane. Their overall lipid composition appears to be quite stable in vitro and their associated protein moieties are thought to be characteristic for each class. Some basic methodology has been elaborated which allows for the isolation and analysis of various lipoproteins. Lipoproteins seem to be manufactured by the body in relatively large amounts and their turnover time is brief and variable compared to the uncomplexed serum proteins. When lipoproteins are exposed to other similar complexes (other lipoproteins, erythrocytes, etc.) or to some lipids, exchange and transfer of certain lipid moieties has been demonstrated by isotopic techniques (5,6). We have proposed that the lipid composition of the various lipoprotein classes is due to information contained in the protein portion of the molecules (7) and that, therefore, some of the problems of lipid-protein interaction could be investigated in such a system. We have chosen to study the mechanisms which are involved in the biosynthesis of rat plasma lipoproteins. By the use of labelled amino acids and lipid precursors, information has been obtained which may elucidate some of the mechanisms by which a lipoprotein complex is assembled.

MATERIALS AND METHODS

Rats were fasted 18–24 hr prior to experimental procedures. Isotopic compounds were administered by stomach tube, dissolved or emulsified with sesame oil, U.S.P., or in mineral oil, U.S.P. Heparinized blood was obtained from ether anesthetized rats by cardiac puncture. Smaller quantities of blood were obtained from the tail which had been cut with a razor blade about 2 mm from the tip, and the plasma was removed after centrifugation of the blood in a Wintrobe-type hematocrit tube.

Radioisotopes and Counting

The following isotopic materials were employed in this study: glyceryl tripalmitate-1-C^{14}, 13.1 mc/mM; palmitic acid-1-C^{14}, 1 mc/

mM; palmitic acid-16-C^{14}, 18.7 mc/mM; palmitic acid-9, 10-H^3, 360 mc/mM; d,l-methionine-2-C^{14}, 4.17 mc/mM; l-methionine-S^{35}, 44.8 mc/mM; l-lysine-U-C^{14}, 220 mc/mM; d,l-leucine-1-C^{14}, 36.4 mc/mM; l-proline-C^{14}, 3.0 mc/mM; l-glutamic acid-6-C^{14}, 10.0 mc/mM.

Blood plasma or lipoprotein samples were counted in a liquid scintillation system. The samples were digested in 0.5 or 1.0 ml of 1.0 M hydroxide of Hyamine[1] for one to three days at room temperature in closed vessels. The dissolved samples were counted in toluene-DPO-POPOP fluid (2,5-diphenyloxazole ("DPO"), 0.4%; p-bis, [2-(5-phenyloxazolyl)] benzene ("POPOP"), 0.01%, in toluene).

Simultaneous radioassay of two isotopes (C^{14} and H^3 or S^{35} and H^3) was performed after optimum counting conditions had been established for each isotope. Percentage of isotope in each sample was calculated using the channel ratio system. Corrections for quenching and for decay time were applied as necessary.

Ultracentrifugation

Plasma was fractionated successively at densities 1.006, 1.019, 1.063, and 1.21 g/ml in the 40.3 rotor of the Spinco Model L preparative ultracentrifuge at temperatures between 13 and 17C, at a speed of 39,000 rev/min (108,800 × g avg). The tubes were filled with no more than 5 ml of plasma or plasma subfraction. A mixed NaCl and KBr salt solution of equivalent density (8) was carefully layered over the plasma to fill the remaining volume. Centrifugation was carried out for 16 hr for all fractionations, except for the density of 1.21 run which required 22 hr.

After centrifugation, the tubes were sliced 1.5 cm (1.006) or 2.1 cm (1.019–1.21) below the meniscus at the clear zone by a standard tube slicer. The tube cap was rinsed with salt solution of equivalent density and the washings combined with the upper fraction.

To increase the density of the plasma and successive fractions, a volume of higher density salt solution (usually a density of 1.085 or 1.21) was added to the bottom fraction, as described by Havel et al. (8), and centrifugation repeated. Solid KBr was added to the density of 1.063 bottom to increase the density to 1.21 for the last run.

The ultracentrifugal fractions yielded different classes of lipoproteins, as described by Bragdon et al. (9) and Fredrickson (10).

[1] p-(diisobutyl - cresoxyethoxyethyl) - dimethylbenzylammonium hydroxide, 1 Molar solution in methanol, Packard Instrument Co., La Grange, Ill.

[2] Resins obtained from Phoenix Precision Instrument Company, Philadelphia, Pa.

Dialysis

Depending upon the subsequent experimental procedures, the plasma fractions were dialyzed against 0.85% (w/v) NaCl or 0.85% (w/v) NaCl containing 0.05 M sodium phosphate buffer and 0.10% (w/v) EDTA, adjusted to pH 7.0, for 8 to 36 hr at 4C, to remove excess salts and any free, labelled amino acids or fatty acids. Electrophoresis by the method of Lees and Hatch (11) and strip scanning procedures were performed on 60 μl samples after dialysis.

Lipid Analysis

Total lipid content of samples was determined on lyophilized material. Dried aliquots were extracted with chloroform: methanol 2:1 (vol/vol), the extract washed with water and the organic phase brought to dryness. Total lipid content was estimated by the dichromate oxidation method (12). Equal weights of cholesterol, palmitic acid and tristearin did not vary in optical density more than 11%. Lipid extracts were separated into TG, FFA and PL (phospholipids) by elution from silicic acid as described elsewhere (13).

Amino Acid Analysis

Amino acid composition was determined on an automatic analyzer (Phoenix, Model K-8000). A 150 cm column with Blend X-150 resin[2] was eluted with pH 3.25 buffer at 30C followed 11 hr later by pH 4.25 buffer at 50C for separating the neutral and acidic amino acids. A 50 cm column was used with Blend X-50 resin, pH 4.25 buffer at 30C for 11 hr, followed by elution at 50C, for separating the basic amino acids. All buffer flow rates were adjusted to 30 ml per hour. Amino acid indentification and quantitation were established by comparison with standard mixtures.

Samples containing from 0.3 to 30 mg protein, as determined by the method of Lowry et al. (14), were prepared by acid hydrolysis in 6N HCl for 24 hr at 105C in evacuated sealed tubes. After hydrolysis, the samples were transferred to small beakers and allowed to dry at room temperature over KOH and P_2O_5 in a vacuum desiccator. The residue was dissolved in about 2 ml of water, filtered through Whatman No. 54 filter paper to remove the humin, and followed by 2 to 4 ml of water wash. The filtrate was washed twice with about 5 volumes of ether to remove any residual lipids from the hydrolyzed lipoproteins. The lipid-free aqueous phase was then lyophilized to dryness and stored at −12C. Total free amine nitrogen determination was made according to Moore and Stein (15) prior to application to

the automatic analyzer. In order to relate amine nitrogen values to lipoprotein-protein content, yield of amino nitrogen was compared to protein values obtained by the Lowry method before and after delipidation and to the protein dry weight. Dry weight could not be determined accurately for the low density fractions because the amounts of material were insufficient for gravimetric determination. For the d <1.063 and d <1.21 fractions, dry weight exceeded the Lowry values by 23% and 3%, respectively. Delipidation of the lipoproteins was accompanied by an apparent loss of protein. The average losses for the fractions were as follows: d <1.006 (38%); d <1.019 (11%); d <1.063 (30%) and d <1.21 (8%). Amino acid composition, as reported here, has been normalized to unit content of glutamic acid. This allows only relevant comparisons within each density class. Since the per cent destruction of individual amino acids during hydrolysis was not estimated and since a meaningful correlation of liberated amino nitrogen to protein values obtained by other methods had not been established, the above method of presentation was chosen.

RESULTS

Separation of Lipoproteins by the Ultracentrifuge

Heparinized rat plasma was separated into various fractions by successively adjusting the density of the plasma solutions according to Havel, Eder and Bragdon (8). Electrophoretic studies showed that the d < 1.006 (μ = 0 with a trace of $\mu = 1.95 \times 10^{-5}$ cm^{2}v^{-1} sec^{-1}) and d < 1.019 ($\mu = 1.22 \times 10^{-5}$ cm^{2}v^{-1} sec^{-1}) fractions were fairly homogeneous. The d <1.063 fraction ($\mu = 8.84 \times 10^{-6}$ cm^{2}v^{-1} sec^{-1}) occasionally contained traces of one band generally associated with the d <1.21 fraction (μ = 1.83 and 2.38 $\times 10^{-5}$ cm^{2}v^{-1} sec^{-1}).

In Table I it is shown how the protein and lipid concentrations in the various lipoprotein changed over a 4-hr period when either a triglyceride load (2 ml of sesame oil) or mineral oil was given by stomach tube. As expected, the most significant changes had taken place in the very low density fractions (VLDL). It is noteworthy that there was a marked disproportionality between the increase in protein content and the increase in lipid content of the d <1.006 fraction; this effect was obtained following the administration of mineral oil as well as after a TG load. We have observed an increase in plasma lipid levels after large doses of mineral oil repeatedly and the mechanisms leading to a postprandial lipemia after mineral oil are being investigated. Total plasma lipids increased approximately 100% per cent during this time after TG and about 60% after mineral oil. As shown in Table II, plasma triglycerides increased almost linearly from 30 to 240 min while there was a marked decrease in the relative and absolute phospholipid content.

Amino Acid Content

Amino acids were determined on hydrolysates of proteins as described above. The values shown in Table III are averages of one or three determinations on pooled rat plasma lipoproteins. The amino acid composition was found to be quite similar for the various lipoprotein fractions. In rat lipoproteins, aspartic acid, glutamic acid, glycine and leucine

TABLE I

Composition of Lipoprotein Fractions in Rat Plasma after Feeding of Triglycerides or Mineral Oil (Values in mg Lipid or Protein per ml Plasma)

	Triglyceride fed				*Mineral oil fed*			
Time (min)	30	60	120	240	30	60	120	240
d <1.006								
Protein	0.089	0.048	0.166	0.113	0.048	0.037	0.070	0.080
(% of Total)	(13.7)	(10.3)	(5.4)	(4.8)	(17.4)	(19.8)	(18.9)	(7.5)
Lipid	0.558	0.415	2.910	2.250	0.230	0.150	0.300	0.980
d <1.019								
Protein	0.045	0.084	0.039	0.952	0.090	0.071	0.118	0.103
(% of Total)	(32.4)	(56.7)	(32.5)	(33.1)	(35.3)	(41.7)	(40.3)	(34.6)
Lipid	0.094	0.064	0.081	0.105	0.165	0.099	0.175	0.195
d <1.063								
Protein	0.152	0.120	0.141	0.126	0.145	0.079	0.160	0.125
(% of Total)	(28.0)	(25.9)	(27.7)	(27.7)	(27.9)	(25.6)	(28.9)	(26.0)
Lipid	0.392	0.343	0.368	0.329	0.375	0.230	0.393	0.355
d <1.21								
Protein	0.77	0.74	0.77	0.74	0.75	0.61	0.75	0.74
(% of Total)	(53.1)	(51.4)	(52.8)	(52.1)	(57.3)	(49.6)	(56.8)	(54.8)
Lipid	0.680	0.70	0.69	0.68	0.56	0.62	0.57	0.61
d >1.21								
Protein	53.2	50.4	53.5	55.3	55.1	45.1	58.0	52.1
(% of Total)	(99.36)	(99.31)	(99.33)	(99.38)	(99.47)	(99.34)	(99.33)	(99.33)
Lipid	0.339	0.358	0.368	0.342	0.295	0.304	0.398	0.359

TABLE II

Lipid Content and Percent Distribution of Nonsteroid Lipids

Time, min	TG	FFA	PL
	Lipids after 2 ml oral sesame oil		
	mg lipids per ml plasma	%	%	%
30	1.881	48.5	20.0	30.8
60	2.362	65.6	24.7	7.9
120	3.330	69.1	19.3	6.8
240	3.438	56.8	16.2	5.6
	Lipids after 2 ml mineral oil			
30	1.805	51.0	15.4	28.8
60	1.888	67.4	22.5	8.1
120	1.748	61.8	27.5	7.4
240	2.905	64.4	22.5	8.0

are relatively abundant amino acids. The low density lipoproteins (LDL, d <1.063) contained relatively more glycine and serine than the high density lipoproteins (HDL, d = 1.063–1.21), while the latter contained higher amounts of methionine, lysine and histidine. An attempt was made to study changes in amino acid composition in the lipoprotein fractions when the rats were subjected to a triglyceride load. For this purpose, the amino acid spectrum of fasted (mineral oil) rats was compared to that of rats in the postabsorptive state, i.e. 4 hr after the oral administration of 2 ml of sesame oil. It was found that few changes in amino acid composition had occurred in the higher density lipoprotein fractions in response to feeding. In the low density fractions, the relative amounts of threonine, serine, valine, leucine, phenylalanine and methionine had increased in the 1.006 fraction. In the 1.019 fraction, a relative decrease in the proportion of proline, alanine and valine was noticeable. An increased amount of isoleucine, leucine and phenylalanine was found.

The changes in the methionine content, even though this amino acid occurs only in small amounts, were thought of interest. In rats fed mineral oil only, methionine could not be detected in the low density fractions, while, in response to TG feeding, this amino acid was recovered in measurable amounts from the protein hydrolysate. Although this change in methionine content is of borderline significance, it was considered noteworthy, especially, in view of the fact that methionine was found to be well utilized in a series of isotopic tracer studies.

Lipoprotein Synthesis from Radioactive Precursors

Isotope content of rat plasma was followed after oral administration of various labelled precursors by sampling blood from the tail vein at intervals. The values shown in Table IV are indicative of the general labelling pattern found with a number of precursors. The highest radioactivity of plasma was usually obtained about 4 hr after feeding. At that time, the acid-

TABLE III

Amino Acid Distribution in Lipoprotein Fractions after Mineral Oil and Triglyceride Administration

	d <1.006		d <1.019		d <1.063		d <1.21	
	M.O.[a]	T.G.	M.O.	T.G.	M.O.	T.G.	M.O.	T.G.
Cysteic acid	.06	.03	.10	.11	.03	.01	.01	.01
Taurine	.18	.25	.31	.63	.05	.11	.01	.02
Aspartic acid	.58	.69	.67	.71	.65	.62	.67	.69
Threonine	.33	.60	.41	.48	.38	.57	.33	.46
Serine	.52	.79	.66	.72	.38	.45	.24	.33
Proline	.20	.16	.52	.28	.28	.22	.27	.30
Glutamic acid	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Glycine	.68	.63	1.17	.92	.49	.47	.33	.38
Alanine	.52	.63	.86	.56	.50	.53	.57	.62
Valine	.35	.49	.37	.24	.41	.45	.38	.48
½ cystine	0	0	0	0	0	0	.06	0
Methionine	tr.	.07	tr.	.03	.04	.07	.11	.16
Isoleucine	.27	.35	.28	.43	.29	.29	.19	.23
Leucine	.59	.86	.50	.70	.73	.76	.71	.85
Tyrosine	.15	0	0	0	.06	0	.05	0
Phenylalanine	.16	.23	.12	.24	.23	.19	.23	.28
Ornithine	.15	.17	.20	.06	.08	.02	.02	.03
Lysine	.36	.43	.29	.23	.39	.56	.56	.58
Histidine	0	.08	0	tr.	.05	.48	.14	.15
Arginine	.14	.45	0	tr.	.13	.15	.34	.38
Ratio of glutamic acid N recovered to total free-amine nitrogen recovered.	.066	.065	.046	.058	.090	.11	.13	.12

[a] Mineral oil values averaged from three determinations, triglyceride values from a single determination.
tr. = Trace

TABLE IV

Isotope Content of Rat Plasma after Oral Administration
(All values in $\mu c \times 10^3$ per ml plasma. Values in parentheses indicate per cent of isotope soluble in 5% trichloracetic acid)

Isotope	Dosage per rat	Minutes after administration				
		10	30	60	120	240
Methionine -2-C^{14} + 1 ml TG	50 μc	9.28	45.05	64.70	87.02	139.20
Methionine -S^{35}	43.5		23.0	61.0	177.0	201.0
+ 2 ml TG			(33.9)	(24.9)	(11.8)	(6.08)
Methionine -S^{35}	43.5		22.5	88.20	186.0	201.0
+ 2 ml Min. Oil			(52.0)	(37.3)	(18.7)	(4.60)
Lysine-U-C^{14}						
+ 1 ml TG	50	2.26	4.57	7.48	13.38	16.13
		(92.0)	(78.0)	(20.0)	(4.30)	(3.03)
Palmitic acid-9, 10-H^3 + 2 ml TG	120		824	1,890	6,930	7,200
Palmitic acid-9, 10-H^3 + 2 ml Min. Oil	120		899	3,325	5,940	10,100

soluble fraction was sufficiently low to suggest that the bulk of the precursor had been utilized in the synthetic process. Dilution of the methionine-S^{35} with TG or mineral oil seemed not to affect its rate of entrance into the plasma pool over a 4 hr period. The amount of methionine incorporated into plasma proteins was increased with an increase in the diet volume. Lysine utilization was of a lower order of magnitude. At 30 min and 240 min only one tenth as much lysine had been incorporated as methionine. When all the plasma lipoproteins of a density of less than 1.21 were floated together, it was observed that only a small percentage of the bound amino acids had been utilized for the synthesis or labelling of these lipoproteins (Table V). Lipid precursors went predominantly to those same classes. Most precursors were utilized less in a mineral oil vehicle.

In a separate experiment, lipoprotein synthesis from radioactive amino acids and lipids was measured 4 hr after intragastric administration of the precursor. The values in Table VI express the specific activities of the various lipoprotein fractions and the effect of a 2 ml diet of triglyceride was compared to that of mineral oil. All the lipoprotein fractions from d <1.063 to d greater than 1.21 showed essentially similar isotope incorporation irrespective of the diet used. The only exceptions noted in this group were palmitic acid-C^{14} and methionine-S^{35}. Double the amount of these precursors was incorporated into the d <1.063 fraction in the postabsorptive state than after mineral oil. In the VLDL fraction (d <1.006) it was found that a TG load markedly stimulated isotope utilization with the striking exception of methionine-S^{35}. It is conceivable that in the absorptive process, induced by the TG load, the supply of isotopic methionine for intestinal lipoprotein synthesis had been exhausted after a 4-hr period. This amino acid though, was well utilized in animals which had received mineral oil only. In general, it can be stated that methionine appears to be an excellent precursor for lipoprotein synthesis. The specific activities of the VLDL fractions relative to protein content were usually higher than those of the HDL. This is thought to be indicative of a high turnover rate for VLDL.

DISCUSSION

The series of experiments reported here are exploratory in view of the premises set forth in the introduction. The use of radioactive tracers in combination with floatation methods appears to be the most feasible approach to the elucidation of the assembly process for entire lipoprotein molecules. Paper electrophoresis of plasma lipoproteins was very limited in its application to this study. Electrophoretic mobility of the lipoproteins varied considerably as a function of their concentration and in

TABLE V

Isotope Incorporation into d <1.21 Lipoproteins, Four Hours after Oral Administration, in Per Cent of Total Plasma C^{14}

	Precursors					
	Leucine	Proline	Methionine	Glutamic acid	Triglyceride	Free fatty acid
TG	6.2	6.0	5.2	15.0	62.0	72.7
Mineral oil	6.6	5.0	2.5	12.3	44.0	34.6

TABLE VI
Lipoprotein Synthesis as Measured by the Incorporation of Radioactive Precursors
(Values in moles × 10^{11} per mg protein[a])

Precursor[b]	Lipoprotein Fraction									
	d <1.006		d <1.019		d <1.063		d <1.21		d >1.21	
	TG	MO	TG	MO	TG	MO	TG	MO	TG	MO
d,l-leucine-C^{14}	77.8	9.62	12.5	10.2	26.7	37.2	20.7	23.4	5.0	6.25
l-proline-C^{14}	79.0	31.4	3.67	11.8	22.5	17.1	17.4	17.1	5.73	8.7
l-methionine-S^{35}	0.62	72.50	20.2	37.1	97.4	52.5	66.2	56.6	14.8	15.8
l-glutamate-C^{14}	79.4	17.0	19.6	5.76	15.8	15.1	11.1	15.6	1.75	1.70
glyceryl tripalmitate-C^{14}	327.0	101.0	12.2	18.0	8.85	9.34	6.5	5.17	0.18	0.19
palmitic acid-C^{14}	74,800	14,700	2,240	3,280	4,370	2,020	907.	938.	34.1	50.4

[a] The specific activities were measured 4 hr after diet.
[b] The isotopes were given in 2 ml of sesame oil (TG) or 2 ml of mineral oil (MO).

relation to other proteins present. A substantial increase in lipoprotein concentration, either by the application of larger plasma volumes to the paper strips, or by concentration of the lipoproteins by ultracentrifugal floatation generally led to decreases in mobility, presumably due to aggregation into larger complexes.

Preparative isolation of the lipoproteins by floatation in the ultracentrifuge at different densities proved to be an expedient method, though a rather time-consuming one. The densities chosen to float the various lipoprotein classes were adjusted arbitrarily to those recommended by Havel et al. (8) for human lipoproteins. Electrophoretic studies indicated that in some density classes several lipoproteins could be separated by their variation in mobility and that some overlap occurred in the higher density lipoprotein fractions. It might be useful to establish, eventually, a lipoprotein profile for rat plasma by methods suggested and utilized by Oncley (16,17).

The analysis of the amino acid pattern of the various lipoprotein fractions did not reveal any striking differences between classes. Comparison of amino acid composition of rat lipoproteins with that of human lipoproteins can be made, but may be of questionable significance. Isolation techniques developed for human studies have been used here, but as pointed out above, they may not be adequate for studies on the rat. Species differences and differences in metabolic pathways could preclude useful comparisons. Shore (18) and Levy and Fredrickson (19) reported leucine, aspartic acid and glutamic acid to be the most abundant acids present. Levy and Fredrickson found a marked difference in the aspartic/glutamic ratio between the LDL and HDL classes. In our studies the aspartic/glutamic ratio is similar for all classes. It is difficult at the present time to evaluate the significance of the amino acid spectrum and its possible contribution to the final lipid composition of the various lipoproteins. We think that determinants of lipid composition are just as likely to be associated with certain amino acid sequences as with secondary structure. The present method of amino acid analysis will be expanded to include determination of individual specific activities after administration of isotopic precursors.

In exploring various approaches to the study of lipoprotein synthesis, it was thought that the measure of the rate at which fatty acids and amino acids were incorporated might serve as indices of catabolic and anabolic rates. At the present time, it has not been well established whether or not the lipid and protein portions of the lipoprotein molecule are assembled (and synthesized) simultaneously. Apo-lipoprotein carriers have been postulated in a recycling mechanism which serves in lipid transport (20). Elsewhere, it has been suggested that the synthesis of the entire lipoprotein molecule is a de novo process (21,22).

The catabolism of the various lipoproteins, also, is only partially understood. The half life of certain lipid moieties has been investigated and was found to range from a few minutes for the VLDL to several days for the HDL. A correlation of the metabolism of the protein portion of the molecule with that of the lipid portion has not been established.

Certain phases of lipid metabolism or lipid transport are thought to depend upon the availability of labile methyl groups (23,24). It has been presumed that the contribution of methionine or choline in lipid metabolism was essentially one of a precursor relationship to the phospholipids and especially phosphatidylcholine. Because of the position of the isotopic label, our data give no indication of the fate of the labile methyl groups of methionine, but we consider the obvious utilization of both the 2-C^{14} and the S^{35} labeled methionine as sub-

stantial evidence for utilization of methionine itself in lipoprotein biosynthesis. Florsheim et al. (25) in a study of β-lipoprotein synthesis in the rooster had employed S^{35} labeled methionine. Their data on the specific activity of β-lipoproteins obtained by a precipitation method showed that methionine was utilized more extensively than tyrosine or a mixture of amino acids. These observations are thought noteworthy, in view of the fact that methionine occurs in relatively small amounts or is not detectable (18,26) in the lipoproteins. It is conceivable that methionine not only plays an essential role in phospholipid synthesis, but that it may serve as an important link in the assembly of the lipid and protein moieties in lipoprotein synthesis (7).

In conclusion, we consider it unlikely that lipoprotein-lipid composition can be related solely to total amino acid composition. On the basis of the amino acid analyses and the isotope incorporation experiments it might be speculated that methionine could play a significant role in the biosynthesis of the low density lipoproteins. In what fashion the proteins or amino acids serve as determinants of the lipid composition in the lipoproteins will be the subject of further studies.

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[Received January 31, 1966]

The Occurrence of Methyl Methoxystearate Isomers in the Methyl Esters Prepared from Sheep Perinephric Fat

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ABSTRACT

A fraction has been isolated from sheep perinephric fat and identified by techniques which included mass and infrared spectrometry, as a mixture of the 8 to 14- methoxyoctadecanoic acid isomers. It is postulated that these isomers are artifacts produced by rigorous esterification with methanol and concentrated H_2SO_4 of a large sample of sheep perinephric fatty acids which are presumed to have contained trace amounts of constituent hydroxy fatty acids. It is estimated that these methoxystearic acid isomers represented approximately 0.08% of the total weight of fatty acids.

INTRODUCTION

METHOXY ACIDS do not appear to have been reported as constituents of natural lipids, except for β-mycolic acid from human and bovine strains of *Mycobacterium tuberculosis* described by Asselineau and Lederer (1). Recent work by Lough (2,3) demonstrated that methoxy acids were formed as artifacts when oleic acid and other olefinic acids were esterified with methanol containing BF_3 as catalyst. Coppock, Daniels and Eggitt (4) also reported the occurrence of methoxy acids which they considered to be artifacts formed when aged or oxidized flours were interesterified with methanol and BF_3 catalyst.

The purpose of this paper is to report in detail the isolation from sheep perinephric fat of a fraction identified as a mixture of methoxyoctadecanoic acid isomers, the methoxy group being located on carbons, 8,9,10,11,12,13 and 14. It is postulated that these methoxystearic acid isomers were formed as artifacts by esterification with methanol and H_2SO_4, of sheep perinephric fatty acids which are presumed to have contained trace amounts of constituent hydroxy acids. A brief communication on this investigation was reported earlier (5).

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EXPERIMENTAL

The sample (N/558) of sheep fat used in this investigation was obtained from the region of the kidneys of a line of ewes. It was prepared by finely mincing the tissues, extracting in hot water to separate the fat from the connective tissue, filtering, washing repeatedly with hot water to remove non-glyceride material, and drying by heating in vacuo on a water bath. Of the extracted fat, 6328 g was saponified with methanolic KOH, the soaps were acidified with a 10% excess of 40% (v/v) H_2SO_4 and the fatty acids were dried in vacuo on a water bath. Esterification of the fatty acids (wt 6037 g) was effected by refluxing on a water bath for 3 hr with 2 vol methanol containing 2% (w/v) concentrated H_2SO_4. Approximately half of the methanol was then distilled off in vacuo on a water bath, thus prolonging the heating by 3–4 hr during which time the concentration of the H_2SO_4 would have increased to approximately 4% (w/v). The solution was then made alkaline with 10% (w/v) K_2CO_3 and extracted with ethyl ether. This ether extract was washed with 10% K_2CO_3 and with water; the ether was distilled off and the methyl esters were dried in vacuo on a water bath. In order to effect separation into saturated and unsaturated constituents, the methyl esters (5972 g) were crystallized three times from 10 vol acetone at −40C and yielded 2547 g "liquid" esters (sap. equiv. 291.1, iodine value 81.6, unsaponifiable matter 1.31%), and 3438 g "solid" esters (sap. equiv. 288.3, iodine value 6.2, unsaponifiable matter 1.72%). Of the "liquid" esters, 1288 g were hydrogenated in ethyl alcohol with PtO_2 as catalyst, then crystallized repeatedly at −40C, initially from 20 vol acetone and subsequently from 20 vol light petroleum (bp 60C–80C). Fractions soluble in these solvents under the conditions specified were combined (wt 111.4 g) and denoted H100. Fractional distillation in vacuo of H100 methyl esters was carried out in a 50 × 1.8 cm fractionating column fitted with a closely coiled nichrome spring, and provided twenty fractions and a residue. The fraction pertaining to this study (H100 L18, wt 3.91 g) was freed of unsaponifiable matter by resaponification and extraction with ethyl ether,

reconverted to fatty acids, and reesterified by refluxing for 4 hr with methanol containing 3% (w/v) H_2SO_4 and yielded 3.67 g methyl esters. Gas-liquid chromatography (GLC) of these methyl esters indicated the presence of the C_{20} multibranched fatty acid 3,7,11,15- tetramethylhexadecanoic acid, together with several unknown constituents. Preparative GLC at 200C on a column impregnated with 60% polyethylene glycol adipate liquid phase was employed in an attempt to isolate and collect these components, but considerable losses were incurred with the chromatograph. One fraction (wt 0.26 g) which GLC indicated to be approximately 95% pure, was in part (0.11 g) injected into the preparative GLC for further purification but the recovery from the chromatograph was only about 5%. Accordingly, the remainder of the fraction (wt 0.15 g) was freed, in the manner described above, of unsaponifiable matter which was thought to have bled off the preparative chromatograph. Reconversion of the soaps to fatty acids and then to methyl esters was effected by acidification with 40% (v/v) H_2SO_4, followed by refluxing the vacuum-dried fatty acids for 4 hr with methanol containing 3% (w/v) H_2SO_4. The resulting methyl esters (denoted H106, wt 0.14 g) were then decolourized by being dissolved in light petroleum (bp 60C–80C), mixed with activated animal charcoal, warmed and filtered. As the decolourized liquid methyl esters (wt 0.13 g) were not completely homogeneous and appeared to contain a small amount of gelatinous material, they were chromatographed through a column (11.5 × 1.5 cm) containing 10 g activated silicic acid (Bio-Rad Laboratories, USA). After applying the fraction to the column, it was eluted with 8 × 5 ml light petroleum (bp 60C–80C), 2 × 5 ml light petroleum containing 5% (v/v) ethyl ether (AR), 5 × 10 ml light petroleum containing 10% ethyl ether, and finally by 4 × 10 ml light petroleum containing 20% ethyl ether. Full recovery was achieved. The main fraction, the eleventh, was eluted with light petroleum containing 10% ethyl ether, and was denoted H108 (wt 0.06 g).

The analytical GLC used was constructed in the laboratory of the Food Chemistry Division, Department of Scientific and Industrial Research, Wellington, and was fitted with a 90 Sr detector as described by Lovelock, James and Piper (6). The glass columns employed were 2.4 meters in length and 6.5 mm in internal diameter and were packed with celite (30–80 mesh, BDH, England) impregnated with (a) 20% (w/w) polyethylene glycol adipate and (b) 5% (w/w) Apiezon L. Polyester columns, but not Apiezon L columns, were siliconized. Argon was used as carrier gas and the operating temperature was 207C. Retention volumes (VR) were determined on methyl esters and are relative to methyl stearate.

The preparative GLC was a model A-700 "Autoprep" (Wilkens Instrument & Research, Inc., California USA), accommodating a thermal conductivity detector. The column used, 3 meters in length and 8 mm in internal diameter, was made of copper and was packed with celite impregnated with 60% (w/w) polyethylene glycol adipate. The operating column temperature was 200C, and the carrier gas was hydrogen.

The mass spectrometer used was a 60° sector, 30 cm radius, single-focussing instrument of the Inghram type, and was constructed in the Division of Chemical Physics, Chemical Research Laboratories, C.S.I.R.O., Melbourne. The sample was introduced at 200C by means of an all-glass greaseless inlet system, and the mass spectrum was recorded using magnetic scanning of the mass scale.

The infrared analysis was made on a thin film of the methyl ester between two KBr discs, using a Perkin-Elmer model 137E spectrometer.

The methoxy value was determined by A.D. Campbell, Microanalytical Laboratory, Chemistry Department, University of Otago, New Zealand.

RESULTS

Fraction H108

Methyl ester, wt 0.06 g; colorless liquid at room temperature; mp −13.0C to −11.5C (noncrystalline solid); iodine value (Wijs) 1.8 (theoretical 0.0); methoxy value 19.02% for methyl ester (calculated for methyl methoxy stearate, $C_{20}H_{40}O_3$, 18.89%, i.e. 9.45% for the side methoxy).

Gas-Liquid Chromatographic Analyses

Fraction H108, when examined by GLC using both adipate and Apiezon L columns, revealed only one component although a slight "tailing" of the peak suggested the possibility of isomers being present. With the adipate liquid phase the VR was 2.17 (carbon number 20.75 [Ref. 7]) while with Apiezon L the VR was 1.53 (carbon number 19.05).

Infrared Analysis

The infrared spectrum of methyl ester fraction H108 was characteristic of that of the

methyl ester of a long chain fatty acid, but in addition it displayed a strong absorption at 1100 cm^{-1} which, according to page 8, implies an equatorial methoxyl group.

Mass Spectrometric Examination

Methyl ester fraction H108 when examined in the mass spectrometer yielded the spectrum shown in Figure 1.

As the compound was known to be the methyl ester of a saturated fatty acid, the molecular weight of 328 suggested a methoxy, hydroxy or dibasic constituent. The two prominent series of peaks in the high mass region (187 + 14n) and (185–14n), n = o . . . . . 6, were a puzzling feature of the spectrum since strong peaks in this region indicate the bonds most likely to break. These series, however, would be explained if the sample were an isomeric mixture in which the structural feature causing the large peaks occurred at different positions in the chain. Thus it is apparent that the pair of peaks at m/e 185 and 187 correspond to

$$CH_3\text{–}(CH_2)_9\text{–}\underset{\displaystyle OCH_3}{\underset{|}{CH}}\text{–} \quad \text{and} \quad \text{–}\underset{\displaystyle OCH_3}{\underset{|}{CH}}\text{–}(CH_2)_6\text{–}\underset{\displaystyle O}{\underset{\|}{C}}\text{–}OCH_3$$

from methyl -8- methoxyoctadecanoate. Similary, the 171 and 201 pair of peaks would be yielded by methyl -9- methoxy- octadecanoate, the 157 and 215 pair by the 10- methoxy isomer, and so on. The spectrum of methyl -11-methoxyoctadecanoate has been reported by Ryhage and Stenhagen (9) and the pair of peaks 143 and 229 referred to by these authors as being due to ions formed by 10,11- and 11,12-cleavage, respectively, are evident in sample H108 (see Fig 1). So also are the peaks corresponding to the loss of one or two molecules of CH_3 OH which are a feature of the spectra of this class of compound. These peaks are present for the other prominent ions, e.g. 215, 183, 151, etc. Further evidence for H108 being an isomeric mixture was gained from the relative prominence of the low mass peaks, compared with the high mass peaks. In all published spectra of this type of compound (cf. Ryhage and Stenhagen [Ref. 9]) the peaks corresponding to fragmentation on either side of the substituted carbon are by far the most prominent ions observed, compared to the usually abundant low mass end of the spectrum. However, as most of these peaks are common to all isomers the additive effect would explain this apparent anomaly. In addition, in the spectrum of the highly purified sample H108, the pair of peaks 185 and 187, while still preserving their mutual ratio, had decreased in intensity relative to the other members of the high mass series, when compared with earlier runs on more impure fractions. To a lesser extent this was true also for the 171 and 201 pair, and at the other end of the series for the 101 and 271 pair. The extensive purification

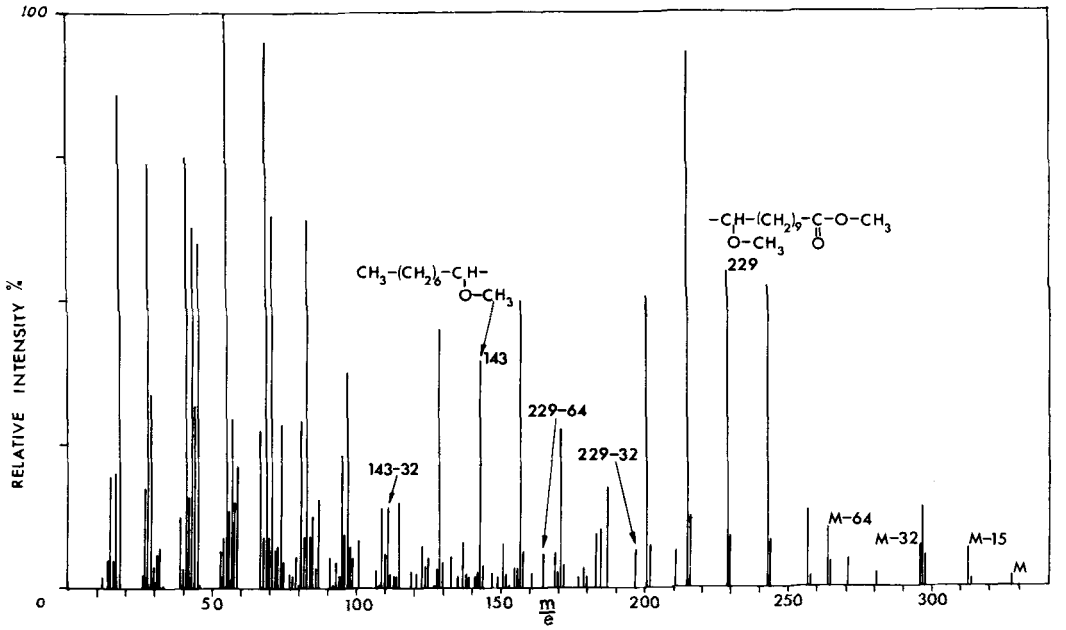

FIG. 1. Mass spectrum of methyl ester fraction H108.

undergone by this sample would be expected to preferentially erode the end members of the series. It was therefore concluded that the unknown (fraction H108) was a mixture of methyl methoxyoctadecanoates in which the methoxy group occurred in the positions from 8 to 14 inclusive.

DISCUSSION

The fraction (H108) isolated in this investigation of sheep perinephric fat, has been identified as a mixture of the methyl esters of 8-, 9-, 10-, 11-, 12-, 13-, and 14-methoxy isomers of octadecanoic acid. Mass spectrometry, apart from establishing the molecular weight of the methyl ester as 328 (calc. for $C_{20}H_{40}O_3$, 328.5) also indicated the presence of methoxy isomers in positions 8 to 14 inclusive. Confirmation of a side methoxy group was provided by the methoxy value (9.51% for 1 side methoxy; calc. for 1 side methoxy in methyl methoxyoctadecanoate, 9.45%), and the infrared spectrum which showed strong absorption at 1100 cm -1 implying the presence of a methoxy grouping (8). This absorption was also observed by Lough (3) and by Coppock et al. (4) for methoxystearic acid and methyl methoxystearate respectively. A negligible iodine value (1.8) was evidence of saturation. GLC showed fraction H108 to be apparently pure, only one peak emerging with both polyester and Apiezon L columns. Slight "tailing" of the peak, however, was consistent with the presence of isomers. The relative retention volumes of fraction H108 obtained with adipate (VR 2.17) and with Apiezon L (VR 1.53) liquid phases, when expressed as carbon numbers (7) are equivalent to 20.75 and 19.05, respectively, and are of the same order as those recorded with corresponding liquid phases for methyl 10-methoxystearate, viz. 21.5 with polyester (3) and 19.1 (3) and 19.07 (4) with Apiezon L. They also approximate the carbon numbers obtained with polyester and Apiezon L, respectively, for methyl -12- methoxystearate (VR 2.26, carbon number 20.9 with adipate polyester; and VR 1.70, carbon number 19.4 with Apiezon L) produced from authentic 12-hydroxystearic acid (Hormel Institute) by reaction with CH_3I and Ag_2O (10).

In the present investigation, the mixed fatty acids from sheep perinephric fat were esterified by refluxing for 3 hr with 2 vol methanol containing 2% (w/v) concentrated H_2SO_4. Approximately half of the methanol was then distilled off in vacuo on a water bath, so that the esterification mixture was heated overall for 6–7 hr, during the latter hours of which the concentration of the H_2SO_4 would have increased to approximately 4% (w/v). The subsequent isolation and identification from this material of a fraction of methoxystearic acid isomers, suggested that these methoxy isomers could have been produced as artifacts under the relatively rigorous conditions of esterification referred to above, if hydroxy fatty acids had been present in the natural fat.

That hydroxy fatty acids yield methoxy acids under such conditions was confirmed by refluxing for 6 hr authentic 12- hydroxystearic acid (Hormel Institute > 99% pure) with 10 vol methanol containing 4% (w/v) H_2SO_4, when approximately 1% of the esterification products which registered on the GLC chart of a polyester column, was identified as methyl methoxystearate. These conditions were intended to simulate those employed in the preparation of methyl esters from the original bulk sample of sheep perinephric fat (N/558). When, however, pure 12- hydroxystearic acid was refluxed for 10 hr with 10 vol methanol containing 20% (w/v) H_2SO_4, the GLC chart of a polyester column indicated approximately 26% of 12- methoxystearate being present in the resulting product.

In order to establish that the fraction of methoxystearic acid isomers isolated in this work was not derived directly from oleic acid by reaction with methanol and H_2SO_4 in the same way that Lough (3) found methyl -10-methoxystearate to be produced from oleic acid by esterification with methanol and BF_3 catalyst, a sample of nearly pure methyl oleate separated by fractional distillation in vacuo from the original "liquid" esters of the sample of sheep perinephric fat under investigation (N/558), was hydrolyzed and the resulting fatty acids were esterified by refluxing for 10 hr with 10 vol methanol containing 20% (w/v) H_2SO_4. No methoxy acid esters were formed. Similarly, methoxy acid esters were not produced when pure oleic acid (Hormel Institute) was esterified under the same conditions.

Wallen, Benedict and Jackson (11) demonstrated that a microorganism (pseudomonad) isolated from fatty material, hydrated oleic acid at the position of its double bond and produced 10- hydroxystearic acid. Although the activity of this microorganism on oleic acid isomers with the double bond in positions other than 9–10 was not investigated, probably it is capable of converting any of these isomers to the corresponding hydroxy acids. The biosynthetic relationship between oleic acid and hydroxy acids has also been established by James,

Hadaway and Webb (12) who showed that oleic acid is the direct precursor of ricinoleic acid.

Hydroxy fatty acids are widely distributed in nature and their sources include wool wax, beeswax, certain seed oils, animal cerebrosides, human fecal lipids, some leaf waxes and a number of microorganisms (cf 13–15). Hydroxy fatty acids have not been shown to be present in animal body or milk fats[3] although Boldingh and Taylor (16) cited circumstantial evidence suggesting their presence in butterfat in amounts of the order of 10 ppm. Nevertheless, it is probable that small proportions of these acids are present, but due to their thermolabile nature (cf 17,18,14), methods of fractional distillation normally employed for the concentration of trace fatty acid constituents would be liable to cause some decomposition. Horn and Pretorius (18) reported that the higher molecular weight hydroxy acids from wool wax also formed nonvolatile polyesters on heating.

James, Webb and Kellock (19) discovered that human fecal lipids contained 10- hydroxystearic acid as a major fatty acid component and that 6-, 7-, 8-, and 9- hydroxystearic acids occurred as minor components. They also found present a series of isomeric octadecenoic acids with the double bond in positions 4,5,6,7,8,9, 10,11 and 12. In butterfat, isomers of oleic acid are known to occur with the double bond in positions other than 9 (cf 13,20,21) including 4,11,13 (21), 11 (22), 9,10,12 (23), 11,16 (24), 16 (25), 14,15 (26). Although the same isomers of oleic acid have not all been identified in muttonfat, it is nevertheless probable that they are present there and in all ruminant fats. Accordingly, it is postulated that the mixture of methoxystearic acid isomers (8 to 14) isolated in this investigation of sheep fat, comprised artifacts produced by rigorously esterifying the corresponding hydroxy acids which are presumed to have been present in the natural fat. The occurrence of these hydroxy acids is considered to be due to microbial or enzymatic action on the isomers of octadecenoic acid. However, as in this particular study, hydrogenation was used to facilitate the separation of "liquid" from "solid" methyl esters, there exists the possibility that hydroxy acids were present as the result of hydrogenation of peroxide groups formed by autoxidation (cf 27–29).

In the current investigation, the residue (excluding unsaponifiable matter) resulting from fractional distillation in vacuo of the "solid" methyl esters (sample N/558) was decolourized with activated charcoal and examined to see if it contained high molecular weight hydroxy fatty acids. The techniques employed included adsorption chromatography, thin-layer chromatography, acetylation, etherification, low temperature crystallization from solvents, and examination of all products by GLC. Although a number of broad unidentified peaks were disclosed, in addition to all the odd- and even- numbered *normal* fatty acids from C_{18} to C_{26}, positive identification of hydroxy fatty acids was not obtained. A feature which pointed to the presence of hydroxy acids, however, was that the chart areas of a number of peaks became substantially increased after acetylation of the solid "residue," and these peaks appeared with retention times appropriate to the suspected acetoxy derivatives. Also, deposits of charred material on the sample capsule of unacetylated "solid" esters after being applied to the gas chromatograph indicated that some decomposition took place as it did with authentic hydroxy esters. Detection of hydroxy fatty acid esters by means of the ionization detector, was found to be inefficient and erratic. For example, when pure methyl -12- hydroxystearate was applied there was a 54.1% loss in weight with the polyester column and a 59.4% loss with the Apiezon L column. Acetylation and etherification appeared to afford a measure of protection for some of the unknown peaks in the "solid" residue which were suspected of representing hydroxy components. Consistent with this was the observation that when methyl 12- methoxystearate, prepared from pure methyl 12- hydroxystearate by the method of Kishimoto and Radin (10) was applied to the gas chromatograph, only 5.7% loss was recorded with the polyester column. The Apiezon L column, however, showed a 23.7% loss.

It is estimated from relevant GLC charts that the content of 8- 14- methoxystearic acid isomers present in the sample of sheep fat examined, represented approximately 0.08% of the total weight of fatty acids.

In an attempt to obtain conclusive evidence for the presence of hydroxy fatty acid constituents in sheep fat, further investigations have been planned.

ACKNOWLEDGMENT

Technical assistance by Miss G. E. M. Wills of the Food Chemistry Division, Department of Scientific and Industrial Research, Wellington.

[3] After this manuscript was prepared, the authors sighted the paper entitled "Occurrence of Esterified Hydroxy Fatty Acids as Precursors of Lactones in Butter" by G. Jurriens and J. M. Oele (Nature, London, *207*, 864–5 [1965]).

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[Received June 6, 1966]

Effect of Isoessential Fatty Acid Lipids from Animal and Plant Sources on Cholesterol Levels in Mature Male Rats[1]

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ABSTRACT

Isopolyunsaturated lipids isolated from plant and animal sources were included in the diets of mature male rats. Liver and blood serum cholesterol lowering effects were noted only in the lipid from the vegetable source. The authors suggest that the cholesterol lowering effect of vegetable oils is associated with the generally beta-unsaturated triglycerides found therein.

INTRODUCTION

CONFUSION EXISTS as to the effects of varying the dietary intake of polyunsaturated fatty acids on liver and blood serum cholesterol concentrations of many animal species. Some authors have reported that dietary fats have no effect on liver cholesterol levels. According to other studies (3–7), diets high in polyunsaturated fatty acids cause increased concentrations of cholesterol in the liver. The ingestion of polyunsaturated fatty acids has also been reported to lower liver cholesterol levels (8). Blood serum cholesterol concentrations have been lowered by the inclusion of essential fatty acids (EFA) in the diet (9–11). Kritchevsky et al. (12) indicated the complexity of the mechanism when they reported that rats fed unsaturated fat showed a relative hypocholesterolemia despite increased hepatic synthesis and decreased hepatic oxidation.

The present study was designed to investigate the effects of lipids of comparable fatty acid composition from animal and plant sources on cholesterol concentrations in the livers and blood serum of mature male rats.

EXPERIMENTAL

Male Caworth albino rats, approximately 20 months of age, were fed diets furnishing 76 cal daily. The basal diet (13) obtained from Nutritional Biochemicals Corporation included the following: "Vitamin Free" casein, 21.10%; "Alphacel" cellulose, 16.45%; sucrose, 58.45%; salt mixture, USP XIV, 4.00%; and the following vitamin supplements (g/100 lb): choline chloride, 272.500; nicotinic acid, 27.250; inositol, 13.750; vitamin A concentrate (200,000 units per gram), 4.500; vitamin D concentrate (400,000 units per gram), 3.000; alpha tocopherol, 10.225; menadione, 0.1025; thiamine hydrochloride, 1.000; pyridoxine hydrochloride, 1.000; riboflavin, 1.000; calcium pantothenate, 2.050. This diet was formulated per Wooley and Sebrell (14). Lipids from plant and animal sources were added to supply 40% of the calories. The lipid material for one group was obtained from a pig fed a 20% corn oil ration for 17 days. The *panniculus adiposus* was finely chopped and rendered under nitrogen gas in an autoclave at 121C for 2 hr. The lipid layer was centrifuged at 2100 rpm for 15 min to remove water and any protein that might have been present. Refined soybean oil was fractionally crystallized from acetone (10% w/w) at −11C. Differential cooling curves (15) indicated that the resulting lipids contained neither wholly saturated nor wholly unsaturated triglycerides. Thin-layer chromatography of the lipids on Anasil B followed by spraying with 50% sulfuric acid in 50% ethanol with 2% ferric chloride and charring at 110C produced no evidence of cholesterol or cholesterol esters. Five percent phosphomolybdic acid in ethanol also failed to detect any traces of cholesterol or cholesterol esters in samples of up to 10 mg of the lipid. These systems were used to detect 2 μg cholesterol in trial runs. Trace amounts of diglycerides were noted. The vegetable lipids were crystallized from acetone on alternate days during the trial. The lipids were stored in brown bottles under nitrogen at 4C throughout the experiment.

Methyl esters of the lipids were prepared according to the method suggested by McGinnis and Dugan (16). Each lipid contained approximately 33% essential fatty acids (EFA) by gas chromatographic analysis (Table I). A 6 ft by ¼ in. copper column packed with 60/70 mesh Anakrom A coated with 12% ethylene glycol succinate was used in a Barber-Colman Model 20 gas chromatograph equipped with an argon ionization detector. Methyl esters of fatty acids were identified by comparing their retention times with standards and by semilogarithmic plots of retention volume against carbon number.

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Each diet was fed to 3 individually caged rats for a 2-week period. In addition, 3 rats received only the basal ration, again at the 76 cal level. The feeders were cleaned daily and feed consumption was recorded. The rats were fasted 24 hr and sacrificed. Tissue samples were taken, blotted on filter paper and stored at −26C under nitrogen. Blood samples were centrifuged at 4C and the serum samples were stored awaiting analysis.

Total and free cholesterol measurements were made by the digitonin precipitation method (17). Modifications in the procedure in order to determine liver cholesterol levels were as follows: The total liver lipid extract (19) was dissolved in 100 ml diethyl ether and duplicate 10 ml samples were taken. The solvent was removed from each sample under reduced pressure. The residue was dissolved in 10 ml isopropyl alcohol and one half milliliter was taken for analysis. This sample was increased to a volume of 5 ml with isopropyl alcohol. The Ferro and Ham (17) color development procedure was followed. A standard curve was prepared using cholesterol concentrations in the range of 0.1 to 1.0 mg in 5 ml isopropyl alcohol. The values obtained from the liver lipid extract were converted to mg/g wet tissue.

RESULTS

In Table II, an analysis is given of the concentrations of total, free and esterified cholesterol in the livers and blood serum from the rats receiving the animal fat (Diet A), vegetable fat (Diet V) and the nonfat controls (Diet C).

Highly significant differences were found between animals ingesting diets V and A for total liver cholesterol ($P < .01$), liver free cholesterol ($P < .01$) and blood serum free cholesterol ($P < .001$). The blood serum total cholesterol difference was nearly significant at $P = .0533$. In all cases, Diet V produced the lower level. No differences were noted between rats fed Diet A and Diet C. Of interest was the lack of effects of the dietary regime on the fatty acid composition of various fractions of tissue lipids. Generally, all lipid fractions of the blood serum, i.e., monoglycerides, diglycerides, triglycerides, free fatty acids and phospholipids, contained a higher proportion of saturated fatty acids than did those from the liver and arterial wall.

TABLE I

The Fatty Acid Composition of the Dietary Lipids in Diets A[a] and V[b]

Fatty acid[c]	Methyl esters as percentage of total methyl esters	
	Diet A	Diet V
14:0	0.7	1.1
16:0	18.2	21.0
16:1	9.7	0
18:0	7.2	15.1
18:1	30.1	27.3
18:2	26.4	29.5
18:3	7.8	5.9
20:4	Trace	0
Percent polyunsaturates	34.2	35.4

[a] Lipid from animal source.
[b] Lipid from vegetable source.
[c] Fatty acid abbreviation system suggested by Dole et al. (18).

Although caloric intake was the same for all individuals, weight losses over the 14-day trial amounted to 37.7 ± 4.9 g for rats fed diet A, 42.7 ± 4.4 g for rats fed diet V and 63.7 ± 7.6 g for the controls.

DISCUSSION

Lipids from soybean oil have a cholesterol-lowering effect that is not found with equally unsaturated lipids from lard. These reductions occur only in the free cholesterol fraction and concurrently in the total cholesterol levels of rat liver and blood serum lipids. It would appear that a factor(s) is present in the vegetable lipid since its inclusion in the diet significantly lowered levels as compared to the controls whereas no cholesterol raising factor was noted in the lard diet when compared with the controls.

Furthermore, the diet of the pig supplying the lard contained no added hormones. The rendering procedure would have removed any water-soluble material.

Although chromatographic techniques did not reveal the presence of plant sterols in diet V, it is possible that minute amounts may have been present in the refined soybean oil and may have contributed a cholesterol lowering effect. In tests with a limited number of sterols,

TABLE II

Cholesterol Concentrations of Mature Rat Liver and Blood Serum

Diet	Liver mg/g			Blood serum mg/100 ml		
	Total	Free	Esterified	Total	Free	Esterified
A	21.9±1.1	17.5±1.9	4.4±1.6	132.1± 4.7	44.1±3.8	87.9±1.5
V	**10.8±1.8**	**6.6±0.6**	4.2±0.7	103.5± 9.3	**19.8±1.4**	83.9±7.6
C	21.4±1.3	14.4±1.1	6.6±1.7	125.8±10.5	46.9±8.5	78.9±2.0

TABLE III

Unsaturated Fatty Acid Content of Feces Lipids

	Monoglyceride	Diglyceride	Triglyceride
Diet A	12.3± 5.6	18.6±1.0	25.6±3.6
Diet V	**42.0±20.1**	15.8±3.8	29.2±0.8

no precipitation from acetone was observed at −15C. The lipids were checked at intervals for oxidative changes. The absence of extraneous peaks on gas chromatograms indicated relative freedom from oxidation products.

The authors suggest that a more likely factor might be the triglyceride configuration. Although in vitro pancreatic lipase hydrolysis data are not available, the composition of the feces monoglycerides is suggestive of structural differences between the lipids (Table III). The contribution of intestinal flora to feces lipids is uncertain; however, the similarity of the diglycerides and triglycerides would indicate that the flora are not responsible for the very significant differences in the monoglyceride composition. The presence of 3.5 times greater amounts of unsaturated acids on the monoglycerides in the feces of animals fed diet V (Table III) supports the evidence of Mattson and Volpenheim (20) who reported that vegetable glycerides are predominantly beta-unsaturated. The low unsaturated acid content of feces monoglycerides of animals fed diet A indicates the probability of the dietary lipid being primarily beta-saturated. Mattson et al. (21) have previously reported that lard glycerides from pigs fed a highly unsaturated vegetable oil diet were primarily beta-saturated as are all lard glycerides.

The authors, therefore, suggest that the cholesterol lowering effects of dietary EFA are seriously decreased if the EFA are not esterified at the beta position on the glyceride.

ACKNOWLEDGMENTS

Specific glycerides for preliminary analytical work were donated by R. J. Vander Wal, Armour and Company Research Laboratories, Chicago, Illinois.

Supported in part by NIH Graduate Training Grant 8 Tl ES 16.

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[Received Nov. 29, 1965]

Compositional Variation in Seed Oils of the *Crepis* Genus

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ABSTRACT

Seed oils from eight species of the genus *Crepis* (family Compositae) fall into three groups differing in chemical composition. Besides conventional fatty acids the oils contain either vernolic acid (47–68%), crepenynic (36–65%), or both (18–35% vernolic and 7–11% crepenynic). Within any one section of the genus, the oils are chemically similar, among the limited groups of samples examined.

INTRODUCTION

Workers at the Northern Laboratory have reported that seed oil of *Crepis foetida* L. contains a predominant percentage of an acetylenic fatty acid, which they structurally characterized as *cis*-9-octadecen-12-ynoic acid (1) and to which they assigned the trivial name, crepenynic acid. Under the influence of alkali, heat, or both, crepenynic acid undergoes an interesting reaction sequence, leading through isolable enallene and conjugated triene intermediates to cyclic structures (2,3). The novel structure and reactivity of this vegetable oil constituent suggest further basic research on its chemistry and biosynthesis and, perhaps, extension to consideration of its practical utility. Another *Crepis* species (*C. vesicaria*) contains vernolic acid as a principal fatty acid constituent of its seed oil (4). Consequently, seed oils of additional species in the *Crepis* genus were examined to determine compositional variability and to permit selection of the most practical plant sources of crepenynic and vernolic acids.

In this paper we report chemical data on eight species of the *Crepis* genus, information on their plant characteristics, and the relationships observed between classification by classical taxonomic methods and the chemical differences found.

BOTANICAL DESCRIPTION AND CROP POTENTIAL

Crepis is a large genus comprising nearly 200 species of annual, biennial, or perennial herbs belonging to the tribe Chicoreae of the family Compositae. The genus includes natives of Eurasia, Africa, and North America with the majority of species occurring in the northern hemisphere of the Old World. The 12 indigenous New World species of *Crepis* are all found in the western half of North America; one of these species also occurs on the coasts of Labrador and Newfoundland (5).

Although the members of *Crepis* have practically no economic importance, the genus is well known to botanists because of the classic taxonomic and evolutionary studies of *Crepis* by Babcock and Stebbins (5–7). To nonbotanists, however, *Crepis* is virtually unknown. The genus includes no crop plant, and only one species, *C. rubra,* is cultivated to any extent, and this for ornamental purposes.

Many obstacles exist for the plant breeder or agronomist who attempts to fashion a new oilseed crop from *Crepis*. Too often, the species are poor in habit, indeterminate in flowering and fruiting, and produce light achenes in small heads which shatter readily. In our limited experience, two native American species, *C. occidentalis* and *C. intermedia,* possess some of the best agronomic characteristics in having an erect habit, more determinate flowering and fruiting, larger achenes, and good achene retention. These perennial species, however, are known to have specialized soil and climatic requirements and slow rates of development (5). In spite of these seemingly unfavorable aspects, we have only scratched the surface in our preliminary investigations of *Crepis*. There remains in the genus a vast and uninvestigated resource of genetic material from which a new crop might ultimately be developed.

MATERIALS AND METHODS

One seed sample was bought from a commercial seed house in this country; the rest were collected from the wild in Yugoslavia, Spain, Turkey, Pakistan, and the U.S.A. by botanists under the direction of the New Crops Research Branch, ARS, USDA. Collections outside the U.S.A. were supported by grants of funds under Public Law 480.

Seed oil from each sample was analyzed for oxirane oxygen essentially by the AOCS Method Cd 9–57 (8). Those oils containing the equivalent of 5% or more epoxyoleic acid were transesterified with $NaOCH_3$-methanol and the

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TABLE I

Analytical Data on *Crepis* Seeds and Oils

Species	Section of genus	Origin	Seed analysis			Oil Properties			Composition of methyl esters, % (area percentage by GLC)								
			Wt./ 1000, g	Oil content, % DB	Protein content N X 6.25, % DB	Iodine value	Refractive index n^{40}_D	HBr reactive acids as epoxy-oleic, %	Crepenynic	Epoxy-oleic	16:0	18:0	18:1	18:2	Other	
															No.	Total
C. biennis L.	*Berinia*	Yugoslavia	1.0	34	23	91	1.4694	65	0.1	68	3.1	2.6	11	15	1	0.1
C. aurea (L.) Cass. subsp. *aurea*	*Brachypodes*	Yugoslavia	1.0	31	30	99	1.4698	53	2.4	54	3.7	1.7	11	22	7	5.1
C. vesicaria L. subsp. *taraxacifolia* (Thuill.) Thell. ex Sch. & K.	*Lepidoseris*	Spain	0.2	20	24	105	1.4705	49	1.1	47	5.6	2.6	11	31	9	1.8
C. intermedia Gray	*Psilochaenia*	U.S.A.	3.2	19	22	114	1.4706	36	10	35	4.4	1.7	15	34	5	1.0
C. occidentalis Nutt.	*Psilochaenia*	U.S.A.	2.0	5	13	115[a]	.....	22	7.2	18	5.0	1.0	28	32	9	8.7
C. occidentalis Nutt.	*Psilochaenia*	U.S.A.	5.0	22	....	119[a]	.....	34	11	30	3.6	2.1	19	34	1	0.4
C. foetida L.	*Hostia*	Turkey	0.2	13	22	153	1.4743	....	51	....	5.3	2.8	5.3	32	7	3.6
C. foetida L. subsp. *rhoeadifolia* (M.B.) Sch. & K.[b]	*Hostia*	Turkey	0.6	24	24	152	1.4745	3	60	....	4.0	2.9	5.6	27	2	0.9
C. foetida L. subsp. *rhoeadifolia* (M.B.) Sch. & K.	*Hostia*	Turkey	0.3	6	....	.....	.....	....	36	....	6.8	2.6	8.1	40	11	7.7
C. rubra L.	*Hostia*	U.S.A.	1.5	22	....	154	. .....	2	55	....	4.6	2.8	4.5	28	8	5.5
C. thomsonii Babc.[b]	*Hostia*	Pakistan	0.4	16	19	159	1.4746	2	65	....	4.6	1.7	3.7	24	9	1.2

[a] Calculated, assuming that 1 mole of halogen reacts with vernolic acid and 2 moles with crepenynic acid.
[b] Compositional results on this line represent one of two similar accessions.

rest with HCl-methanol. Other analyses were made as previously described (9,10).

RESULTS AND DISCUSSION

Among the current group of samples analyzed (Table I), the species of *Crepis* fall into three categories with respect to the amounts of acetylenic and epoxy acids in their seed oils. One group of species is high in vernolic acid, another high in crepenynic acid, and a third group is intermediate in chemical composition. The species comprising these groups show, in part, a marked agreement with Babcock's sectional classification of *Crepis* based on such criteria as comparative morphology, chromosome number and morphology, genetics, cytogenetics, and geographic distribution (6,7). The species high in crepenynic acid all belong to section *Hostia* and those in the intermediate group belong to section *Psilochaenia*. The three other species making up the group high in vernolic acid each belong to different sections of the genus; namely, sections *Berinia, Brachypodes,* and *Lepidoseris*.

Such chemico-botanical information can provide valuable leads for further screening in the genus *Crepis*. The occurrence of high concentrations of vernolic acid in three species in three distantly related (6) sections would appear to indicate a large potential reservoir of *Crepis* species rich in this epoxy acid. Also, the logical place to encounter other species high in crepenynic acid would be among the remaining members of section *Hostia* and in the closely related section *Paleya* (6).

Two accessions of *C. foetida* var. *rhoeadifolia* and of *C. occidentalis* are reported because of the marked differences in the analyses of both the seed and the oils. The lower amounts of oil and of unusual acid in one sample of each species may indicate immaturity of the sample, but such immaturity is not obvious on inspection. Other than crepenynic and vernolic acids, *Crepis* oils contain primarily the four common fatty acids as shown in Table I. Small amounts, about 1% or less, of the following acids are in most oils: 14:0, 16:1, 18:3, 20:0, and 20:1. Traces of C_{22} acids were found in two oils. The low-oil *C. occidentalis* seed is unique in containing oil with 6.4% 16:1, whereas no other oil contains more than 0.3%. These minor acids are reported together in the table as "Other" with an indication of the number of components and their total amount.

The amounts of epoxy acid as determined by gas-liquid chromatography (GLC) and by HBr titration are in reasonable agreement for those oils containing 18% or more of this acid. Infrared absorption confirms the presence of the epoxy structure in these oils, and the component acid has been identified as vernolic in oils from *C. biennis, C. aurea, C. vesicaria,* and *C. occidentalis* (11). The identity of the HBr-reactive material in those oils containing less than 10%, calculated as vernolic acid, has not been investigated.

As used in these preliminary investigations, infrared spectrophotometry did not detect crepenynic acid because the acetylene bond is located near mid-chain and is not conjugated. In the ultraviolet, however, there are sometimes weak absorption bands in the 227–231 mμ and 268–274 mμ regions which suggest the presence of traces of conjugated enynes and conjugated trienes, respectively. Both of these structures might be formed by isomerization of crepenynic acid.

The occurrence of vernolic acid in some *Crepis* species, and of both vernolic and crepenynic acids in others, adds support to the suggestion of Mikolajczak et al. (1) that crepenynic acid may be biosynthetically related to vernolic and linoleic acids. The suggestion is further strengthened by the definite inverse relationship between linoleic acid and the sum of vernolic and crepenynic acids in the oils. No evidence for a dihydroxy acid intermediate has been found in this group of samples.

ACKNOWLEDGMENTS

R. W. Miller, R. Kleiman, and J. W. Hagemann provided the GLC analyses.

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[Received May 11, 1966]

Metabolism of Alpha-Alkoxy Glyceryl Monoethers in Rat Liver, in Vivo and in Vitro[1]

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ABSTRACT

An investigation of the metabolism of ^{14}C and ^{3}H labeled α-isomers of C_{16} and C_{18} alkoxy monoethers, administered intravenously and added to liver slices, showed extensive cleavage of the ether bond in rat liver. Approximately 99% cleavage of the $C_{16:0}$ ether bond and approximately 94% cleavage of the $C_{18:0}$ ether bond occurred in rat liver within 6 hours after intravenous injection. With doubly labeled chimyl alcohol (^{3}H and ^{14}C), acetylation and subsequent acetolysis demonstrated that less than 0.92% of the phosphatides and less than 1.52% of total lipid radioactivity were in the form of alkoxy ethers. Long-chain fatty alcohols and fatty acids were the principal products of the ether cleavage in the liver. The relative rate of ^{14}C incorporation from chimyl alcohol and batyl alcohol into triglycerides and phospholipids, respectively, demonstrates that the palmitic (from chimyl alcohol) and stearic (from batyl alcohol) acids formed after cleavage enter the free fatty acid pool. The liver contained most of the radioactive label in the lecithin and cephalin of the microsomal fraction. Incubation of the labeled batyl or chimyl alcohols with liver slices resulted in the same products as in the in vivo experiments. Less than 1.4% of the C_{16} and C_{18} alkoxy ethers was oxidized to $^{14}CO_2$ during a 3-hour incubation. In view of the extensive cleavage of the ether bond by liver, the hemopoietic and radioprotective activities reported for the alkoxy ethers should be reevaluated in terms of their metabolic products.

INTRODUCTION

THE α-ALKOXY GLYCERYL monoethers (GE) exist in nature as free ethers, fatty acid esters, and phospholipids. Their distribution in mammalian tissues has been reported by several investigators (1–3), but their origin and metabolic significance in mammalian cells are unknown. Thompson and Hanahan (4) have shown, however, that radioactivity from glucose-6-^{14}C is incorporated predominantly into the α-carbon of the glycerol moiety of glyceryl ethers of bovine bone marrow in vitro. They (4) suggest that the biosynthesis of glyceryl ethers proceed analogously to that of glyceryl esters, i.e., from L-α-glycerophosphate. Considerable attention has been focused on the alkoxy glyceryl ethers because of their structural similarity to vinyl glyceryl ethers, found primarily as plasmalogens, and because of their reported biological activities related to hemopoiesis. For an up-to-date review of the α-glyceryl ethers, including references to their hemopoietic and radioprotective actions, the reader is referred to articles by Hanahan and Thompson (5) and Brohult (6).

In vivo studies in mammals with labeled GE have been concerned with their gastrointestinal absorption. First Bergström and Blomstrand (7) and Blomstrand and Ahrens (8) reported that the ether bond of chimyl alcohol and its diester (9) was extensively cleaved in the mucosal cells of rats and humans. The hydrolytic product of the chimyl-1-^{14}C alcohol was found to be cetyl alcohol, which was further oxidized to palmitic acid. More recently, Swell and co-workers (10) reported that the β-isomers of batyl alcohol were absorbed into the lymph (as the diacyl ethers) more efficiently than the α-isomers; however, they found that the α-isomer was metabolized to a greater extent in the intestine.

Enzymatic studies of the cleavage of the ether bond in rat liver by Tietz et al. (11) revealed the existence of a pteridine-requiring enzymatic system capable of oxidizing batyl alcohol to free glycerol and fatty acid. They postulated that the reaction proceeds by hydroxylation to form a hemiacetal that spontaneously yields a long-chain aldehyde; the aldehyde being further oxidized to the acid.

Our paper reports the tissue distribution and metabolic products found after the intravenous administration of ^{14}C and ^{3}H-labeled α-alkoxy glyceryl monoethers. In addition, the metabolic transformations of GE in liver, bone marrow, and spleen were investigated in vitro.

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METHODS

Synthesis of ^{14}C- and ^{3}H-Labeled Glyceryl Ethers

The batyl-1-^{14}C alcohol was synthesized by refluxing the potassium salt of 1,2-isopropylidene glycerol with octadecyl bromide in xylene, whereas two preparations of the doubly labeled α-chimyl-1-^{14}C (2-^{3}H-glyceryl) alcohol were synthesized from hexadecyl-1-^{14}C bromide and isopropylidene-2-^{3}H-glycerol. The ^{14}C specific activities (μc/mg) of the glyceryl ethers obtained were 1.97 for the batyl-1-^{14}C alcohol and 2.20 and 4.11 for the chimyl-1-^{14}C (2-^{3}H-glyceryl) alcohol. The ratio of $^{3}H/^{14}C$ in the doubly labeled chimyl alcohol samples was 3.5 for the former preparation and 0.12 for the latter preparation. Using the TLC procedure described later (system B), we found the synthesized racemic mixtures to have R_f's identical to that of batyl alcohol isolated from shark liver oil (Western Chemical, Ltd., Vancouver, Canada). The radiopurity of these labeled ethers was greater than 99% (Fig. 1) as determined by thin-layer chromatography (TLC). Gas-liquid chromatography of their trifluoroacetate derivatives (12) on 5 ft × 1/8 in. glass columns containing 15% EGSSX liquid phase demonstrated that the molecular purity of the l-isomer was greater than 99% for the chimyl alcohol and greater than 98% for the batyl alcohol (Fig. 1). A separate report in collaboration with Drs. Piantadosi, Oswald and Anderson of the University of North Carolina will describe the detailed syntheses and other purity evaluations.

In Vivo Experiments

A total of 15 female Carworth Farm Nelson's strain rats weighing between 160 and 190 g was used in these experiments. The labeled glyceryl ethers were given intravenously (10 μc/100 g body weight) as a fat emulsion (Abbott Laboratories) containing safflower oil (10% w/v), glycerine (8% w/v), and lecithin (0.5% w/v). The animals in these experiments were not fasted. Total lipid extracts were prepared (13), and the quantity of radioactivity in the various tissues was calculated as percentage of the original dose administered. In a few experiments $^{14}CO_2$ (14), urine and feces were collected for radioassay.

In another group of 3 rats, the emulsion of the doubly labeled chimyl alcohol was given intravenously to determine its subcellular distribution in the liver. Nuclear (700 × g), mitochondrial (5000 × g), and microsomal (31,000 × g) fractions were obtained from rat liver homogenates prepared in 0.25 M sucrose solution according to conventional techniques (15). The morphological purity of the subcellular fractions was not checked.

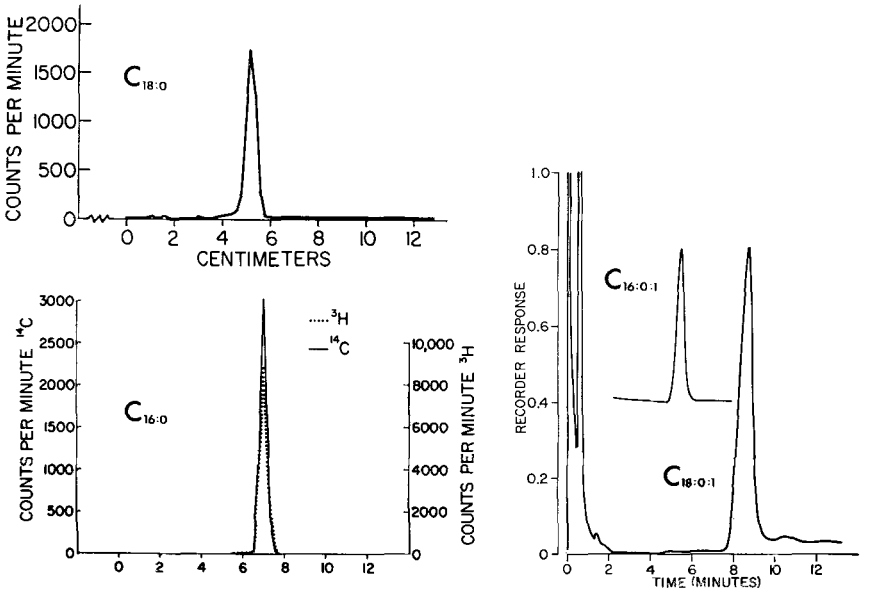

FIG. 1. TLC zonal scans in Solvent System B (*left*) and gas-liquid chromatograms (*right*) of the l-isomers of batyl and chimyl alcohols used in the biological experiments (see Methods section for details). The zonal scan for chimyl alcohol ($^{3}H/^{14}C = 0.124$) had the same degree of purity as the preparation ($^{3}H/^{14}C = 3.5$) depicted above.

In Vitro Experiments

The 18 female Carworth Farm Nelson's strain rats used in these experiments (weight, 150–200 g) were not fasted. All glassware was cleaned with nitric acid, and all solutions and glassware were sterilized. Twenty micrograms of doubly labeled chimyl alcohol containing 0.082 μc ^{14}C and 0.66 μc ^{3}H or 17 μg of batyl-1-^{14}C alcohol containing 0.045 μc ^{14}C were incubated with liver slices (100 mg per flask) in 1 ml Tyrode's solution adjusted to pH 7.3. In a few instances, bone marrow cells and spleen slices were incubated with chimyl alcohol. The Tyrode's solution contained 1% (v/v) propylene glycol to solubilize the glyceryl ethers. The propylene glycol was chosen to solubilize the glyceryl ethers in the in vitro experiments because it is split by liver enzymes to enter the formate and lactate metabolic pools (16). The glyceryl ethers were first solubilized in undiluted propylene glycol before addition of Tyrode's solution. Incubation blanks were prepared in the same manner with the omission of the liver slices. Penicillin (400 units) and streptomycin (40 μg) were added to each milliliter of Tyrode's solution to prevent microbial growth. At various time periods (0.5 to 5 hr) the reaction was stopped by adding methanol or 6 N HCl (to release $^{14}CO_2$) after which the total lipids were extracted (13) according to the procedure described later. In some flasks $^{14}CO_2$ was collected in Hyamine for subsequent liquid scintillation radioassay (17).

The liver slices actively incorporated K-palmitate-1-^{14}C acid into triglycerides and phospholipids under the same incubation conditions as GE. The fatty acid incorporation was unaffected by the lipid solubilizer (1% propylene glycol). A 2% level of the propylene glycol gave results identical to those observed for the 1% level, but the 5% level of propylene glycol in Tyrode's solution was found to be inhibitory.

Total Lipid Extraction

All livers obtained in the in vivo experiments were perfused with saline and homogenized in distilled water before freeze-drying. The total lipids from the various tissues were extracted by the procedure of Bligh and Dyer (13). The tissues used in the in vitro experiments were not perfused nor freeze-dried before lipid extraction.

Chromatography and Radioassay of Lipid Extracts

Three solvent systems were used for the development of the thin-layer chromatograms.

Solvent System A. Hexane:diethyl ether:acetic acid (80:20:1 v/v/v) was used for separation of the triglycerides, fatty acids, and di- and monoesters of glyceryl ethers.

Solvent System B. Hexane: diethyl ether: methanol:acetic acid (80:20:10:1 v/v/v/v) was used primarily for separating unesterified glyceryl ethers, monoglycerides, and diglycerides.

Solvent System C. Chloroform:methanol:acetic acid:saline (50:25:8:4 v/v/v/v) was used for separating phospholipids.

All chromatograms for zonal scans were prepared on 2 × 20 cm glass plates coated with a 250 μ layer of Silica Gel G (in water) for systems A and B, and Silica Gel HR (in 0.01 M Na_2CO_3) for system C.

The lipid extracts from each tissue were concentrated to appropriate volumes containing sufficient radioactivity (greater than 500 dpm) for the preparation of thin-layer chromatographic zonal scans (2-mm) using an automatic zonal scraper (18) and Packard liquid scintillation spectrometers. Lipid standards were chromatographed simultaneously on a lane adjacent to the lipid extract. The quantity of ^{14}C and ^{3}H present in each of the doubly labeled samples was determined from the simultaneous solution of equations designed for determining the fractional quantities of isotopes present in the mixture of these two isotopes.

Visualization of the lipids was accomplished by exposing the chromatograms to iodine vapor sublimed from crystals of iodine in a beaker on a hot plate; residual iodine on the silica does not quench during liquid scintillation assay. Occasionally, separations would be visualized by charring after spraying with concentrated sulfuric acid and heating on a hot plate.

Acetylation and Acetolysis of Phospholipids

The quantity of intact alkoxy ethers incorporated into the alkoxy ether phosphatides of the liver was assessed after acetylation and acetolysis, which was used to cleave the PO_4-nitrogenous base moiety, and the fatty acids of lecithin (PC) and cephalin (PE).

This procedure regenerates the original glyceryl ether, since the alkoxy ether bond is not split under these conditions (19, 20), whereas the phosphate and acid esters are cleaved. Normally, the phosphate ester bond of glycerol is not split by saponification alone. It is thought that vinyl ethers are cleaved to form glycerol and fatty aldehydes under the acidic condition (21) of acetylation.

After isolating the phospholipids by preparative TLC, we eluted them with ten 10-ml portions of $CHCl_3$:CH_3OH (2:1 v/v). The residual silica gel after elution contained less than 4% of the total phospholipid radioactivity present in the original sample.

Acetylation and acetolysis of the total lipids of the liver and synthetic standards (GE and their esters) were carried out essentially according to the procedure of Bevan (22). The lipids, freed of solvents, were acetylated by refluxing the sample (less than 15 mg) in 5 ml acetic acid:acetic anhydride (3:2 v/v) for 12 hr. The acetates obtained after acetylation were hydrolyzed by refluxing the sample for 2 hr in 5 ml of ethanolic 1 N KOH. Thin-layer chromatography and ^{14}C and ^{3}H radioassay were performed on the final products of this reaction.

RESULTS AND DISCUSSION

At the outset it is important to recall that all labeled glyceryl ethers used in our experiments were the racemic mixtures and not the naturally occurring D forms. It is not our intent in the discussion that follows to imply that both optical isomers are attacked equally well. Since initial experiments in our laboratories confirmed earlier studies (7–9) showing that the racemic forms of alkoxy ethers were markedly altered in the gastrointestinal tract, we examined the metabolism of labeled batyl or chimyl alcohols after intravenous administration, thereby bypassing the tract's ether-cleaving enzymes. Under our conditions, approximately 28% of the ^{14}C from chimyl alcohol and 13% of the ^{14}C from batyl alcohol was eliminated as $^{14}CO_2$ within 6 hr after intravenous administration. During the same period the urine contained 6.5% of the ^{14}C from the $C_{16:0}$ ether and 1% of the ^{14}C from the $C_{18:0}$ ether. Approximately 6 to 13% of the radioactivity was accounted for as lipid in the liver (Table I). Apparently, the remainder of the ^{14}C activity was diluted in adipose tissue or transferred into a nonlipid metabolic pool.

The data in Table I also show metabolic transformations of the labeled glyceryl ethers that occurred in the liver. The greatest portion of liver radioactivity was found in the phospholipid fraction, primarily as phosphatidyl choline and phosphatidyl ethanolamine. A considerable amount of radioactivity from the chimyl alcohol (40%) and a lesser quantity from the batyl alcohol (9%) was also found in the triglyceride fraction (Fig. 2, Table I). Long-chain alcohols, fatty acids, diglycerides, and glyceryl ether diesters contained less than 10% of the total liver radioactivity from batyl or chimyl alcohols.

After the administration of the doubly labeled chimyl alcohol with a $^{3}H/^{14}C$ ratio of 3.5, the $^{3}H/^{14}C$ ratio of all lipid fractions isolated by TLC was reduced below unity. Even in the nonsaponifiable liver lipids, the ratio of $^{3}H/^{14}C$ was much lower than in the labeled chimyl alcohol injected. The subcellular distribution of ^{14}C and ^{3}H of the liver after an intravenous injection of doubly labeled chimyl alcohol is shown in Table II. The microsomal fraction contained 40% and the mitochondrial fraction only 10% of the total liver radioactivity. The nuclear and soluble fractions contained 29 and 21%, respectively, of the total radioactivity. Lecithin followed by cephalin accounted for most of the phospholipid radioactivity in all of the subcellular fractions. The high radioactivity content of the microsomal fraction is of considerable interest, since the enzymatic studies by Tietz et al. (11) indicated that the microsomal (catalyzes the oxidative step) and soluble supernatant fractions (for utilization of the pteridine cofactor) of the liver were needed

TABLE I

Percentage of ^{14}C in Lipid Classes of Liver 6 Hr After Intravenous Administration of Labeled Batyl and Chimyl Alcohols

GE diester	Triglyceride	Fatty acid	Fatty alcohol	GE monoester	GE	Lecithin	Cephalin
Batyl-1-^{14}C Alcohol[a] (6 female rats)							
1.7 ±0.27	8.8±1.2	1.7±0.50	1.5 ±0.57	1.1 ±0.40	6.2 ±1.5	58±3.7	18±1.2
Chimyl-1-^{14}C (2-^{3}H-glyceryl) Alcohol[b] (6 female rats)							
0.88±0.21	40 ±6.0	4.1±0.68	0.85±0.13	0.51±0.17	0.64±0.20	34±3.9	16±1.8

Only small portions of tissues other than liver were assayed and they contained negigible quantities of ^{14}C or ^{3}H per sample.

[a] 6.35±1.53% of the injected dose present in liver; 13.8% was eliminated as $^{14}CO_2$ and 1.05±0.24% was eliminated in the urine.

[b] 13.6±1.68% of the injected dose present in liver; 29.7% was eliminated as $^{14}CO_2$ and 6.5±0.98% was eliminated in the urine.

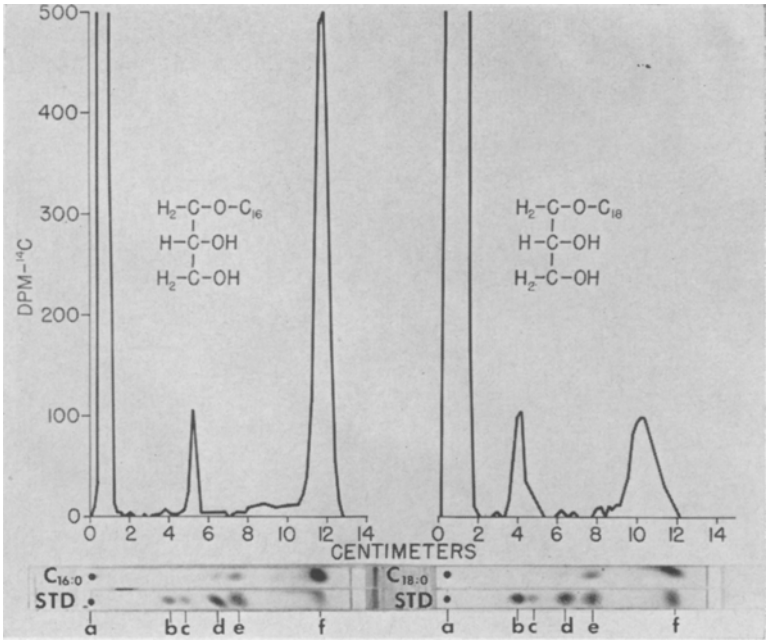

FIG. 2. Zonal scans (2-mm) of thin-layer chromatograms of total liver lipids 6 hr after intravenous chimyl-1-^{14}C (2-^{3}H-glyceryl) alcohol (*left* scan) or batyl-1-^{14}C alcohol (*right* scan) injections. The arabic letters designate lipid classes based on known standards (Std): (a) phospholipids, (b) monoglycerides, (c) glyceryl ethers, (d) free fatty acids, (e) fatty alcohols, (f) triglycerides.

Chromatograms were developed in Solvent System B.

for maximal activity of the ether-cleaving enzyme.

The ratios of lipid ^{3}H/^{14}C were essentially the same in all subcellular fractions, but the ratios were lower than that of the original chimyl alcohol injected. Under the conditions of this experiment the liver contained 13% of the ^{14}C and 2% of the ^{3}H injected: these percentages and the decrease in the ^{3}H/^{14}C ratio demonstrate that a much greater quantity of GE glycerol than GE hydrocarbon chain is lost from the lipid metabolic pool.

Acetylation and acetolysis of liver lipids obtained after intravenous injection of doubly labeled chimyl alcohol revealed that less than 1% of the phosphatide radioactivity and less than 1.6% of the total lipid activity were in the form of alkoxy ethers (Table III). These data suggest that the dietary α-alkoxy ethers do not serve, to any great extent, as precursors of phospholipid-containing alkoxy ethers. Since labeled long-chain fatty aldehydes were not seen on any of the zonal scans after acetolysis, we also conclude that the labeled alkoxy ethers were not precursors of vinyl ether lipids. This conclusion is based on evidence by Farquhar (21), who has shown that under acidic conditions, the vinyl ethers yield long-chain fatty aldehydes. The ^{14}C from batyl alcohol (yielding stearic acid) was greater in the phospholipids and less in the triglycerides than the ^{14}C from chimyl alcohol (yielding palmitic acid). This distribution of radioactivity incorporated into triglycerides and phospholipids is consistent with what one finds after the administration of labeled stearic and palmitic acids, respectively. The large amount of radioactivity in the liver suggests that this organ rapidly cleaves the ether linkage and allows the fatty acid product to enter the free fatty acid pool.

The observations made after intravenous in-

TABLE II

Distribution of Radioactivity from Chimyl Alcohol (^{14}C, ^{3}H) in Subcellular Fractions of Liver[a]

Fraction	^{14}C %	^{3}H %	^{3}H/^{14}C ratio[b] of total lipids in fraction	Lecithin ^{14}C %	Lecithin ^{3}H %	Lecithin ^{3}H/^{14}C	Cephalin ^{14}C %	Cephalin ^{3}H %	Cephalin ^{3}H/^{14}C	% Lipid per fraction
Nuclear	29	29	1.57	20	29	1.30	11	22	1.41	17
Mitochondrial	10	10	1.57	15	20	1.33	11	13	1.43	16
Microsomal	40	40	1.57	31	39	1.31	15	22	1.39	46
Soluble	21	21	1.56	17	23	1.36	7	13	1.38	21

[a] Six hours after intravenous injection; the liver contained 13% of ^{14}C and 2% of ^{3}H injected dose.
[b] The ^{3}H/^{14}C ratio of the chimyl alcohol injected was 3.5; the ratio of ^{3}H/^{14}C of the total liver lipids was 1.2 and the ratio of the nonsaponifiable fraction (containing 3.16% of the total ^{14}C of the liver) was 0.7.

TABLE III

The $^{3}H/^{14}C$ Ratio of Lipid Fractions of Liver Before and After Acetylation and Saponification Following the Administration of Chimyl-1-^{14}C (2-^{3}H-glyceryl) Alcohol

Sample	Ratio of $^{3}H/^{14}C$			
	In vivo (6 rats)	In vitro (10 samples at each time period[a])		
	6 hr	0.5 hr	3.0 hr	5.0 hr
Before Acetylation and Saponification				
Chimyl alcohol injected	0.124±0.002	0.124±0.002	0.124±0.002	0.124±0.002
Total lipids	0.033±0.010	0.101	0.093	0.092
Total phospholipids	0.049±0.014	0.183	0.063	0.066
Lecithin	0.016±0.006	0.065	0.045	0.010
Cephalin	0.021±0.006	0.085	0.074	0.051
After Acetylation and Saponification				
Chimyl alcohol injected	0.124±0.002	0.124±0.002	0.124±0.002	0.124±0.002
Alkoxy GE isolated from total lipids of liver	0.122±0.003[b]	.....	.....	.....
Alkoxy GE isolated from total phospholipids of liver	0.120±0.008[c]	0.189[d]	0.161[e]	0.139[f]

[a] All 10 samples combined.
[b] 1.52±0.14% of total lipids.
[c] 0.92±0.17% of total phospholipids.
[d] 12.5% of total phospholipid radioactivity as GE.
[e] 4.04% of total phospholipid radioactivity as GE.
[f] 3.31% of total phospholipid radioactivity as GE.

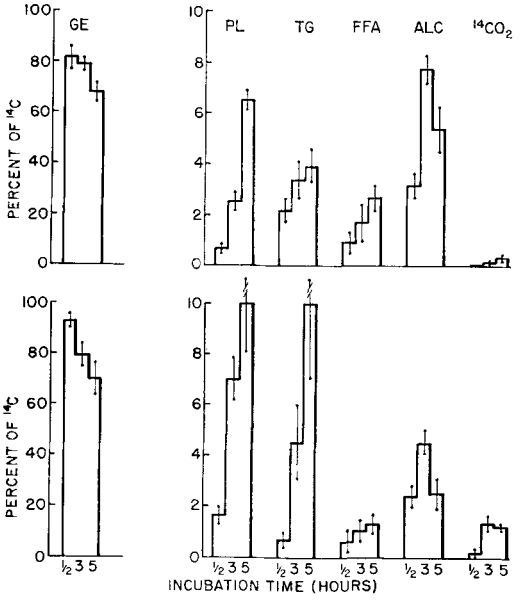

FIG. 3. The metabolism of batyl alcohol (*upper graph*) and chimyl alcohol (*lower graph*) by rat liver slices. The numbers on the ordinate refer to percent of ^{14}C added to the flasks. The numbers below each bar graph refer to hours of incubation, and the letters above each bar graph designate components assayed for ^{14}C. GE—glyceryl ethers; PL—phospholipids; TG—triglycerides; FFA—free fatty acids; ALC—fatty alcohols. Each mean value shown was based on the ^{14}C radioassay of samples obtained from 10 different flasks. The vertical line bisecting each bar represents ± one standard deviation.

jections of the glyceryl ether were confirmed qualitatively in the in vitro liver slice system (Fig. 3, Table III). The ^{14}C from batyl and chimyl alcohol was found in triglycerides, free fatty acids, fatty alcohols, glyceryl ether monoesters, lecithin, and cephalin. Less than 0.2% of the batyl alcohol and less than 2% of the chimyl alcohol was oxidized to $^{14}CO_2$ during the 3-hr incubations.

Bone-marrow cells and spleen slices were also studied and found to be less active than the liver slices in metabolizing the labeled chimyl alcohol. Less than 6% of the ^{14}C from chimyl alcohol was incorporated into phospholipids by marrow cells or spleen slices during the 3-hr incubation. The marrow cells did actively incorporate palmitic-1-^{14}C acid into triglycerides (25%) and phospholipids (45%) under identical conditions.

Acetylation and acetolysis of the total labeled liver phospholipids isolated from these in vitro experiments demonstrated that there was only a small amount of radioactivity in phospholipid ether linkages (Table III). The ratio of $^{3}H/^{14}C$ in the GE area after acetolysis was slightly higher than that of the original chimyl ether. This finding is difficult to interpret; perhaps biological exchange occurred or new ether linkages were formed under the in vitro conditions. We did not observe this difference in the $^{3}H/^{14}C$ ratio obtained for the GE fraction after acetolysis of the liver lipids in the in vivo study.

Our experiments have revealed that the

alkoxy ether bond is even more rapidly and completely cleaved after their intravenous administration than when given orally, as reported by other investigators (7–10). The cleavage yield the alcohols and acids of the hydrocarbon chain that existed in ether linkage with glycerol. Although we could not detect aldehydes on TLC, such an intermediary step has been postulated in the conversion of the hydrocarbon chain of the ether to the acid (11,23). The essential 100% splitting of the alkoxy ethers after their intravenous injections indicates that if hemopoietic and radio-protective actions thought to be inherent in the α-alkoxy glyceryl ethers exist, they most probably reside with one of their metabolic products.

ACKNOWLEDGMENTS

Expert technical assistance during this investigation by Edgar A. Cress and Nelson Stephens.

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[Received April 4, 1966]

Identification and Distribution of Epoxyacyl Groups in New, Natural Epoxy Oils[1]

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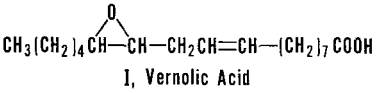
ABSTRACT

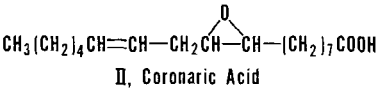
New high-epoxy vegetable oils from nine species representing three plant families and four genera have been investigated. The epoxyacyl moiety in at least one oil from each genus was characterized and shown to be the (+)-vernoloyl (*cis*-12,13-epoxy-*cis*-9-octadecenoyl) group. Intraglyceride distribution studies revealed a general preference of the (+)-vernoloyl groups for the β-position of triglyceride molecules. Interglyceride distribution of (+)-vernoloyl groups was studied in three oils and found not to agree with predictions based on either 1,2,3-random or 1,3-random-2-random distribution. A striking exception to the general intraglyceride distribution pattern was discovered in the monoepoxy triglyceride fraction from *Euphorbia lagascae* seed oil.

INTRODUCTION

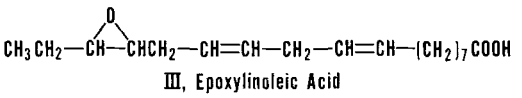
FOUR EPOXY ACIDS (I-IV) are known to occur as fatty acyl groups in natural oils (1–5). Sometimes two or more of these occur in the same oil (6,7). In addition, vernoloyl groups occur in both enantiomorphic forms (8) and epoxystearoyl groups in both geometric forms (4,9). The (+)-vernoloyl groups in *Vernonia anthelmintica* seed oil are present almost exclusively in trivernolin molecules, a fact which has both theoretical implications and useful consequences (10). It is therefore of interest to determine both the nature and distribution of epoxyacyl groups in new, natural epoxy oils.



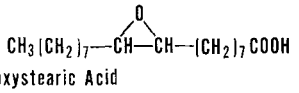
I, Vernolic Acid



II, Coronaric Acid



III, Epoxylinoleic Acid



IV, Epoxystearic Acid

In the course of an extensive seed screening program being conducted in this laboratory, evidence has been obtained indicating that epoxy-containing vegetable oils are more widely distributed than we had previously believed. The 10 seed oils found to contain the largest quantities of epoxyacyl groups are given in Table I, along with *Vernonia anthelmintica* seed oil for comparison. Our colleagues have already identified the vernoloyl group in *Euphorbia lagascae* seed oil (12); and the positive optical rotation of the oil (17) suggests that it is the dextrorotatory enantiomorph, i.e., the (+)-vernoloyl group, that is present. We now report further characterization of *Euphorbia lagascae* seed oil and studies on the other new high epoxy oils.

EXPERIMENTAL

Seed cleaning, grinding, and extraction were performed as previously described (18). For determination of epoxyacyl content of new seed oils, hydrogen bromide titrations were performed by the procedure of Harris et al. (19), and methyl esters for GLC were prepared from the oils by sodium methoxide catalyzed methanolysis (20). In all other instances preparation of methyl esters involved saponification with ethanolic potassium hydroxide and esterification with diazomethane (21). Gas-liquid chromatography (GLC) was carried out as described by Miwa et al. (22,23). Short (50–125 cm) columns, preferably with SE-30 as stationary phase, gave the best quantitative results for epoxide-containing esters for reasons discussed by Herb et al. (24).

In general, 20 × 20 cm plates coated to a thickness of 250 μ with Silica Gel G were used for thin-layer chromatography (TLC). The chromatograms were developed with *n*-hexane-diethyl ether-acetic acid (80:20:1), and the spots were made visible with iodine vapor. The modifications described in reference 13 improved resolution of the components of epoxy oils.

[1] Presented in part at the AOCS Meeting in Houston, April, 1965.

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TABLE I

Epoxyacyl Groups in Natural Epoxy Oils

Source of seed oil	Percent in oil[a] GLC	HBr titration	Proportion in β-position[b]
Euphorbiaceae			
Euphorbia lagascae Spreng.	58	60	43
Compositae			
Vernonia anthelmintica (L.) Willd.	68	71	[33][c]
Crepis biennis L.	68	68	43
Crepis aurea (L.) Cass. var. *aurea*	54	56	54
Crepis vesicaria L. ssp. *taraxacifolia*	47	50	
Crepis intermedia Gray	34	35	...
Crepis occidentalis Nutt.	18	23	50
Dipsacaceae			
Cephalaria joppica (Spreng.) Beg.	27	32	56
Cephalaria leucantha (L.) Schrad.	24	28	....
Scabiosa sp.[d]	22	22	54
Valerianaceae			
Valerianella radiata (L.) Dufr.	31	30	64

[a] Calculated as vernoloyl groups. The presence of significant amounts of epoxide in these oils was qualitatively confirmed by IR (11) and TLC (12, 13).

[b] Proportion in β-position $= \frac{\text{Mole percent in monoglycerides}}{3 \times \text{Mole percent in oil}} \times 100$. See reference 14.

[c] Predicted from the fact that essentially all the vernoloyl groups in *Vernonia anthelmintica* seed oil are present in trivernolin molecules (12, 15, 16).

[d] An unidentified *Scabiosa* species collected by a botanist in Turkey.

Infrared (IR) spectra were obtained with a Perkin-Elmer Infracord spectrophotometer, Model 137B. Melting points were determined on a Kofler micro hot stage.

For determination of intraglyceride distribution of vernoloyl groups, samples of oils or fractions thereof were treated with pancreatic lipase (Nutritional Biochemicals Corp., Cleveland, Ohio) according to the procedure of Mattson and Volpenhein (25). After removal of solvent from diethyl ether extracts of the acidified reaction mixtures, 5- to 20-mg samples of the residues were treated with diazomethane. Following a method to be described in detail elsewhere (26), pyridine solutions of the resulting mixtures of partial glycerides and methyl esters were treated with hexamethyldisilazane and trimethylchlorosilane, and the products were analyzed by temperature-programmed GLC.

For isolation of methyl esters of epoxy acids, samples of mixed methyl esters weighing approximately 3 g were subjected to countercurrent distribution (CCD) in a 200-tube Craig-Post apparatus with *n*-hexane-acetonitrile (1:1) as the solvent system. Single withdrawal fractions (40 ml) of upper phase were collected. The nonepoxy esters appeared in the first 70 withdrawal fractions, and the epoxy esters appeared in fractions 160–250, with a peak at fraction 205. Alternatively, approximately 1-g samples of mixed methyl esters were chromatographed on columns prepared from 30 g of Adsorbosil (100–140 mesh). After the nonepoxy esters were eluted with benzene, the epoxy esters were eluted with 10% ether in benzene (v/v).

Conversion to dihydroxy acids was accomplished by refluxing solutions of epoxy methyl esters (300–500 mg) or oils (1–2 g) in 15 ml of glacial acetic acid under nitrogen for 5 hr. After removal of the solvent in vacuo in a rotating evaporator, the residues were saponified. In several cases, portions of the dihydroxy acids from epoxy methyl esters were crystallized from mixtures of cyclohexane and ethyl acetate.

The crude products obtained by acetolysis and saponification of oils contained both dihydroxy and nonoxygenated acids. In a simplification of the Bharucha-Gunstone procedure (27), these mixtures were dissolved in 20-ml portions of the lower layer of a solvent system prepared by mixing 20 ml of water, 80 ml of methanol, and 100 ml of *n*-hexane. The solutions were washed once with 20 ml and three times with 10-ml portions of the upper layer of the solvent system. Removal of the solvent from the final water-methanol solutions afforded residues composed mainly of the desired dihydroxy acids.

Hydrogenations were effected in ethanol solutions at room temperature and atmospheric pressure in the presence of 10% palladium on charcoal. In several instances portions of the hydrogenated products were crystallized from cyclohexane-ethylacetate. The crystalline samples melted at 97–99C, either alone or mixed with authentic 12,13-dihydroxystearic acid prepared from *Vernonia anthelmintica* oil (1). Mixed melting points with 9,10-dihydroxy-

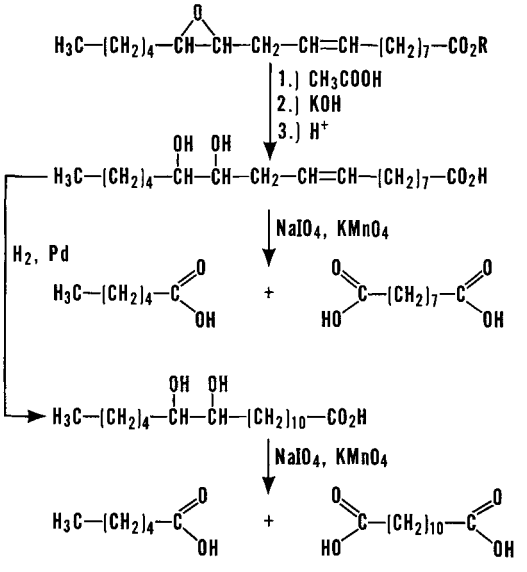
stearic acid (mp 94–96C) prepared from *Chrysanthemum coronarium* oil (5) were depressed.

Oxidative cleavages were carried out by the procedure of von Rudloff (28).

DISCUSSION

Location of Double Bonds and Epoxide Rings

The sequence of reactions used to locate double bonds and epoxide rings in epoxyacyl groups, as illustrated for vernoloyl groups, is shown below. The monocarboxylic acids



R = CH_3 or a diacyl glyceryl moiety

obtained in the oxidative cleavage reactions were determined as such by GLC, and the dicarboxylic acids were converted to dimethyl esters before identification by GLC.

The nature of the starting material for the degradation sequence varied. The epoxyacyl moieties in *Crepis vesicaria* and *Cephalaria joppica* seed oils were isolated as methyl esters by CCD. For *Crepis aurea, Crepis biennis,* and *Valerianella radiata* seed oils a slightly simplified version of the Bharucha-Gunstone method (27) was used. This method involves subjecting the oil to acetolysis conditions and then saponifying the product. A separation of the dihydroxy acids formed from the non-oxygenated acids is then made by solvent partitioning. Hydrogenated and unhydrogenated portions of the dihydroxy acid fraction were used for oxidative cleavage.

The Bharucha-Gunstone method bypasses isolation of the methyl esters of the epoxy acids. This is a disadvantage for the determination of stereochemistry. Hence, a simple chromatographic procedure was developed for separating epoxy methyl esters from their non-oxygenated counterparts. This procedure was used to isolate methyl vernolate from *Crepis aurea, Crepis biennis,* and *Valerianella radiata* seed oils for the determination of stereochemistry and from *Crepis occidentalis* and *Scabiosa* sp. seed oils for both oxidative cleavage and stereochemical studies.

In order not to miss evidence of the possible presence of two or more different epoxyacyl groups, use of crystallized samples for oxidative cleavage was deliberately avoided. Either total crude preparations of dihydroxyoctadecenoic acid and dihydroxystearic acid or residues of mother liquors from crystallization of these compounds were oxidized. Nevertheless, the only cleavage products detected in significant amounts were those expected from 12,13-dihydroxyoleic acid and 12,13-dihydroxystearic acid. Therefore, in all oils examined the only epoxyacyl group present had a 9,10-double bond and a 12,13-epoxide ring.

Stereochemistry of Epoxyacyl Groups

Because of the partial stereospecificity of the acetolysis reaction, the *threo*-12,13-dihydroxyoleic acid from (+)-vernoloyl groups is levorotatory (8,29). An indication of the extent of the stereospecificity of the reaction can be seen by comparing the magnitude of the rotation reported by Chisholm and Hopkins (8) for the more highly purified (+)-enantiomorph from a (−)-vernoloyl-containing oil (*Malope trifida*) with that of levorotatory products given in Table II. The negative sign of the specific rotation of dihydroxyoleic acid from *Euphorbia lagascae* seed oil is in agreement with the positive rotation of the oil itself (17). Both indicate that it is the dextrorotatory enantiomorph, i.e., the (+)-vernoloyl group, that is present in *Euphorbia lagascae* seed oil. The sign of the specific rotation of 12,13-dihydroxyoleic acid samples from three other oils indicated that they also contained (+)-vernoloyl groups. In these three cases the conclusion was confirmed by determining the sign of the optical rotation of methyl vernolate isolated from the same oil. In addition, the specific rotation of isolated methyl vernolate provided the desired stereochemical information for four oils from which crystalline samples of 12,13-dihydroxyoleic acid were not obtained. The numerical value of the specific rotation of the methyl vernolate from *Crepis occidentalis* was relatively low. An ether solution of this sample had been treated with Dowex 1 (OH-

TABLE II

Methyl Vernolate and 12,13-Dihydroxyoleic Acid from Natural Epoxy Oils

Source of oil	Methyl vernolate[a]			12,13-Dihydroxyoleic acid[a]			
	Method of isolation	$[\alpha]_{450}^{25 \pm 2}$	$[\alpha]_{D}^{25 \pm 2}$	Prepared from	mp	$[\alpha]_{450}^{25 \pm 2}$	$[\alpha]_{D}^{25 \pm 2}$
Malope trifida (8)				Oil	61–62		+18.9
Vernonia anthelmintica (29)			[+2.1][b]	Oil	52–55		− 5.7
Euphorbia lagascae				Oil	51–54	−2.2	− 1.0
Crepis biennis	Chromatography	+2.7	+1.9	Epoxy ester	53–55		− 2.7
Crepis aurea	Chromatography	+2.5	+1.8	Oil			
Crepis vesicaria	CCD	+3.1	+2.1	Epoxy ester	54–56	−9.1	− 4.5
Crepis occidentalis	Chromatography	+0.9	+0.8	Epoxy ester			
Cephalaria joppica	CCD	+3.8	+2.1	Epoxy ester			
Scabiosa sp.	Chromatography	+2.0	+1.5	Epoxy ester	54–55		− 4.3
Valerianella radiata	Chromatography	+2.3	+1.8	Oil			

[a] All samples of these compounds gave acceptable C and H analyses and had IR (neat for methyl vernolate and chloroform solutions for dihydroxyoleic acid) spectra identical to those of authentic samples of the same substances prepared from *Vernonia anthelmintica* oil. For determination of specific rotations, ethanol solutions were used for dihydroxyoleic acid samples and *n*-hexane solutions for methyl vernolate. Concentrations were near 2% in the former case and 2% to 8%, depending on the amount of sample available, in the latter. Where rotations at two different wavelengths are given, the rotations were determined with a Cary Model 60 Spectropolarimeter.

[b] Calculated from the reported (16) specific rotations in *n*-hexane of trivernolin ($[\alpha]_{D}^{25} + 2.2$) and vernolic acid ($[\alpha]_{D}^{25} + 2.0$).

phase) to remove a small percentage of free fatty acids that had escaped methylation. The sample was apparently partially racemized in this procedure, which did not cause any change detectable by IR, GLC, or TLC.

So far as the possibility of geometrical isomerism is concerned, the IR spectrum of all methyl vernolate samples isolated was identical to that of authentic methyl *cis*-12,13-epoxy-*cis*-9-octadecenoate from *Vernonia anthelmintica* oil. The absence of a distinct band near 10.35 μ in the spectra proves that the double bond is *cis* in all the samples (11). The melting point of crystalline 12,13-dihydroxyoleic acid samples was in agreement with that reported for the *threo* isomer (29) and therefore confirmed the *cis*-configuration of the epoxide rings in the esters from which they were prepared. The corresponding ester with a *trans* epoxide ring would have given *erythro*-12,13-dihydroxyoleic acid, mp 87–88C (29,30). In addition, the melting point of 12,13-dihydroxystearic acid prepared in several instances corresponded with that reported (29) for the *threo* rather than the *erythro* isomer. Available evidence therefore supports the conclusion that the epoxyacyl groups in all the oils examined are (+)-vernoloyl groups. Morris and Wharry (31) have shown that the absolute stereochemistry of the (+)-vernoloyl group, designated according to the Cahn-Ingold-Prelog system (32), is 12*S*, 13*R*.

Intra- and Interglyceride Distribution of Vernoloyl Groups

Isolation and analysis of monoglycerides produced from epoxy oils by treatment with

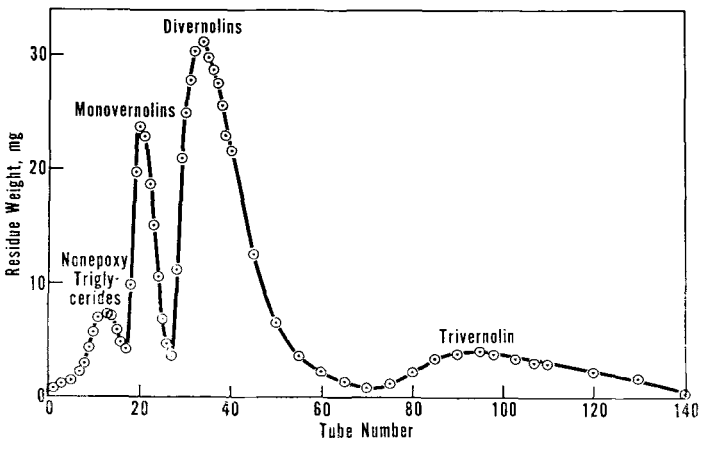


FIG. 1. Chromatographic separation of triglyceride fractions of *Euphorbia lagascae* oil. Column = 125 g of silicic acid (Mallinckrodt No. 2847); solvent = *n*-hexane-diethyl ether (4:1); sample = 1.082 g. Each tube referred to on the abscissa contained 5 ml of eluate. The peaks were identified by TLC (12). In addition, a sample of the product from combined fractions 19–25 (monovernolins) was saponified, treated with diazomethane, and analyzed by GLC. The sample contained 31% vernoloyl groups. By a similar analysis the combined divernolins (fractions 29–55) contained 65% vernoloyl groups.

TABLE III

Fractionation of Natural Epoxy Oils

Oil and fraction[a]	Mole percent in triglycerides				
	Fraction, found	Fraction, calculated[b]		Vernoloyl groups	
		1,2,3-Random	1,3-Random-2-random	Found[c]	Calcd.[d]
Euphorbia lagascae				58	61
Nonepoxy triglycerides	10	7	6		
Monovernolins	15	31	31		
Divernolins	56	42	44		
Trivernolin	19	20	19		
Crepis aurea				52	56
Nonepoxy triglycerides	13	11	7		
Monovernolins	18	36	42		
Divernolins	59	39	41		
Trivernolin	10	14	11		
Cephalaria joppica				27	28
Nonepoxy triglycerides	37	38	36		
Monovernolins	40	43	47		
Divernolins	22	16	15		
Trivernolin	0	2	2		

[a] Mono- and divernolins and trivernolin are triglycerides containing, respectively, one, two, and three vernoloyl groups. They may also be described as mono-, di-, and triepoxy triglyceride fractions. The plural terms "monovernolins" and "divernolins" should not be confused with the singular ones, "monovernolin" and "divernolin," which refer to mono- and diglycerides, respectively, containing one and two vernoloyl groups.

[b] Calculated with the aid of formulas given in references 34 (1,2,3-random) and 35 (1,3-random-2-random) by treating all acids other than vernolic as a single group.

[c] Average of titration and GLC values in Table II converted to mole percent.

[d] From fraction yields; e.g., for *Euphorbia lagascae* oil: $100 \times [(0.15 \times 0.33) + (0.56 \times 0.67) + (0.19 \times 1.00)] = 61$.

pancreatic lipase (25) were complicated by the properties conferred by the epoxy group. These complications prompted the development of a new method to be described elsewhere (26) for analyzing lipolysis products. This new method, which avoids isolation of monoglycerides, involves conversion of free hydroxyl groups in the total lipolysis product of an oil to trimethylsilyloxy groups. The resulting mixtures are then analyzed by temperature-programmed GLC. The data given in the last column of Table I were calculated from GLC curves obtained in this manner. These data show that the vernoloyl group exhibits a general preference for the β-position in the triglyceride molecules of the oils examined. The results of Sampugna et al. (33) argue strongly against the possibility that our evidence for this preference is due to discrimination against the vernoloyl group by pancreatic lipase. They found the enzyme to act at about the same rate on trivernolin to produce β-monovernolin as on triolein to produce β-monoölein.

As illustrated in Figure 1, column chromatography provided a method for separating epoxy oils into fractions based on the number of vernoloyl groups present in the triglyceride molecules. Results of such fractionation are given in Table III for one oil from each of three of the four plant families represented in Table I. *Valerianella radiata* seed oil, representing the fourth family, was compared with the oils in Table III by TLC and had approximately the same relative amounts of monovernolins, divernolins, and trivernolin as *Cephalaria joppica* seed oil.

The percentage of trivernolin found in *Euphorbia lagascae* seed oil by column chromatography is almost exactly the same as that previously determined by preparative TLC (12), and the satisfactory agreement of values in the last two columns of Table III provides further assurance of the validity of the experimental results presented. In general, the amounts of the fractions are not in agreement with predictions based on either of two mathematical distribution patterns. This unpredicted interglyceride distribution of vernoloyl is not, however, unprecedented. The already mentioned fact that vernoloyl groups in *Vernonia anthelmintica* seed oil occur almost exclusively in trivernolin molecules represents the ultimate in deviation from the two mathematical distribution patterns.

A more surprising discovery was made when the distribution of vernoloyl groups within the epoxy triglyceride fractions was determined in the same manner as indicated above for the parent oils. As shown in Table IV, the monoepoxy triglyceride fraction from *Euphorbia lagascae* seed oil presents a striking exception to the general preference of vernoloyl groups for the β-position. In this fraction the vernoloyl groups are more concentrated in the α-

TABLE IV

Distribution of Vernoloyl Groups in Fractions from Natural Epoxy Oils

Oil and fraction[a]	Monovernolin in monoglycerides, mole percent[b]	Proportion of vernoloyl groups in β-position[c]
Euphorbia lagascae		
Monovernolins	20	20
Divernolins	92	46
Oil (calcd.)[d]	74	42
Oil (found)	75	43
Crepis aurea		
Monovernolins	61	61
Divernolins	98	49
Oil (calcd.)[d]	79	51
Oil (found)	84	54
Cephalaria joppica		
Monovernolins	57	57
Divernolins	95	47
Oil (calcd.)[d]	44	54
Oil (found)	46	56

[a] See footnote a, Table III.

[b] Determined by temperature-programmed GLC of the trimethylsilylated lipolysis product of the oil or fraction (26).

[c] See footnote b, Table I.

[d] Calculated from the amounts of mono-, di-, and triepoxy triglyceride fractions (Table III) and the monovernolin content of the monoglycerides from them. For example, for *Euphorbia lagascae*, $100 \times [(0.15 \times 0.20) + (0.56 \times 0.92) + (0.19 \times 1.00)] = 74$ mole percent of vernoloyl groups in the β-position.

than the β-positions. The diepoxy triglycerides from the same oil and the mono- and diepoxy triglycerides from the other two oils fractionated do not show this anomaly. Moreover, the difference in distribution of vernoloyl groups in monovernolins is not simply related to the percentage of such groups in the parent oils because the epoxide contents of *Crepis aurea* and *Euphorbia lagascae* seed oils are about the same. The agreement between the value calculated in footnote d of Table IV and the experimentally determined monovernolin content of the monoglycerides from the whole oil substantiates the unexpected result for *Euphorbia lagascae* monoepoxy triglycerides. A similar preference for the α-positions by vernoloyl groups in monovernolins from a *Cephalocroton* seed oil has been found by another investigator (36). Like *Euphorbia*, *Cephalocroton* is a genus of the family Euphorbiaceae.

ACKNOWLEDGMENTS

Arrangements for collection and botanical identification of seeds by Quentin Jones, Crops Research Division, ARS, USDA, Beltsville, Maryland; elemental analyses by Mrs. Clara E. McGrew and her associates; some of the optical rotations by R. G. Powell; 12,13-dihydroxyoleic acid from *Euphorbia lagascae* by C. R. Smith; authentic *threo*-9,10-dihydroxystearic acid, *threo*-12,13-dihydroxyoleic acid, and *threo*-12,13-dihydroxystearic acid by M. O. Bagby; and authentic vernolic acid (for preparation of authentic methyl vernolate) by C. F. Krewson.

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[Received May 3, 1966]

Isomeric Monoethylenic Fatty Acids in Herring Oil

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ABSTRACT

Monoethylenic fatty acids from herring oil were concentrated by chromatography by chromatography on silver nitrate-silicic acid columns. Examination of consecutive fractions by open tubular gas chromatography confirmed the preferential elution of longer chain length esters and of esters within one chain length with the double bond closer to the terminal methyl group. Isomeric monoethylenic fatty acids with double bonds in the positions closer to the carboxyl group than the approximate midpoint of the even-numbered fatty acid chains could not be adequately separated by gas chromatography and were determined by ozonolysis. The isomers observed are consistent with primary formation from saturated acids through the action of an enzyme specifically removing hydrogen atoms in positions Δ^9 and Δ^{10} relative to the carboxyl group. Chain extension of particular monoethylenic isomers by two carbon atoms in the C_{20} and longer chain lengths is apparently influenced by the position of the double bond.

INTRODUCTION

A NUMBER OF MONOETHYLENIC fatty acids of various chain lengths from marine lipids have been characterized by classical procedures (1–3). With the development of recent techniques for chromatographic separation and oxidative fission, supplemented by gas chromatography, further research has indicated that mixtures of isomers would probably occur in each of the C_{16}, C_{18}, C_{20} and C_{22} chain lengths (2,4–11). The recognition of different isomers in gas chromatographic analyses has, however, not been possible with conventional packed columns, since the column efficiencies are too low. The separation of certain monoethylenic isomers of one chain length has recently been shown to be feasible with high efficiency open tubular (capillary or Golay) polar columns (12,13). The present report shows that it is possible to determine the occurrence of most, but not all, of the isomers of monoethylenic fatty acids of marine lipids in the even chain lengths through separation of the monoethylenic fatty acids as a class followed by open tubular gas chromatography. Several previously unreported fatty acids have been tentatively identified in herring oil by this procedure combined with ozonolysis.

EXPERIMENTAL

Methyl esters (ca. 200 mg) from a British Columbia herring oil (a commercial oil principally from *Clupea pallasii* Valenciennes) were subjected to column chromatography on silicic acid impregnated with silver nitrate as previously described (14). Following elution of the major part of the saturated acid esters with 4% diethyl ether in petroleum ether (40–60C bp) three further fractions (3.6, 4.7 and 10.0 ml) were usually collected for these studies. No detectable *trans* material was found in an examination of the infrared spectra of these fractions.

Open tubular gas chromatography was carried out with a column 150 ft in length and 0.01 in. I.D., coated with butanediol-succinate polyester. The apparatus was a Perkin-Elmer Model 226, operated isothermally at 170C, with an injection port temperature of 260C and helium as the carrier gas at 40 psig. Output was recorded on a Honeywell ElectroniK 16 (−0.05 to +1.05 mv) recorder, fitted with a Model 227-S Disc Instruments Inc. integrator, operated at a chart speed of 15 in./p hr. Other gas chromatographic equipment used in this work included an Aerograph A-90 (semi-preparative, SE-30 column) and an Aerograph Hy-FI with both polyester and SE-30 columns. Areas of peaks of different chain lengths were corrected to weight percent (15).

RESULTS

The three fractions usually obtained from the chromatography on silver nitrate-silicic acid columns represented a gradation from a fraction containing only a modest proportion of monoethylenic fatty acids to a fraction containing traces of saturated fatty acids but no polyethylenic fatty acids (Analysis 1, Table I). The saturated fatty acids were present in similar relative proportions in the three fractions, but the composition of monoethylenic fatty acids differed markedly for the several chain lengths, the longer chain materials eluting first as reported by Bhatty and Craig (16) and Kishimoto and Radin (17). The total recoveries of various monoethylenic fatty acids

[1] This work was carried out in partial fulfillment of MSc requirements at Dalhousie University.

TABLE I

Proportions of Monoethylenic Fatty Acids of Even Chain Lengths Recovered in Fractions from Silver Nitrate-Silicic Acid Chromatography

	Fractions from Analysis 1			Totals from this study		Totals from complete oil
	1	2	3	Analysis		analysis
Weight recovery (mg) each fraction	25	90	42			
Percent monoethylenic acids each fraction	14.8	58.6	98.2	1[a]	2[b]	(18)
16:1	.....	7.1	28.0	15.7	12.9	14.1
18:1	9.8	40.4	54.0	44.9	45.0	41.9
20:1	27.3	23.2	11.5	18.4	18.5	19.8
22:1	60.2	28.5	6.5	20.5	23.5	22.3
24:1	2.7	0.7	.....	0.5	.....[c]	1.8

[a] From 3 separate fractions, open tubular GC.
[b] From pooled fractions, packed column GC.
[c] 24:1 not determined.

were approximately in agreement with the results of a previous analysis of the esters of whole herring oil carried out by conventional means (18).

Interpretation of Gas Chromatograms

The preliminary identification of the components indicated by gas chromatography was based in part on the known isomers of monoethylenic fatty acids isolated by classical techniques (1–3). These procedures, including extensive crystallization and other purification steps, generally indicated only one component in each chain length. More reliable data (Table II) based on modern chromatographic procedures, but also usually including some concentration steps, clearly showed that at least two isomeric monoethylenic fatty acids could be expected in each chain length.

Each of the three fractions was co-chromatographed with rapeseed oil esters to identify the acids of ω9 and ω7 series[2] (13,19). A plot of log retention time against chain length was drawn based on these components (20). Additional lines then joined points for other components of presumed common *w* values, resulting in a system of lines for esters of the ω11, ω9, ω7, ω5 and ω3 monoethylenic fatty acids. The 24:1 acid was identified as the ω9 isomer both by the log plot and by the mixed chromatogram with rapeseed oil esters.

The lines in the linear log plot were all virtually parallel, although subsequent detailed study with more efficient columns has indicated that some divergence may be observed with increasing chain length and larger ω values (21). There was in the present instance very little deviation of the points from the idealized lines and the separations between lines were adequate to place the point for a 17:1 ester (largely in fraction 3, not shown in Figure 1) between the ω9 and ω7 lines in agreement with presumption of an ω8 structure (9). The average separations between lines (longer retention times divided by shorter) for the series of ratios of end carbon chains 9/11, 7/9, 5/7 and 3/5 were respectively 1.022, 1.032, 1.052 and 1.077. The progressive nonlinear increase in these *average* values is similar to those for isomers of polyethylenic fatty acids (20,22).

[2] The shorthand notation of chain length:number of double bonds is used in this paper. The addition of an "ω" value defines the double bond position through the number of carbon atoms from the center of the double bond to and including the terminal methyl group. The symbol Δ is conventionally used to indicate the position of the double bond relative to the carboxyl group where this is discussed. In monoethylenic fatty acids chain length = Δ + ω.

TABLE II

Proportions of Isomers of Monoethylenic Fatty Acids of Even Chain Lengths from Marine Sources Reported in the Literature

Fatty acid	Isomer structures	Isomer ratios	Origin of sample and reference
16:1	ω7, ω8	6:1	Menhaden oil (5)
	ω7, ω9, ω10	11:1:2	European herring oil (4)
	ω7, ω9 (trace)	?	North Sea plankton (10)
18:1	ω9, ω7	5:1	Tuna meat (7)
	ω9, ω7	2.5:1	Dogfish liver oil (8)
	ω9, ω7	1.2:1	Mullet oil (9)
20:1	ω11, ω9	1.2:1	Tuna meat (7)
	ω11, ω9	0.8:1	Dogfish liver oil (8)
	ω9, ω7	0.6:1	Mullet oil (9)
22:1	ω11, ω9	14:1	Tuna meat (7)
	ω11, ω9	4:1	Dogfish liver oil (8)
	ω11, ω9	?	Dogfish liver oil (11)

TABLE III

Proportions of Isomeric Monoethylenic Fatty Acids of Even Chain Lenghts in Silver Nitrate-Silicic Acid Chromatographic Fractions (Analysis 1) as Indicated by Open Tubular GC and in Fraction 3 by Ozonolysis

Chain length and isomer	Isomer (GC) area percent in fraction			Isomer weight percent by ozonolysis in fraction 3 only
	1	2	3	
16:1 ω9	.....	...	1.7	1.7
16:1 ω7	.....	14.7	96.5	97.0
16:1 ω5	.....	82.4	1.7	2.3
16:1 3	.....	2.9	0.1	.....
18:1 ω11	33.3	69.5	89.8	1.0
18:1 ω9				87.3
18:1 ω7	54.5	26.9	8.5	10.3
18:1 ω5	11.1	3.1	1.7	1.4
18:1 ω3	1.0	0.5	0.2	.....
20:1 ω13	7.3	29.7	66.7	1.6
20:1 ω11				65.0
20:1 ω9	83.9	66.1	30.6	29.9
20:1 ω7	7.3	3.3	2.8	2.9
20:1 ω5	1.5	0.9	.....	0.6
22:1 ω13	83.3	92.2	95.5	29.8
22:1 ω11				65.2
22:1 ω9	13.6	5.9	4.5	3.3
22:1 ω7	3.1	1.9	.....	1.7
24:1 ω9?	100	100	. .	.....

The actual values for the 7/9 and 5/7 separations are very close to those averaged from data for bacterial fatty acids separated on a Carbowax open tubular column (12,13,21).

The results of the preliminary identifications based on GC separations only (Table III) appeared reasonable in view of the data in the literature. The major isomers were qualitatively and quantitatively similar to those previously reported and the additional isomers could be fitted reasonably into known metabolic pathways for monoethylenic fatty acids (see discussion). In subsequent work of this type on the same sample, traces of 18:1ω6 have been tentatively identified by GC only (21).

The observations of Bhatty and Craig (16) that the isomers of monoethylenic fatty acids of common chain length are further subfractionated in the course of chromatography on silver nitrate-silicic acid to give an initial enrichment in isomers with the shorter end carbon chains (ωvalues) supported these identifications. This effect is clearly illustrated in Figure 1 and quantitatively assessed in Table III but since they would not be well defined on reproduction the C_{16} acids and the trace 18:1ω3 acid have been omitted from Figure 1.

Oxidative Fission Studies

Subsequent to these preliminary identifications qualitative and quantitative ozonolysis studies (Table III) were carried out on the materials of even chain length isolated from fraction 3 of Analysis 1 by semipreparative gas chromatography (23). The results from oxidative fission generally confirmed the tentative identifications based on GC alone, but with some minor differences and one major difference. The failure of ozonolysis to detect the tentatively identified 16:1ω3 and 18:1ω3 acids is probably primarily due to the structure and to the very low proportions in fraction 3. Thus in the ozonolysis product GC the esters of the product monoacid would be masked by solvents, and the ester of the diacid product would have a very inconspicuous peak with a long retention time. Conversely the recognition in fraction 3 of 20:1ω5 and 22:1ω7 by ozonolysis but not in the open tubular GC is due to the relatively minor amounts of the latter isomers in terms of the low proportion of 22:1 sample analyzed by GC (compare Tables I and III, Figure 1). The failure of of 22:1ω13 and 22:1ω11 to separate was unexpected, but close scrutiny of the peak in question failed to show any evidence of two materials both on the column used in the present study and on more efficient columns subsequently employed in detailed studies of the effect of double bond position on such separations (21). The latter work does show that there are grounds for predicting that separation will probably be very slight in the pairs 18:1ω11 and 18:1ω9, and 20:1ω13 and 20:1ω11.

Owing to the effect of subfractionation within each chain length it was possible that the

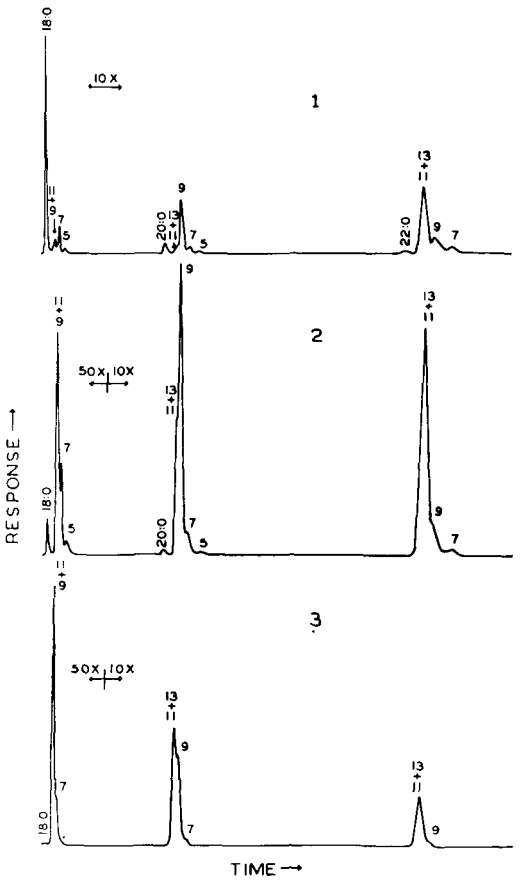


FIG. 1. Partial gas-liquid chromatograms (C_{18}-C_{20}-C_{22}) of fractions 1, 2 and 3 from herring oil methyl ester chromatography. Open tubular column 150 ft × 0.01 in I.D., BDS coated, isothermal operation at 170 C and 40 psig helium. Saturated acids are labelled as to chain length, monoethylenic peaks (excepting trace 18:1 ω3) denoted by ω values. Attenuations as marked.

22:1ω13 acid was particularly concentrated in fraction 3, but the bulk (75%) of the 22:1 acids had been in fraction 2, which was no longer available for oxidative fission. Accordingly a fresh isolation (Analysis 2) of the monoethylenic fatty acid esters of herring oil was carried out by chromatography on silver nitrate-silicic acid. The three fractions were pooled and the proportions of saturated and monounsaturated acids in the various chain lengths determined by conventional packed-column GC (Table I). Preparative GC separation of each chain length and ozonolysis were carried out as before (Table IV). This established the overall content of 22:1ω13 as about 13% of the 22:1 acids. On this basis the composition of fraction 2 in analysis 1 would be approximately 12% 22:1ω13, as compared with approximately 30% in fraction 3. This degree of enrichment appears reasonable in view of the proportions in each fraction and the nature of the isomers involved.

DISCUSSION

Analytical Techniques

The overall results of the analyses of the sample of herring oil (Table IV) by two somewhat different approaches are in reasonable quantitative agreement for the proportions of most components. The subfractionation effect of the silver nitrate-silicic acid technique (initial elution of the longer chain lengths and of the isomers with the lesser ωvalues) is particularly advantageous in the open tubular GC analyses where minor components such as 18:1ω3 are readily recognized when thus concentrated. In terms of convenience, the employment of open tubular GC alone has obvious advantages, but the failure to separate those isomers of even chain length with double bonds occurring from approximately the midpoint of the fatty acid chain to a Δ^6 position would be a handicap if such isomers were significant (21). The study of isomers of monoethylenic fatty acids of odd chain length, which are very minor components of most marine lipids, could also be largely accomplished by open tubular GC.

It should be noted that monoethylenic fatty acids with double bonds sufficiently close to the carboxyl group for interaction to occur (e.g., *trans* 16:1ω13) have increased retention times and therefore could be confused with isomers with low ωvalues (21, cf 24). This is a further limitation on the use of open tubular GC alone, and the risk of confusion is compounded by the observation that in chromatography on silver nitrate-silicic acid columns (elution with benzene in petroleum ether) the *trans* isomers precede the *cis* isomers of the same chain length (16,25). In open tubular GC on polar substrates the separation of *trans* and *cis* fatty acids with the same chain length and double bond positions may be negligible, although if such a separation occurs the *trans* isomer will appear first (21,26). In the present study, infrared analysis of the esters of whole herring oil, and of the individual fractions (Analysis 1) did not show any detectable *trans* absorption. *Trans* material of unspecified nature has been concentrated from menhaden oil (27), and appears in certain fractions from mullet oil where it is indicated that this is probably due to artifact formation during distillation (9).

TABLE IV

Proportions of Isomers of Monoethylenic Even Chain Length Fatty Acids in Herring Oil as Indicated by Open Tubular GC and Partial Ozonolysis (Analysis 1) and by Packed Column GC and Ozonolysis (Analysis 2)

Chain length and isomer	Isomers as percent of chain length		Isomers as percent of net C_{16}-C_{24}[a] monoethylenic acids	
	Analysis 1	Analysis 2	Analysis 1	Analysis 2
16:1 ω9	1.3	3.2	0.2	0.4
16:1 ω7	76.5	76.7	12.0	9.9
16:1 ω5	21.6	17.3	3.4	2.2
16:1 ω3	0.6	2.8	0.1	0.4
18:1 ω11	75.7	3.2	34.9	1.4
18:1 ω9		70.4		31.6
18:1 ω7	21.6	24.2	9.9	10.9
18:1 ω5	2.4	2.8	1.1	1.3
18:1 ω3	0.3	.....	0.1	.....
20:1 ω13	38.3	1.5	6.9	0.3
20:1 ω11		36.7		6.8
20:1 ω9	57.2	59.0	10.3	10.9
20:1 ω7	3.3	2.8	0.6	0.5
20:1 ω5	1.2	.....	0.2	.....
22:1 ω13	91.6	13.1	18.4	3.1
22:1 ω11		80.9		19.0
22:1 ω9	6.5	6.0	1.3	1.4
22:1 ω7	1.8	.....	0.4	.....
24:1 ω9?	100	.....	0.5	.....

[a] 24:1 not determined in Analysis 2.

Isomers of Monoethylenic Fatty Acid Isomers in Other Animals

Of the less well-known isomers of monoethylenic fatty acids the widespread occurrence of 18:1ω7 in animal lipids is well documented (28–31). In most lipids the proportions of 18:1ω9 and 18:1ω7 are about 10/1, although in specialized lipids of rats the ratio may be lower (29,31). The data from the literature (Table II) and from the present analysis of herring oil (Table IV) suggest that in depot fats of fish a ratio of 3/1 is typical for these two isomers.

Very little is known about the occurrence of other unusual isomers of monoethylenic fatty acids. The detailed study of fatty acids of rat lipids by Sand et al. (28) does provide evidence for the natural occurrence in animal lipids at low levels of some of the acids found in the present study. These authors report, in addition to the major C_{16} and C_{18} monoethylenic fatty acids, 14:1ω7 and 14:1ω5 (ratio of 10/1), 16:1ω10 and 16:1ω8 (cf. Table II), 18:1ω11 and 18:1ω10. From certain organs of the rat the acids recovered were 20:1ω13, 20:1ω9 and 20:1ω7, with very little 20:1ω11. The livers of fat deficient rats are also reported to contain 20:1ω9 and 20:1ω7 acids (32). These results may reflect the dietary status of the rat, since the abolition by fasting of desaturation, but not of elongation, would lower the proportion of 20:1ω11 acid (31). Other studies with rats suggest the presence of 16:1ω5 as well as 16:1ω6 and 18:1ω8 acid (31). In fatty acids of pig brain lipids 22:1ω9 and 22:1ω7 are accompanied by some 22:1ω13, but not by 22:1ω11, and the absence of 20:1ω11 (see above) should also be noted (33).

Origin of Isomers of Monoethylenic Fatty Acids

The composition distribution, rearranged in terms of proportions of isomers (Table V) shows clear trends with both chain length and ω value, yet poses a number of problems, particularly since the longer chain acids are not significant in depot fats of mammals and therefore have not received much attention. These acids are probably formed largely in the herring, since the limited literature on the fatty acid composition of zooplankton suggests that the fat of the primary food of the herring would be low in 20:1 and 22:1 acids (34). This is a generalization and may not hold in some instances (35).

It is possible to explain the various isomeric fatty acids found in the C_{16}-C_{22} even chain lengths of herring oil on the basis of one desaturation enzyme removing two hydrogens from the Δ^9 and Δ^{10} positions of saturated fatty acids. This was originally proposed by Breusch (36) and has been specifically

TABLE V

Comparison of Percent Isomers in Each Chain Length with Structure

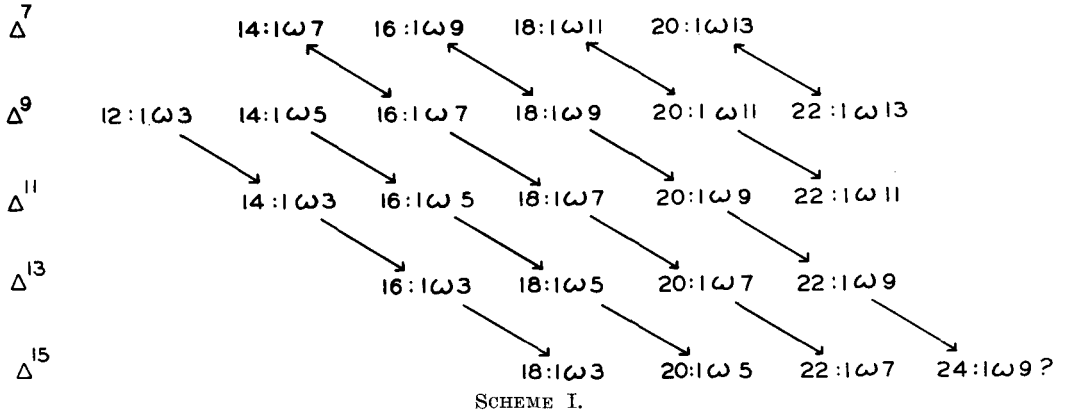
Double bond position from carboxyl	Isomer percentage in each chain length and ω value					
	Herring oil acids[a]					
	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}	C_{26}
Δ^7	1.3(ω9)	3.4(ω11)	1.5(ω13)	.....		
Δ^9	76.5(ω7)	72.3(ω 9)	36.8(ω11)	12.8(ω13)		
Δ^{11}	21.6(ω5)	21.6(ω 7)	57.2(ω 9)	78.8(ω11)		
Δ^{13}	0.6(ω3)	2.4(ω 5)	3.3(ω 7)	6.5(ω 9)		
Δ^{15}	.....	0.3(ω 3)	1.2(ω 5)	1.8(ω 7)	100(ω9)?	
	Pig brain sphingolipid acids (17)					
Δ^{13}				15 (ω 9)	.....	.....
Δ^{15}				85 (ω 7)	78(ω9)	.....
Δ^{17}				.....	20(ω7)	40(ω9)
Δ^{19}				.....	.....	49(ω7)

[a] Composite percentages from Analyses 1 and 2.

demonstrated to apply to 16:0 and 18:0 in rats (29,31). Numerous other workers in this field have discussed this concept (8,9,28,33,37,38,cf.39).

The saturated fatty acids commonly recognized in marine oils normally range from 12:0 to 20:0, and 22:0 has also been noted in concentrates but evidently occurs in very small proportions (14,40,41). The overall reaction, as shown in Scheme I conveniently accounts for the majority of observed isomers by the normal process of two-carbon chain extensions. It is stated that longer chain acids are normally completely broken down to acetate (42). However, formation of 16:1ω12 (as well as 20:1ω12) in small amounts in rats fed on 18:1ω12 clearly indicates that in addition to two carbon chain extensions there may be limited selective removal of two carbon atoms as well as complete chain breakdown (28,cf.31,43).

An anaerobic pathway operative in bacteria can produce the same isomers, including the 16:1ω5 acid (39). The specificity of the Δ^9-Δ^{10} dehydrogenation is also illustrated by the presence of 17:1ω8 and apparent absence of other 17:1 isomers in significant amounts in the fatty acids of the herring oil under study, and also in the unusual oil of the striped mullet (9). In the latter oil 15:1ω6 is also found, with only slightly less 15:1ω8. However, 19:1ω8 is the only isomer reported in the mullet oil. Since 15:0 and 17:0 are present, and 19:0 is virtually absent from this oil, the correlation proposed for the desaturation is excellent, accounting for 15:1ω6, 17:1ω8 and the absence of 19:1ω10. The 15:1ω8 and 19:1ω8 evidently arise by respective shortening and lengthening of the chain of 17:1ω8. There must be considerable metabolic activity in the saturated and monounsaturated acids of these shorter chain lengths, since 16:1ω9 must arise from 18:1ω9 and is fairly common (28,29). This may also account for the 14:1ω9 reported in addition to 14:1ω5 in a number of marine oils (1). There has recently been considerable interest in plant fatty acids with unsaturation in a Δ^5 position relative to the carboxyl group (44). It is therefore possible that 14:1ω9 or a homologous acid could be found in some unknown marine plant source, particularly as 12:1ω7 has been isolated from a marine oil (1). The contrast between the complex mixture of isomers found in the fatty acids of herring oil (Table IV) and in other oils (Table II), and the apparent simplicity of marine oils based on earlier isolations of single



SCHEME I.

fatty acids by classical techniques, suggests that speculation on the shorter chain fatty acids should be limited pending further data.

To account for the wide range of isomers of monoethylenic fatty acids in pig brain lipids an additional dehydrogenation enzyme has been suggested operating on the carbon atoms Δ^6 and Δ^7 relative to the carboxyl group (33). This could account for the 16:1ω10 reported for herring oil (4) and in lipids of the rat (28), for the 17:1ω11 accompanying 17:1ω8 in menhaden oil (27), and if operating on 15:0 and 13:0 could also be invoked to explain the 19:1ω9 and 19:1ω7 observed in minor amounts in a shark liver oil (45).

The apparent poor recovery of 24:1, as compared with the analysis of the whole oil (Table I), may be deceptive as this acid was previously estimated by difference from coincident components, and the level found was somewhat higher than found in other herring oil analyses (18). The presence of isomers other than 24:1ω9 is reported in lipids of brain tissue (17,33,46). They may also occur in the herring oil although this is the only known isomer from marine sources (1). The origin of 24:1ω9 by chain extension from 18:1ω9 has been shown in rat brain, and 20:1ω9 and 22:1ω9 were indicated as intermediate precursors (46), although direct multistep elongation from 18:1ω9 is a significant process in this particular system (cf.47). In herring oil, a depot fat, it is quite possible that the stepwise two carbon chain extension process is the principal synthetic route to 24:1.

Some support for this view is provided by the fatty acids of shark liver oils, which were reported as very rich (10%) in 24:1 (1,48). However, more recent analyses of some species indicate 24:1 levels of 1–3% varying roughly in proportion to the amount of 20:1 and 22:1 acids present (11,49,50). It will be noted that the proportion of 22:1ω9, the potential precursor to 24:1ω9, is higher relative to 22:1ω11 in dogfish liver oil than in tuna lipids (Table II) or in the herring oil under study (Table IV). Studies with rat liver enzymes have explored the conditions for the conversion of 22:1ω9 into 24:1ω9 in this animal (47).

Structure and Chain Elongation in Isomers of Monoethylenic Fatty Acids

The proportions of the isomers of different chain lengths (Table V) indicate that chain length and structure influence the probability of either two carbon chain extension or direct multistep elongation to a given end product. The presumed stoppage of chain extension at 22:1ω11 and 22:1ω13 suggests that either the proximal Δ^{11} or terminal ω11 and ω13 structures make this step less probable. The relatively high levels of 20:1ω11 and 22:1ω13 in comparison with the low levels of 20:0 and 22:0, as related to total saturates, point to these ω11 and ω13 structures as relatively inert. Monoethylenic acids with ω values greater than 11 are found in the even chain acids of pig brain lipids, but only as minor components (33). On the other hand the progressive accumulation of the Δ^{11} acids as chain length increases in the herring oil suggests that in the longer chain lengths the Δ^{11} acids are increasingly inert. The high proportion of 20:1ω11 could then be a feedback effect from accumulated 22:1ω11 due to the nonreactive Δ^{11} structure of the latter.

If Δ^{11} or ω11 and ω13 give effective blockage of chain extension in the C_{22} acids, then preferential structures might behave similarly in the C_{24} acids. It is in fact indicated by the proportions of these acids in pig brain sphingolipids (Table V) that 22:1ω9 is possibly elongated in preference to 22:1ω7, but 24:1ω7 in preference to 24:1ω9. The comparison is not specific since the monoethylenic fatty acids of longer chain length in the pig brain sphingolipids play a particular role which may not be duplicated in a depot fat and they may be of different origin (see above). The complete pig brain fatty acid analysis for C_{18}-C_{22} monoethylenic acids also shows an increasing disproportionation between ω9 and ω7 isomers in favor of increasing amounts of the latter (33). However, proportions in the C_{22} chain length for fatty acids of whole pig brain are 1.7/1, whereas in the sphingolipid fatty acids (Table V) the proportions are 0.18/1. More than one lipid system is represented in the whole brain analysis and this difference in ratios emphasizes the possibilities of different routes to a given acid in different lipids. The pig brain data, however, support the view that low "ω" values promote chain elongation.

ACKNOWLEDGMENTS

Initial demonstration of the feasibility of separating marine oil monoethylenic fatty acid isomers by L. S. Ettre and F. J. Kabot, the Perkin-Elmer Corp., Norwalk, Conn. Bursary provided by the National Research Council of Canada to J. D. Castell.

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[Received Jan. 17, 1966]

Interconversion of Fatty Aldehydes and Dimethyl Acetals at Low Temperatures

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ABSTRACT

Facile procedures are described for nearly quantitative conversion of saturated and unsaturated fatty aldehydes to their dimethyl acetals, and vice versa, at low temperatures. The methods are based on the chemical behavior of aldehydes and dimethyl acetals in 100% sulfuric acid. Under the experimental conditions described, no side reactions seemed to occur. The purity of the aldehydes and dimethyl acetals was ascertained by thin-layer chromatography, infrared spectra and other techniques.

INTRODUCTION

THE ALDEHYDE MOIETIES of plasmalogens in tissue lipids are usually characterized by gas-liquid chromatography (GLC) of their dimethyl acetals obtained by methanolysis of the lipids (1). Some investigators have found it necessary first to separate the resulting products into fatty acid methyl esters and dimethyl acetals by thin-layer chromatography (2–4) or by chemical means (5). The plasmalogen aldehydes have also been isolated as their 2,4-dinitrophenylhydrazones, and the aldehydes regenerated by the levulinic acid procedure (6).

For GLC, the free aldehydes are usually converted to the dimethyl acetals by refluxing with anhydrous methanolic HCl. The aldehydes that remain unchanged are removed by washing a petroleum ether solution of the products with a saturated solution of sodium bisulfite (1).

Analysis of dimethyl acetals by GLC has been fraught with many difficulties, including partial decomposition on the GLC columns (1,3,7). Other methods for characterization of the dimethyl acetals have therefore been used. These methods involve hydrolysis of dimethyl acetals to aldehydes, which are then converted to the methyl esters of the corresponding fatty acids following oxidation of the aldehydes to acids, or to the acetyl esters of the corresponding alcohols following reduction of the aldehydes to alcohols. The dimethyl acetals in these cases are hydrolyzed to the aldehydes with 90% acetic acid for 8 to 24 hr in a sealed ampule (5). The hydrolysis of the dimethyl acetals to the aldehydes, and their reconversion to the dimethyl acetals, is frequently used in the analysis of aldehydogenic lipids.

Reaction conditions for the complete methanolysis of aldehydes and of aldehydogenic lipids with BF_3-methanol and methanolic-HCl are rather rigorous and time-consuming (1,3). BF_3-methanol reagent is also known to cause undesirable side reactions with unsaturated compounds (8). Further, both reagents are mainly used for the conversion of aldehydes to the dimethyl acetals and not for the reverse reaction which is usually effected by prolonged hydrolysis with acetic acid as discussed before.

This paper reports a simple procedure for the conversion of fatty aldehydes to dimethyl acetals, and vice versa, in almost quantitative yields at rather low temperatures (−30C). Further, the time for reaction in either direction is only 30–40 min. The method is based on the chemical behavior of aldehydes and dimethyl acetals in 100% sulfuric acid. The reactions of fatty acid esters, and of mixtures of organic acids and alcohols in 100% sulfuric acid at low temperatures have been reported by McGinnis and Dugan (9) and Newman (10).

PROCEDURES AND RESULTS

Saturated fatty aldehydes (C_{12}, C_{14}, C_{16}, C_{18}) and C_{18} unsaturated aldehydes (oleyl, linoleyl and linolenyl) were prepared by oxidation of the mesylates of the corresponding alcohols by dimethyl sulfoxide as described by Mahadevan et al. (11). Trimerized palmitaldehyde was separated from commercial palmitaldehyde (Columbia Organic Chemicals Co., Inc., Columbia, S. C.) by preparative thin-layer chromatography (TLC) as described by Tuna and Mangold (12) and used as control. One hundred percent sulfuric acid was prepared by mixing appropriate amounts by weight of 96% sulfuric acid and 20% fuming sulfuric acid, as described by Newman (10).

For the analysis of aldehydes and dimethyl acetals by TLC, glass plates (20 × 20 cm) were coated as usual with Silica Gel G to give a layer approximately 250 μ in thickness and activated at 110C for 1 hr. The samples were applied in petroleum ether solution and the plates were developed with toluene. The spots were visualized by charring after spraying with 50% H_2SO_4.

GLC analysis of the aldehydes and dimethyl

acetals were carried out with a Beckman GC-2A gas chromatograph equipped with a hydrogen flame detector and a ¼ in. × 12 ft aluminum column packed with 20% β-cyclodextrin acetate on Gas-Chrom R, 30–60 mesh, at 230C. The carrier gas was helium.

Infrared spectra were taken with a Perkin-Elmer Model 21 double-beam infrared spectrophotometer.

The reactions and results were essentially the same in all cases and, therefore, in what follows, only the conversions of an unsaturated and a saturated aldehyde, linoleyl aldehyde and palmitaldehyde, to their dimethyl acetals and the latter's hydrolysis back to the free aldehydes, are described in detail.

Conversion of the Aldehydes to Their Dimethyl Acetals

Linoleyl aldehyde, 50 mg, was dissolved in 20 ml of dry peroxide-free ethyl ether in a 125 ml Erlenmeyer flask. The flask was stoppered after flushing with nitrogen and placed in a dry ice-acetone bath. The contents of the flask were agitated with a magnetic stirrer. When the contents reached −30C, 2 ml of 100% sulfuric acid was added, after which the bath temperature was allowed to rise to 0C in about 10 min. The bath temperature was again lowered to −30C and 25 ml of absolute methanol was added. After stirring for about 2 min, 20 ml of 35% methanolic KOH was added. A gelatinous precipitate formed which interfered with magnetic stirring and, therefore, the flask was swirled by hand while being kept in the cold bath until the nearly solid white mass changed to a milky liquid. The flask was removed from the bath and magnetic stirring was resumed and continued for 15 min. The contents were then transferred quantitatively to a separatory funnel in 300 ml H_2O. Nitrogen was kept bubbling through the mixture. The dimethyl acetal was extracted with three 25 ml portions of ethyl ether, and the ether extracts combined and washed with water until neutral and then dried over anhydrous sodium sulfate. Evaporation of the ether yielded 50 mg of the product (84% theory).

By the same procedure, 50.8 mg of palmitaldehyde yielded 49.6 mg of its dimethyl acetal (82% theory).

Analysis of the products by TLC (Fig. 1) yielded single spots and their R_f values were identical with those of the dimethyl acetals prepared by other methods (1,13). Since the R_f values differ little among the aldehydes and the dimethyl acetals during adsorption TLC, only the TLC behavior of linoleyl aldehyde and its dimethyl acetal is shown in Figure 1. Figure 2 shows the IR spectra of the dimethyl acetals and the aldehydes. Bergmann and Pinchas (14) found that the dimethyl acetals of low molecular weight aliphatic aldehydes possessed characteristic bands in the regions 1158–1190 cm^{-1}, 1124–1143 cm^{-1}, 1063–1098 cm^{-1} and 1038–1056 cm^{-1}. These bands are also seen in dimethyl acetals of linoleyl aldehyde and palmitaldehyde. Bands characteristic of the aldehyde group, 2700 cm^{-1} and 1730 cm^{-1} were absent.

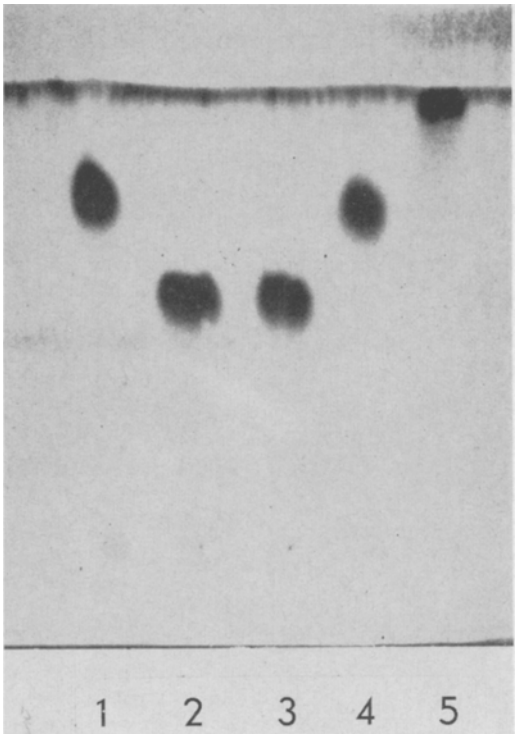

FIG. 1. Thin-layer chromatography of linoleyl aldehyde, its dimethyl acetal and reference compounds: 1) linoleyl aldehyde; 2) linoleyl aldehyde dimethyl acetal obtained from 1 by the present method; 3) linoleyl aldehyde dimethyl acetal standard; 4) linoleyl aldehyde obtained from compound No. 2 by the present method; 5) trimerized palmitaldehyde.

Conversion of the Dimethyl Acetals to the Aldehydes

The dimethyl acetal of linoleyl aldehyde, 40 mg, was dissolved in 20 ml of peroxide-free ethyl ether and subjected to the procedure described above, up to and including the addition of 2 ml of 100% sulfuric acid. The mixture was then stirred for 10 min at 10C and poured over crushed ice. The liberated free aldehyde was extracted thrice with 25 ml portions of

ethyl ether, the ether extracts were combined and washed successively with 5% sodium bicarbonate solution and water, and dried over anhydrous sodium sulfate. The ether was evaporated, leaving 31 mg of the aldehyde (92.3% theory).

By the same procedure, 50 mg of palmitaldehyde dimethyl acetal yielded 38.6 mg of the aldehyde (92% theory). Analysis of the products by TLC (Fig. 1) showed that the aldehydes were free of the dimethyl acetals and trimerized products. The infrared spectra of the aldehydes (Fig. 2) show bands characteristic of the aldehyde group.

DISCUSSION

The saturated and the unsaturated aldehydes used in this study were converted to the corresponding dimethyl acetals in approximately 80–85% yield and the dimethyl acetals to the corresponding aldehydes in 90–93% yield. The procedures described here afford a quick and convenient means for the conversions of the aldehydes and the dimethyl acetals at relatively low temperatures. The reaction in either direction can be completed in the course of an hour. The use of low temperatures helps to prevent oxidative changes. The procedures in all cases yielded essentially pure products as judged by TLC, GLC and infrared spectra.

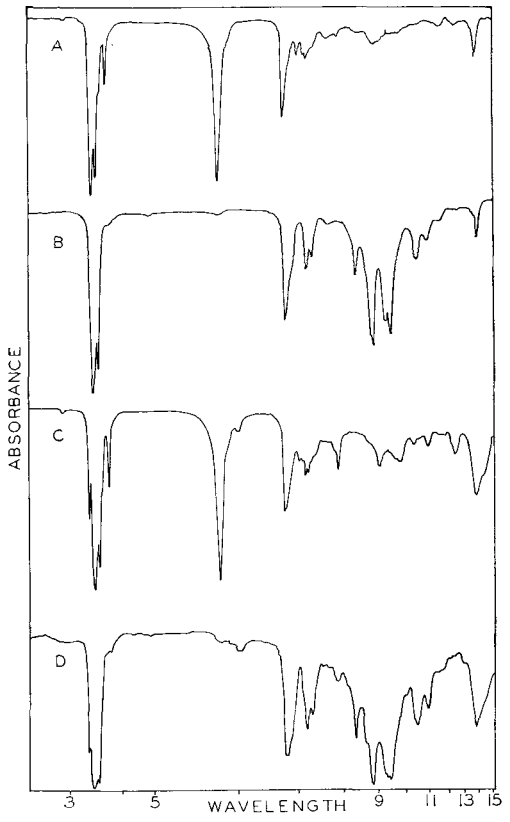

FIG. 2. Infrared spectra of palmitaldehyde, linoleyl aldehyde and their dimethyl acetals: A) palmitaldehyde; B) palmitaldehyde dimethyl acetal; C) linoleyl aldehyde; D) linoleyl aldehyde dimethyl acetal. Solution spectra of A-C in 0.1 mm cell using 10% solutions in CS_2 (2.0 to 4.2 μ, 5 to 6.1 μ, 7.2 to 15.0 μ) and in tetrachloroethylene (4.2 to 5.0 μ and 6.1 to 7.2 μ). Spectrum of D was obtained as liquid film between salts.

Although sulfation of double bonds in unsaturated fatty acids is known to occur with concentrated sulfuric acid, such reactions do not seem to occur with the unsaturated aldehydes or their dimethyl acetals under the present experimental conditions. This was evidenced by complete absence of any spots on the TLC plate at or near the origin (Fig. 1). Being highly polar, sulfated compounds and their hydrolytic products can be expected to remain near the origin. After a complete reaction cycle involving conversion to the dimethyl acetals and their reconversion, the original unsaturated aldehydes were recovered in good yields and unchanged as shown by TLC and infrared spectra. With the relatively low temperatures and with the large excess of diethyl ether, sulfation may not occur with the species, $(C_2H_5)_2OH^+$ OSO_3H^-, present.[1] It is pertinent to point out that in their studies on the analysis of fatty acid composition of highly unsaturated fats using methyl esters prepared by this method, McGinnis and Dugan (9) found no apparent changes in the fatty acids and their results compared favorably with other methods.

The presence of aldehydes as impurities in dimethyl acetals, and vice versa, may be detected by TLC. The aldehydes invariably are found to have a higher R_f value than the dimethyl acetals. On hydrolysis, dimethyl acetals yield the aldehydes as monomers. No trimerized products were detected by TLC. The difference in the R_f values between the aldehyde and its trimer was large enough to detect the latter as an impurity in the aldehyde (Fig. 1). Further, the aldehydes gave single yellow spots with the 2,4-dinitrophenylhydrazine reagent (15) and possessed the same R_f values as those of the original aldehydes. The reference trimer aldehyde also yielded a yellow spot with this reagent but had a higher R_f value than the monomer.

The acid-catalyzed reaction between the aldehyde and the methanol to form the correspond-

[1] Suggested by the reviewer.

ing dimethyl acetal and the latter's hydrolysis to the aldehyde proceed according to the following:

$$RCHO \underset{H_2O,H^+}{\overset{CH_3OH,H^+}{\rightleftharpoons}} RCH(OH)OCH_3 \underset{H_2O,H^+}{\overset{CH_3OH,H^+}{\rightleftharpoons}} RCH(OCH_3)_2$$

Although aliphatic aldehydes were reported to react with methanol without acid catalyst to form their hemiacetals, as shown by UV-spectroscopy (16), their preparation and properties have not been described in the literature. However, the hemiacetals of glucose, methyl α-D-glucopyranoside and its anomer have been characterized by their infrared absorption bands at 844 cm^{-1} and 891 cm^{-1}, respectively (17). The infrared spectra of the dimethyl acetals of the aldehydes prepared by the present technique show no absorption in this region. On the other hand, their spectra are identical with those of the same compounds prepared by methods described in the literature and show characteristic bands of acetals described by Bergmann and Pinchas (14). The infrared spectra of the aldehydes and the dimethyl acetals show no absorptions at 1665 cm^{-1} and 1175 cm^{-1} attributed to the $-CH=CH-O-Me$ group (18) resulting from the demethanolization of the dimethyl acetal or dehydration of the hemiacetal intermediate. The slight absorption in this region seen in linoleyl aldehyde and its dimethyl acetal is due to *cis*-double bonds present in these compounds. This band is virtually absent in the saturated compounds, palmitaldehyde and its dimethyl acetal.

The detection of aldehyde impurities in dimethyl acetals by GLC is not always feasible. Our studies on the behavior of fatty aldehydes and their dimethyl acetals on various stationary liquid phases used in GLC have shown that in EGS columns, for example, both the aldehydes and the dimethyl acetals have identical retention times whereas in other columns a well-defined separation is effected (19). The differences in retention times of the aldehydes and their corresponding dimethyl acetals on the β-cyclodextrin acetate column used in this study were large enough to distinguish them unequivocally. Further, compounds in both classes gave single peaks and no decomposition products when chromatographed on this column.

TLC did not reveal the presence of any corresponding acids in the aldehydes regenerated from the dimethyl acetals according to the procedure described. UV spectroscopy showed no conjugation in the linoleyl or linolenyl aldehydes, nor in the dimethyl acetals, nor did any *cis* to *trans* isomerization occur according to the IR spectra.

The low temperature method for the methanolysis of plasmalogen containing lipids to yield simultaneously mixtures of dimethyl acetals of fatty aldehydes and methyl esters of fatty acids and for the hydrolysis of the separated dimethyl acetals is being used in our laboratories for the analysis of the aldehydes and will be described elsewhere.

ACKNOWLEDGMENT

Supported in part by Grant HE 02772 from USPHS National Heart Institute. Assistance in the determinations of infrared spectra by Jacques Chipault and Werner Deutsch.

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[Received May 16, 1966]

Thermal Reactions of Methyl Linoleate. I. Heating Conditions, Isolation Techniques, Biological Studies and Chemical Changes

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ABSTRACT

Methyl linoleate, diluted with an equal weight of methyl laurate, was heated without exclusion of air at 200C for 200 hours. The reaction mixture was separated by means of molecular distillation, urea adduction, column chromatography, and gas chromatography. Cyclic and aromatic materials were detected in the nonurea adductable monomer fractions. The dimer was separated into polar and nonpolar fractions. Analytical data for the nonpolar dimer are consistent with a cyclic Diels-Alder product. Bioassays showed the nonadductable monomer, the polar dimer, and a fraction of intermediate boiling point to be toxic when administered to weanling male rats. Urea-adductable fractions, nonpolar dimer, and polymer were not toxic. The concentrations of the toxic components were so low that the heated linoleate, before fractionation but after removal of the laurate, was not toxic.

INTRODUCTION

IN RECENT YEARS papers have appeared in the literature reporting observations of toxic effects attributed to heated fatty materials (1–7). These reports have tended to be confusing and contradictory because of differences in starting materials, heating procedures, and effects observed. In some of these studies the fatty materials were severely oxidized, strongly heated, or both. In some cases (1,4,7), the materials formed during abusive treatments were partially fractionated, and then assayed for biologic activity in the weanling rat. The various fractions separated in these studies were mixtures of compounds, and it was not possible to attribute a specific biologic response to a specific compound or group of compounds. In other cases (8–11), heated or oxidized fatty materials were prepared, and chemical compounds were isolated from them, but biological studies were not reported.

The development of an understanding of lipid chemistry in the greatest possible detail requires a complete investigation of the chemical and biological properties of the reaction products formed from fatty materials when heated even under conditions far removed from conditions of practical usage. It is important, of course, that results of such studies not be applied indiscriminately to all situations in which fatty materials are subjected to the action of heat or air. Indeed, there is a sizeable body of literature (12–21) indicating that fats do not undergo nutritionally significant changes when subjected to the heat or oxidation incidental to normal processing and cooking operations. Rather they must be treated by artificially abusive laboratory procedures before they manifest toxicity when fed to animals.

An earlier report (10) from our laboratories dealt with the isolation and characterization of an ester of 11-(2′-methylcyclohex-2′-enyl)undec-9-enoic acid, formed by reacting linseed oil under the anaerobic conditions described by Crampton et al. (1). For the more recent study, of which this paper is a partial report, methyl linoleate was heated under conditions similar to those used by Firestone et al. (22) for the production of toxic materials from cottonseed oil. Firestone et al. found that cottonseed oil was virtually unchanged after heating at 205C in vacuo for 300 hr. However, when it was heated at the same temperature for 40 eight-hour days in an open beaker, there were chemical changes in the oil. Dimers and urea filtrate monomers were separated from the heated oil and found to be toxic.

It seemed not unlikely that the toxic substances detected by Firestone et al. in their cottonseed oil after heating were derived from linoleate. Therefore, we decided to study the chemical changes which take place during the heating of methyl linoleate. The use of methyl ester as starting material made it unnecessary to convert a triglyceride to a distillable derivative after the heating period, with the attendant risk of artifact production. The use of methyl linoleate, rather than a mixture of unsaturated fatty esters in some naturally occurring ratio, promised to simplify the composition of the mixture of products formed on heating, as well as to make the interpretation of results more straightforward. The methyl linoleate was diluted with methyl laurate to avoid excessive viscosity build-up during the heating. It was

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assumed that any transformations of the methyl laurate which might take place under the heating conditions used would be negligible compared to changes in the methyl linoleate.

After the heating period, products were separated by molecular distillation, urea adduction, column chromatography, and gas chromatography. Various fractions were chemically characterized and were bioassayed in a weanling rat acute toxicity test.

EXPERIMENTAL

Methyl esters were prepared from safflower oil by transesterification with methanol and sodium methoxide. To concentrate the linoleate, the mixture of methyl esters was treated with urea in acetone solution four times at 0C and twice at −40C (1). The unadducted methyl linoleate was distilled at 147–150C/0.5 mm. Analysis by gas chromatography showed only linoleate except for 0.25% oleate. Methyl laurate (Matheson, Coleman, and Bell) was purified by distillation (125C/2 mm) before use. The esters contained neither natural nor synthetic antioxidants.

Nine hundred grams each of methyl linoleate and methyl laurate were placed in a 3 liter beaker and heated at 200 ± 3C for 200 hr. The beaker was lightly covered with a watch glass to retard evaporation of the methyl esters; even so it was necessary occasionally to add small amounts of methyl laurate to restore the original volume in the beaker. The smaller quantity of methyl linoleate evaporated was not replaced. Possibly part of the apparent loss of material resulted from loss of volatile oxidation fragments.

After the heating period, the methyl laurate was distilled from the mixture at temperatures up to 115C at 1 mm. The residue from this distillation was then fractionated by molecular distillation at successively higher temperatures in a "Rota-Film" (Asco 2)[1] or a Nester-Faust[2] spinning band molecular still. The various distillation and urea adduction steps applied are outlined in Figure 1.

The monomeric urea filtrate fraction (Filtrate I) and the dimer fraction (Distillate V) were fractionated further by column chromatography on silicic acid (23), Florisil (24), or silicic acid impregnated with silver nitrate (25,26). These chromatographic fractionation steps are outlined in Figures 2 and 3.

Gas chromatographic separation of the monomer and dimer fractions was carried out

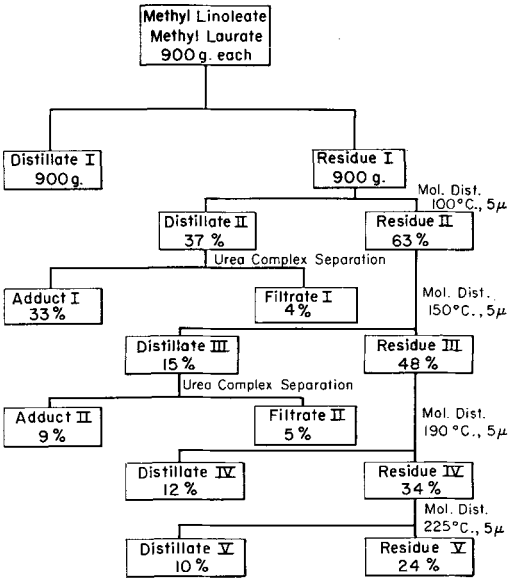


FIG. 1. Distillation and urea adduction of methyl laurate—methyl linoleate mixture after heating at 200C.

on an Aerograph[4] Model A-90-P instrument with a thermal conductivity detector. The monomeric fractions, both adductable and non-adductable, were analyzed on a 10-ft × 3/16 in. I.D. copper column packed with 10% (w/w) ethylene glycol adipate polyester on 80–100 mesh acid-washed Chromosorb W. The following operating conditions are representative: helium pressure 40 psi; flow rate 75 ml per min; oven temperature 205C; injection temperature 295C; detector temperature 350C. The dimer fractions and oxygenated long chain esters were analyzed on a 6-ft × 3/16 in. I.D. stainless steel column packed with 25% silicone rubber SE-30 on 60–80 mesh acid-washed Chromosorb W. The column temperature varied between 200–350C, depending on the type of sample being assayed.

Molecular weights were determined in a Mechrolab Osmometer No. 302.[5] Viscosities were measured with an Oswald-Fenske viscometer. Wijs iodine values, peroxide values, saponification values, and acid values were determined either according to AOCS methods or by modification of these for semimicro analyses. Carbonyl oxygen was determined by a modification of the method of Knight and Swern (27). Hydroxyl values were determined by using acetic anhydride as the acylating agent (28).

[1] A. F. Smith Co., Rochester, New York.
[2] Nester-Faust, Newark, Delaware.

[4] Varian Aerograph Co., Walnut Creek, California.
[5] Mechrolab Instrument Co., Mountain View, Calif.

TABLE I

Gas Chromatographic Analyses of Linoleate and Laurate Mixture and Isolated Monomer Fraction

Identification	Unheated mixture of linoleate and laurate %	Heated mixture of linoleate and laurate %	Distillate II %
C^{12}	48.5	72.2	2.5
C^{14}	0.2	0.2	0.7
C^{16}	....	.....	0.4
$C^{16}=1$	.....	... ..	0.1
C^{18}	.....	0.7	0.3
$C^{18}=1$	0.7	0.7	4.1
$C^{18}=2$	50.6	26.2	89.3
C^{20}			2.6

The heated methyl linoleate and the isolated fractions from it were bioassayed by an acute toxicity method using weanling male Simonsen rats of Sprague-Dawley origin. The animals were distributed randomly into groups of five each on the basis of litter and body weight (50–60 g). Purina Labena and water were supplied ad libitum. Each rat was given 0.5 ml of the designated test material per day for three consecutive days by stomach tube. A 2 ml tuberculin syringe fitted with a blunt-tipped 17 gauge needle and a number 8 French catheter was used for the forced feeding. The animals were observed closely for overt symptoms and diarrhea. Daily body weights were recorded. All rats were autopsied, including those that died during the experiment and those sacrificed after an observation period of ten days. The thymus gland was removed and weighed, because excess atrophy of this organ is a measure of nonspecific stress.

RESULTS

Heating the mixture of methyl linoleate and methyl laurate as described resulted in a reduction in the content of linoleate (Tables I, II, and III).[6] Although the content of methyl laurate (as measured by gas chromatography) appeared to have increased, this must be partially attributed to extra methyl laurate added during the heating, as well as to the conversion of some of the methyl linoleate to materials of low volatility which did not pass through the gas chromatography column under the conditions used. Hence, the values given in the second column of Table I represent the composition, not of the entire mixture of heated esters, but only of the volatile portion of the final mixture. Vacuum distillation removed the methyl laurate (Distillate I in Figure 1). Analytical values on the distillate suggested that small amounts of other materials, possibly oxidative scission products, were removed with the methyl laurate. The residue from this original pot distillation was fractionated further by molecular distillation. Urea adduction of the molecular distillates separated straight-chain from branched-chain or cyclic materials. The monomeric urea filtrate (Filtrate I) amounted to 4% of the original methyl linoleate.

The infrared spectrum of Filtrate I showed, among others, bands at 5.73μ, 10.3μ, 13.8μ, and a relatively weak band at 13.3μ, which were assigned to ester, *trans* double bond, tetramethylene, and possibly 1,2-disubstituted benzene respectively.

The further fractionation of urea Filtrate I

TABLE III

Chemical Analyses of Dimer Fractions

Analysis	Fraction DVB	DVC2
Carbon %	77.0	72.9
Hydrogen %	11.0	10.4
Oxygen %	10.5	16.7
Saponification value	200.0	205.0
Iodine value	84.8	67.3
Carbonyl value	4.5	28.3
Hydroxyl value	.....	35.4
Peroxide value	.....	22.0

TABLE II

Analyses of Methyl Esters Obtained by Fractionation of Thermally Abused Methyl Linoleate

Sample	Molecular weight	Viscosity (centipoise 25C)	Peroxide value	Carbonyl value	Hydroxyl value	Acid value	Sapon. value	Iodine value[a]
Unheated mixture of linoleate and laurate	.....	4.00	1.45	0.26	.....	.....	.....	.....
Heated mixture of linoleate and laurate	....	8.86	.....	6.59	.....	.... .	... ..	.....
Distillate I	218	3.36	.....	4.6	34.4	3.8	260	5.2 (0)
Adduct I	355	5.14	573.0	17.3	0	27.5	174	108.1 (1.5)
Filtrate I	250	13.60	19.6	19.2	23.5	5.4	201	94.3 (1.0)
Adduct II	453	22.90	744.0	28.4	9.2	13.1	256	38.6 (0.7)
Filtrate II	440	40.82	8.5	29.1	7.5	11.3	196	73.6 (1.3)
Distillate IV	586	246.50	46.0	19.6	8.5	8.9	208	70.0 (1.6)
Distillate V	658	.....	6.2	22.4	12.2	11.0	200	72.1 (1.8)
Residue V	1122	4352.0	6.8	21.4	34.4	5.3	205	78.0 (3.5)

[a] Values in parentheses represent the average number of double bonds based upon molecular weight.

[6] Much of the analytical data in Tables I-III are not discussed in detail in this report, but are presented as reference material for future publications in this series.

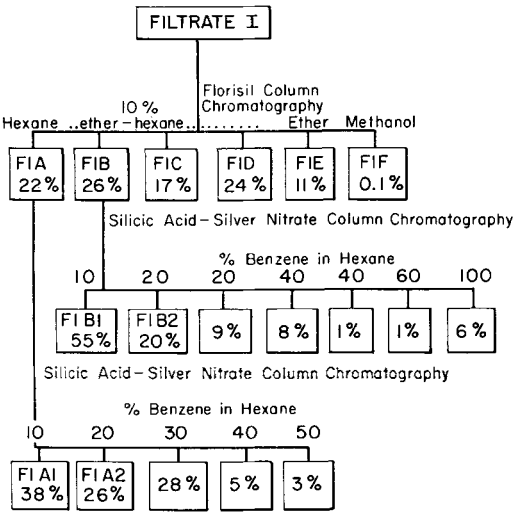


FIG. 2. Column chromatography of distillable nonurea adductable fraction.

is outlined in Figure 2. Much of the material required polar solvent for elution from Florisil. However, the substituted benzene derivatives, the presence of which had been suspected from the infrared spectrum, were eluted from Florisil in the relatively nonpolar fractions FIA and FIB, and were concentrated further in the fraction eluted from silicic acid—silver nitrate with 10% benzene in hexane. The infrared spectra of fractions FIA1 and FIB1 showed strong absorbance at 13.3μ, and also bands at 6.2μ, and 6.7μ, which are indicative of aromatic compounds. A band at 10.3μ suggesting *trans* double bonds was present also. Gas chromatograms confirmed that both of these fractions of distillable nonurea adductable material (DNUA) contained at least two major components. Fractions FIA2 and FIB2, eluted from silicic acid—silver nitrate with 20% benzene in hexane (Fig. 2), showed infrared absorption bands at 3.3μ, 6.0μ and a weak shoulder at 10.3μ, which could be assigned to compounds containing double bonds with only a trace being *trans* isomers. These findings are appropriate for compounds containing an unsaturated ring formed by internal addition across one of the double bonds of the linoleate molecule.

Residue II (Fig. 1) was fractionated further by molecular distillation at 150C and 5μ and the distillate (Distillate III) was analyzed by infrared. The spectrum included bands at 5.72μ, 10.11μ, 10.3μ, and 13.8μ, which were assigned to ester, *trans,trans* conjugated double bonds, *trans* double bond and tetramethylene respectively.

Distillate III was treated with urea to separate straight-chain from branched-chain or cyclic compounds. The adduct fraction had a much higher peroxide value and a lower iodine value than the filtrate fraction. It is not improbable that at least part of the peroxide was formed during the isolation process. Both of these fractions were quite complex and had a higher average molecular weight than the lower boiling filtrate (Filtrate I). Gas chromatography of Filtrate II indicated that it was probably a mixture of monomer and dimer.

Further molecular distillations at increasing temperatures produced Distillates IV and V. Chemical analyses of these fractions indicated that they were largely dimeric in nature. Distillate V was separated further by column chromatography on silicic acid (Fig. 3). Chemical analyses of its subfractions DVB and DVC2 are shown in Table IV. The infrared spectrum of fraction DVB showed bands at 5.72μ, 10.3μ and 13.8μ, while fraction DVC2 showed a band at 5.8μ which indicated that more than one carbonyl function was present in the mixture.

In the acute toxicity test, fractions were judged to be nontoxic when rats dosed with them did not differ appreciably from control animals in body weight or thymus weight after ten days. Dosing with toxic materials usually resulted in the death of one or more animals within three days, and the survivors, if any, showed marked depression in body and thymus weights when sacrificed at ten days. Affected rats often suffered severe diarrhea during the test period. The results of the toxicity tests are shown in Table IV. Note that toxicity was confined to the lower-boiling nonurea adduct-

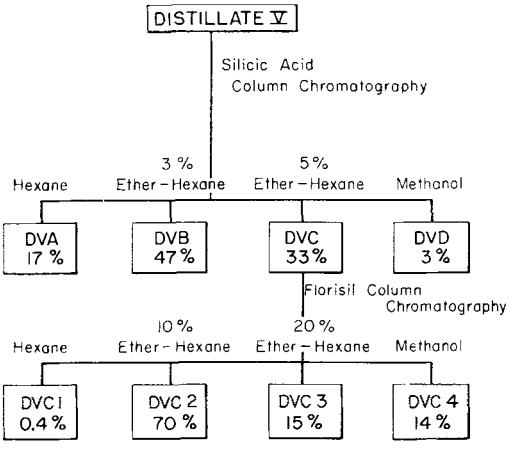


FIG. 3. Column chromatography of high-boiling distillate from heated methyl linoleate.

TABLE IV

Determination of Toxicity of Various Fractions[a]

Nontoxic	Toxic
Adduct I	Filtrate I (DNUA)
Adduct II	Filtrate II
Residue V (Polymer)	Distillate IV (Dimer)
DVA (Dimer)	Distillate V (Dimer)
DVB (Dimer)	DVC2 (Dimer)
DVC3 (Dimer)	DVD (Dimer)
DVC4 (Dimer)	

[a] 0.5 ml fed by stomach tube to weanling rats (50–60 g) for 3 consecutive days.

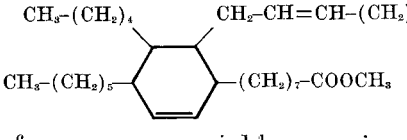
able fractions and the more polar dimer fractions. The straight-chained monomeric material, the nonpolar dimers, and the polymers were innocuous.

DISCUSSION

The chemical changes which occurred in the methyl linoleate during severe heating appear to have resulted from a combination of thermal and oxidative processes. Earlier work of Paschke et al. (29,30) suggested that cyclic monomers are formed during the thermal polymerization of methyl linoleate. Mehta and Sharma (31) obtained a monomeric ester from thermally polymerized safflower oil, and presumed that it was a cyclic product from methyl linoleate. The present work indicates that cyclic and aromatic monomers are formed under the conditions described above, and that such compounds may be partially responsible for the toxicity which has been observed by previous workers.

The presence of aromatic monomers in the reaction mixture of a thermally abused fatty material has not been reported previously. The mechanism of their formation may involve a proton extraction followed by ring closure, and subsequent transhydrogenation to form the aromatic ester. The isolation, characterization, and syntheses of the aromatic compounds will be reported in a subsequent paper.

After the separation of dimeric material into fractions of varying polarity, it was generally found that the nonpolar fractions were innocuous to rats under the test conditions used. Analytical data for the nonpolar dimer are consistent with the structures proposed by Paschke et al. (29,30), of which the following structure is representative. This structure is,



of course, accompanied by many isomers which differ from it in the lengths of the side chains, the positions of the double bonds, and the orientation of the hydrocarbon and ester chains on the ring.

The polar dimer material contains more oxygen than the nonpolar dimer, and has a lower iodine value. The analytical data lend support to the belief that it is a mixture of compounds containing hydroxy, peroxy, and/or carbonyl groups. Gas chromatography was of no value in separating the polar dimeric compounds, because no distinct peaks were found at temperatures up to 350C. The components probably decomposed at such temperatures.

Toxicity studies were in good agreement with those reported by Kaunitz et al. (32–34). The monomeric urea filtrate was toxic when administered to weanling rats at a level of 25 g/kg. The nonadductable portion of the intermediate-boiling fraction was also toxic when administered at the same level. The dimer was found to be nontoxic, but after fractionation, the oxygenated polar portion of the dimer mixture was toxic when administered at the 25 g/kg level, while the nonpolar portion showed no evidence of toxicity.

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[Received March 28, 1966]

Thermal Reactions of Methyl Linoleate. II. The Structure of Aromatic C_{18} Methyl Esters

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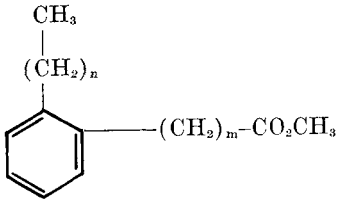
ABSTRACT

This second report describes the characterization of C_{18} aromatic esters from the heated linoleate and the independent synthesis of two of them. The esters were isolated by a combination of molecular distillation, urea adduction, column chromatography, and gas chromatography. They were characterized by infrared, ultraviolet, NMR, and mass spectroscopy. The analytical data for the isolated esters were compared with the data for the synthetic esters, methyl 11-(2′-methylphenyl)undecanoate, methyl 7-(2′-pentylphenyl)heptanoate, and methyl 8-(2′-butylphenyl) octanoate. The latter two compounds were found to be components of the aromatic fraction isolated from heated linoleate, and their synthesis is described in detail.

INTRODUCTION

METHYL LINOLEATE was heated at 200C for 200 hr and the reaction mixture was separated by a combination of molecular distillation, urea adduction, column chromatography, and gas chromatography (1). As reported previously, a fraction whose infrared spectrum showed it to be rich in aromatic compounds was concentrated from the urea filtrate monomers by column chromatography. The quantity of this fraction isolated corresponded to about 0.6% of the methyl linoleate heated. This fraction was further purified by preparative gas chromatography. The analytical data were consistent with the empirical formula, $C_{17}H_{27}CO_2CH_3$. The NMR, mass spectroscopy, and infrared data indicated that the material was a mixture of isomers different from methyl 11-(2′-methylphenyl)undecanoate (I) described by Hutchinson and Alexander (2). On this basis the following structure was proposed:



where n = 3–4 and n + m = 10

The two compounds postulated to be present in the mixture, methyl 7-(2′-pentylphenyl) heptanoate (IIa) and methyl 8-(2′-butylphenyl)octanoate (IIb), were independently synthesized. Figure 1 is an outline of the synthetic scheme, which comprised the following steps. Butyl bromide was allowed to react with magnesium, and the resulting Grignard reagent was treated with cadmium chloride to give dibutyl cadmium (IIIa). 2-Bromobenzoyl chloride was added to the cadmium reagent to yield a ketone (IVa) which was reduced by the Haung-Minlon (3) modification of the Wolff-Kishner reaction to 2-amylbromobenzene (Va). The bromide was converted to the corresponding cadmium reagent (VIa) which, upon interaction with the ester chloride of pimelic acid (VIIa) (4) afforded the keto-ester (VIIIa). The keto-ester was reduced to the acid by the Wolff-Kishner reaction. The acid was esterified by the Fisher procedure (5) to give methyl 7-(2′-pentylphenyl)heptanoate (IIa). Methyl 8-(2′-butylphenyl)octanoate (IIb) was made by following the same procedure but using propyl bromide and suberic acid in place of butyl bromide and pimelic acid. The aromatic esters were hydrogenated to cyclohexane derivatives (IXa, IXb) with a platinum oxide catalyst and a trace of acid (6). The mixture of esters isolated from heated methyl linoleate was similarly hydrogenated. Comparison of the physical properties of the synthetic material IIa and b, and IXa and b, with the properties of the isolated material before and after hydrogenation, respectively, confirmed that the isolated esters consisted largely of IIa and b, with possibly small traces of IIc.

The synthesis of methyl 11-(2′-methylphenyl) undecanoate was reported previously (2,7). Comparison of its properties with those of the isolated mixture showed that the mixture contained none of this substance.

EXPERIMENTAL

Isolation of Aromatic Esters

The preparation of the methyl linoleate, the heating conditions, and the isolation procedures used in this investigation were described previously (1). The fractions from the silicic acid—silver nitrate columns which were richest

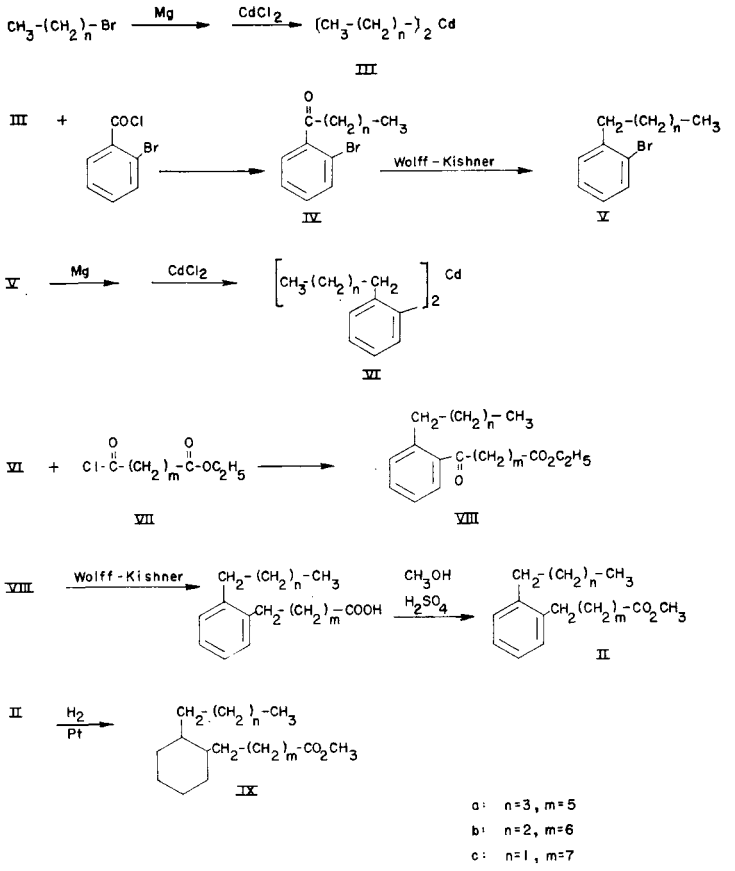


FIG. 1. Syntheses of aromatic esters.

in aromatic compounds were combined. The aromatic components were purified by preparative gas chromatography. The materials collected from the gas chromatograph were again injected, fractionated, and collected until the final product showed only one peak upon reinjection.

The instrument used for the gas chromatography was an Aerograph Model A-90-P. The column was a 3/8 in. (O.D.) × 5 ft copper tube packed with Chromosorb W (30–60 mesh) containing 25% ethylene glycol adipate. The following operating conditions are representative: helium pressure 60 psi; helium flow rate, 200 ml/min; oven temperature 205C; injection temperature 295C; detector temperature 350C; injection volumes 50–100 μl. The aromatic component of the mixture had a retention time of about 70 minutes. The material was collected in a 16-mm tube containing glass wool wet with methanol.

Absorption bands were observed in the infrared spectrum of the isolated material at the following wavelengths: 3.21, 6.22, 6.70 μ (aromatic); 13.34 μ (1,2-disubstituted benzene); 5.72, 8.50 μ (ester); 13.75 μ (tetramethylene). The ultraviolet spectrum showed absorption maxima at 263, 267, and 270 mμ; this spectrum was consistent with an *ortho* substitute benzene (8). The NMR spectra were obtained on a Varian A-60 spectrometer. The NMR spectrum gave the following τ values: 3.02 ppm (aromatic protons); 6.40 ppm ($-OCH_3$); 7.20–8.00 ppm (α-methylene protons);[1] 8.20–8.90 ppm (methylene protons); 8.90–9.30 ppm (terminal methyl protons).

Anal. Calc. for $C_{19}H_{30}O_2$: C, 78.6; H, 10.4. Found: C, 78.2; H, 10.7.

2-Amylbromobenzene (Va)

To a suspension of 7.5 g (0.30 g-atom) of magnesium and 20 ml of dry ether was added slowly with stirring 52 g (0.37 mole) of *n*-butyl bromide in 200 ml of dry ether. When

[1] α-Methylene designates methylene protons α to an electron withdrawing substituent.

the addition of the *n*-butyl bromide was complete, the reaction was stirred at reflux temperature for an additional 15 min. The reaction was carried out in a nitrogen atmosphere.

The reaction flask was cooled in an ice bath and 30 g (0.26 mole) of cadmium chloride was added over a period of 5–10 min. The reaction mixture was stirred and heated under reflux for 3 hr. The ether was distilled and 300 ml of anhydrous benzene was added. A solution of 58 g of 2-bromobenzoyl chloride (prepared from 2-bromobenzoic acid and thionyl chloride at 60C) in 100 ml of anhydrous benzene was added over a period of 5 min. The reaction mixture was then heated under reflux for 4 hr. Approximately 300 g of crushed ice and 100 ml of ice-cold 20% sulfuric acid were added to the reaction mixture. The benzene and aqueous layers were separated and the aqueous layer was extracted with 100 ml of benzene. The benzene layers were combined and washed with water, 20% sodium carbonate, water, and saturated sodium chloride solution. The benzene layer was dried over anhydrous sodium sulfate and the benzene was removed by distillation. The 2-bromovalerophenone (IVa) distilled at 124–134C (4.5–5 mm). The yield was 40 g (62% based on 2-bromobenzoyl chloride).

The ketone (IVa) (65 g) was heated under reflux with 80 g of potassium hydroxide and 60 ml of 85% hydrazine hydrate in 600 ml of ethylene glycol for 1 hr. The water formed was removed by distillation and the temperature of the solution was allowed to rise to 175C; after 16 hr at this temperature, the reaction mixture was poured into one liter of ether and the layers were separated. The ether layer was washed with water. The solvent was removed on a rotary evaporator to leave a light yellow liquid. The 2-amylbromobenzene (Va) was collected by distillation at 80–120C (0.3–0.5 mm). The yield was 28 g (45% based on the ketone (IVa)).

Anal. Calc. for $C_{11}H_{15}Br$: C, 58.2; H, 6.6; Br, 35.2 Found: C, 58.8; H, 6.9; Br, 31.8.

Methyl 7-(2′-Pentylphenyl)heptanoate (IIa)

Twenty-eight grams (0.123 mole) of 2-amylbromobenzene (Va) in 200 ml of dry ether was added slowly to a flask containing 4 g (0.162 g-atom) of magnesium in 20 ml of dry ether under a nitrogen atmosphere. When the addition of the 2-amylbromobenzene was complete, the reaction mixture was stirred at reflux temperature for an additional 15 min.

The reaction flask was cooled in ice and 12 g (0.108 mole) of cadmium chloride was added over a period of 5–10 min. The reaction mixture was stirred and heated under reflux for 3 hr. The ether was distilled off and 300 ml of anhydrous benzene was added. A solution of 4 g (0.194 mole) of monoethyl pimeloyl chloride [prepared by the method of Cason and Rapoport (4)] in 100 ml of anhydrous benzene was added over a period of 5 min. The reaction mixture was heated under reflux for 4 hr. The reaction work-up was identical with that described for 2-bromovalerophenone. The resulting keto-ester (VIIIa) was distilled at 155–165C (0.1–0.2 mm), and 21.7 g of product was obtained.

The keto-ester (VIIIa) (21.7 g) was reduced by the Wolff-Kishner method described above with 40 g of potassium hydroxide, and 30 ml of 85% hydrazine hydrate in 200 ml of ethylene glycol. After the initial one-hour heating, water was removed by distillation and the temperature was allowed to rise to 175C where it was maintained for 16 hr. The product obtained from the reaction was heated under reflux with 100 ml of methanol and 600 mg of sulfuric acid for 3 hr. The yield of ester (IIa) was 15.2 g (42% based on 2-amylbromobenzene). A 1.25 g portion of the product was chromatographed on silicic acid (20 g, ½ in. column). Elution with benzene:hexane (1:1 vol) afforded a product having n_D^{25} 1.4882.

Anal. Calc. for $C_{19}H_{30}O_2$: C, 78.6; H, 10.4. Found: C, 78.7; H, 10.7.

Absorption bands were observed in the infrared spectrum of the isolated material at the following wavelengths: 3.21, 6.22, 6.71 μ (aromatic); 13.40 μ (1,2-disubstituted benzene); 5.72, 8.50 μ (ester); 13.80 μ (tetramethylene). The ultraviolet spectrum gave absorption maxima at 263, 267, and 270 mμ. The NMR spectrum gave the following τ values: 3.02 ppm (aromatic protons); 6.40 ppm ($-OCH_3$); 7.20–8.00 ppm (α-methylene protons); 8.20–8.90 ppm (methylene protons); 8.90–9.30 ppm (terminal methyl protons).

2-Butylbromobenzene (Vb)

This intermediate was prepared using the same reaction sequence and conditions described above for 2-amylbromobenzene.

2-Bromobutyrophenone (IVb) was synthesized from 58.9 g (0.475 mole) of propyl bromide, 12 g (0.50 g-atom) of magnesium, 45 g (0.25 mole) of cadmium chloride, and 83.4 g (0.368 mole) of 2-bromobenzoyl chloride. The ketone IVb (62.5 g) was distilled at 115–120C (4.5–5 mm).

The ketone (IVb) (62 g) was reduced by the Wolff-Kishner procedure with 40 g of potassium hydroxide and 30 ml of 85% hydrazine

hydrate in 300 ml ethylene glycol. The product was distilled at 74–84C (0.5 mm). The yield was 18 g (23% based on 2-bromobenzoyl chloride). The refractive index was n_D^{25} 1.5313.

Anal. Calc. for $C_{10}H_{13}Br$: C, 56.4; H, 6.1; Br, 37.5. Found: C, 56.7; H, 6.3; Br, 35.1.

Methyl 8-(2′-Butylphenyl)octanoate (IIb)

The aromatic-keto ester VIIIb was prepared from 13 g (0.061 mole) of 2-butylbromobenzene, 1.5 g (0.0625 g-atom) of magnesium, 6 g (0.033 mole) of cadmium chloride, and 19.7 g (0.0895 mole) of monoethyl suberyl chloride. The ester (3.5 g) was purified by distillation at 180–195C (0.25 mm).

Methyl 8-(2′-butylphenyl)octanoate (IIb) was prepared from 3.5 g (0.0124 mole) of the keto-ester (VIIIb) by the Wolff-Kishner procedure with 20 g of potassium hydroxide and 20 ml of 85% hydrazine hydrate in 100 ml ethylene glycol. The isolated acid was esterified and the resulting 2.75 g (15% based on 2-butylbromobenzene) of methyl 8-(2′-butylphenyl)octanoate (IIb) was purified by distillation at 155–165C (0.20 mm). The refractive index was n_D^{25} 1.4888.

Anal. Calc. for $C_{19}H_{30}O_2$: C, 78.6; H, 10.4. Found: C, 78.6; H, 10.7.

The infrared and NMR spectra were identical with those of methyl 7-(2′-pentylphenyl) heptanoate.

Hydrogenation of Aromatic Esters

A 124 mg quantity each of the synthetic esters and the isolated esters was hydrogenated by the method of Brown, Durand, and Marvel (6). Three moles of hydrogen were consumed per mole of ester. The molecular weights of the hydrogenated materials as determined by mass spectroscopy were 296.

Mass Spectroscopy

Mass spectra of the isolated and synthetic esters were obtained on a Bendix Time-of-Flight mass spectrometer. The spectra are reproduced in Figure 2.

All of the spectra showed parent peaks at m/e 290, as expected for aromatic C_{18} methyl esters, as well as the expected peaks at m/e 259, corresponding to loss from the molecule of the elements CH_3O.

The following peaks were ascribed to loss of part or all of the hydrocarbon side chain (C_nH_{2n+1}) from the molecule: for IIa, 233 (corresponding to n = 4) and 219 (n = 5); for IIb, 247 (n = 3) and 233 (n = 4); and for the isolated ester, 247, 233, and 219 (n = 3, 4, 5, respectively). The following peaks were attributed to loss both of the hydrocarbon side chain and of the elements CH_4O from the ester group: for IIa, 201 and 187 (n = 4, 5); for IIb, 215 and 201 (n = 3,4); and for the isolated esters, 215, 201, 187 (n = 3, 4, 5). Additional peaks apparently arose from loss of the hydrocarbon side chain, the elements CH_4O, and the elements H_2O as follows: for IIa, 183, 169 (n = 4, 5); for IIb, 197, 183 (n = 3, 4); for the isolated esters 197, 183, 169 (n = 3, 4, 5).

Another series of peaks in each spectrum was attributed to the loss of part or all of the ester chain, $C_nH_{2n}CO_2CH_3$, as follows: for IIa, 175, 161 and 147 (corresponding to n = 4,5,6, respectively); for IIb, 161, 147, 133 (n = 5,6, 7); for the isolated esters, 175, 161, 147, 133 (n = 4,5,6,7).

Intense peaks at m/e 91 and 105 were seen in all three spectra. These peaks are common to the mass spectra of dialkylbenzenes, and correspond to the ions $C_7H_7^+$ and $C_8H_9^+$.

Figure 3 shows the mass spectra of the hydrogenated esters. Parent peaks at m/e 296 and peaks corresponding to loss of CH_3O at 265 were seen as expected.

Peaks apparently related to the loss of hydrocarbon side chain (C_nH_{2n+1}) from the ring were the following: for IXa, 225 (corresponding to n = 5), for IXb, 239 (n = 4); for the isolated esters, 253, 239, 225, (n = 3, 4, 5). The following peaks were attributed to loss of the side chain plus the elements CH_4O: for IXa, 193 (n = 5); for IXb, 207 (n = 4); for the

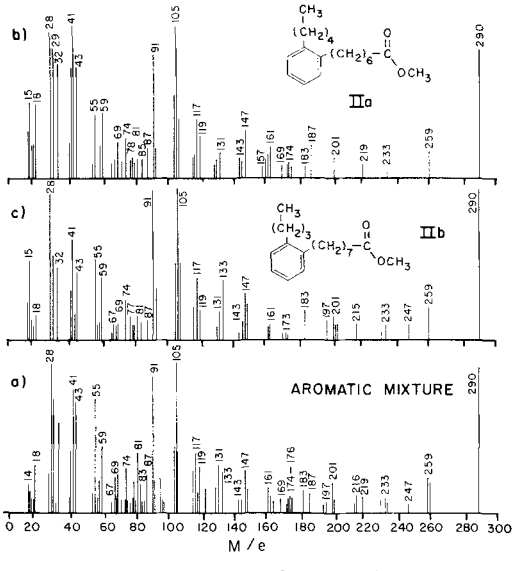


FIG. 2. Mass spectra of synthetic esters and isolated mixture.

isolated esters, 221, 207, 193 (n = 3, 4, 5). Peaks which apparently arose from loss of the hydrocarbon side chain, the elements CH_4O, and the elements H_2O were these: for IXa, 175 (n = 5); for IXb, 189 (n = 4); and for the isolated esters, 203, 189, 175 (n = 3,4, 5). Note that the hydrogenated synthetic esters, unlike their aromatic counterparts, did not give peaks corresponding to fragmentation within the hydrocarbon chain. Peaks resulting from the loss of the hydrocarbon side chain, therefore, gave an indication of the length of the chain and of the numbers of components in the isolated mixture.

Peaks which apparently arose through the loss of the ester chain; $C_nH_{2n}CO_2CH_3$, were the following: for IXa, 153 (n = 6); for IXb, 139 (n = 7), for the isolated esters, 153, 139, 125 (n = 6, 7, 8). Again it appeared that fragmentation did not occur within the ester side chain, and that the peaks seen for the mixture indicate the number of components in the mixture and the lengths of their side chains.

Bioassay of Aromatic Esters

The toxicity of IIa was checked by the bioassay method described previously (1). One-half milliliter per day for 3 days was administered by stomach tube for 5 weanling rats. All rats died on the third day.

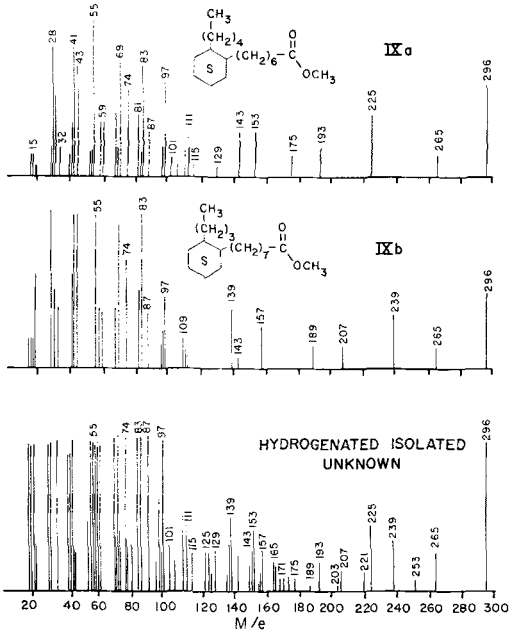


FIG. 3. Mass spectra of hydrogenated synthetic esters and isolated mixture.

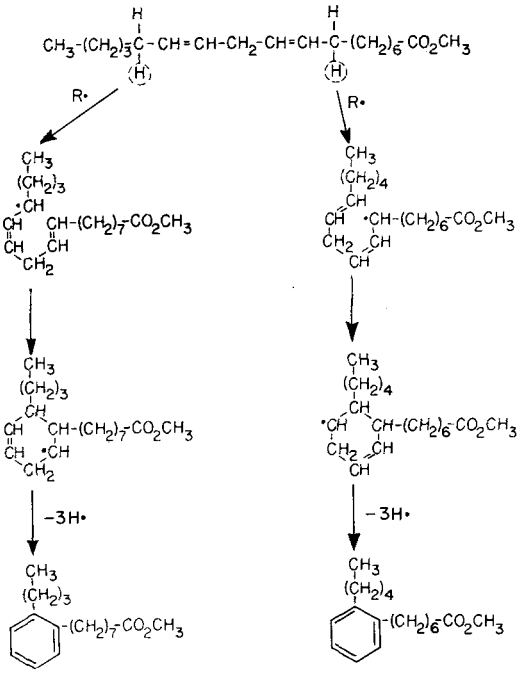


FIG. 4. Possible mechanism for formation of cyclic esters.

DISCUSSION

The analytical data for the isolated ester fraction are consistent with the empirical formula for the proposed structure $C_{17}H_{27}CO_2CH_3$. The final determination of structure for the fraction depends upon the interpretation of the infrared, ultraviolet, NMR, and mass spectral data. The spectra of the synthetic compounds methyl 11-(2′-methylphenyl)undecanoate (I), methyl 7-(2′-pentylphenyl)heptanoate (IIa), and methyl 8-(2′-butylphenyl)octanoate (IIb) were compared with the spectra obtained from the isolated mixture.

The infrared spectra and ultraviolet spectra of the synthetic materials were indistinguishable from the spectrum of the isolated material.

The NMR spectra for all four materials showed peaks at 3.02 ppm corresponding to the 4 protons on the aromatic nucleus. Each compound and the isolated material showed a peak at 6.40 ppm which corresponded to the three protons of the methyl ester. Compound I differed from compounds IIa and IIb and from the mixture in the regions 7.20–8.00 ppm and 8.90–9.30 ppm. For Compound I a strong peak at 7.68 ppm corresponding to the methyl protons adjacent to the benzene ring was found. The other two synthetic compounds (IIa and IIb) and the isolated mixture showed only peaks of low intensity which would be expected

for the α-methylene protons adjacent to the benzene ring and adjacent to the carboxyl group. In the 8.90–9.30 ppm region each of the latter compounds showed a triplet corresponding to the three methyl protons. This triplet was not present in the spectrum of compound I. A triplet at 8.90–9.30 ppm corresponds to a terminal methyl group of a hydrocarbon chain, as would be expected if the proposed structure (II) were correct. Thus it was apparent from the infrared and NMR data that the isolated ester fraction was a 1,2-disubstituted benzene with an ester chain of more than two and less than 11 carbons and a hydrocarbon chain of more than one and less than 10 carbons.

The mass spectra of the four materials were compared to determine the chain lengths of the radicals attached to the benzene ring. The spectrum of the isolated fraction was much more complex than the spectra of the three synthetic materials (Fig. 2). It was immediately apparent that this was not one pure compound but a mixture of isomers differing from each other by the length of the chains attached to the benzene ring. Comparisons of the mass spectra of synthetic IIa and IIb with the mass spectra of the isolated mixture strongly indicated that IIa and IIb were components of the mixture. Comparions of the mass spectra of IXa and IXb with the mass spectrum of the hydrogenated mixture confirmed that conclusion. The peaks at m/e 253, 221, 203, and 125 in the mass spectrum of the hydrogenated mixture suggested the presence of IXc; from this it was concluded that methyl 9-(2′-propylphenyl)nonanoate (IIc) was a component of the mixture of aromatic esters isolated from heated methyl linoleate.

Aromatic esters have not hitherto been isolated from the reaction products of heated fats. A mechanism based upon a single ring closure via a free radical allylic proton abstraction (Fig. 4) may account for the presence of these esters in the urea filtrate monomers. This mechanism, in part, predicts that nonaromatic cyclic compounds should be formed during the heating process. Indeed, this does happen, and the isolation and characterization of such materials are reported in a subsequent paper in this series.

In an earlier paper (1), the urea filtrate monomers were reported to be toxic to weanling rats. Methyl 7-(2′-pentylphenyl)heptanoate, shown to be a component of these monomers, was found to be toxic when administered to weanling rats at a level of 25 g/kg.

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[Received March 28, 1966]

Thermal Reactions of Methyl Linoleate. III. Characterization of C_{18} Cyclic Esters

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ABSTRACT

This third paper presents the isolation and characterization of nonaromatic cyclic monomers formed from the heated linoleate. The esters were isolated by a series of column chromatographic separations, followed by repeated gas chromatography to obtain fractions containing C_{18} cyclic esters. Characterization of the esters was achieved by use of infrared, NMR, mass spectroscopy, and standard chemical analyses. Also characterized were the isomers found in a complex mixture of cyclic monomers which had been partially separated by column chromatography. Use of both physical and chemical methods of analyses permitted characterization of the mixture of isomers without their having been separated from each other.

INTRODUCTION

THE FIRST REPORT (1) of this series described the heating of methyl linoleate and the separation procedures used to concentrate groups of structurally related transformation products from the heated ester. The second report (2) gave details of the isolation and characterization of aromatic products from the heated linoleate. The aromatic products were shown to be methyl esters of ω-(*o*-alkylphenyl) alkanoic acids having 18 carbon atoms. The present report describes the isolation, from the same reaction mixture, of nonaromatic cyclic products and their characterization.

During the past 15 years, evidence has been presented by several laboratories that cyclic monomers are formed when unsaturated fats and fatty acids are oxidized and polymerized. Crampton, in a series of papers (3–5), showed that linseed oil which had been heated at 275C in an inert atmosphere caused growth depression in rats. The toxic substances were found in the non-urea-adductable fraction of the distillable ethyl esters formed from the heated oil. Wells and Common (6) suggested that these materials contained a nonterminal ring structure. Paschke and co-workers (7,8) have isolated, from thermally polymerized linoleate, monomers which did not hydrogenate to stearate and were presumed to be cyclic. The non-urea-adductable fraction from polymerized safflower oil contained materials presumed to be cyclic (9). MacDonald and co-workers (10) isolated cyclic monomers from heated linseed oil and partially characterized the mixture of materials by use of a combination of infrared spectroscopy and oxidation for double bond location. Rivett's (11) characterization of cyclic monomers formed during polymerization of methyl eleostearate made use of infrared and ultraviolet spectroscopy. In a more recent study by Hutchison and Alexander (12), the isolation and characterization of a cyclic monomer from linseed oil urea filtrate material was described. The study utilized gas chromatography, NMR spectroscopy, mass spectroscopy and the standard techniques of structure determination.

EXPERIMENTAL

Formation and Isolation of Products

As previously reported (1), methyl linoleate, diluted with an equal volume of methyl laurate, was heated 200 hr at 200C in an open beaker. The laurate was first distilled off, then the linoleate and monomeric transformation products were distilled. Urea treatment separated the cyclic monomers, which were then chromatographed on Florisil and on silicic acid—silver nitrate. The fraction which was previously designated F1B2 was subjected to further chromatographic steps, as shown in Figure 1. Aromatic and nonaromatic components of the final concentrate were separated by gas chromatography (2). Gas chromatographic purification of the nonaromatic fraction was repeated until only one peak was seen when the collected material was re-injected. The final purified material was designated Fraction I.

Hydrogenation and Dehydrogenation of Monomer

Part of the collected product was hydrogenated over 10% Pd on charcoal in methanol at room temperature and 50 psi. One mole of hydrogen was consumed per mole of ester. Another portion of the collected product was dehydrogenated by treatment with Pd on carbon as described by Hutchison and Alexander (12).

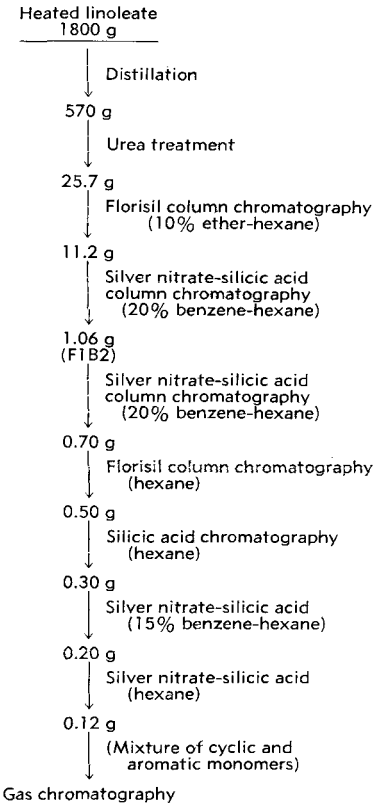


FIG. 1. Concentration of cyclic monomer (Fraction 1) from methyl linoleate.

Infrared Spectra

The infrared spectrum of the collected product showed bands at 3.20 μ (double bond) and 5.72 and 8.55 μ (ester). There was no band at 10.35 μ (trans double bond). After the dehydrogenation reaction the material showed bands at 3.21, 6.22 and 6.70 μ (aromatic) and at 13.28 μ (disubstituted benzene). The absence of characteristic ester bands suggested that decarboxylation as well as aromatization had occurred during the dehydrogenation reaction.

NMR Spectrum

The NMR spectrum of the isolated monomer showed shifts at the following τ values: 4.45 ppm (vinyl protons), 6.40 ppm ($-OCH_3$), 7.20–8.00 ppm (α-methylene protons[1]), 8.67 ppm (methylene protons) and 9.00–9.10 ppm (terminal methyl protons).

Anal. Calc. for $C_{19}H_{34}O_2$: C, 77.5; H, 11.6. Found: C, 78.3; H, 12.2.

[1] α-Methylene designates methylene protons α to an electron withdrawing substituent.

Mass Spectrometry

The mass spectrum (Fig. 2A) of the isolated monomer showed a peak at m/e 294, as expected for the parent peak of the methyl ester of a C_{18} acid containing one ring and one double bond. The expected peak at m/e 263, corresponding to loss from the molecule of the elements CH_3O, was also seen. Many of the other mass peaks seemed not to come from fragmentation of a single compound, but rather fell into several series, each of which represented one mode of fragmentation for several isomeric compounds.

Thus the following peaks were attributed to the loss from a disubstituted cyclic molecule of part or all of the hydrocarbon side chain, C_nH_{2n+1}: 251, 237, 223, and 209 (corresponding to n = 3,4,5 and 6, respectively). The following peaks were ascribed to loss both of the hydrocarbon side chain and of the elements CH_4O from the ester groups: 219, 205, 191, and 177 (n = 3,4,5,6).

Peaks which were apparently formed by the loss of the hydrocarbon side chain and the unsaturated ring, $C_nH_{2n-1}C_6H_5$, were seen at 171, 157, 143, and 129 (n = 3, 4, 5, 6).

Another series of peaks apparently arose through the loss of all or part of the ester-containing side chain, $C_nH_{2n}CO_2CH_3$. These

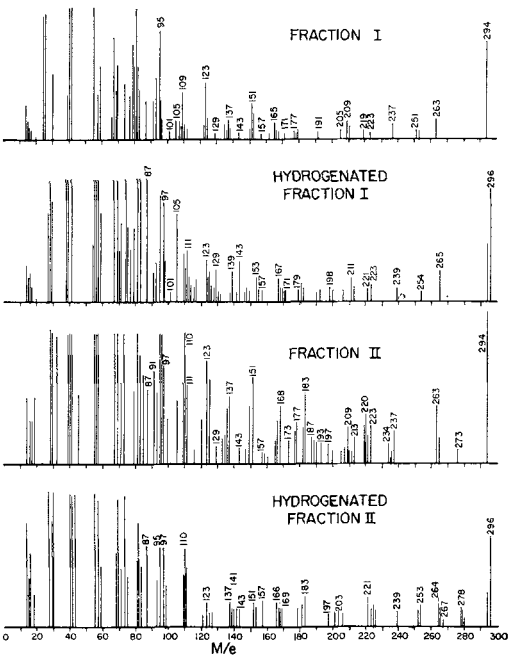


FIG. 2. Mass spectra of (A) fraction I, (B) hydrogenated fraction I, (C) fraction II, and (D) hydrogenated fraction II.

were at m/e = 165, 151, 137, and 123 (n = 5, 6, 7, 8).

Figure 2B shows the mass spectrum of that portion of the isolated product which had been hydrogenated. This spectrum was very similar to the spectra of the hydrogenated aromatic esters described previously (2).

A parent peak at m/e 296 and a peak corresponding to loss of CH_3O at 265 were seen as expected. Peaks apparently related to the loss of the hydrocarbon side chain, C_nH_{2n+1}, from the ring were the following: 253, 239, 225, and 211 (n = 3, 4, 5, 6). The following peaks were attributed to simultaneous loss of the hydrocarbon side chain plus the elements CH_4O: 221, 207, 193, 179 (n = 3, 4, 5, 6). Peaks which apparently arose through the loss of the ester chain, $C_nH_{2n}CO_2CH_3$, were at 167, 153, 139, and 125 (n = 5, 6, 7, and 8).

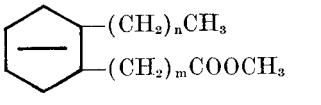
Related Compounds

Extensive work was done also with another fraction of the heated linoleate. This fraction, designated F1B1 in the previous paper, differed from the one described above in that it was eluted from the silicic acid—silver nitrate column with 10% benzene in hexane, rather than 20%, and in that it received fewer subsequent chromatographic purification steps. Like Fraction I described above, this one was finally freed of aromatics by gas chromatography. The collected material, designated Fraction II, differed from Fraction I also in some of its properties; most notably its infrared absorption spectrum showed a band at 10.35 μ, indicating *trans* unsaturation in an aliphatic chain, and its NMR spectrum showed chemical shifts at τ values of 4.21–4.40 and 4.50–4.80 ppm, indicating that at least two different kinds of vinyl protons were present. When subjected to oxidative cleavage with periodate-permanganate according to the procedure of Kuemmel (13), it gave hexanoic and azelaic acids (molar ratio 3:2) as the only identifiable products. The total quantity of volatile products produced in the oxidation was only 45% of the amount which would have been expected for a C_{18} ester containing one double bond, and it was assumed that the remainder of the oxidation products were tricarboxylic acids or keto acids resulting from the cleavage of esters containing a double bond in a ring.

Mass spectra of Fraction II (Fig. 2C) and its hydrogenated derivative (Fig. 2D) were similar to those described in Fraction I, but more complex. Most of the peaks could be accounted for by assuming that Fraction II was similar to Fraction I but contained both species having their double bond in the ring and species having their double bond in one or the other of the side chains. It also appeared likely that lengths of the side chains covered wider ranges than those of the more highly purified fraction previously described.

DISCUSSION

The structure of the compounds comprising Fraction I are deduced from the following considerations. Analytical values and the infrared spectrum indicate that the material is the ester of a C_{18} acid containing one double bond and one ring. Dehydrogenation to a 1,2-disubstituted benzene derivative indicates that the ring is 6-membered and vicinally disubstituted. Absence of infrared absorption at 10.35 μ indicates that the double bond is either *cis* or in the ring; the NMR line at $\tau = 4.45$ ppm suggests that the double bond is in the ring. The fragmentation patterns observed in the mass spectra of Fraction I and its hydrogenated derivative indicate that the hydrocarbon chain varies in length from 3 to 6 carbon atoms and that the ester chain varies from 6 to 9 carbon atoms. Since each of the peaks attributed to the loss of either side chain from the hydrogenated derivative was 2 mass units heavier than the corresponding peak in the spectrum of the original ester, it is concluded that the double bonds in the original ester were located exclusively in the ring. There is no evidence to fix the position(s) of the double bond in the ring, however. Thus the following generalized formula is given for Fraction I: where n = 2–5,



m = 5–8, and n + m = 10, and where (—) indicates a double bond whose position is not established. It is not certain whether or not there are also isomers present having n and m outside these ranges.

Fraction II was similar to Fraction I, having come from the same source by nearly the same separation procedure. It had, however, been less extensively purified by chromatography, and apparently contained a wider range of structural types. The structural evidence is similar to that offered for Fraction I with the following exceptions. The infrared absorption at 10.35 μ indicates *trans* double bonds, which would have to be in the side chains, rather than in the ring. The oxidative cleavage results indicate that the double bonds occurred in both of the side chains. There is a suggestion that

about half of the double bonds were in the ring and half were in the side chains. This conclusion was confirmed by the finding of NMR peaks of approximately equal intensity at 4.38 and 4.60 ppm. The lengths of the two side chains were indicated by the mass spectroscopic fragmentation patterns; the hydrocarbon chain contained 2 to 11 carbon atoms, and the ester side chain must have contained 0 to 9 carbons in addition to the carboxyl group.

A single mechanism to describe the transformation which occurred during the heating of linoleate would be difficult to conceive. The one that best fits the products isolated and described in this report would involve a free radical allylic proton extraction, randomization of double bonds, and ring closure. This type of mechanism was proposed in part by Hutchison and Alexander (12) and could be used to explain the products isolated by MacDonald (10).

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[Received March 28, 1966]

SHORT COMMUNICATION

Cholesterol Ester in Degenerating Nerve: Origin of Cholesterol Moiety

NORMAL PERIPHERAL nerve does not contain cholesterol esters, however, cholesterol esters do appear in nerves undergoing degeneration. The source of the cholesterol ester has been a subject of speculation. Rossiter and co-workers (1,2) upon observing a decrease of cholesterol and an increase of cholesterol ester in degenerating nerve suggested that the cholesterol moiety of cholesterol ester might arise from the cholesterol in the nerve. Later Berry and Cevallos (3) after noting the failure of in vitro studies to demonstrate labeling of cholesterol ester even when cholesterol itself was labeled, suggested that cholesterol esters might be carried into the degenerating nerve by invading cells. In view of the absence of direct evidence and two opposing suggestions, it was decided to undertake an in vivo study of the problem using C^{14}-cholesterol.

Cholesterol (4-C^{14}) (10–20 mc/m mole) was purified before use by separation on a silicic acid column. The purity was checked by autoradiography of 1 μc of purified cholesterol developed on a thin-layer plate with redistilled hexane/ether/acetic acid (80/20/5) as the solvent.

A normal saline suspension of (4-C^{14}) cholesterol (0.2 ml containing 2 μc of C^{14}) was injected once every other day into 3 groups of 9 myelinating rats from the 10th to the 18th day of life. Five months after the final injection a right sciatic section was performed and the animals sacrificed 16 days post-section.

The distal stump of the sectioned nerve and the contralateral normal nerve were removed and weighed. The nerves and all additional organs checked for radioactivity were homogenized in chloroform/methanol (C/M) 2:1, the suspension centrifuged and the extract decanted. The residue was extracted two more times with C/M 2:1, once with C/M 1:2 and finally with C/M 4:1 with 25 ml concentrated NH_4OH per liter. The combined extracts were evaporated to dryness, and the lipids purified on Sephadex (4). The purified lipids were applied to a silicic acid column, the cholesterol esters (eluted with hexane/ether 99:1) and the cholesterol (eluted with hexane/ether 85:15) were obtained free of contaminants. The purity and identity of the various lipid fractions were checked by TLC. The radioactivity was determined by liquid scintillation counting and cholesterol was quantitated colorimetrically. The specific radioactivity of the cholesterol moiety of the cholesterol ester was further checked after hydrolysis.

TABLE I

Average Specific Activity (cpm/μmole) and Quantity (μmoles) in Sterol Fractions
(Figures represent average values of 3 pools of animals.)

	Normal nerve		Sectioned nerve	
	Specific activity	μmole	Specific activity	μmole
Cholesterol	610	8.25	1260	3.15
Cholesterol ester	0	0	1070	1.15

No radioactivity was found in the lipid extract of muscle, heart, liver or blood while 150,000 counts per minute were present in the whole brain. It therefore seems unlikely that the radioactivity found in the degenerating nerve could have been carried there from other sources. The relative specific activity (cpm/μmole chol. ester/cpm/μmole chol.) was 0.85 (Table I). This would indicate that at least 85% of the cholesterol moiety of the cholesterol ester found in degenerating nerve is derived from the cholesterol of that nerve, especially in light of the fact that with the exception of brain no radioactive cholesterol was found elsewhere in the animal. The specific activity of the cholesterol of the contralateral normal nerve compared to that of the cholesterol of the degenerating nerve was 0.5. Since the C^{14} cholesterol was injected only during the first 8 days of the myelination period, one would expect the myelin laid down first to be the most heavily labeled and the myelin laid down latter in the myelination period to contain much less label. With this in mind the data listed in this table could be explained if it were assumed that the myelin is broken down in an orderly, sequential manner, starting with the myelin that was laid down last and progressing towards the myelin laid down early in the myelinating period.

The above evidence strongly indicates that the cholesterol moiety of cholesterol ester in degenerating nerve arises from the cholesterol of the nerve.

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ACKNOWLEDGMENT

This work was supported by grants NB 06113–01 and NB 05020–2 from the National Institute of Neurological Diseases and Blindness.

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[Received June 27, 1966]

Mass Spectrometry of Lipids. II. Monoglycerides, Their Diacetyl Derivatives[1] and Their Trimethylsilyl Ethers

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ABSTRACT

The mass spectra of 1- and 2-monoglycerides, their diacetyl derivatives and their trimethylsilyl (TMS) ether derivatives were recorded at high (80 eV) and low (6–13 eV) voltages. The fatty acid components of these derivatives included the even-numbered saturated acids from capric to arachidic acid plus oleic, linoleic and linolenic acids. Differences between isomeric 1- and 2-monoglycerides were not sufficient to provide a basis for the analysis of these isomers. Mass spectra of the monoglycerides were very similar to the corresponding methyl esters. Mass spectra of the diacetyl derivatives were qualitatively similar to triglycerides of long-chain fatty acids, but parent ions were not observed. The spectra of diacetyl derivatives may be used for distinguishing 1- and 2-monoglycerides, but the spectra of the TMS ethers are better in this regard. The latter derivatives have fragmentation patterns distinct for the 1- and 2-monoglyceride isomers, particularly at low electron voltages.

INTRODUCTION

MASS SPECTRA of a wide range of saturated and unsaturated fatty acids and esters, both substituted and unsubstituted, have been investigated (1–4). With less volatile compounds, such as glycerides, difficulties associated with decomposition of the sample (5) and pumping out of the sample from the spectrometer are experienced when a conventional inlet system is used (3). This problem has been overcome by the development of a direct injection inlet in which the sample is placed at the entrance to the ion source. With this system mass spectra of triglycerides up to tribehenin have been reported (6,7). The fatty acids in the one and three positions of the triglyceride molecules could be identified by the presence of corresponding acyloxymethylene peaks in the spectra (3,6). Thus, 1- and 2-oleo-distearin could be readily distinguished by these peaks (6). A similar distinction might be made between 1- and 2-monoglycerides on either the parent compounds or their derivatives. If so, gas chromatography of volatile derivatives such as the diacetyl and the trimethylsilyl ethers of monoglycerides might be combined with mass spectrometry for the separation and identification of monoglycerides (8–10).

Thus far, the mass spectra of monoglycerides and their derivatives have not been reported. It seemed likely that mass spectra might be useful for characterization of the structures of these compounds. Therefore, 1- and 2-monoglycerides were prepared from straight-chain saturated fatty acids of even carbon number from capric acid to arachidic acid, and from the unsaturated acids, oleic, linoleic and linolenic acids. Diacetyl derivatives and trimethylsilyl (TMS) ethers of the monoglycerides were also prepared. The mass spectra were recorded with use of a direct inlet system in which pyrolysis is minimized.

EXPERIMENTAL

Fatty acids of purity >99% were obtained from The Hormel Institute, Austin, Minnesota. 1-Monoglycerides were prepared by acylation of *dl*-1,2-isopropylidene glycerol with fatty acid chlorides (11), followed by removal of the isopropylidene group (12). 2-Monoglycerides were prepared in a similar manner via 1,3-benzylidene glycerol (11). They were purified either by crystallization from diethyl ether-hexane or by thin-layer chromatography on borate-impregnated silica gel (13) with chloroform:acetone (75:25) as the solvent. The borate complexes were eluted from the plates with diethyl ether, these were decomposed by washing the solution with distilled water, and the monoglycerides were recovered. The purity of the monoglycerides was checked by TLC on borate-impregnated plates or by GLC of the TMS derivatives (9).

Diacetyl derivatives were prepared by acylation of the monoglycerides with acetyl chloride in pyridine (11). Reaction of the monoglycerides with hexamethyldisilazane in the presence of trimethylchlorosilane and pyridine was used for the preparation of the trimethylsilyl ether derivatives (9).

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A mass spectrometer (Hitachi-Perkin Elmer Model RMU-6D) equipped with a direct evaporation system (MG-150) was employed. The sample in a small cup on the tip of a rod was inserted through a vacuum lock into a heating block just outside the ionization chamber. The sample evaporated from the cup directly into the ion source at about 70C, and a spectrum was recorded when the evaporation, as measured by a total-ion monitor, settled to a constant rate. Sample pressure was in the order of 5×10^{-7} torr, measured in the source housing.

Thermal decomposition of the monoglycerides in this system was minimized as evidenced by the small M-18 peak (loss of water) in the spectra. Mass spectra were obtained at both high (80 eV) and low electron voltages. The higher electron voltage spectra were more reproducible, though more complex, than those recorded at the low voltages. In the latter case the actual voltage was the lowest that produced a countable spectrum, and this was usually between 6 and 13 eV. Peaks of high mass were more prominent in the low voltage spectra, whereas extensive breakdown of the molecules to short hydrocarbon and oxygen-containing chains was observed in spectra at the high voltage.

RESULTS AND DISCUSSION

Monoglycerides

The mass spectra of 1- and 2-monomyristin are shown in Figure 1, and a summary of significant peaks is given in Table I. The spectra can be divided into hydrocarbon and oxygen-containing peaks, the latter being more prominent in the low electron voltage spectra. Significant differences exist between the spectra of the saturated and unsaturated monoglycerides. Within each of these series, the intensities of many of the peaks changed progressively according to the chain length or the degree of unsaturation of the fatty acid. Differences between the spectra of 1- and 2-monoglycerides were not sufficiently large or consistent to provide a basis for the analysis of these isomers. A series of peaks was observed corresponding to ions of structure

$$-CH{=}CH-CH_2-O-CO(CH_2)_n$$

for 1-monoglycerides or

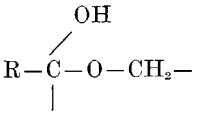
$$CH_2{=}C(CH_3-)-O-CO-(CH_2)_n$$

for 2-monoglycerides where

$n = 0$ to 9. These structures were assigned on the basis of similar peaks in the spectra of methyl esters (14).

Saturated Monoglycerides

Spectra at High Electron Voltage. Peaks corresponding in mass to fatty acid and methyl ester were present in the spectra of monoglycerides. These ions were probably formed by fragmentation of the glycerol moiety with prior migration of a hydrogen atom, to give in the latter instance a probable transient structure of



The peak corresponding to the fatty acid ion plus one was of greater intensity than that expected from isotope ratios, and is probably formed by an acyloxy-fragmentation with a rearrangement involving two hydrogen atoms (2). In the spectra of monoglycerides containing the shorter chain fatty acids, the peak for fatty acid + 1 was more intense than the acid ion peak. This phenomenon was also observed in the mass spectra studied of short-chain ethyl esters and long-chain esters of propyl and higher alcohols and formates but not of long-chain methyl or ethyl esters (2,4,14,15).

Increasing the chain length of the fatty acid resulted in an increase in the intensity of the acid ion peak, whereas the intensity of the acylium ion decreased. The latter change paralleled a decrease in the P-31 peak which signifies the loss of hydroxymethylene group ($-CH_2OH$) from the parent ion, P. A metastable peak corresponding to the transition [P-31] $\longrightarrow$ $CH_3(CH_2)_nCO-$ was prominent in each spectrum, indicating that the acylium ion was derived to some extent from the P-31 ion.

In each of these spectra, the base peak was due to a three-carbon fragment (C_3H_7- m/e = 43) which, however, was absent or of very low intensity at the low voltages. This fragment was third in prominence, behind those corresponding to $CH_3-O-C(OH){=}CH_2$ and $CH_3-O-CO-CH_2-CH_2-$, in the mass spectra of the corresponding methyl esters (14).

Spectra at Low Electron Voltage. Peaks in the high mass regions were of slightly greater intensities than in the corresponding high voltage spectra. Main differences arose in the low and medium mass ranges of the spectra, and a change in the base peak from one corresponding to a hydrocarbon fragment to one of an oxygen-containing ion. The base peak of a monoglyceride containing a short-chain fatty acid was the acylium ion. This peak decreased in intensity with increasing chain

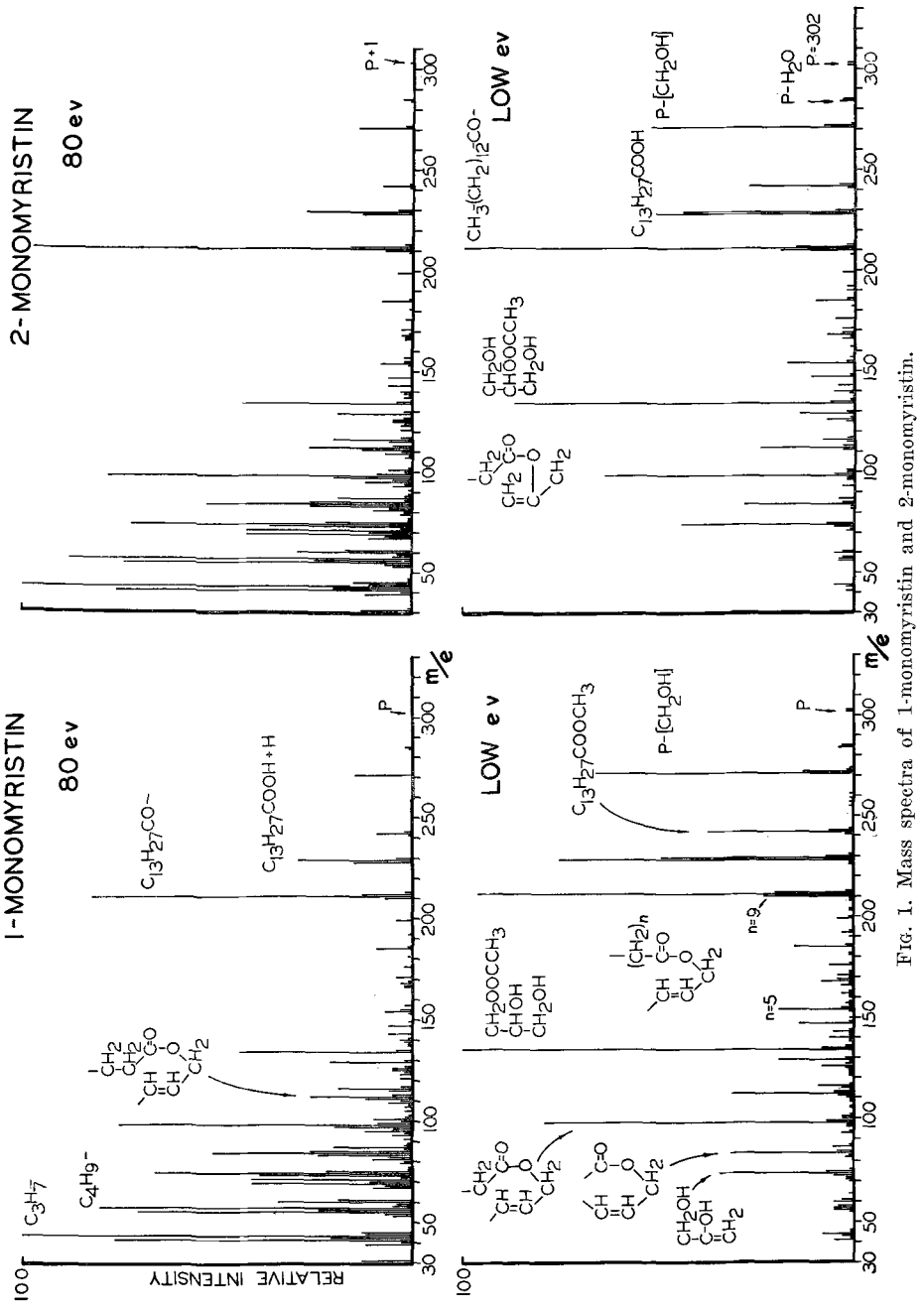


FIG. 1. Mass spectra of 1-monomyristin and 2-monomyristin.

length of the fatty acid, whereas that for the acid ion increased to become the base peak. Peaks due to hydrocarbons were not measurable in most cases.

In some instances, especially in the spectra of monoglycerides containing short-chain fatty acids, the peak of the rearranged monoacetin ion (m/e = 134) became prominent, analogous to the rearranged methyl acetate ion peak at m/e = 74 in spectra of methyl esters (14), and was of an intensity equal to or slightly greater than the acylium peak. It is evident that the glycerol part of the ion is susceptible to the loss of water or a hydroxy-methylene group to produce peaks of m/e = 116 or m/e = 103, respectively. These losses take place either

TABLE I

Monoglyceride Mass Spectra

Fatty acid ion	MG[a]	10:0		12:0		14:0		16:0		18:0		20:0		18:1		18:2		18:3	
		H.V.[b]	L.V.[c]	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.
Parent ion (P)	1	....	....	0.8	1.1	....	1.9	....	8.5	0.7	1.2	1.0	8.3	1.5	18	2.7	32	2.4	100
" "	2	0.7	....	0.9	1.5	....	1.5	0.8	1.0	1.2	7.7	0.7	8.5	1.2	10.2	1.4	34	15	100
P—[OH]	1	....	....	2.1	1.7	2.2	2.6	1.2	5.4	1.6	2.5	1.3	11	....	....	1.0	2.8	0.5	0.7
" "	2	1.3	4.1	2.6	2.6	3.2	2.9	....	5.0	0.9	3.1	0.7	....	....	....	0.6	2.5	0.5	0.8
P—[H_2O]	1	....	....	0.6	1.3	1.0	3.9	1.0	11	1.4	3.7	0.9	16	1.2	2.9	1.9	9.3	....	0.7
" "	2	....	7.1	0.9	3.7	14	3.5	1.8	....	1.5	9.2	1.2	6.6	3.4	3.7	1.1	8.6	0.7	3.0
P—[CH_2OH]	1	7.6	....	15	44	15	66	5.5	65	7.2	19	3.2	52	1.9	8.1	0.6	....	1.4	0.5
" "	2	9.3	62	20	94	14	52	4.0	42	7.5	44	4.5	15	2.9	7.9	...	....	4.8	0.9
Acyl $CH_3(CH_2)_nCO$	1	62	45	93	100	82	96	31	68	40	28	16	53	20	20	29	22	3.9	10
" "	2	94	100	98	100	96	100	37	92	43	46	22	19	29	24	21	21	14	8.4
$CH_2(CH_2)_nCO$	1	1.1	5.9	4.6	12	6.6	23	4.3	32	9.1	15	4.8	45	39	100	55	100	2.6	0.5
" "	2	4.4	20	6.6	27	7.0	19	4.1	26	10	30	7.2	14	51	100	39	100	5.5	....
Acid $CH_3(CH_2)_nCOOH$	1	7.7	19	8.2	25	15	75	11	100	51	100	12	100	1.4	15	8.8	41	0.9	4.0
" "	2	3.4	37	10	46	13	51	4.2	39	25	100	27	100	1.4	6.0	3.3	25	3.4	11
Acid + H	1	18	14	30	44	29	49	10	41	21	27	6.8	40	0.5	4.4	2.2	10	1.1	1.9
" "	2	18	44	29	47	27	44	9.0	29	16	32	11	25	0.6	1.8	0.7	5.1	2.0	3.5
Methyl ester	1	2.9	....	8.8	25	8.9	37	3.8	34	5.4	11	2.3	28	....	....	....	1.6	....	1.0
" "	2	4.0	29	10	40	7.5	27	2.8	23	5.3	22	3.4	8.0	....	0.9	....	....	....	....
Monoacetin	1	12	14	36	78	44	100	21	94	31	21	20	80	3.8	....	3.6	....	6.5	3.1
"	2	21	60	40	100	43	87	30	100	35	56	25	4.7	5.8	1.6	3.5	0.7	7.8	....
C_3H_7	1	100	23	100	1.1	100	2.5	100	3.1	100	1.1	100	1.4	56	....	29	....	67	....
"	2	100	12	100	....	100	1.7	100	6.0	100	....	100	2.0	66	....	36	....	33	....
$-CH_2CH_2CH=CH_2$	1	70	22	72	0.5	70	0.8	62	6.8	83	....	62	0.5	100	....	73	....	10	....
" "	2	55	4.7	66	3.1	73	....	69	....	71	....	69	6.8	100	....	78	....	82	....
$-CH_2CH=CHCH=CH_2$	1	9.0	4.6	9.1	....	10	....	8.0	....	15	....	8.6	....	4.3	....	100	....	79	....
" "	2	6.7	2.9	8.0	....	11	....	12	....	12	....	11	3.2	65	....	100	....	86	....
$-CH=CH-CH=CH-CH=CH_2$	1	2.7	3.2	2.3	....	2.9	....	1.8	...	4.6	....	1.8	....	8.4	....	42	....	100	....
" "	2	2.1	....	2.0	....	2.7	....	3.2	....	3.2	....	2.6	....	21	....	42	....	100	....
m/e = 98	1	30	100	82	4.6	75	79	44	75	71	14	48	54	46	....	9.5	....	21	0.7
" "	2	26	85	52	45	77	64	71	74	71	34	62	6.2	49	5.1	7.8	....	12	....

[a] MG = monoglyceride.
[b] H.V. = high electron voltage.
[c] L.V. = low electron voltage.

from the parent ion or after the loss of the hydrocarbon fragment from the parent ion.

Unsaturated Monoglycerides

Spectra at High Electron Voltage. The base peaks of these spectra were the unsaturated hydrocarbon fragments of m/e 55 for oleate ($\cdot CH_2-CH_2-CH=CH_2$), 67 for linoleate ($\cdot CH_2-CH=CH-CH=CH_2$) and 79 for linolenate ($\cdot CH=CH-CH=CH-CH=CH_2$). These were the major hydrocarbon peaks observed in the spectra of the corresponding fatty acid methyl esters (16). Compared with the spectra of 1- and 2-monostearins, significant decreases in the intensities of the peaks corresponding to the methyl ester, acid ion and acid-plus-one ion were observed, with a smaller decrease in intensity of the acylium ion. The intensities of the peaks in the high mass regions of these spectra were similar to those of corresponding peaks of the monostearins. However, a metastable peak for the transition [P-31] ⟶ acylium ion was not observed.

Spectra at Low Electron Voltage. A large increase in intensity of the parent ion peak was observed with increase in unsaturation of the fatty acid component, and this peak was the base peak in the spectra of the monolinolenins. Such parent ion peaks have been observed in the spectra of the corresponding methyl esters (16). Peaks corresponding to the loss of a hydroxyl group or water from the parent ion were of similar intensity to the corresponding peaks in spectra of the monostearins, but the intensity of the peak caused by the loss of a hydroxymethylene group was much less. The intensities of the methyl ester ion, acid ion, acid-plus-one ion and monoacetin ion peaks showed similar patterns, and this is probably a reflection of the greater stability of the parent ions.

The base peaks in the monoolein and monolinolein spectra were those corresponding to the acylium fragment minus one, i.e., the loss of glycerol from the parent ion rather than the glyceryl group, as is the case for saturated monoglycerides. In the mass spectrum of methyl oleate (16), the base peak corresponded to the loss of methanol from the parent ion (P-32), whereas this peak was insignificant in the spectrum of methyl linolenate where the P-31 peak (loss of methoxy group) becomes the more prominent of the two. Thus, the unsaturated monoglycerides and the corresponding methyl esters have similar fragmentation patterns.

Diacetyl Derivatives of the Saturated Monoglycerides

Spectra at High Electron Voltage. The high voltage spectra of 1,2-diacetyl-3-myristin and 1,3-diacetyl-2-myristin are shown in Figure 2. Intensities of peaks relevant to this study are shown in Table II. Because the diacetyl derivatives are triglycerides, similarities in the spectra of diacetins and other triglycerides should be apparent. Parent ion peaks were not observed in these spectra, as was the case for triacetin and other mono-acid triglycerides studied in this and other laboratories (3,17). Triglycerides having three different long-chain fatty acid moieties have been reported to yield parent ion peaks in their spectra (6,7), the intensity of which probably depends on the sample insertion system and instrument used.

Unlike the triglycerides containing only long-chain fatty acids, the base peaks of spectra of the diacetins corresponded to fragments of m/e = 43 as was the case with the monoglycerides. Because this peak was very small or nonexistent in the low voltage spectra, it is probably a hydrocarbon radical arising by secondary fragmentation of the long-chain acid moiety. As in other triglyceride spectra, loss of the long-chain acyloxy groups to produce a charged residue of m/e = 159 was, in general, the second most intense peak, especially with the 2-monoglyceride derivatives. The acyl ions and ions resulting from the loss of methylene acetate (CH_3COOCH_2-) from the parent ions were also prominent. The loss of the long-chain acyloxymethylene groups was more prominent in the derivatives of the 1-monoglycerides. These differences, though too small for the analysis of monoglyceride mixtures, can be used for distinguishing between isomers. In general, the intensity of these peaks increased with increase of the chain length of the fatty acid. An ion, formed by the loss of the long-chain fatty acid was also prominent in both groups of spectra. Loss of the acetyloxy group, acetic acid or methylene acetate, in most instances, resulted in small peaks in the spectra.

Spectra at Low Electron Voltage. No generality may be stated regarding the position of the base peak in these spectra. However, peaks corresponding to fragmentations described immediately above provided similar patterns, and the differences between the spectra of isomeric saturated monoglyceride derivatives were similar to those described above for spectra at high electron voltage. Peaks due to the loss of the acetyloxy group were more intense in these than in the high electron voltage spec-

tra, especially for monoglycerides containing long-chain fatty acid moieties.

Diacetyl Derivatives of the Unsaturated Monoglycerides

Spectra at High Electron Voltage. The base peak in all cases was the hydrocarbon fragment of m/e = 43 which was absent in the low voltage spectra. Other than short-chain fragments of m/e <200, there were few peaks of significance. Those present showed a pattern similar to the corresponding peaks in the spectra of the monostearin derivatives.

Spectra at Low Electron Voltage. In all cases, the m/e = 159 peak indicating loss of the long-chain acyloxy group from the parent ions was prominent. The base peaks for the 2-monoolein and 2-monolinolenin derivatives were the acyl ion minus one and the acyl ion, respectively. Other peaks in the spectra were, in general, insignificant compared to these.

Trimethylsilyl (TMS) Ether Derivatives

The mass spectra of the trimethylsilyl ether derivatives of 1- and 2-monomyristin are shown in Figure 3 and the intensities of relevant peaks are listed in Table III.

The spectra may be divided into hydrocarbon and silicon-containing peaks, the former being absent in the spectra at low electron voltage. The spectra of TMS ethers resembled those of the parent monoglycerides, but there was no similarity in the structures of the oxygen-containing ions of the monoglyceride spectra and the silicon-containing ions of the derivative spectra. Isomeric saturated monoglyceride TMS ethers gave spectra which were sufficiently different and characteristic to allow ready identification of the compounds, this being especially so at low electron voltages. Differences in the spectra of TMS derivatives of isomeric unsaturated monoglycerides were not so marked.

No parent ion peaks were observed in these spectra. The highest mass present in the spectra was the P-15 peak corresponding to the loss of a methyl radical from the parent ion. This has been found to be true for TMS ethers of relatively simple hydroxy compounds (5,18). In the case of large cyclic compounds (19) and some steroids (20), a parent ion peak may appear in the spectra of their TMS ether derivatives. This is probably because the positive charge is distributed over the cyclic system rather than being present on the oxygen-containing functional groups, providing greater stability to the parent ion. Reasoning from this basis, and from the spectra of the unsaturated monoglycerides, parent ion peaks might be expected to occur in the spectra of unsaturated monoglyceride TMS derivatives, but this was not found to be so.

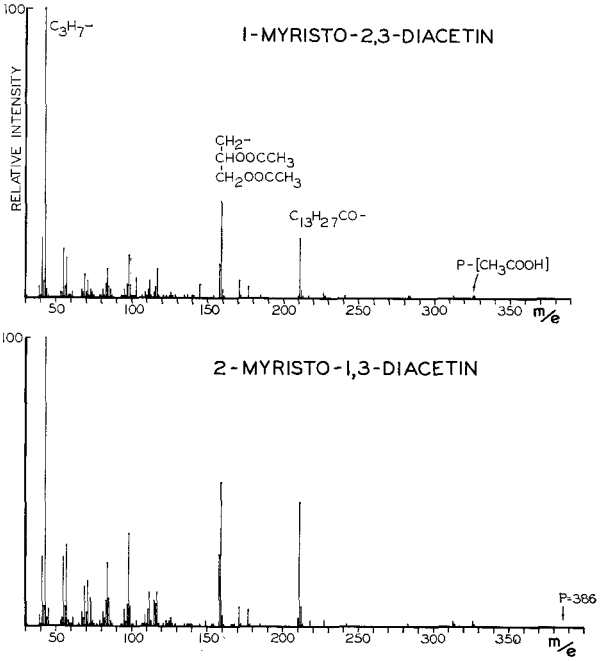


FIG. 2. Mass spectra of 1,2-diacetyl-3-myristin and 1,3-diacetyl-2-myristin.

TABLE II

Mass Spectra of Monoglyceride Diacetins

Fatty acid ion	MG[a]	10:0		12:0		14:0		16:0		18:0		20:0		18:1		18:2		18:3	
		H.V.[b]	L.V.[c]	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.
P—[CH_3COO]	1	1.7	2.5	....	....	0.9	40	2.7	10	27	100	...	7.3	0.5	3.6	....	....	....	....
" "	2	....	1.7	....	....	1.0	12	....	7.0	2.3	26	0.5	3.7	0.4	2.0	....	2.0	....	....
P—[CH_3COOH]	1	....	2.5	0.7	1.0	1.4	60	2.0	....	2.3	20	....	4.4	1.0	15	....	1.5	....	4.0
" "	2	....	4.6	....	....	2.2	52	0.6	12	6.4	95	0.6	8.7	0.9	3.0	....	30	....	....
P—[CH_3COOCH_2]	1	1.2	7.8	0.5	0.5	1.0	8.8	1.2	2.8	0.7	4.2	....	....	....	0.5	....	....	....	....
" "	2	2.7	22	0.7	....	2.4	37	....	....	0.6	21	....	1.4	....	1.3	....	....	....	....
P—[Long-chain acyloxy]	1	8.1	21	7.0	1.0	34	100	100	100	63	64	44	100	24	100	16	100	1.0	100
" "	2	14	25	5.5	1.1	50	100	24	100	71	100	71	100	16	85	7.8	100	11	90
P—[Long-chain acyloxy methylene]	1	2.2	7.2	2.0	0.5	4.5	10	11	12	6.0	6.2	3.9	7.8	1.6	2.5	2.9	12	2.0	8.0
	2	....	0.6	....	2.0	1.0	2.8	....	....	0.8	....	1.4	2.2	....	1.0	0.6	....	0.8	....
Acyl $CH_3(CH_2)_nCO$	1	13	19	6.6	1.0	21	48	35	28	21	18	1.8	11	3.6	11	....	2.5	....	....
" "	2	33	53	8.2	....	43	94	14	55	34	68	4.9	37	4.4	29	....	6.0	....	3.0
Acyl + 1	1	1.5	3.0	1.0	2.2	3.3	7.0	6.2	5.3	3.9	4.6	0.5	2.5	0.7	1.6	....	3.8	....	..
" "	2	3.9	7.0	1.3	1.0	6.6	15	2.4	10	6.6	16	1.2	8.1	0.8	4.0	....	....	1.4	100
$(CH_2)_nCO$	1	0.8	3.4	0.8	4.1	1.4	17	2.9	8.0	2.3	8.6	....	3.5	3.7	40	....	8.0	....	3.0
" "	2	1.0	5.5	0.6	3.5	2.5	18	1.0	7.0	4.8	30	0.6	5.4	3.4	100	....	35	....	4.0
Triacetin	1	....	2.5	4.4	4.8	....	....	1.1	....	1.3	5.5	....	2.4	....	..	....	1.2	..	4.0
"	2	0.5	5.5	0.5	2.5	1.6	21	0.5	35	3.0	25	0.6	2.3	0.7	0.8	....	....	...	2.0
CH_3COOCH_2	1	100	100	100	100	3.1	2.0	6.8	....	29	....	18	15	1.5	....	5.4	3.6	2.9	....
" "	2	68	100	100	100	9.7	1.1	2.3	17	28	15	5.4	....	2.4	0.8	3.1	....	3.6	2.5
C_3H_7	1	67	....	40	....	100	....	81	..	100	....	100	4.6	100	....	100	6.5	100	5.0
"	2	100	4.6	34	....	100	....	100	43	100	....	100	2.5	100	....	100	....	100	3.5

[a] MG = monoglyceride.
[b] H.V. = high electron voltage.
[c] L.V. = low electron voltage.

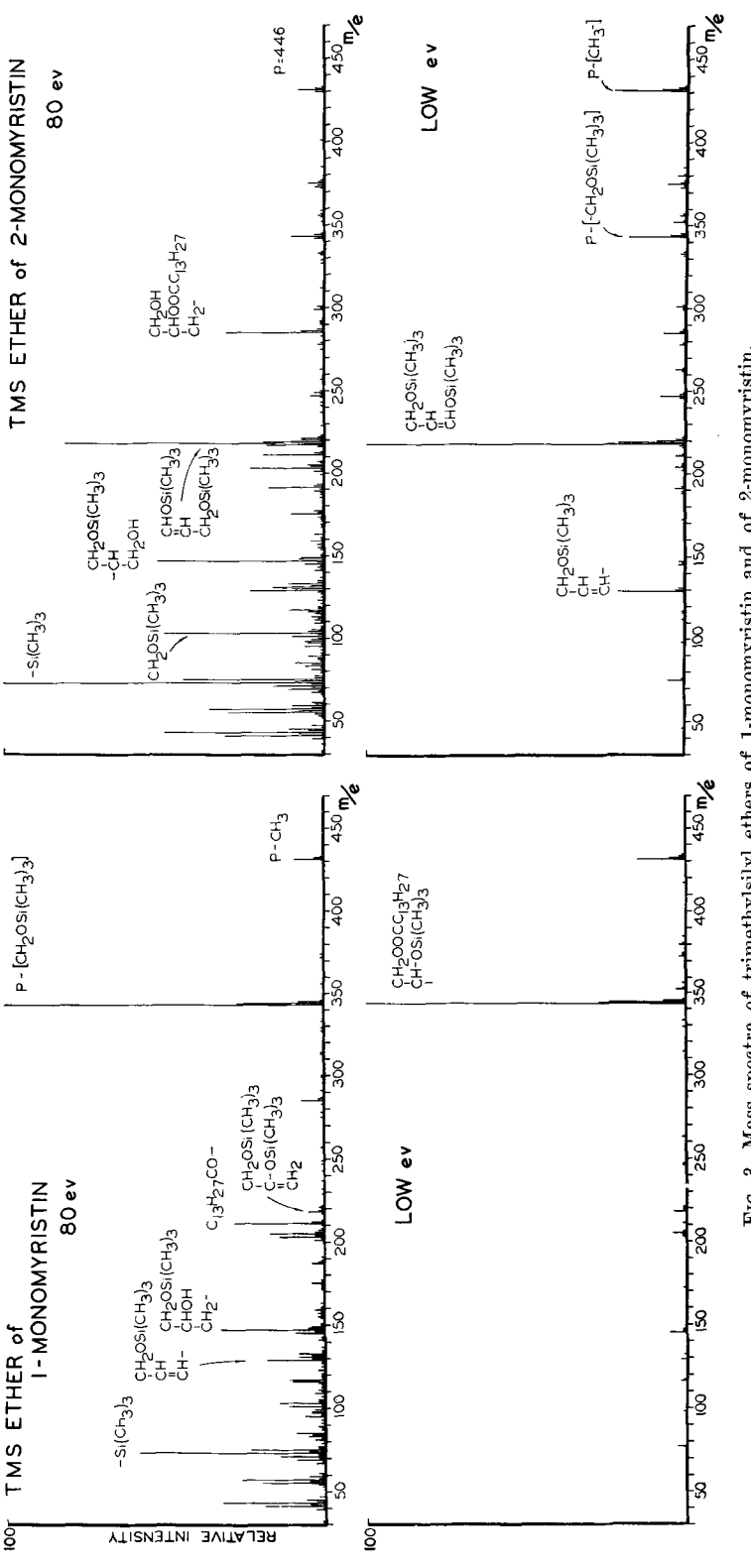


FIG. 3. Mass spectra of trimethylsilyl ethers of 1-monomyristin and of 2-monomyristin.

TABLE III

Mass Spectra of Monoglyceride TMS Derivatives

Fatty acid ion	MG[a]	10:0		12:0		14:0		16:0		18:0		20:0		18:1		18:2		18:3	
		H.V.[b]	L.V.[c]	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.	H.V.	L.V.
P—[CH_3]	1	7.9	14	8.8	12	8.6	15	3.5	15	8.3	17	3.3	12	4.4	31	2.3	11	0.6	....
" "	2	4.8	17	7.2	18	8.3	23	4.5	12	4.2	33	....	11	2.0	4.6	0.9	3.8	....	....
P—[CH_2O $Si(CH_3)_3$]	1	100	100	100	100	100	100	42	100	92	100	32	100	31	49	8.0	35	1.5	....
" "	2	3.7	10	4.0	12	10	18	3.8	7.8	3.2	2.2	0.7	18	2.9	8.2	....	0.8	....	....
m/e = 41	1	23	....	24	....	19	....	55	....	33	....	50	....	55	....	....	....	72	4.5
" "	2	21	....	28	....	31	....	33	....	23	....	39	....	62	1.0	61	....	100	....
m/e = 43	1	38	....	39	....	32	....	91	....	68	....	100	....	51	....	....	....	63	9.5
" "	2	33	....	42	....	50	....	57	....	45	....	65	....	52	1.5	34	....	70	....
m/e = 75	1	43	4.5	68	....	23	....	30	10	33	....	34	1.0	25	48	32	38	52	100
" "	2	38	22	40	3.5	44	4.8	33	....	25	....	28	....	39	17	43	2.3	47	100
m/e = 218	1	6.9	9.1	5.2	6.9	4.7	3.5	4.5	15	6.1	4.0	2.7	....	1.5	....	2.0	10	2.0	2.0
" "	2	53	100	82	100	81	100	63	100	76	100	63	100	11	33	8.8	27	3.5	3.0
m/e = 129	1	22	4.2	19	2.5	18	1.7	26	21	39	1.2	31	6.6	62	100	35	100	19	16
" "	2	74	15	98	26	23	21	92	7.0	100	5.1	100	43	22	100	72	71	20	26
$(CH_3)_3Si$	1	100	....	98	....	58	....	100	2.0	100	1.0	74	11	100	15	100	3.0	100	10
"	2	100	....	100	....	100	....	100	....	82	....	73	....	100	6.0	100	1.5	87	16
Acyl $CH_3(CH_2)_nCO$	1	63	1.9	38	0.8	28	1.0	23	22	23	1.0	14	4.1	13	28	6.3	24	2.4	3.0
" "	2	25	2.5	23	3.5	19	2.9	12	3.0	8.6	7.0	9.8	7.9	13	19	50	25	....	1.5
$(CH_2)_nCO$	1	....	....	....	....	0.7	....	....	1.2	0.8	....	....	....	2.6	23	6.3	70	2.1	3.0
" "	2	....	....	....	....	....	....	....	....	....	....	0.9	3.2	10	33	6.0	100	....	2.0
Acid	1	....	....	....	....	....	....	....	0.6	1.1	1.9	....	....	....	10	0.8	4.1	2.8	2.0
"	2	0.6	....	....	....	....	....	....	....	....	....	....	....	2.7	1.3	0.6	6.7	....	2.0
Acid + 1	1	9.5	1.4	8.3	....	7.2	7.0	1.3	2.2	7.4	1.3	....	....	....	9.0	16	3.8	5.9	3.0
" "	2	26	4.8	32	7.8	30	....	18	14	16	....	....	....	1.7	1.0	1.2	3.1	1.0	3.0

[a] MG = monoglyceride.
[b] H.V. = high electron voltage.
[c] L.V. = low electron voltage

Trimethylsilyl Ether Derivatives of Saturated Monoglycerides

Spectra at High Electron Voltage. Except for 1-monostearin, the base peak of TMS derivatives of 1-monoglycerides corresponded to fragments caused by the loss of methylene trimethylsilyl ether radicals, $-CH_2OSi(CH_3)_3$, from the parent ions. In the spectra of TMS derivatives of 2-monoglycerides, this peak was, in general, less than 10% of the base peak which was the trimethylsilyl radical (m/e = 73). The latter peak was also prominent in the spectra of the 1-monoglyceride derivatives. Loss of the acyloxy group to form an ion of m/e = 218 was more prominent in the spectra of the 2-monoglyceride derivatives than in those of 1-monoglycerides. Ions containing one silicon atom, having m/e = 219 and 147, were present in these spectra.

The intensity of the acyl ion was less in these spectra than in those of the parent monoglycerides. Peaks corresponding to the fatty acid and methyl ester were absent, though usually an ion was present corresponding to the acid-plus-one.

Spectra at Low Electron Voltage. The only peaks of significance in the spectra of TMS derivatives of 1-monoglyceride were those corresponding to the loss of methylene trimethylsilyl ether (base peak) or the methyl group from the parent ions. The base peaks in the spectra of 2-monoglyceride derivatives corresponded to the loss of the acyloxy group (m/e = 218). Three peaks of approximately equal intensity were present, corresponding to $P-[CH_3]$, $P-[CH_2-O-Si(CH_3)_3]$, and m/e = 129.

Trimethylsilyl Ether Derivatives of Unsaturated Monoglycerides

In all cases the base peaks in the spectra at high electron voltage corresponded to the trimethylsilyl radical, as was the case for the saturated monoglyceride TMS derivatives. No generalization may be made for the position of the base peaks at low electron voltages. Differences in the spectra of isomers were similar to those described above for the saturated compounds, though they were not as great.

Possible Analytical Applications of the Spectra

Because the differences in the spectra of 1- and 2-monoglycerides themselves were small and variable, this precludes the use of these for the analysis of monoglyceride mixtures. This was also found to be true for the diacetin derivatives of the monoglycerides. The trimethylsilyl ether derivatives show more promise in this respect, though the marked differences in the spectra of the saturated and unsaturated compounds is a disadvantage.

Spectra of TMS derivatives of monoglycerides can be used to distinguish the isomers. Moreover, the content of each isomer can be estimated, for the $P-[CH_2OSi(CH_3)_3]$ is a measure of the 1-monoglyceride, and the m/e = 218 is a measure of the 2-monoglyceride. The TMS derivatives of saturated 1-monoglycerides have comparable spectra and analysis of their mixtures is feasible by mass spectrometry. The same is true for TMS derivatives of saturated 2-monoglycerides. The analysis of monoglyceride mixtures by GLC-mass spectral analysis should be feasible.

Complete mass spectra of all compounds mentioned in this study are available upon request.

ACKNOWLEDGMENTS

Technical assistance by H. W. Hayes; recording and measuring of the spectral data by Marlys Clementson.

Supported in part by Grant HE 03559 from the National Institutes of Health.

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[Received May 19, 1966]

An Unidentified Lipid Prevalent in Tumors

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ABSTRACT

An unidentified lipid was found in five different tumor sources. It was not found in liver, bone marrow or plasma from tumor-bearing animals, nor in the extracellular fluid supporting growth of Ehrlich ascites cells. The polarity of the unidentified component was similar to that of a glyceryl ether diester, and it was isolated in milligram amounts by preparative thin-layer chromatography. Neither methyl esters of fatty acids, vinyl ether diesters nor quinones were found in the structural makeup of this lipid. Thin-layer chromatography of the purified unidentified tumor lipid on Ag-impregnated silica layers revealed two main components of intermediary unsaturation. Saponification of the unidentified tumor lipid, when water washing was omitted, yielded two components that migrated at R_f's identical to those of free fatty acids and dihydroxy glyceryl monoethers. Neither acetate-1-^{14}C nor palmitic-1-^{14}C acid (single injections) was found to be incorporated into the unidentified lipid of a mature rat tumor.

INTRODUCTION

OUR LABORATORY HAS FOUND a significant quantity of an unidentified lipid component in total lipid extracts isolated from a variety of tumors. Its polarity is such that it migrates directly above triglycerides on thin-layer chromatograms (TLC). The presence of such a compound has not been reported in previous studies of the lipid composition of tumors (1–6), nor had we ever observed it in total lipid extracts from other tissues. The lipid component that we find in tumors is unrelated to "malignolipin," purportedly a sphingolipid unique in malignant tissues (7), but never confirmed (8–10). Lindlar and Wagenar (5) show the TLC lipid patterns of five different mouse tumors, each revealing a component above the triglycerides that the authors refer to as fatty acid methyl esters. The evidence for this identification, however, was only the TLC R_f in a single solvent system, so it is quite possible that the spot in question was not a fatty acid methyl ester at all, but rather *the same component* we have observed in TLC of tumor lipid extracts. Its occurrence in rat tumors, Ehrlich ascites cells, and a human tumor is documented in this paper.

EXPERIMENTAL

The rat tumors used in this study were two transplantable tumors (Walker-256 and R-3259) and a tumor that occurs in rats approximately one year after exposure to 800 R total-body irradiation. The Walker-256 and irradiated rat tumors grew in Carworth Farms Nelson's strain rats, whereas the R-3259 tumor was transplanted in Fischer strain rats. Sodium acetate-1-^{14}C (in saline) or palmitic-1-^{14}C acid (as an albumin complex) was injected intravenously into some irradiated rats bearing tumors, to determine the ^{14}C incorporation pattern into the tumor lipids. The rats were killed 6 hr after injection of the ^{14}C-labeled compounds. Liver, plasma and femoral bone marrow were also taken from a few rats for lipid analysis. A total of 34 rat tumors were analyzed.

The Ehrlich ascites cells (EAC) were grown in the peritoneal cavity of several hundred white Swiss mice. After a week of growth, the ascites fluid was removed and the EAC were separated from extracellular fluid by centrifugation for 15 min at 1100 × g at 2C. The EAC cell-packed volume was approximately 30% (v/v) under these conditions. Livers from these mice were also removed for lipid analyses.

The tumor tissue from a 66-year-old man with lymphosarcoma was obtained at autopsy from an axillary lymph node.

Lipid Extraction and TLC

All the homogenized solid tumors, EAC, EAC extracellular fluids, livers, plasmas, and marrow samples were freeze-dried before extraction and purification according to the procedure of Folch et al. (11). The water-washed lipid extracts were concentrated to 20 mg/ml in chloroform, and a 5λ aliquot of this solution was used for TLC. All solvents used for lipid extraction and TLC were redistilled or purified by passage over activated alumina columns. The adsorbent for TLC was Silica Gel G (Brinkman) and the solvent system for

[1] Under contract with US Atomic Energy Commission.
[2] An operating unit of Oak Ridge Associated Universities.

chromatographic development was hexane: diethyl ether:acetic acid (70:30:1, 80:20:1, 85:15:1, 90:10:1, 95:5:1 or 100:0:1 v/v/v). Both equilibrated (filter-paper-lined) and non-equilibrated (unlined) solvent chambers were used. Silver-ion TLC (12), which separates lipids according to the number of double bonds, was also used. Under these conditions, chloroform:carbon tetrachloride:methanol:acetic acid (50:50:2:1 v/v/v/v) was used as the developing solvent. The separated components were visualized by H_2SO_4-charring, methylene blue (13), dichlorofluorescein, or iodine vapor, and the radioactivity distribution along the entire chromatographic lane was obtained by zonal scan analysis (14). The glyceryl alkoxy monoethers and a plasmalogen were obtained from Claude Piantadosi of the University of North Carolina. H. K. Mangold and Wolfgang Baumann of the Hormel Institute provided us with glyceryl alkoxy di- and triethers (15). Other lipid standards were purchased from the Hormel Foundation and Applied Science Laboratories.

Thin-layers of Silica Gel G (250 μ) were also used to prepare pure quantities of the unidentified tumor lipid. Development of the preparative plates in nonequilibrated chambers of hexane: diethyl ether (90:10 v/v) was found to provide maximal separation of the unidentified tumor lipid from the triglyceride fraction. Preliminary results had shown that breakdown on silicic acid occurred to some extent during scraping and elution on highly activated plates or during prolonged exposure to air; this was avoided by activating preparative TLC plates at 110C for only 15 min. Storage of the tumor lipids in solution at −20C was found to be wise, since the component appears to be readily oxidized. Two-

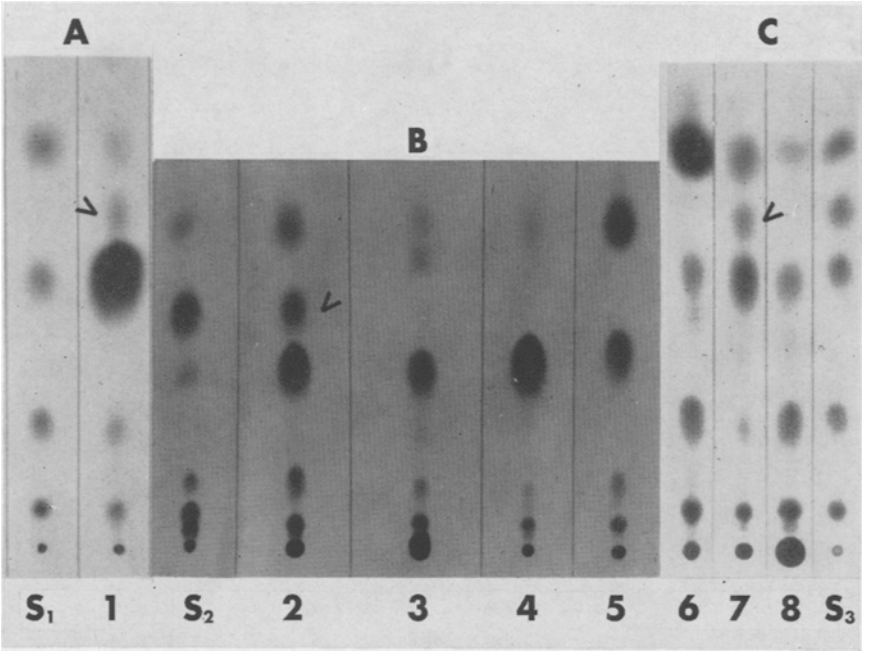

FIG. 1. Thin-layer chromatograms of total lipid extracts from (A) human lymphosarcoma (Lane 1), (B) irradiated rat tumor (Lane 2) and (C) Ehrlich ascites cells (Lane 7). The < designates the unknown lipid component (above triglycerides) prevalent in all tumors examined. TLC patterns of total lipids from other tissues or fluids are: *Plate B*, rat liver (Lane 3), rat femoral bone marrow (Lane 4), and rat plasma (Lane 5); *Plate C*, EAC extracellular fluid (Lane 6) and mouse liver (Lane 8). S_1 is a mixed lipid standard (from bottom to top) containing lecithin, cholesterol, oleic acid, triolein and cholesterol oleate. S_2 is a mixed lipid standard containing S_1 components plus 1-hexadecanol (3rd component from bottom) and dipalmityl batyl alcohol (6th component from bottom at same R_f as unknown tumor lipid). S_3 is a mixed lipid standard containing S_1 components plus dipalmityl batyl alcohol (5th component from bottom at same R_f as unknown tumor lipid). Although the lipid patterns from the Walker-256 sarcoma and the R-3259 tumor (both tumors transplantable in rats) are not shown in the chromatogram, they, too, exhibited an unidentified spot having an identical R_f to that observed in the tumor lipids shown above.

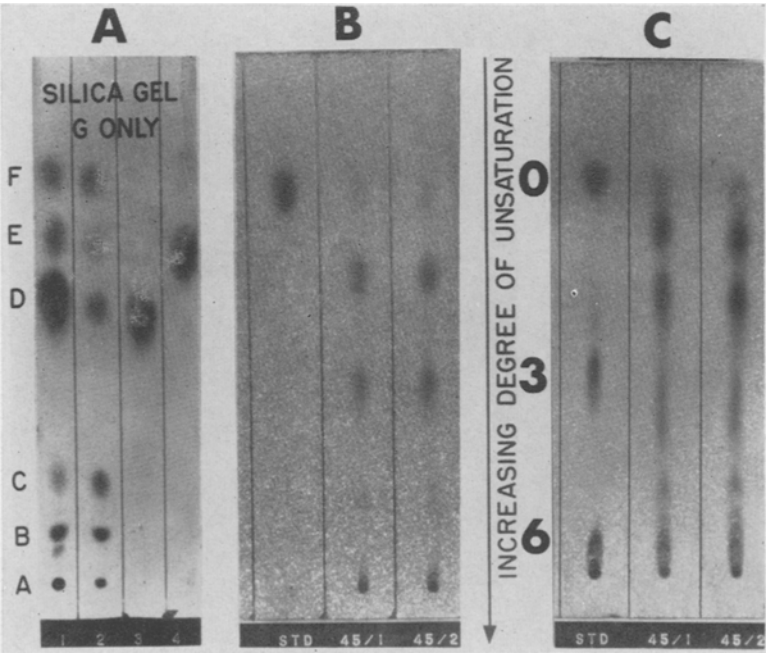

FIG. 2. Silver ion thin-layer chromatography of unidentified tumor lipid. *Plate A* (Silica Gel G only)—Lane 1 represents a mixed lipid standard containing lecithin (A), cholesterol (B), oleic acid (C), triolein (D), dipalmityl batyl alcohol (E) and cholesterol oleate (F). Lane 2 represents rat tumor lipids. Lane 3 represents tumor triglycerides purified by preparative TLC. Lane 4 shows the unidentified tumor lipid purified by preparative TLC. *Plate B* (Ag-layer) STD is dipalmityl batyl alcohol; Nos. 45/1 and 45/2 are purified unidentified lipid component of tumors (same sample as in Lane 4 on *Plate A*); *Plate C* (Ag-layer) STD refers to tripalmitin (O double bonds), triolein (3 double bonds) and trilinolein (6 double bonds); Nos. 45/1 and 45/2 are purified triglycerides of tumors (same sample as in Lane 3 on *Plate A*).

dimensional chromatography of the EAC total lipids in the same solvent system produced a chromatogram with all components of the mixture on a diagonal between the two solvent fronts, indicating that no decomposition occurred during TLC. The unidentified lipid on the preparative plates could be visualized simply by looking at the pattern of translucent lipid bands. Chloroform was used to elute the tumor lipid from the preparative adsorbent scrapings of the TLC plate.

No attempt was made to determine the percentage loss during the isolation procedure, but comparisons of the R_f's of the eluted lipid and the unknown component in the original total-lipid extract were always made on analytical TLC plates as described in the previous paragraph.

The purified unidentified lipid component from EAC and the irradiated rat tumor was checked for the presence of vinyl ethers by the $HgCl_2$-diphenylcarbozone method of Norton (16). A known C_{12} plasmalogen (17) obtained from Claude Piantadosi gave a positive purple test, whereas highly purified samples of selachyl alcohol, batyl alcohol, linolenyl alcohol, and lecithin were *negative*. A vinyl ether test developed by Schmid and Mangold (18) was also tried. The latter test involved TLC of the lipid component in one direction, exposure to HCl fumes for 5 min above a hot plate, followed by TLC in the second direction in the same solvents (hexane:diethyl ether 90:10 v/v). We did not detect any "neutral plasmalogens" by this method. Aliquots of the purified unknown lipid from EAC and the irradiated rat tumor were checked for methyl esters of fatty acids by gas-liquid chromatography (GLC) on an ethylene glycol succinate column at 190C. No peaks were observed even 18 hr after injection of the sample.

Saponification

The purified unidentified tumor lipid was saponified for 1 hr with 2 ml 0.5 N ethanolic KOH. During initial experiments the saponified mixture was acidified and washed several times with equal volumes of hexane and water.

In other experiments the saponified mixture was acidified with 6 N HCl, but not washed. Aliquots of either the hexane extract or of the acidified ethanolic mixture were chromatographed as before, but a solvent system of hexane:diethyl ether:methanol:acetic acid (80:20:10:1 v/v/v/v) was used for development.

RESULTS AND DISCUSSION

In a survey project to establish TLC patterns for neutral and phospholipids of various tumors, we soon became aware of a prominent characteristic spot that always appeared directly above the class of triglycerides but below fatty acid methyl esters in an equilibrated solvent system of hexane:diethyl ether:acetic acid (90:10:1 v/v/v) (Fig. 1). This lipid component has been visualized on TLC plates for all tumor tissues that we have examined. Lipids known to have an R_f in the region of that of the unidentified component include fatty aldehydes, fatty acid esters of glycol, fat-soluble vitamin derivatives, glyceryl monoacyl diethers, glyceryl diacyl monovinyl ethers, glyceryl diacyl monoalkoxy ethers, glyceryl triethers, and methyl esters of fatty acids.

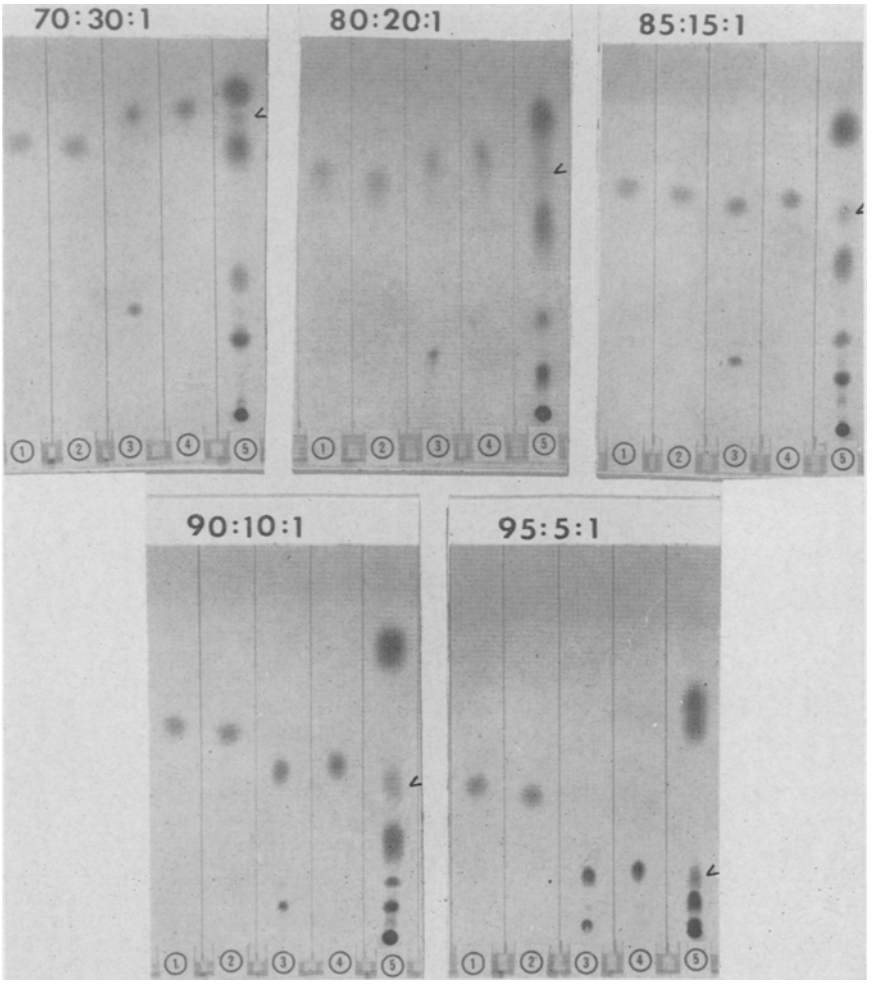

FIG. 3. Thin-layer chromatograms of fatty acid methyl esters, glyceryl ether diesters, and total lipids from Ehrlich ascites cells (Silica Gel G used as adsorbent). Numbers above each chromatogram refer to solvent ratio v/v/v of hexane:diethyl ether:acetic acid. Lane 1, methyl stearate; Lane 2, methyl oleate; Lane 3, dipalmityl batyl alcohol (upper spot) and monopalmityl batyl alcohol (lower spot); Lane 4, distearyl batyl alcohol; and Lane 5, total lipids from Ehrlich ascites cells. The < designates the position of the unidentified tumor lipid. The polarity of the solvent systems decreases from top to bottom and from left to right.

Chromatographic separation of this purified unknown lipid on silica gel containing silver ion has revealed at least two major components exhibiting an intermediate unsaturation (Fig. 2). In the same figure we show the different pattern of unsaturation exhibited by tumor triglycerides; it appears that the two most prominent tumor triglycerides exhibit unsaturation of the same type found in the unidentified tumor lipid. The unidentified tumor lipid has an R_f identical with that of a glyceryl ether diester in certain solvent systems (Figs. 1 and 3). Saponification and purification by hexane extraction did not yield the alkoxy monoether; however, TLC of the total saponified mixture that had been acidified but not washed revealed a component at the same R_f as the dihydroxy glyceryl ethers, e.g. batyl alcohol and a second component at the same R_f as free fatty acids (Fig. 4). Apparently the minute quantities of glyceryl ethers liberated were solubilized in the water phase under these conditions. The tests for vinyl ethers were negative. Glyceryl monoacyl diethers, glyceryl triethers and waxes all had higher R_f's when co-chromatographed with the purified unknown tumor lipid. The unidentified lipid exhibited no color change upon charring a H_2SO_4 sprayed layer on a hot plate, nor did sterols have similar R_f's on TLC.

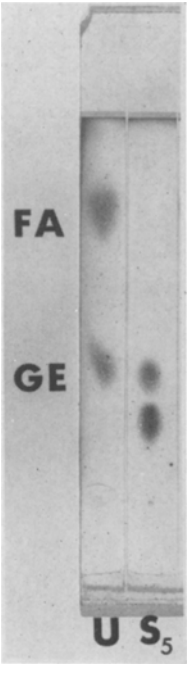

FIG. 4. Saponification of purified unidentified lipid (U) from Ehrlich ascites carcinoma cells. S_5 contained monopalmitin (lower R_f) and batyl alcohol (GE) (upper R_f). FA refers to fatty acid position. Solvent development for TLC was hexane:diethyl ether:methanol:acetic acid (80:20:10:1 v/v/v/v).

Fatty acid methyl esters and glyceryl ether diesters having R_f's similar to the tumor lipid reverse their positions of migration on TLC when the polarity of a solvent system is changed by varying the solvent ratios (Fig. 3). The unknown tumor lipid behaved in a manner similar to that of the glyceryl ether diesters in the various solvent systems tested (Fig. 3). Figure 3 also shows that slight differences in migration caused by unsaturation (methyl stearate and methyl oleate) and slight differences in migration caused by chain length (dipalmityl batyl alcohol and distearyl batyl alcohol) occurred in all solvent systems. GLC of the purified unidentified tumor lipid from EAC and the irradiated rat tumor confirmed that it was neither a methyl ester of a fatty acid nor a long-chain fatty aldehyde.

Work to pinpoint the chemical structure of this tumor lipid continues. We have found that the component can readily be purified by preparative TLC for such studies (Fig. 2). The purified spot has a slight fluorescence under ultraviolet light if visualized immediately after spotting on a TLC plate, although this fluorescence disappears with time. It could not be visualized with leucomethylene blue, indicating that its redox potential, if any, is less than that of methylene blue.

The unidentified tumor lipid has never been found in other tissues from normal or tumor-bearing rats at *normal* chromatographic loads, nor was it found in the extracellular fluid supporting the growth of Ehrlich ascites cells or in the liver of the EAC-bearing mice (Fig. 1). We do not mean to imply that the lipid component we have isolated from tumors does not exist in normal tissues, since it may be present at much lower concentrations. In fact, Schmid and Mangold (19) have recently found that human perinephric fat contained small amounts of glyceryl ether diesters (approximately 0.3%) with an alkoxy:alkenyl ratio of approximately 1:1.

It would indeed be of interest to determine if the unidentified tumor lipid is in any way associated with cell proliferation, a proposal that Price (20) has previously suggested. In our work, the Ehrlich ascites carcinoma cells were harvested during their rapid period of growth. In contrast, the majority of the rat tumors we examined were relatively large and slow growing at the time of their removal. The proportion (Fig. 1) of unidentified lipid to

total fat differed from tumor to tumor, and there was no correlation between size of solid tumors and total fat content. In the EAC, the total lipids represented 8.3 ± 2.3% of the dry weight, and the unidentified lipid was estimated to represent about 1–2% of that.

Until more is known about the unequivocal structural configuration of this class of tumor lipid, it is difficult to speculate on either its biochemical origin or its biological significance. Nevertheless, we gave some common lipid precursors as tracers to a couple of tumor-bearing irradiated rats and examined the incorporation of acetate or fatty acid into lipids 6 hr after single injections. The ^{14}C from palmitic acid was primarily incorporated into the triglycerides (63%), and the ^{14}C from acetate was primarily incorporated into the phospholipids (70%) of rat tumor lipids. However, we could never demonstrate any of the ^{14}C-label in the area above the triglycerides. It is obvious that multiple injections in combination with more rapidly proliferating tumors need to be explored.

The results so far, especially the absence of the unidentified tumor lipid in the extracellular ascites fluid, leads us to the rather interesting hypothesis that the origin of the unknown lipid might be in a biosynthetic pathway unique to tumor cells. However, an alternate explanation is that the tumor cells only concentrate the unidentified lipid from another tissue.

ACKNOWLEDGMENT

Assistance in this work by Miss Judy Malone is appreciated.

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[Received April 18, 1966]

The Prevalence of Aliphatic Delta-Lactones or Their Precursors in Animal Fats

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ABSTRACT

Data are presented to show the occurrence of saturated aliphatic delta lactones, namely the δ-C_{10}, δ-C_{12}, δ-C_{14}, and δ-C_{16}, in numerous ruminant and monogastric animal fats. These trace components were isolated by silicic acid adsorption chromatography followed by identification employing gas chromatography. The general prevalence of the delta-lactones or their precursors in animal depot fat, mammary tissue, blood serum lipids and milk fat is suggestive that they occur commonly in animal fats and are related to lipid metabolism.

INTRODUCTION

NUMEROUS INVESTIGATIONS from our own and other laboratories have provided evidence of γ- and δ-aliphatic lactones in bovine milk fat (1,6,8,14,17). The occurrence of these minor components has been particularly associated with the flavor of heated and stored forms of fat-containing dairy products. Evidence indicates that the precursors are monohydroxyalkanoic acids in esterified glyceride form (1,6,7,11) and that the lactones result by a spontaneous nonoxidative mechanism (1,15) involving hydrolysis and lactonization of these hydroxy acids. For better definition of the metabolic origin and significance of these lactones (hydroxy acid), it was of interest to determine whether their occurrence is limited to ruminants. The following experiments indicate general prevalence in animal fats.

EXPERIMENTAL PROCEDURE

A description of the samples analyzed and the methods of lipid extraction is presented in Table I. In order to isolate the lactone-rich fraction, silicic acid adsorption chromatography was employed. A similar technique has been reported (6). Twenty grams of Mallinckrodt silicic acid (5) was slurried onto an 18-mm I.D. glass column in ethyl ether. The column packing was washed thoroughly with 500 ml of petroleum ether (bp 35–42C). Two to six grams of lipid, dissolved in petroleum ether, was applied and washed into the packing. Elution was carried out by adding 250 ml of 100% petroleum ether, 250 ml of 10% ethyl ether in petroleum ether and lastly 300 ml of 100% ethyl ether. The first fraction eluted contained hydrocarbons and traces of sterol esters. The second fraction contained essentially all the triglycerides and some nonesterified fatty acids. The 100% ethyl ether fraction was composed of traces of triglycerides, diglycerides, monoglycerides, sterols, nonesterified fatty acids, free lactones and "lactone precursor (7)." This whole fraction was evaporated to dryness on a steam bath under N_2 and saponified with 2.5 ml 7.8% KOH in ethanol by refluxing 20 min. The solution was diluted with 2.5 ml H_2O, rewarmed to a boil, cooled and extracted

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TABLE I

Description of Samples and References to Methods of Lipid Extraction Used in This Survey of δ-Lactones and Their Precursors

Samples		Source	Reference
Cow	Milk fat	Mixed herd	(11, 16)
	Mammary tissue	Individual cow[a]	(16)
	Depot fat	Individual steer[a]	(3)
	Whole blood	Individual cow[a]	(13)
	α-lipoprotein β-lipoprotein	Holstein cow	(12)
Goat	Milk fat	Toggenburg goat	(16)
Sheep	Milk fat	Hampshire ewe	(16)
	Depot fat	Individual sheep[a]	(3)
Swine	Milk fat	Hampshire sow	(16)
	Depot fat	Individual swine[a]	(3)
Human	Milk fat	Mixed[b]	
		Individual	(3)

[a] History of source not precisely known.
[b] Supplied by J. B. Brown, Laboratory of Physiological Chemistry, The Ohio State University, Columbus, Ohio.

TABLE II

Comparison of Retention Times Between δ-Lactones of Human Milk Fat and Those Reference Lactones on Two GC Columns

Reference lactone	Retention time relative to δ-C_{12} (actual time, min)[a]			
	Polyester column[b]		Apiezon column[c]	
	Unknown	Reference	Unknown	Reference
δ-C_{10}	0.52(4.4)	0.53(4.5)	0.45(6.5)	0.44(6.3)
δ-C_{12}	1.00(8.5)	1.00(8.5)	1.00(14.4)	1.00(14.4)
δ-C_{14}	1.98(16.8)	1.99(16.9)	2.27(32.7)	2.26(32.6)
δ-C_{16}	3.86(32.8)	3.88(33.0)[d]	5.08(73.1)	5.07(73.0)[d]

[a] Actual retention times are measured from solvent front, samples and references run consecutively.
[b] 10% diethyleneglycol adipate plus 2% H_3PO_3 at 172C, argon pressure of 16 psig, Barber-Colman Model 10 GC equipped with radium 226 detector source. Detector cell voltage 750.
[c] 20% Apiezon-L column at 207C, argon pressure of 30 psig, Barber-Colman Model 10 GC equipped with a radium 226 detector source. Detector cell voltage 1000.
[d] Reference unavailable; time evaluated from semilog plot of retention data for homologous series of δ-lactones.

3 times with hexane to remove nonsaponifiable material. The resulting soap solution was decomposed with 6 N HCl and extracted 3 times with hexane. Upon evaporation the residue had a strong fatty acid and coconut-like (lactone) odor. This residue, dissolved in petroleum ether, was then applied to a second 20-g silicic acid column and fractions eluted as before. The bulk of the fatty acids were eluted with the 10% ethyl ether in petroleum ether fraction and the lactones were eluted from the column with pure ethyl ether. This lactone-rich fraction was then evaporated to 200 μl and 10 μl amounts were used for the gas chromatographic (GC) analysis (2). When sufficient lipid material (100 g) was available, the lactones were also isolated by steam deodorization as previously reported (17).

Identification of the lactones was accomplished by comparing GC retention times for authentic compounds (kindly supplied by J. Boldingh, Unilever Ltd., Vlaardingen, The Netherlands) with those for the unknowns on both polar (polyester) and nonpolar (Apiezon) coated column packings (for representative data, see Table II). Coincidence of the lactone odor with the emerging peak was also used as evidence of identity. However, δ-C_{16}, being unavailable to us and apparently nonodorous, was implicated by a plot of retention data for the homologous series of δ-lactones and by previous identification of it from bovine milk fat (6).

RESULTS AND DISCUSSION

Analyses of the various fats proved positive for the occurrence of δ-aliphatic lactones (Table III, Figures 1 and 2). No attempt was made to quantitate the lactones in this survey; however, a quantitative visual comparison of the lactone peaks is justified and can be seen in Figure 1 for milk fats from the ruminants

TABLE III

Methods Employed in Demonstrating the Major δ-Lactones or Their Precursors in Lipids of Various Species

Sample	Lactones			
	δ-C_{10}	δ-C_{12}	δ-C_{14}	δ-C_{16}
Cow				
Milk fat	1[a], 2[b]	1, 2	1, 2	1, 2
Mammary tissue	1	1	1	1
Depot fat	1, 2	1, 2	1, 2	1, 2
δ-lipoprotein	1	1	1	
β-lipoprotein	1	1	1	1
Goat				
Milk fat	1, 2	1, 2	1, 2	1, 2
Sheep				
Milk fat	1	1	1	1
Depot fat	1	1	1	1
Swine				
Milk fat	1	1	1	1
Depot fat	1	1	1	1
Human				
Milk fat	1, 2	1, 2	1, 2	1, 2

[a] Refers to silicic acid adsorption chromatography. For details of column, see text.
[b] Refers to steam deodorization of 100 g of fat.

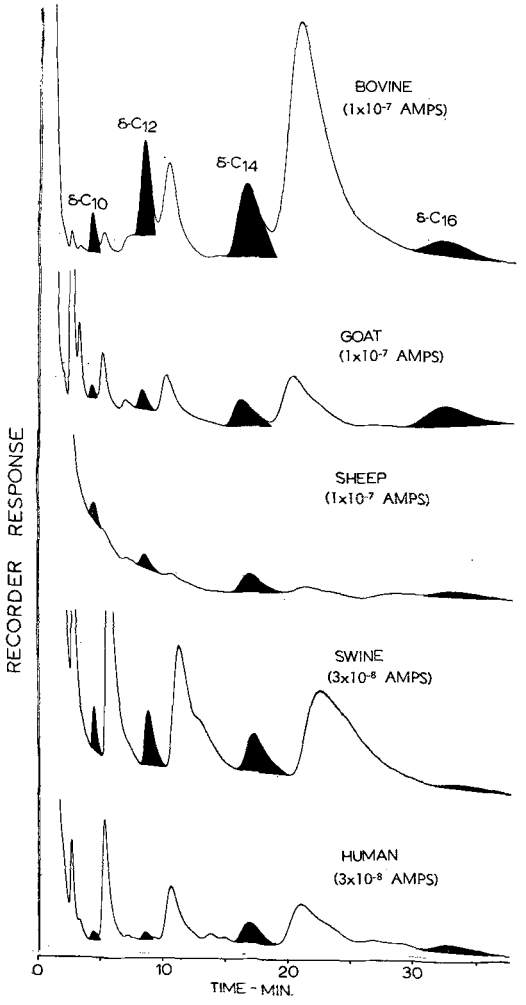

FIG. 1. Gas chromatograms of lactones isolated from the milk fats of cow, goat, sheep, swine, and human on a 6-ft by 6-mm column packed with 10% diethyleneglycol adipate treated with 2% phosphoric acid on 60–80 mesh Gas-Chrom P (Applied Science Laboratories, State College, Pennsylvania).

(cow, goat, sheep); and the monogastrics (swine, human). The initial amount of lipid material in each analysis was 6.0 ± 0.4 g, and conditions for isolation, concentration and GC were held constant. Published data (1,2,6) indicating that the δ-C_{10}, δ-C_{12}, δ-C_{14}, and δ-C_{16} lactones occur in the range of 10 to 60 ppm for cow milk fat may also provide a useful frame of reference.

The occurrence of lactones or their precursors in the milk fat of monogastric animals tends to eliminate the rumen as an exclusive source of the lactone precursors. Admittedly the human diet consists of large quantities of animal fats which could conceivably account for the presence of these compounds in mother's milk. However, with the identification of lactones in milk fat and depot fat of the swine in which the diet was completely devoid of animal fat, it is reasonable to conclude that these compounds are not unique to ruminants.

The δ-C_{10} and δ-C_{12} lactones have been detected in bovine tallow but at considerably lower levels than in bovine milk fat (1). Similarly in this investigation, traces of lactones were found in steer, sheep and swine depot fat. Interestingly, even though the depot fats of the animals analyzed are composed mainly of long chain fatty acids, namely 16 and 18 carbon acids (13); identifiable amounts of δ-C_{10}, δ-C_{12}, and δ-C_{14} lactones were evident. A similar inconsistency was evident in the swine milk fat. It is therefore assumed that the corresponding hydroxyalkanoic acid precursors, and their keto glyceride analogs (18), may be involved in a unique synthesis or degradation of fatty acids by four-carbon units. The use of intact four-carbon units (β-hydroxybutyrate) has been demonstrated in milk fat synthesis (9,10).

It was not surprising to find lactones in whole blood of the cow since they occur in depot fat, mammary tissue and milk fat. Analyses of equal amounts of lipid (2.5 g) from α- and β-lipoproteins of blood serum indicated a greater proportion of the lactone potential was present in the β-fraction. This is of particular interest since these β-lipoproteins are major contributors of lipids to milk fat (4).

From these data it is evident that the lactone precursors occur commonly in animal fats and are related to general lipid metabolism. The mechanism involved in formation of the hydroxyalkanoic acid precursors is presently under investigation.

ACKNOWLEDGMENT

Authorized for publication on April 13, 1966 as Paper No. 3127, Journal Series of the Pennsylvania Agricultural Experiment Station. Supported in part by Agricultural Research Service, U.S.D.A., Grant No. 12-14-100-7980 (73).

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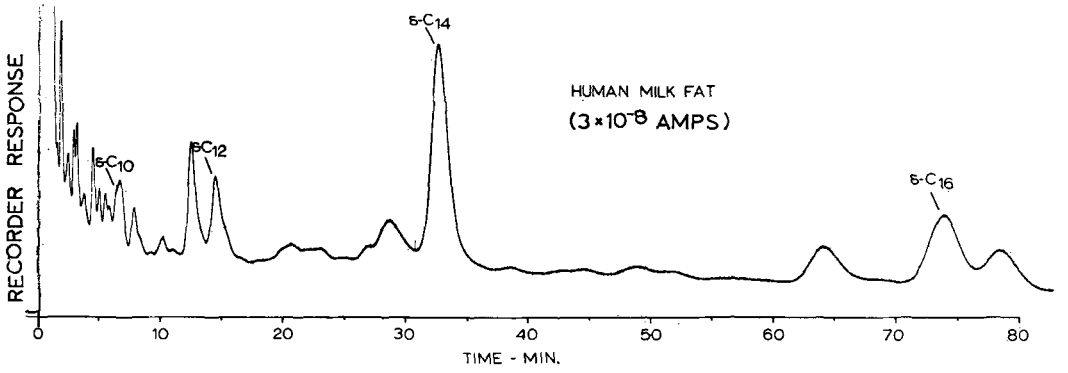

FIG. 2. Typical gas chromatogram showing the separation of lactones (human milk fat) on 6-ft by 4-mm column packed with 20% Apiezon-L on 120–140 mesh Anakrom AB (Analabs Inc., Hamden 18, Connecticut).

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[Received May 11, 1966]

Fatty Acids in Phospholipids Isolated from Human Red Cells[1,2]

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ABSTRACT

Total lipids of packed erythrocytes from healthy men 22 to 25 years old were extracted with chloroform-methanol mixture. Phospholipid classes were separated from neutral lipids and pigments on a silicic acid column. Phosphatidyl inositol (PI) was freed of its contaminants phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS) on an aluminum oxide column. Additional silicic acid columns with modified solvent systems were needed for complete separation of other overlapped phospholipid classes. The identification of phospholipids in each eluted fraction was accomplished by TLC, using the appropriate spray tests and reference compounds, and confirmed on each of the isolated phospholipids by IR spectrophotometry.

The total content of phospholipids as determined by phosphorus analysis was found to be 2.63 mg/ml of packed cells. These phospholipids were found to have the following composition (in per cent of total phospholipid): PI, 2.3; PE, 13.4; ethanolamine plasmalogen (EP), 14.5; PS, 3.9; lecithin (L), 34.2; choline plasmalogen (CP), 1.4; sphingomyelin (Sph), 28.4 and lysolecithin (LL), 1.7. The fatty acid composition of each phospholipid was determined by GLC. The average number of double bonds per fatty acid in the isolated phospholipids was found to be as follows: PI, 1.5; PE, 1.9; EP, 3.6; PS, 2.1; L, 1.0; CP, 2.0; Sph, 0.2 and LL, 0.5. The positional distribution of fatty acids in both L and PE was ascertained by selective enzymatic hydrolysis with phospholipase A. Saturated fatty acids of L were esterified predominantly in the α'-position, whereas in PE only 63.9 mole per cent of the saturated fatty acids were found in this position.

[1] Presented in part at the AOCS Meeting in Los Angeles, April 1966.

[2] The following abbreviations are used; L, lecithin; CP, choline plasmalogen; LL, lysolecithin; PE, phosphatidyl ethanolamine; EP, ethanolamine plasmalogen; PS, phosphatidyl serine; PI, phosphatidyl inositol; Sph, sphingomyelin; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; IR, infrared; α', denotes fatty acids in the 1-, and β in the 2-position of phosphoglycerides.

[3] Dept. of Health, Education and Welfare, USPHS.

INTRODUCTION

A NUMBER OF INVESTIGATORS have contributed information in recent years on the composition of the phospholipids of human red cells employing the techniques of column (6–8,14,22,23,29), paper (2,10,11,17,24,30,31) and thin-layer chromatography (3), or successive chemical hydrolyses followed by an examination of the separated water-soluble fragments (4,5). Only a few reports have appeared on the fatty acid composition of isolated phospholipid classes. These studies have dealt with the major phospholipid components of red cells: phosphatidyl ethanolamine, phosphatidyl serine, lecithin (7,8,14,29), and sphingomyelin (8,14,29) and have neglected the minor components. In addition, the fatty acids were derived from the phospholipid classes which were not completely resolved. In two of the investigations (8,14) only the major fatty acids are given. Also, the fatty acids in phosphoglycerides described in previous studies were given as the total of the acetal and the ester forms rather than separately (7,8,29) or the values for fatty acids were even not corrected for the presence in the methyl ester mixtures of dimethyl acetals arising from the plasmalogens (14). Therefore, it appeared worthwhile to carry out further investigations on the fatty acids in human red cells of each of the individual classes of phospholipids.

This study was aimed at the complete chromatographic separation of phospholipid classes in red cells by column fractionation and refractionation of overlapping components until the isolated preparations were free of contaminants, as justified by TLC and IR spectrophotometry, so that the fatty acids could be determined by GLC. Other objectives of this study were 1) to describe the fatty acids of the plasmalogens separately from the fatty acids of plasmalogen-free glycerophosphatides, and 2) to compare the positional distribution of fatty acids in highly unsaturated phosphatidyl ethanolamine, free of plasmalogen, with the positional distribution of fatty acids in plasmalogen-free lecithin in which saturated and unsaturated acids were expected to be present in about equal proportions.

EXPERIMENTAL

Materials

A total of 3,844 ml of blood was collected from the same 8 students who donated blood for investigation of phospholipids in serum (32). Each bottle for the collection of 250 ml of blood contained 37.5 ml of an anticoagulant with the following composition per 100 ml: 0.73 g citric acid, 2.20 g sodium citrate, and 2.45 g dextrose. Cells were centrifuged out, separated from plasma, and washed twice with saline solution. The volume of the packed cells in the blood of donors was 46 to 50% averaging 47.6%.

Other materials and reference compounds of both phospholipids and methyl esters are described elsewhere (32).

Lipid Extraction

The antioxidant -4-methyl-2,6-di-tert-buthylphenol (BHT) (33) was added to the solvents and was present throughout the procedures of extraction, separation, and storage of lipids at a concentration of 0.005%. One volume of water was added to each volume of packed red cells in order for lysis to take place. The lysed red cells were suspended in methanol and chloroform in a Waring blender during extraction of the lipids. The extract was filtered on a Buchner funnel and the residue re-extracted twice with chloroform. Chloroform, methyl alcohol, and water present in the combined extract were in the ratio 8/4/3, v/v/v, respectively (9). This amount of water includes that present in the cells. The extract was left overnight in the cold room for the phases to separate, and the lower layer contained the lipid extract. The lipid extracts were pooled at this point. The total phosphorus (1) in the lipid extract was 192.04 mg, or 2.63 mg of phospholipid per one ml of packed cells.

Chromatographic Separations

Lipids in chloroform solution were chromatographed on 250 g of silicic acid in a 4.5 cm diameter column. A load of no more than 0.04 mg of phospholipid phosphorus was applied per gram of silicic acid. The thin-layer chromatographic procedures for identifying the phospholipids in each fraction and for following the elution of each phospholipid class are given elsewhere (32). First, neutral lipids were eluted with two liters of chlorofrom and pigments with 4 liters of acetone. Then the phospholipids were eluted according to the fractionation scheme outlined in Table I.

Appropriate fractions were combined into groups, and lipid phosphorus was determined on all groups. Aliquots of groups of fractions containing more than one component were chromatographed on thin-layer plates in the solvent system of Wagner (28) and the separated components determined quantitatively as lipid phosphorus after visualization by charring with 50% sulfuric acid (1). These data served for the calculation of the percentage of each component within the mixture and for computation of total amount of phosphorus in each phospholipid component.

The bulk of the phospholipids which were present in mixtures in the above groups were then subjected to further separation by column chromatography. Phosphatidyl inositol in Group II (Table I) was separated from phosphatidyl ethanolamine and phosphatidyl serine on an aluminum oxide column (13). Lecithin in Group IV was separated on a silicic acid column using 20% methanol in chloroform (v/v) to elute cephalins and methanol to elute lecithin. The combined total mixture of phosphatidyl ethanolamine and phosphatidyl serine was separated on a hydrated silicic acid silicate column (25). Most of the lecithin in Group VII was separated from sphingomyelin on silicic acid column using 30% methanol in chloroform to elute lecithin and methanol to elute sphingomyelin. The separation was completed on another silicic acid column with the

TABLE I

Scheme for Chromatographic Separation of Phospholipids on Silicic Acid Column

Group	Fraction number (50 ml each)	Eluant	Components eluted
I	1–2		None
II	3–16		PI, PE, PS
III	17–51	20% Methanol in chloroform	PE, PS
IV	52–65	(v/v)	PE, PS, L
V	66–72		L
VI	73–101	20% Methanol in chloroform	L
VII	102–150	containing 1.35% water	L, Sph
VIII	151–191	(v/v/v)	Sph
IX	192–224	5% Water in methanol (v/v)	L, Sph, LL

solvent system described by Phillips (21). The separation of the components in Group IX was achieved in a similar way on a silicic acid column (21).

Identification of Phospholipids

The identification and establishment of the purity of the isolated phospholipids by TLC and IR spectrophotometry are described elsewhere (18,32).

Hydrolysis of Plasmalogens

Aliquots of the lecithin, phosphatidyl ethanolamine and phosphatidyl serine fractions were each incubated at 38C in 90% acetic acid in order to liberate plasmalogen aldehydes. This procedure was adopted from Gray (12). The separation and quantification of the hydrolysis products were previously described (32).

Enzymatic Hydrolysis of Lecithin and Phosphatidyl Ethanolamine

Enzymatic hydrolysis of lecithin free of plasmalogen was achieved with *Crotalus adamanteus* venom in diethyl ether solution by the procedure of Tatrie (27). Apparently the enzymatic reaction was completely quantitative since no phosphorus was detected in the supernatant ether phase after removal of the lysolecithin by centrifugation. The procedure for the enzymatic hydrolysis of phosphatidyl ethanolamine free of plasmalogen and for the separation of the products of the reaction is described elsewhere (19). This enzymatic hydrolysis also was complete since unhydrolyzed phosphatidyl ethanolamine was not detected.

Preparation of Methyl Esters

The methyl esters of phosphoglycerides fatty acids were prepared by Hornstein procedure (15,16). Sphingomyelin fatty acids were hydrolyzed and esterified with 5% anhydrous methanolic hydrochloric acid (19).

Gas-Liquid Chromatography

Gas-liquid partition chromatography, identification of fatty acids and calculation as mole per cent of each fatty acid is described elsewhere (32).

RESULTS AND DISCUSSION

The phospholipid composition of normal human red cells is presented in Table II. The proportions of lecithin, sphingomyelin and the phosphatidyl ethanolamine fraction are in general agreement with those reported by other workers (3,7,11,14,24,29). The content of phosphatidyl serine obtained in this study is in agreement with that obtained by paper chromatography (10) or by chemical hydrolysis (4) but lower than that found by several other investigators (2,3,7,14,24,29). The fact that a clean separation of phosphatidyl serine from a phospholipid mixture is difficult to achieve in a single chromatographic separation may offer an explanation for the marked differences in the proportion of phosphatidyl serine reported in the phospholipids of red cells by various investigators.

TABLE II

Phospholipid Content of Human Red Cells (as per cent of phosphorus)	
Phosphatidyl inositol	2.5
Phosphatidyl ethanolamine	13.4
Ethanolamine plasmalogen	14.5
Phosphatidyl serine	3.9
Serine plasmalogen	0.0
Lecithin	34.2
Choline plasmalogen	1.4
Sphingomyelin	28.4
Lysolecithin	1.7

Other workers (3,11,22,23,24) have reported a low content of lysolecithin which is in agreement with the proportion found in this publication. Phosphatidyl inositol in red cells has been listed as a component of the phosphatidyl serine (6) or lysolecithin fractions by some investigators (17,30). Other workers have either failed to observe this phospholipid (5) or have found it at twice the level given in Table II (2).

In the present study the proportion of plasmalogen in the phosphatidyl ethanolamine fraction was found to be 52% whereas other workers have reported about 36 (5,14,29) or 67% (7). In agreement with Dawson et al. (5) serine plasmalogen could not be detected in the present study although others have reported from 1 to 8% plasmalogen in this fraction (4,7,14,29). The finding that choline plasmalogen made up only 3.9% of the lecithin fraction is in agreement with the observations of several other investigators (5,14,19) but is lower than the 10% plasmalogen reported by Farquhar (7). The discrepancies between different reports concerning the proportions of phospholipid fractions and the contents of plasmalogen in phosphoglycerides in normal human red cells are probably due to different analytical methods used and difficulties in achieving clean separations of the phospholipid components present.

The composition of the fatty acids of the phosphatidyl ethanolamine fraction, ethanolamine plasmalogen, plasmalogen-free phos-

phatidyl ethanolamine, including positional distribution of fatty acids, is presented in Table III. The phosphatidyl ethanolamine fraction showed a high degree of unsaturation with over 75 mole per cent of unsaturated fatty acids. The chief unsaturated fatty acids were arachidonic and oleic acids which were present at levels of 26 and 20%, respectively. The high content of 2.4 double bonds per molecule of acid was due to the presence of about 54% of polyunsaturated fatty acids which were primarily of chain lengths of 20 and 22 carbons. Palmitic acid contributed half of the saturated fatty acids.

Ethanolamine plasmalogen fatty acids were predominantly unsaturated as reflected in the fact that 93% of the acids present were unsaturated. The remaining 7% was scattered among nine saturated fatty acids. The main unsaturated fatty acids were arachidonic, docosatetraenoic and docosahexaenoic fatty acids. The ratio between mono- and polyunsaturated fatty acids was almost 1 to 9 and the amount of double bonds per molecule of fatty acid in the ethanolamine plasmalogen was 3.6.

Two-thirds of the fatty acids in plasmalogen-free phosphatidyl ethanolamine were unsaturated with 1.9 double bonds per average molecule of acid. The major unsaturated fatty acids were oleic and arachidonic acids. Palmitic acid contributed about two thirds of the total amount of the saturated fatty acids. The latter showed a high tendency to be esterified in α' position. Only about 10% of the saturated fatty acids were present in β position, and these acids were mainly palmitic and stearic acids. Since the ratio of unsaturated to saturated fatty acids in total plasmalogen-free phosphatidyl ethanolamine was 2 to 1, it is obvious that a part of unsaturated fatty acids had to be present in the α' postion. Among unsaturated fatty acids in the α' position, about two thirds were monounsaturated. There was a general tendency for the percentage of an unsaturated acid in the β position to increase with the degree of unsaturation.

TABLE III

Fatty Acids of Phosphatidyl Ethanolamine of Human Red Cells
(mole per cent)

	PE Fraction		Plasmalogen fatty acids	Plasmalogen-free PE			
				Total			
Fatty acid	Found	Calculated[a]		Found	Calculated[b]	α'	β
10:0	0.4		0.2	0.5		0.3	0.4
11:0	0.3		0.3	0.5		0.5	....
12:0	0.4		0.3	0.3		0.3	0.6
14:0	0.2		0.5	0.4		0.4	0.3
15:0	0.3		0.7	1.4		1.2	0.8
ISO 16:0	0.2		1.6	....		....	....
16:0	12.4	13.8	2.2	20.1	23.3	42.3	4.3
16:1	0.9		0.8	0.9		1.3	1.6
17:0	0.2		....	0.5		1.0	....
ISO 18:0	0.7		....	....		....	....
18:0	7.3	5.8	1.0	8.4	8.9	16.5	1.2
18:1	20.2	19.0	8.9	24.4	23.5	20.6	26.4
18:2	7.6	6.4	3.8	7.8	8.0	6.1	9.8
18:3	0.1		....	....		....	....
20:0	1.4		....	0.3		1.4	0.1
21:0	0.5		....	0.2		....	0.8
20:3	0.7		1.0	1.3		0.7	1.3
20:4	26.4	27.1	43.5	18.3	16.1	1.0	31.1
22:1	0.4		0.9	0.7		1.0	0.9
22:4	7.3	8.4	14.6	5.2	5.3	....	10.6
24:0	0.3		0.6	....		....	....
22:5	4.3	5.6	8.3	4.1	4.1	3.0	5.2
22:6	7.5	6.8	10.8	4.7	3.5	2.4	4.6
Saturated	24.6		7.4	32.6		63.9	8.5
Unsaturated	75.4		92.6	67.4		36.1	91.5
Double bonds per molecule	2.4		3.6	1.9		0.7	2.7

[a] The values listed as calculated were obtained from the fatty acid (A) composition of the plasmalogen-free phosphatidyl ethanolamine (PE) and that of the ethanolamine plasmalogen (EP) using the following formula: Per cent calculated A in PE fraction = (%A in PE × 48.0 × 2 + %A in EP × 52.0)/(48.0 × 2 + 52.0). The formula is based on the observation that the phosphatidyl ethanolamine fraction is composed of 48.0% PE containing 2 fatty acids per molecule and 52.0% EP having only 1 fatty acid.

[b] The calculated fatty acid composition of plasmalogen-free PE was obtained by dividing the sum of the per cent acid in the α' and β positions by 2.

Since the data presented in Table III were obtained after many steps of treatment of phosphatidyl ethanolamine such as acid hydrolysis of the plasmalogen aldehydes in the phosphatidyl ethanolamine fraction, separation of hydrolysis products on a silicic acid column, enzymatic hydrolysis of plasmalogen-free phosphatidyl ethanolamine, and separation of enzymatic hydrolysis products, the exact arithmetic balancing of these data could not be expected, particularly in the case of the fatty acids which were minor components. As a check on the possible deterioration of these fractions during these processes, a calculation was made of the fatty acid content of the phosphatidyl ethanolamine fraction from the fatty acid content of the two fractions, plasmalogen-free phosphatidyl ethanolamine and ethanolamine plasmalogen, which were derived from the original fraction. A similar calculation was made for the total fatty acids in the plasmalogen-free phosphatidyl ethanolamine from the amounts of fatty acid in the α' and β positions which were determined independently of the total fatty acid. The calculation was carried out only for eight of the major fatty acids in each fraction which, however, represent 93% of the total amount of fatty acid present. The details of the calculation are given in Table III.

As can be seen in Table III, there was good agreement between the calculated and observed values for the eight fatty acids in both the phosphatidyl ethanolamine fraction and the plasmalogen-free phosphatidyl ethanolamine. This result indicated that despite the highly unsaturated nature of these phospholipids it was possible to obtain a valid description of their fatty acid composition under the conditions of these experiments.

In Table IV are listed the fatty acids of the lecithin fraction, choline plasmalogen, plasmalogen-free lecithin and the positional distribution of fatty acids within plasmalogen-free lecithin. Almost equal proportions of saturated and unsaturated fatty acids were found in plasmalogen-free lecithin. This lipid furnishes a different model for study of positional distribution of fatty acids as compared with plasmalogen-free phosphatidyl ethanolamine in which the proportions of saturated to unsaturated fatty acids were one to two. Palmitic acid provided 73% of the saturated fatty acids, stearic acid over 20% and the remaining 4% was scattered among 7 other saturated fatty acids of the plasmalogen-free lecithin. The 2 major unsaturated fatty acids were linoleic and oleic acids. The unsaturated fatty acids with chain lengths of 20 and 22 carbon constituted only about one sixth of the total

TABLE IV

Fatty Acids of Lecithin of Human Red Cells
(mole per cent)

	Lecithin fraction		Plasmalogen fatty acids	Plasmalogen-free lecithin			
				Total			
Fatty acid	Found	Calculated[a]		Found	Calculated[a]	α'	β
12:0	0.4		....	0.3		0.8	0.2
14:0	0.5		0.4	0.4		0.9	0.2
15:0	0.2		0.5	0.2		0.7	0.3
ISO 16:0	0.1		0.5	0.1		0.2	....
16:0	32.9	36.1	17.3	36.5	35.5	63.2	7.7
16:1	1.4		1.2	1.2		1.6	0.7
17:0	0.5		....	0.5		1.2	0.1
ISO 18:0	0.1		....	0.1		0.3	0.1
18:0	9.7	10.1	3.2	10.2	9.7	18.4	1.0
18:1	18.9	18.6	21.7	18.5	18.3	8.3	28.2
18:2	23.5	23.2	26.1	23.1	22.0	1.8	42.2
20:0	0.2		....	0.4		....	0.5
20:2	0.1		....	0.1		....	0.2
20:3	1.4		2.7	1.4		0.8	2.6
20:4	6.3	5.2	19.6	4.9	5.8	1.8	9.8
22:4	0.6		1.2	0.4		....	1.1
24:1	0.2		....	0.1		....	0.2
22:5	0.9		1.1	0.5		....	1.5
22:6	2.1		4.5	1.1		....	3.4
Saturated	44.6		21.9	48.7		85.7	10.1
Unsaturated	55.4		78.1	51.3		14.3	89.9
Double bonds per molecule	1.2		2.0	1.0		0.2	2.0

[a] The values listed as calculated were obtained from the fatty acid composition of other fractions as described in Table III.

unsaturated fatty acids present, and therefore the amount of double bonds per molecule of acid was only 1.0. Despite an almost equal proportion of saturated to unsaturated fatty acid in lecithin free of plasmalogen, 7% of the total unsaturated fatty acids still were found in α' position and 5% of the saturated acid in the β position. In lecithin a trend, similar to that prevailing in phosphatidyl ethanolamine, was noted for the amount of the acid esterified in the β position to increase with degree of unsaturation.

The fatty acids of choline plasmalogen contained a high percentage of unsaturated fatty acids, a result similar to that found with ethanolamine plasmalogen, although the number of double bonds per molecule of fatty acid in choline plasmalogen was only 2.0. The major unsaturated fatty acids in this phospholipid were linoleic, oleic and arachidonic acids. Since there was only 3.9% of choline plasmalogen in the lecithin fraction, the composition of the fatty acids in the lecithin fraction is rather similar to that of the plasmalogen-free lecithin despite large differences in degree of unsaturation between the choline plasmalogen and plasmalogen-free lecithin composing the lecithin fraction.

Calculations, similar to those discussed for the phosphatidyl ethanolamine, were made of the fatty acid composition of the lecithin fraction and the plasmalogen-free lecithin from the observed fatty acid content of the lipids derived from these fractions. As can be seen in Table IV, there was excellent correlation between the calculated and the observed content of the major fatty acids. This agreement indicates that artifacts could not have significantly influenced the fatty acid composition reported for the lecithin fractions.

As shown in Table V, the fatty acids of phosphatidyl inositol were divided equally between unsaturated and saturated fatty acids. The fatty acids of plasmalogen free of lecithin were distributed in a similar manner but the number of double bonds per molecule was 50% higher in the fatty acids of phosphatidyl inositol than in the fatty acids of lecithin free of plasmalogen. This greater degree of unsaturation was due primarily to the 21% arachidonic acid present in the phosphatidyl inositol.

The composition of the fatty acids in phosphatidyl serine resembles that in phosphatidyl ethanolamine. The major unsaturated acids are arachidonic and oleic acids. However, the ratio of saturated to unsaturated fatty acids in phosphatidyl serine of one to two is similar to the ratio in the plasmalogen-free phosphatidyl ethanolamine. On the other hand, the level of 6.6% of acids with carbon chain shorter than 16 in phosphatidyl serine was 3 to 4 times higher than in phosphatidyl ethanolamine.

TABLE V

Fatty Acids of Human Red Cells Phosphatidyl Inositol, Phosphatidyl Serine, Lysolecithin and Sphingomyelin (mole per cent)

Fatty acid		PI	PS	LL	Sph
	10:0	....	0.3	....	....
	11:0	....	0.2	....	....
	12:0	0.7	0.9	2.7	....
	13:0	....	0.2	....	....
	14:0	0.8	2.4	3.8	0.5
	15:0	2.7	2.6	3.1	.
ISO	16:0	0.1	0.2	1.0	.
	16:0	10.4	12.3	37.9	45.7
	16:1	0.8	0.2	2.5	1.1
Anteiso	17:0	0.4	0.8	0.8	....
	17:0	0.6	0.2	0.5	0.6
ISO	18:0	0.3	1.1	....	....
	18:0	24.0	10.2	7.7	7.3
	18:1	13.5	19.1	15.4	1.1
	18:2	4.6	6.2	14.0	0.3
ISO	20:0	0.7	....	....	....
	20:0	7.3	0.7	3.9	1.3
	20:2	0.9	..	..	....
	20:3	2.1	0.8	1.3	....
	20:4	21.1	22.0	0.6	....
	22:0	0.4	0.3	0.5	8.7
	22:1	0.1	0.4	....	....
	23:0	..	....	2.1	0.9
	22:4	3.4	8.1	....	....
	24:0	1.1	0.6	0.9	17.1
	24:1	..	..	1.3	15.4
	22:5	1.7	5.2	....	....
	22:6	2.3	5.0	....	....
Saturated		49.5	33.0	64.9	82.1
Unsaturated		50.5	67.0	35.1	17.9
Double bonds per molecule		1.5	2.1	0.5	0.2

The fatty acids in lysolecithin were two times as saturated as the fatty acids in plasmalogen-free lecithin. Palmitic acid contributed 58% of the total saturated fatty acids. Seventy-two per cent of the unsaturated fatty acids were present in oleic and linoleic acids.

The fatty acids of sphingomyelin were the most saturated among the acids of the various phospholipids isolated in this study. Palmitic acid at a level of 46% made the main contribution to the saturation of these fatty acids. Appreciable amounts of lignoceric, behenic and stearic acids also were present. The 15% of nervonic acid, which was present, accounted for 86% of the unsaturated acid. Lignoceric, nervonic and behenic acids occurred as major acids only in sphingomyelin.

It is of interest to compare the composition of the fatty acids of human red cells phospholipids described in this study with that reported by other workers. The degree of unsaturation and fatty acid compositions in lecithin

fraction is in good agreement with the results of previous workers (7,8,14,29). The fatty acids of the phosphatidyl ethanolamine fraction resemble those found by some other investigators (7,8,29), but in contrast to the findings in the present publication, a high content of palmitic acid and equal proportions of saturated and unsaturated fatty acids in phosphatidyl ethanolamine also has been reported (14). Also, the proportion of two to one of unsaturated to saturated fatty acids in phosphatidyl serine which can be observed in Table V is in disagreement with results of other workers (7,8,14,29) who have found equal proportions of these acids. These discrepancies in the content of fatty acids in these phosphoglycerides might be due partially to incomplete resolution of phospholipids in the single chromatographic separation utilized by these investigators (7,8,14,29), and also might be attributable to known difficulties in the handling of highly unsaturated phospholipids.

On the other hand, the composition of sphingomyelin fatty acids, characterized by a high content of palmitic acid and a major contribution of behenic, lignoceric and nervonic acids, is in general agreement with the results of others (14,29). However, the presence of 2 to 4% of linoleic acid in these previously described preparations of other workers (14,29) may indicate the admixture of lecithin in the sphingomyelin fraction (32).

As far as we are aware, there are no published data on red cell phospholipids with which to compare the fatty acid composition found in the present study for human red cell lysolecithin, phosphatidyl inositol, choline plasmalogen or ethanolamine plasmalogen. The same thing is true for the studies of the positional distribution of fatty acids in plasmalogen-free lecithin or plasmalogen-free phosphatidyl ethanolamine.

The fatty acids in the serum phospholipids of the men in the present study have been described in a previous publication (32). It is of interest to compare the fatty acids of the red cell phospholipids with those of the serum phospholipids of these men. Red cell sphingomyelin had two times more lignoceric and nervonic acid than did the serum lipid. Similarly, the content of oleic acid in red cell phosphatidyl ethanolamine was threefold that of the corresponding serum lipid. Also, different ratios of docosapolyenoic acids were observed for this lipid in the two tissues. The fatty acid composition of red cell phosphatidyl serine was markedly different from that of the corresponding serum lipid. This difference was evident particularly in the higher content of polyunsaturated fatty acids in red cell phosphatidyl serine. The average number of double bonds per fatty acid molecule was twofold higher in the red cell phosphatidyl serine than in the serum lipid. In both tissues the amounts of fatty acid with carbon chains less than 16 was several times higher in phosphatidyl serine as compared with the percentages of these acids in the corresponding phosphatidyl ethanolamine. Otherwise, the fatty acid composition of the phospholipids of the red cells and serum appeared to be similar.

The fatty acids of the lysolecithin of red cells are of an entirely different composition from those of the fatty acids of the choline plasmalogen or the fatty acids found in either the α' or β position in the lecithin of the red cells. These facts indicate that the small amount of lysolecithin in human red cells is a naturally occurring phospholipid and not an artifact arising from the decomposition of choline plasmalogen or lecithin. These observations also support the findings of other investigators of the presence of lysolecithin in normal red cells (2–6,11,17,22–24,30,31).

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[Received May 12, 1966]

The GLC and TLC Resolution of Diastereoisomeric Polyhydroxystearates and Assignment of Configurations[1]

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ABSTRACT

Trifluoroacetate (TFA) derivatives of methyl 12-hydroxystearate, methyl ricinoleate, five positional isomers of methyl *threo-* and *erythro*-dihydroxystearate, four diastereoisomeric methyl 9,10-12-trihydroxystearates, and four racemic diastereoisomeric methyl 9,10-12,13-tetrahydroxystearates were prepared and analyzed by gas-liquid chromatography (GLC). The isomeric *threo-* and *erythro*-dihydroxystearates that had not previously been resolved by GLC were separated. Excellent resolution of the diastereoisomeric pairs of methyl *threo-* and *erythro*-9,10-12-tri- and methyl *erythro, erythro-* and *threo, threo*-9,10-12,13-tetrahydroxystearates was obtained by GLC of their TFA derivatives. Analyses of these high-molecular-weight compounds were carried out on polar and nonpolar packed columns used routinely for methyl ester analysis.

The various methyl mono-, di-, tri-, and tetrahydroxystearate esters were also analyzed by thin-layer chromatography (TLC) on Silica Gel G adsorbent layers and on Silica Gel G impregnated with sodium arsenite.

Relative and absolute configurations were assigned to the various diastereoisomeric tri- and tetrahydroxystearates based on information obtained from GLC, TLC, synthetic ratios, and molecular-models.

A micro hydroxylation method that gives quantitative yields of *threo-* and *erythro*-dihydroxy acids from various concentrations of C_{18} monoene geometrical isomers was developed. Subsequent GLC analysis of the isomeric methyl dihydroxy TFA derivatives allows the quantitative determination of double-bond configuration on small samples without expensive or specialized equipment.

INTRODUCTION

THIN-LAYER CHROMATOGRAPHY (TLC) is routinely used for the separation and isolation of lipid classes followed by gas-liquid chromatography (GLC) for the quantitative analysis of closely related members of a homologous series in each class. Analysis of long-chain polyhydroxy acids by these complementary analytical methods is an exception. The TLC of long-chain mono- and poly-hydroxy acids has been investigated extensively and most successfully by Morris and co-workers (1–3) on silica gel impregnated with boric acid and sodium arsenite and on silica gel adsorbent layers and by other investigators (4–5). On the other hand, GLC analysis of long-chain polyhydroxy acids has been very limited, primarily due to the polarity of these compounds and the lack of suitable derivatives to raise their vapor pressure. Derivatives that have been used for the GLC analysis of long-chain mono- and dihydroxy acids have been briefly reviewed (6,6A). The trimethylsilyl ether derivatives of methyl mono- di-, tri-, and tetrahydroxystearates have been found to give quantitative GLC results by Wood et al. (6). This was the first reported GLC analysis of tri- and tetrahydroxy acids; however, the method did not resolve the diastereoisomeric di-, tri-, or tetrahydroxystearates satisfactorily. A quantitative GLC method for the analysis of diastereoisomeric di- and tetrahydroxy acids would be applicable for determining configuration of geometrical isomers of unsaturated acids. The isomeric hydroxy acids resulting from the hydroxylation of the unsaturated acids could be analyzed quickly and quantitatively by GLC. Such a method would obviously be applicable for determining configuration of long-chain isomeric dihydroxy acids that occur in various seed oils (7), in addition to furthering our understanding of the physical and chemical properties associated with configuration.

Each of the geometrical isomers of linoleic (*cis cis, cis trans, trans cis,* and *trans trans*), yields a racemic diastereoisomeric pair of 9,10-12,13-tetrahydroxystearic acids and ricinoleic and ricinelaidic acids yield an optically active diastereoisomeric pair of 9,10-12-trihydroxystearic acids upon hydroxylation. The configuration of the vicinal hydroxyl groups becomes apparent when the geometrical configuration of the unsaturated acids, from which they are prepared, and the stereochemistry of the hydroxylation reaction are known. However, the spatial relation between the hydroxyl groups on carbon atoms 10 and 12 of the diastereoisomeric pairs has not been established

[1] Presented at the AOCS Meeting, Los Angeles, April 1966.

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beyond mere speculation. Each member of the four racemic diastereoisomeric 9,10-12,13-tetrahydroxystearic acid pairs was prepared by McKay and Bader (8) who assigned configurations based on the assumption that hydroxyl groups *threo* to each other on carbon atoms 10 and 12 gave a compound with a lower melting point than one in which the hydroxyl groups were *erythro*. The eight optically active 9,10-12,13-tetrahydroxystearic acids were prepared by an alternative approach by Bharucha and Gunstone (9) who were reluctant to assign configurations other than by pairs. All the optically active diastereoisomeric 9,10-12-trihydroxystearic acids were first prepared by Kass and Radlove (10) and more recently by Morris (1). These workers declined to assign configurations. Recently Morris and Wharry (11) prepared an optically active diastereoisomeric pair of *threo* 9,10-12-trihydroxyoctadecanols. They (11) suggested that the hydroxyl groups *erythro* to each other on carbon atoms 10 and 12 might have the lower melting point. The problem is briefly summarized by saying that until now there has been insufficient data to assign configurations to the members of each diastereoisomeric pair.

This report describes the resolution of several diastereoisomeric polyhydroxystearate trifluoroacetate (TFA) derivatives by GLC, assignment of absolute and relative configurations, and the applicability of the method for the determination of configuration of monounsaturated fatty acids.

EXPERIMENTAL

Nomenclature

Confusion and misunderstandings arising from terminology and nomenclature applied to polyhydroxy acids exist in the literature (8,12,13). We do not propose a new system of nomenclature, but only wish to make it clear which system is being used. The terms *threo* and *erythro* as set forth by Bharucha and Gunstone (9) for open-chain polyhydroxy acids are used. By definition *threo* compounds result by *trans* addition to a *cis*, or by *cis* addition to a *trans* double bond, whereas *erythro* compounds are the result of *cis* addition to a *cis*, or *trans* addition to a *trans* double bond. *Threo* and *erythro* can also be used to describe the spatial relation of hydroxyl groups interrupted by a methylene unit as in 9,10-12-tri- and 9,10-12,13-tetrahydroxystearic acids. Sgoutas and Kummerow (4), without elaboration, have made this use of *threo* and *erythro*. This system of nomenclature, along with Fischer formula projections showing the relative and absolute configurations of the di-, tri-, and tetrahydroxystearic acids, appear in Table I. Each of the diastereoisomeric hydroxy acids used in this study has been given a number (Column 1, Table I), which is used to compare GLC data, TLC behavior, and other physical and chemical properties with assigned configurations. The Cahn-Ingold-Prelog (14) system of specifying asymmetric configurations was applied to the diastereoisomeric trihydroxystearic acids.

Materials

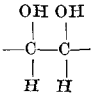
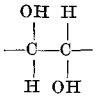
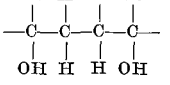
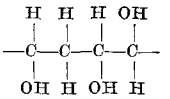
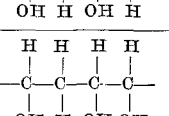
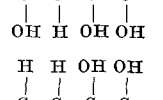
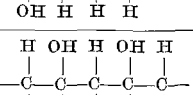
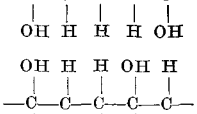
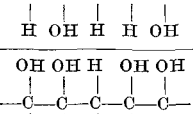
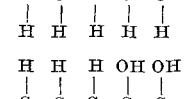
Purified oleic, elaidic, petroselenic, *cis*- and *trans*-vaccenic, linoleic, *trans,trans*-linoleic, ricinelaidic and 12-hydroxystearic acids of greater than 98% purity were obtained from the Hormel Institute, Austin, Minnesota. Ricinoleic acid was prepared from castor oil esters by preparative TLC as previously described (6). The castor oil used in this study was packaged by Welton Laboratories, Inc., New York. Solvents and other reagents were reagent grade or better and were used without further purification.

Hydroxylation

Polyhydroxystearic acids were prepared by alkaline permanganate oxidation of the corresponding unsaturated acids (50–100 mg) according to the procedure of Wiberg and Saegebarth (15). This procedure was later modified, (see sections on Quantitative Micro Hydroxylation). High purity racemic *erythro*- and *threo*-9,10-, *erythro*- and *threo*-11,12- and *erythro*-6,7-dihydroxystearic acids were obtained by preparative TLC (solvent system chloroform-methanol 95:5 v/v). The uncorrected melting points were (127.3–127.7C), (92.5–93.1C), (125.2–126.3C), (92.2–93.0C) and (118.5–119.6C), respectively. Melting points of the other hydroxy acids are given in Table 1. Diastereoisomeric trihydroxystearic acid pairs were purified by preparative TLC (solvent system chloroform-methanol 90:10 v/v). Each member of the pairs was isolated by preparative TLC on layers impregnated with sodium arsenite (solvent system chloroform-methanol 98:2 v/v) as described by Morris and Wharry (11) for trihydroxyoctadecanols. The racemic diastereoisomeric tetrahydroxystearic acid pairs were purified by extraction of the unreacted and partially unreacted unsaturated acid impurities with hexane. The racemic diastereoisomeric tetrahydroxystearic acid pairs were separated by preparative TLC on layers im-

TABLE I

Physical and Chemical Properties of Diastereoisomeric Polyhydroxystearates Resulting from Alkaline Permanganate Oxidation of C_{18} Unsaturated Fatty Acids[a]

Cpd. no.	Parent acid[b]	Di-, tri- and tetrahydroxystearate	Relative & absolute configuration	Rel. spot position on Silica Gel G	Rel. spot position on Silica Gel G (10% $NaASO_2$)	GLC elution order of TFA deriv.	Melting points[d] °c	Percent in synthetic mixture
1.	18:1 (9c)	*erythro*-9,10-		Lower	Lower	1st	127.5	....
2.	18:1 (9t)	*threo*-9,10-				2nd	92.8	....
3.	18:1 (9t) 12-OH	(9-S, 10-S, 12-R)[c] *threo*-9,10-*threo*-10,12-		Lower	Upper	2nd	106.5	50
4.	18:1 (9t) 12-OH	(9-R, 10-R, 12-R)[c] *threo*-9,10-*erythro*-10,12-				1st	85.1	50
5.	18:1 (9c) 12-OH	(9-R, 10-S, 12-R)[c] *erythro*-9,10-*erythro*-10,12-		Upper	Lower	2nd	110.7	42
6.	18:1 (9c) 12-OH	(9-S, 10-R, 12-R)[c] *erythro*-9,10-*threo*-10,12-				1st	135.8	58
7.	18:2 (9t, 12t)	*threo*-9,10-*erythro*-10,12-*threo*-12,13-		Upper	Lower	1st	122[e]	43
8.	18:2 (9t, 12t)	*threo*-9,10-*threo*-10,12-*threo*-12,13-				2nd	148[e]	57
9.	18:2 (9c, 12c)	*erythro*-9,10-*erythro*-10,12-*erythro*-12,13-		Upper	Lower	2nd	156[e]	40
10.	18:2 (9c, 12c)	*erythro*-9,10-*threo*-10,12-*erythro*-12,13-				1st	177[e]	60

[a] Lines separate synthetic diastereoisomeric pairs.
[b] The number and letter in parenthesis represent location and configuration of double bond.
[c] Cahn-Ingold-Prelog system (14) of designating configuration.
[d] Melting points represent the uncorrected means of the ranges of three determinations.
[e] Literature melting points.

pregnated with sodium arsenite (solvent system chloroform-methanol 96:4 v/v). However, yields were poor and the isomers were impure owing to their high solubility in water and the difficulty encountered in the hydrolysis of the arsenite complexes, a problem also encountered by Morris and Wharry (11). The lack of absolute purity of these diastereoisomers in no way affects the validity of the results and conclusions reached.

Quantitative Micro Hydroxylation

Eight to 12 mg samples varying in concentration of oleic and elaidic acids (see Table II) were placed in a 50-ml Erlenmeyer flask with 3 ml of sodium hydroxide (4 mg/ml), 1 ml of tertiary butyl alcohol, and 18 ml of water. The clear solution was stirred rapidly and 1.9 ml of freshly prepared potassium permanganate solution (10 mg/ml) was quickly added, after which stirring was continued for 10 min. At this time the solution was decolorized with sulfur dioxide. After the addition of 0.5 ml of concentrated hydrochloric acid the solution was cooled at 0C for one hour, filtered in a fritted glass funnel, washed with cold water and dried in vacuo at 85C for 2 hr. Hydroxy acids were then methylated, trifluoroacetylated, and analyzed by GLC.

Preparation of Derivatives

Trifluoroacetate (TFA) derivatives of the hydroxy acid esters were prepared by the method recently described by us (16) for isomeric glyceryl monoethers. Methyl esters were prepared by reacting the acids with an etheral solution of diazomethane. The diazomethane was prepared according to the procedure of DeBoer and Backer (17).

Gas-Liquid Chromatography

An Aerograph Model 600D Hy-Fi Gas Chromatograph (Wilkens Instruments and Research, Inc., Walnut Creek, Calif.) equipped with a hydrogen flame ionization detector, was used in this study. The oven temperature was controlled (±0.1C) with a Wilkens Model 32S isothermal temperature controller. The signal from the chromatograph was recorded by a 1.0 MV Brown recorder (Minneapolis-Honeywell Reg. Co., Philadelphia, Pa.). Analyses were carried out on a 5 ft × ⅛ in. O.D. stainless steel column packed with 5% methyl silicone polymer (SE-30) coated on 60–80 mesh Chromosorb W (obtained from Wilkens prepacked), operating at 187C. A high resolution 5 in. × ⅛ in. O.D. pyrex 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, Pa.), operating at 180C was also used. Columns were packed, tested, thermally and trifluoroacetic anyhydride conditioned as previously described (16).

Column pressures ranging from 12–15 psi were used to maintain a helium flow rate of 80–100 ml/min through each of the columns. The flash evaporator was always operated between 240 and 250C. The operational temperature of the flame ionization detector located in the forced-air oven was equal to that of the column.

Thin-Layer Chromatography

Sodium arsenite impregnated plates were prepared by mixing an aqueous sodium arsenite solution (5.8%) with Silica Gel G (2:1 w/w) as described by Morris (1). Unimpregnated plates were prepared from Silica Gel G. Uniform 0.50-mm preparative and 0.25-mm analytical layers were spread on 2- × 20- and 20- × 10-cm glass plates with a Colab applicator modified by us (18), (Colab Laboratories, Inc., Chicago Heights, Ill.). After the chromatoplates had air dried for 30 min they were activated for 30 min at 110C and placed in a desiccator. Development of the chromatoplates was carried out in chambers saturated with the developing solvents. Separations on the preparative plates were visualized by spraying with 0.2% 2′, 7′-dichlorofluorescein in ethanol and viewing under UV light. Analytical plates were charred according to the procedure of Privett and Blank (19), and the results were documented by photography. Selected regions were scraped from the preparative plates into Büchner funnels (fine fritted discs) and eluted with several volumes of diethyl ether-methanol (1:1). Arsenite complexes of the polyhydroxy acids were hydrolyzed as described by Morris and Wharry (11).

TABLE II

Determination of Configuration of Unsaturated C_{18} Monoenes by GLC Analysis of Their Isomeric Dihydroxy TFA Derivatives

Mixture	% of Each Acid		Expt. No. 1 Found[a]	Expt. No. 1 Error	Expt. No. 2 Found[a]	Expt. No. 2 Error
No. 1	Oleic	25	26.6	+1.6	26.4	+1.4
	Elaidic	75	73.4	−1.6	73.6	−1.4
No. 2	Oleic	50	50.4	+0.4	50.5	+0.5
	Elaidic	50	49.6	−0.4	49.5	−0.5
No. 3	Oleic	75	76.0	+1.0	75.5	+0.5
	Elaidic	25	24.0	−1.0	24.5	−0.5

[a] Each experimental value represents the mean of three determinations.

RESULTS AND DISCUSSION

Quantitative Micro Hydroxylation

The alkaline permanganate hydroxylation procedure described in the experimental section was the results of several attempts, some of which are of interest. Hydroxylation of the oleic and elaidic acid mixtures shown in Table II by the procedure of Wiberg and Saegebarth (15) always resulted in incomplete elaidic and com lete oleic acid oxidation, especially in the higher elaidic acid concentrations. Doubled concentration of the permanganate, doubled reaction time and ambient reaction temperature failed to hydroxylate both acids completely or equally. The more rapid and complete oxidation of oleic acid appeared to be associated with solubility differences of the two acids. This was confirmed when l-propanol was used to solubilize the acids. Under these competitive oxidation conditions with insufficient permanganate, elaidic acid was oxidized at a faster rate: however, l-propanol oxidation products increased the water solubility of the di-hydroxy acids. Tertiary butyl alcohol, which is unreactive to alkaline permanganate, was found to solubilize both acids equally effectively. Quantification of the hydroxylation procedure in t-butanol was evaluated by GLC of the TFA derivatives of the hydroxy acids (Table II). Oxidation was virtually complete within 10 min as determined by GLC and TLC. Over-oxidation was not observed to any appreciable degree. Occasionally, a small peak (less than 1%) was found to elute approximately 4 min after the isomeric dihydroxy TFA derivatives, which was assumed to be the monoketo-monohydroxy TFA derivative. The applicability of the method for diunsaturated fatty acids was not investigated, since mixtures of the tetrahydroxy TFA derivatives derived from geometrical isomeric dienes were not sufficiently resolved for quantitative determination by GLC.

GLC of Monohydroxy Acids

The resolution by GLC of most derivatives of methyl 12-hydroxystearate and the corresponding unsaturated analogue methyl ricinoleate is more difficult than the separation of saturated and monounsaturated esters of the same chain length. Methyl 12-hydroxy-stearate and ricinoleate TFA derivatives were not resolved with only indications of two components on the EGSS-X column. On the other hand, the TFA derivatives of methyl 12-hydroxystearate and ricinoleate were resolved sufficiently to allow quantitative measurements on the SE-30 liquid phase.

GLC of Dihydroxy Acids

Chromatograms of methyl *erythro* and *threo*-9,10-dihydroxystearate TFA derivatives: peak 1 and 2, respectively, obtained on SE-30 (top) and EGSS-X (bottom) liquid phases are shown in Figure 1. Resolution was sufficient on the EGSS-X column to allow quantitative determination of each of the isomers, while both isomers were eluted as one peak on the SE-30 column. *Erythro* and *threo* isomers of methyl 11,12- and methyl 6,7-dihydroxystearate TFA derivatives were also resolved on the EGSS-X column. Mixtures of either *erythro* or *threo* positional isomers were not resolved. Resolution of these two diastereoisomers by GLC has not previously been reported to the authors' knowledge. Resolution of the isomeric dihydroxy acids allows the configuration of geometrical isomers of monounsaturated fatty acids to be determined by GLC after quantitative hydroxylation (see Quantitative Micro Hydroxylation). Mixtures varying in proportions of oleic and elaidic acids were quantitatively hydroxylated as described and discussed earlier, methylated, trifluoroacetylated, and analyzed by GLC. The close agreement between experimental values obtained by GLC at all concentrations for each of the acids, and the known values are shown in Table II.

GLC of Tri- and Tetrahydroxy Acids

Shown in Figure 2 are chromatogram tracings of TFA derivatives of two diastereo-

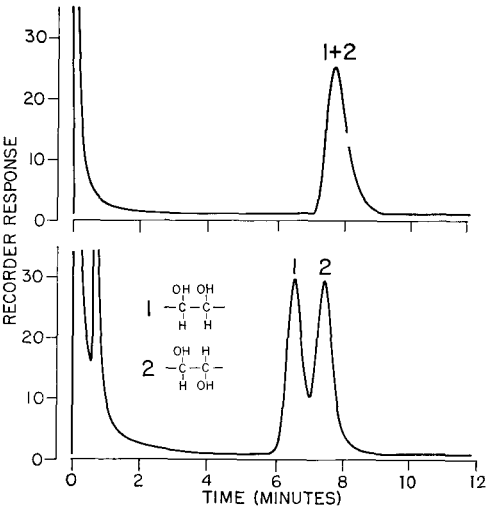


FIG. 1. Chromatograms of methyl esters of *erythro*- and *threo*-methyl 9,10-dihydroxystearate TFA derivatives obtained on 15% EGSS-X (bottom) and 5% SE-30 (top) operating at 180 and 187C.

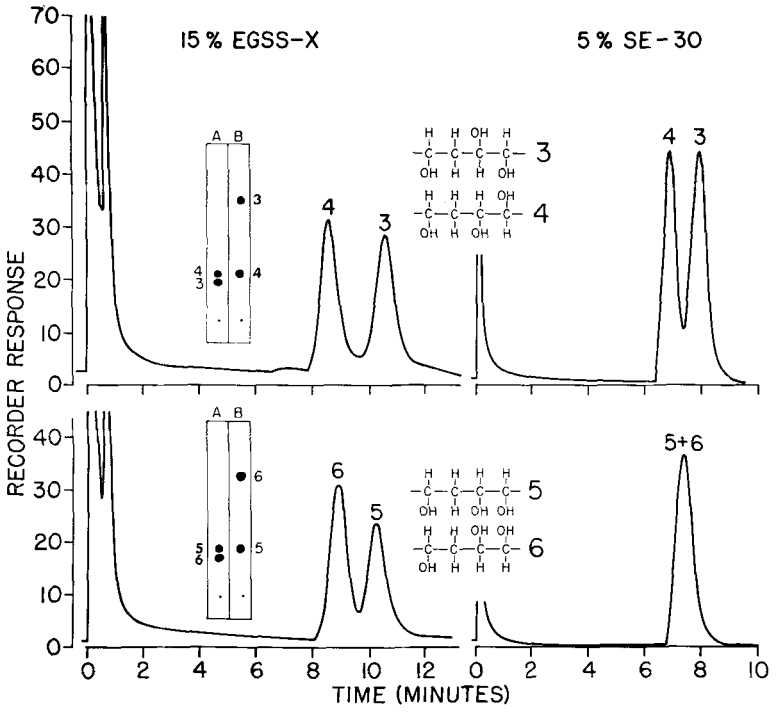


FIG. 2. GLC analyses of the methyl ester-TFA derivatives of diastereoisomeric trihydroxystearic acids produced by alkaline permanganate oxidation of ricinelaidic acid (top) and ricinoleic acid (bottom) on EGSS-X and SE-30 columns. TLC behavior, (A) unimpregnated Silica Gel G and (B) Silica Gel G impregnated with sodium arsenite; GLC data and configuration of each isomer are comparable by number.

isomeric trihydroxystearate pairs, resulting from alkaline permanganate oxidation of ricinelaidic (3 and 4) and ricinoleic (5 and 6) acids obtained on EGSS-X (left) and SE-30 (right) liquid phases. Chromatogram tracings of the TFA derivatives of the two diastereoisomeric tetrahydroxystearic pairs resulting from alkaline permanganate oxidation of *trans, trans*-linoleic (7 and 8) and *cis,cis*-linoleic (9 and 10) acids obtained on EGSS-X (left) and SE-30 (right) liquid phases are shown in Figure 3. The relative TLC migration order of each hydroxy ester pair on Silica Gel G impregnated with sodium arsenite (B) and unimpregnated Silica Gel G (A) is illustrated by the sketching in Figures 2 and 3, for comparison with the GLC elution order of the TFA derivatives and the Fischer formula projections of the configurations assigned to each of the diastereoisomers. The assignment of configurations will be discussed in detail in a later section. Each of the diastereoisomeric tri- and tetrahydroxystearate pairs [(3 and 4), (5 and 6), (7 and 8), and (9 and 10)] were easily resolved on the EGSS-X liquid phase. Pair 5 and 6 eluted as one peak on the SE-30, and, surprisingly, the other three pairs were resolved on this phase. The resolution of pairs that have identical molecular weights on SE-30, which normally separates according to molecular weight, demonstrates that other factors affect GLC separations on this liquid phase. The same elution order on both liquid phases suggests that those factors responsible for the observed separations on SE-30 are also present in the EGSS-X column. Furthermore, the differences in observed resolution between SE-30 and EGSS-X are probably attributable to polarity differences. Differences in vapor pressure and actual physical shape are probably the main contributing factors responsible for the observed resolution of identical molecular weight isomers on SE-30 and to some degree on EGSS-X. Mixtures of all four isomeric tri- or tetrahydroxystearate TFA derivatives could not obviously be separated on either liquid phase.

GLC of a Polyhydroxy Acid Mixture

Mixtures of mono-, di-, tri-, and tetrahy-

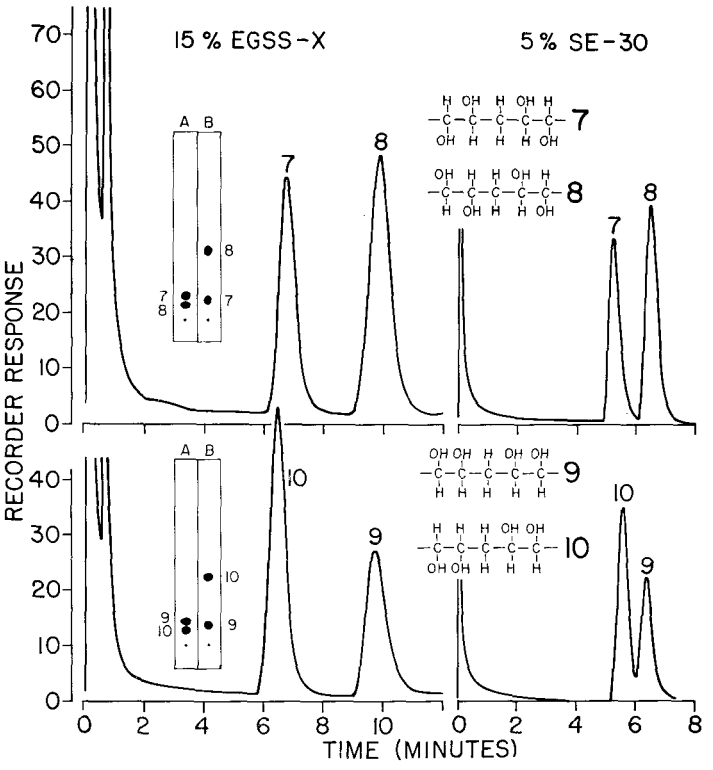


FIG. 3. GLC analysis of the diastereoisomeric tetrahydroxystearic acids obtained by alkaline permangantate hydroxylation of *trans,trans*-linoleic acid (top) and *cis,cis*-linoleic acid (bottom) as methyl ester-TFA derivatives on EGSS-X and SE-30 liquid phases. TLC behavior, (A) unimpregnated Silica Gel G and (B) Silica Gel G impregnated with sodium arsenite; GLC data and configuration of each isomer are comparable by number.

droxystearate TFA derivatives were not resolved sufficiently for quantitative estimation on either column. This was expected by comparison of retention times shown in Figures 1, 2, and 3. More surprising than the resolution of the diastereoisomeric tri- and tetrahydroxystearates on SE-30, was the elution order of a selected mixture of mono-, di-, and tetrahydroxystearate TFA derivatives, shown in Figure 4. The expected elution order was mono, di, tri, and tetra rather than the tetra, tri, di, and mono observed. The molecular basis for the elution pattern observed will be discussed further under Assignment of Configurations. The trihydroxystearate was not added to the mixture shown in Figure 4 because it elutes between peak 1 and 9 and overlaps them. The molecular weight of each successive higher homologue is increased by that of the TFA molecule, which is 112, yet it eluted more quickly than the lower homologue as shown in Figure 4. Alternatively stated, the increased volatility (all the factors responsible for the shorter retention times) of a single trifluoroacetate unit added to the molecule more than compensates for the added molecular weight of itself.

TLC of Mono- and Polyhydroxy Esters

The TLC separation of methylated mono-, di-, tri- and tetrahydroxystearates on Silica Gel G plates impregnated with sodium arsenite and unimpregnated Silica Gel G is shown in Figures 5 and 6. Excellent separation of the methyl hydroxystearates according to the number of hydroxyl groups was obtained on the unimpregnated Silica Gel G plate. Methyl 12-hydroxystearate (lane 11) and methyl ricinoleate (lane 12) were not separated on either plate, as expected. Methyl *erythro*- and *threo*-9,10-dihydroxystearates (lanes 1 and 2, respectively) were resolved on the impregnated layer but not on the unimpregnated. Mixtures of either *erythro* or *threo* positional isomers (i.e., 6,7-, 9,10-, and 11,12-dihydroxystearates) were not sufficiently resolved for quantitative

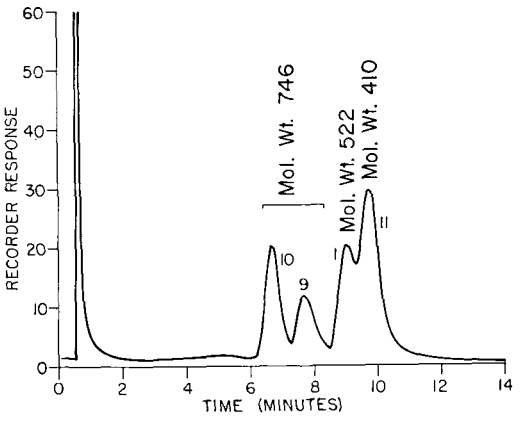


FIG. 4. A chromatogram of methyl esters of mono-, di-, and tetrahydroxystearate TFA derivatives depicting elution order inversely proportional to molecular weight obtained on SE-30. The numbered TFA derivative peaks are: (1) methyl *erythro*-9,10-dihydroxystearate; (9 and 10) methyl tetrahydroxystearates (see Table I for complete names), and (11) methyl 12-hydroxystearate.

estimation on either the unimpregnated or impregnated layers. Each of the diastereoisomeric methyl esters of the tri- and tetrahydroxystearate pairs was separated on both plates, but the separations on the impregnated plate were much greater than on the unimpregnated one. The top spots of each of the isomeric tri- and tetrahydroxystearates on the unimpregnated plate corresponded to the bottom spots on the impregnated plate. This was determined by GLC and TLC analysis of individual diastereoisomers isolated from a sodium arsenite impregnated preparative plate. The chromatoplates also indicate the purity of the hydroxy acids used in this study. More detailed information of each compound identified by a compound number at the top and side of the chromatoplates is given in Table I. The TLC separations described here are similar to those obtained previously by Morris (1).

Assignment of Configurations

The relative and absolute configurations assigned to the various polyhydroxystearic acids used in this study are shown in Table I. The absolute configurations of the trihydroxystearic acids are also shown by "to scale" molecular models in Figure 7. Assignments were made based on the combined information obtained from synthetic ratios of diastereoisomeric pairs, GLC, TLC on impregnated and unimpregnated adsorbent layers, and molecular-model data.

The relation between oleic and elaidic acids and the two racemic *erythro*- and *threo*-9,10-dihydroxystearic acids derived from them by alkaline permanganate oxidation, which proceeds *via* a *cis* addition to yield a cyclic intermediate, has been made clear by Swern (20) and Wiberg and Saegebarth (15). Their relative configurations along with chemical and physical properties are given in Table I, compounds 1 and 2. From this, the spatial relation of the vicinal hydroxyl groups are known.

The remainder of the paper will be concerned with the possible spatial relation of the hydroxyl groups on carbon atoms 10 and 12, and, hence, the absolute configuration of the 9,10-12-tri- and the relative configurations of the racemic 9,10-12,13-tetrahydroxystearic acids.

If the reaction mechanism is understood, the percentage of each isomer in each diastereoisomeric pair obtained from a synthetic mixture can hold information about their configurations. The tri- and tetrahydroxystearic acids are no exception. An attacking permanganate ion would be expected to exhibit a preferred attack on ricinoleic, *cis,cis*-linoleic and *trans,trans*-linoleic acids owing to steric hindrance of another attacking ion on a neighboring position,

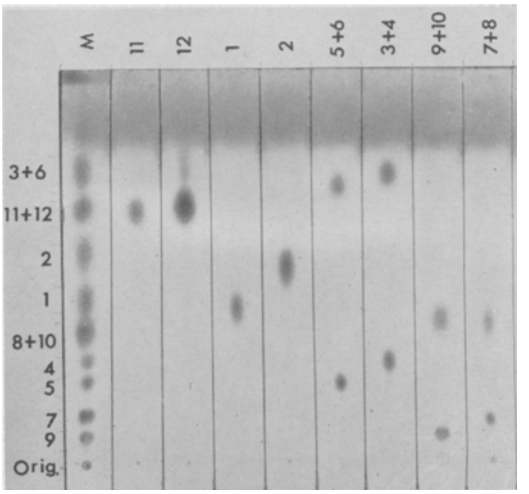


FIG. 5. TLC separations of methyl esters of mono- and diastereoisomeric di-, tri-, and tetrahydroxystearates on Silica Gel G plates impregnated with 10% sodium arsenite. Solvent system was chloroform:methanol (98:2 v/v). The hydroxystearates are: (1) *erythro*-9,10-di-; (2) *threo*-9,10-di-; (3) *threo*-9,10-*threo*-10,12-tri-; (4) *threo*-9,10-*erythro*-10,12-tri-; (5) *erythro*-9,10-*erythro*-10,12,tri; (6) *erythro*-9,10-*threo*-10,12-tri; (7) *threo*-9,10-*erythro*-10,12-*threo*-12,13-tetra-; (8) *threo*-9,10-*threo*-10,12-*threo*-12,13-*tetra;* (9) *erythro*-9,10-*erythro*-10,12-*erythro*-12,13-*tetra;* (10) *erythro*-9,10-*threo*-10,12-*erythro*-12,13-tetra; (11) 12-hydroxystearate; (12) ricinoleate; and (M) mixture.

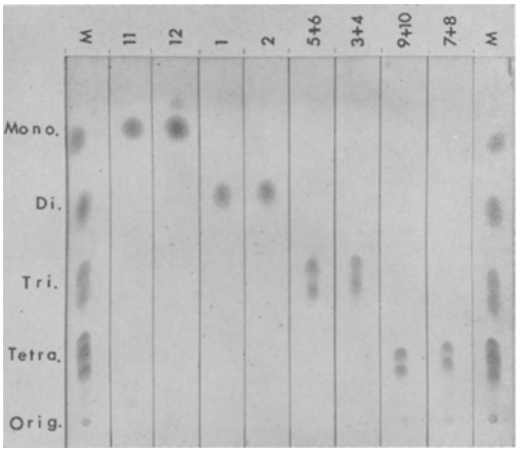


FIG. 6. TLC separation of methyl esters of mono-, and diastereoisomeric di-, tri-, and tetrahydroxystearates on Silica Gel G. Solvent system was chloroform:methanol (92:8 v/v). Numbered lanes are same as those in Figure 5.

for the linoleic acids, or by a neighboring group already present in the molecule, for ricinoleic and the partially oxidized linoleic acids. The proximity of groups *erythro* to each other on carbon atoms 10 and 12 and the influence a group already present at either position could have on an incoming group at the other position becomes apparent from the molecular-model representation of the diastereoisomeric trihydroxystearic acids shown in Figure 7. In accord with these considerations, the more abundant isomer in each diastereoisomeric pair was assigned the configuration where the hydroxyl groups on carbon atoms 10 and 12 are *threo* to each other. The percentages of each isomer for each diastereoisomeric pair as determined by GLC are given in the last column of Table I. The ratio of the two possible conformations is approximately 3 to 2 in favor of the less sterically hindered one. As predicted from a molecular model representation of ricinelaidic acid due to the vulnerability of attack from either direction, an equal molar mixture of compounds 3 and 4 was obtained from the synthetic mixture. Assignment of configurations to this pair was based on the GLC elution order of compounds 7 and 8. Hydroxyl groups *threo* to each other on carbon atoms 10 and 12 in compound 8 had a longer retention time than compound 7; therefore, compound 3, with the longer retention time, was assumed to have the *threo* configuration between hydroxyl groups on carbon atoms 10 and 12. A similar relation exists between compounds 5 and 6 and 9 and 10. Hydroxyl groups *erythro* to each other on carbon atoms 10 and 12 in these pairs had a longer retention time.

With the configurations assigned according to synthetic-mixture ratios and GLC elution order, other physical and chemical properties were examined to confirm or reject the assignments. It quickly became apparent by comparison of the relative TLC spot position of each pair [obtained on Silica Gel G adsorbent layers impregnated with sodium arsenite (column 6, Table I)] with the assigned configurations (column 4, Table I), that the lower spot of each pair corresponded to a configuration where the hydroxyl groups on carbon atoms 10 and 12 were *erythro* to each other. The molecular model representation of the diastereoisomeric trihydroxystearic acids shown in Figure 7 indicates the proximity of the hydroxyl groups *erythro* to each other on carbon atoms 10 and 12, which exists in the tetrahydroxy acids as well. Strong hydrogen bonding between these two hydroxyl groups would render them unavailable for complex formation with the arsenite ion and would migrate less than those with reduced polarity by arsenite complex formation. Hydrogen bonding between the *erythro*-10-12-hydroxyl groups should reduce the polarity somewhat, and, therefore, migrate further on unimpregnated adsorbent

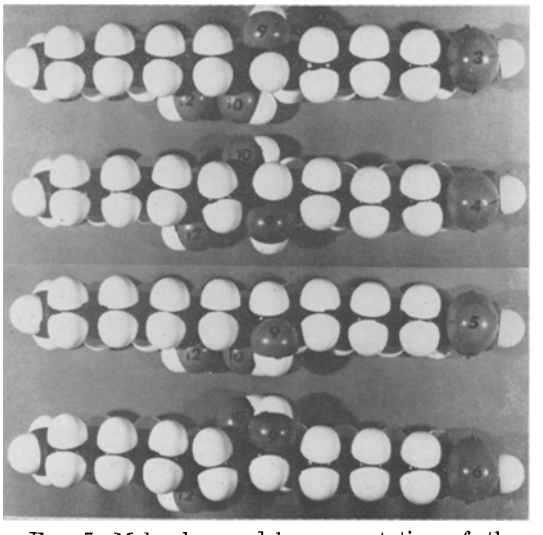


FIG. 7. Molecular model representation of the four diastereoisomeric 9,10-12-trihydroxystearic acids. The numbers on the carbonyl oxygen correspond to the compound number (column 1) in Table I. The numbered hydroxyl groups refer to the carbon atoms they are bonded to. The spatial relation of the vicinal hydroxyl groups both *threo* and *erythro* and the proximity of hydroxyl groups *erythro* to each other on carbon atoms 10 and 12 are illustrated.

layers than those not engaged in hydrogen bonding. This was found to be true, and is summarized in column 5 of Table I.

Our configuration assignments were further supported by comparison of melting points (column 8, Table I) with the assigned configurations. As suggested by Morris and Wharry (11), each isomer of each diastereoisomeric pair (excluding the dihydroxy acids) with the hydroxyl groups *erythro* to each other on carbon atoms 10 and 12 exhibited a lower melting point. This is presumably also due to the hydrogen bonding between these proximal hydroxyl groups (Figure 7).

The configuration assignments to the diastereoisomeric tetrahydroxystearates made by McKay and Bader and co-workers (8,13) are incorrect according to our assignments; therefore, the physical and chemical properties assigned to each isomer of the diastereoisomeric pairs by these investigators and others (4), must be switched. The incorrect McKay and Bader assignments were based on the assumptions that hydroxyl groups *erythro* to each other on carbon atoms 10 and 12 have a higher melting point than those *threo* at the same position. This might appear to be a logical assumption at first by comparison of the melting points of *erythro-* and *threo*-9,10-dihydroxystearic acids (column 8, Table I). However, the pitfall lies in trying to apply inferences drawn from *erythro*-1,2-vicinal to *erythro*-1,3-proximal hydroxyl groups.

Knowledge accumulated over the years for each of the diastereoisomers can now be evaluated in terms of configurations. The physical and chemical properties of the diastereoisomeric trihydroxystearates recorded by Kass and Radlove (10) can be related to their configuration through the melting points. Assignment of configurations to the other four diastereoisomeric 9,10-12,13-tetrahydroxystearic acids not reported in this study can also be made from their behavior on Silica Gel G TLC plates impregnated with sodium arsenite reported by Morris (1) and their melting points given by Bharucha and Gunstone (9). They are: *threo*-9,10-*threo*-10,12-*erythro*-12,13-(mp 156); *threo*-9,10-*erythro*-10,12-*erythro*-12,13-(mp 130); *erythro*-9,10-*threo*-10,12-*threo*-12,13-(mp 165); and *erythro*-9,10-*erythro*-10,12-*threo*-12,13-(mp 112). The tentative assignments of the diastereoisomeric 9,10-12-trihydroxy-octadecanols made by Morris and Wharry (11) are correct according to our criteria of configuration assignment.

The assigned configurations of the trihydroxy acids are absolute, since the absolute configuration of ricinoleic acid from which they were derived has been previously determined by Serck-Hanssen and Stenhagen (21,22). The ricinelaidic acid was prepared from ricinoleic acid without alteration of configuration. The absolute configuration of the eight optically active diastereoisomeric 9,10-12,13-tetrahydroxystearic acids prepared by Bharucha and Gunstone (9) is now known and the Chan-Ingold-Prelog (14) system of specifying asymmetric configuration can be applied.

These configuration assignments, which are in agreement with all our experimental observations, can now be correlated with the physical and chemical properties in the literature and should further our understanding of these compounds at the molecular level.

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[Received April 25, 1966]

Synthesis of Cholesterol and Total Lipid by Male and Female Rats Fed Beef Tallow or Corn Oil

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ABSTRACT

Male and female weanling rats were fed diets containing 2 or 42% of calories as corn oil or 40% as beef tallow plus 2% as corn oil until they were 12 or 18 weeks of age. Incorporation of C^{14}-acetate into lipids of serum and liver and concentration of lipids in serum, liver, and carcass at the end of these periods were determined.

Net synthesis of noncholesterol lipid was repressed by changing the diet from 2% to 42% of calories from either dietary fat in both sexes and at both ages. Cholesterol net synthesis was enhanced 29-fold in males and 22-fold in females fed 42% corn oil compared to 2% corn oil to the age of 12 weeks. It was enhanced only 2.6-fold for males and 3.4-fold for females by 40% beef tallow plus 2% corn oil. At 18 weeks of age cholesterol synthesis in males fed 42% corn oil was 7.3 and in females 9.1 times the value for those fed 2% corn oil. At this age the values for rats fed 40% beef tallow plus 2% corn oil were 1.2 and 3.7 times those for 2% corn oil fed rats of the respective sexes.

INTRODUCTION

IT HAS BEEN REPORTED that rate of cholesterol biosynthesis is responsive to fat in the diet of female rats (1). This observation was supported by a study using liver slices of rats fed diets containing safflower oil (2). These two studies involved use of corn oil (1), a mixture of lard and butter (1) and safflower oil (2). All resulted in increasing cholesterol biosynthesis when fed in amounts from 2 to 40% of dietary calories.

It has recently been reported from this laboratory that beef tallow, when fed to female rats in quantities of 20 to 80% of dietary calories, resulted in increasing cholesterol synthesis. Cholesterol biosynthesis (as indicated by acetate incorporation) exhibited highly significant linear regression upon amount of beef tallow in the diet (3).

It appeared desirable to know whether the effects observed in female rats also occurred in males. It also seemed desirable to further investigate age as a factor in the reported phenomenon. The present study was designed to determine the effects of sex and kind of dietary fat upon cholesterol and total lipid biosynthesis in rats of two ages.

EXPERIMENTAL

Male and female Carworth Farms CFE rats were obtained at weaning (21 days) and upon arrival were caged singly and fed pathogen-free diet biscuits (Price-Wilhoite, Frederick, Maryland) for 24 hr. They were then divided into groups of 8 of each sex and placed on diets formulated to contain 42% of calories as corn oil (high corn oil diet, CO), 40% of calories as beef tallow plus 2% of calories as corn oil (high beef tallow diet, BT), or 2% of calories as corn oil (low-fat diet, LF). Corn oil in the LF and BT diets was near the minimum required to furnish essential fatty acids. The diets contained similar quantities of protein, vitamins, and minerals on a calorie basis as previously described (3). The rats were allowed food and water ad libitum and weight was recorded weekly. Food intake was recorded one week out of each four.

After 9 or 15 weeks on the diets (age 12 or 18 weeks) the rats were subjected to the following regime: Food was removed at 10 pm, then replaced the following morning; each rat was allowed ad libitum access to food for 1 hr, then was given an intraperitoneal injection of 6 μc/100 gm body weight of Na-1-C^{14}-acetate (2.0 mc/mM) in physiological saline solution. After 4 hr exposure to the tracer, rats were anesthetized with amobarbital. A blood sample was obtained by heart puncture, the liver was excised and stored, and the gut was excised and discarded. Sera, livers, and carcasses were stored at −20C until analyzed.

Serum and liver lipids were extracted according to the Folch procedure (4). Total lipid was determined by drying and weighing samples of the washed chloroform-methanol extract. The dried samples were counted in a Sharp Wide-Beta automated counting system (Beckman Instrument Co.) for C^{14} activity. Samples of the lipid extracts were saponified and cholesterol digitonide obtained, assayed and counted as previously described (3).

Carcasses were frozen in powdered dry ice, broken up by pounding, then homogenized in a heavy-duty Waring Blendor as a mixture with dry ice. This procedure resulted in a homogeneous mixture from which samples were taken for lipid extraction. Lipid extraction and

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total lipid and cholesterol analyses were carried out as for serum and liver (3).

Incorporation of labeled acetate into lipid fractions is reported using the following terms: Noncholesterol total activity (TA) indicates per cent of the injected dose of C^{14} in total lipid minus cholesterol; specific activity (SA) of noncholesterol lipid is TA per gram of total lipid minus cholesterol. Total activity of cholesterol is per cent of injected dose of C^{14} in cholesterol and specific activity is TA per gram of cholesterol. Statistical analyses of the data consisted of determination of standard errors of the means and estimation of probability of difference between means using the "t" test (5). Probabilities of 5% or less are considered significant, but greater emphasis is given to differences having a probability of less than 1% of being due to chance.

RESULTS

Lipid Concentration in Serum, Liver, and Carcass

Serum. Cholesterol and noncholesterol lipid concentrations in serum are shown in Table I. Standard errors of the means for noncholesterol lipid indicate wide variation among individual rats, resulting in few significant differences. One difference is that males on BT diet at 12 weeks of age tended to have higher total lipid than other groups. The higher value was significant only in comparison to CO males at 12 weeks and to BT males at 18 weeks. Another significant difference was that the female rats after 18 weeks on LF had a lower value for noncholesterol lipid than BT females the same age or LF females at 12 weeks of age. The groups thus fall into a pattern of high serum noncholesterol lipid in young males fed beef tallow; no age, sex, or diet differences in the majority of groups; and finally an apparent decline in females fed low-fat for a longer time.

Serum cholesterol (Table I) was not nearly as variable among individuals as was noncholesterol lipid. In 12-week-old animals there were no significant differences related to diet in either sex. After another 6 weeks on the diets, however, serum cholesterol of BT rats tended to rise resulting in significantly higher values than occurred with either CO or LF for both sexes.

Serum cholesterol in the LF groups of males and females decreased slightly with age, but

TABLE I

Lipid Composition of Serum, Liver and Carcass

Age and diet		Males		Females	
		Non-cholesterol lipid	Cholesterol	Non-cholesterol lipid	Cholesterol
			Serum		
		mg/100 ml	mg/100 ml	mg/100 ml	mg/100 ml
12 wk	BT[a]	632 ± 109 A[b]	89 ± 4 AD	395 ± 55 AB	87 ± 12 ABD
	CO	366 ± 27 B	99 ± 4 AE	432 ± 75 B	70 ± 2 BCF
	LF	360 ± 67 AB	79 ± 8 AF	395 ± 59 B	68 ± 4 ABF
18 wk	BT	312 ± 32 B	101 ± 4 D	451 ± 71 B	100 ± 6 D
	CO	257 ± 49 B	90 ± 3 E	301 ± 35 BC	76 ± 5 F
	LF	436 ± 80 B	74 ± 2 F	244 ± 26 C	61 ± 7 F
			Liver		
		mg	mg	mg	mg
12 wk	BT[a]	434 ± 25 A[b]	19.6 ± 0.9 A	341 ± 16 C	11.8 ± 0.6 C
	CO	604 ± 57 B	36.8 ± 5.1 B	347 ± 22 C	15.1 ± 0.6 D
	LF	382 ± 19 A	19.8 ± 0.8 A	328 ± 18 AC	12.3 ± 1.0 C
18 wk	BT	529 ± 26 D	24.1 ± 1.8 E	388 ± 30 C	14.0 ± 0.6 D
	CO	530 ± 38 DB	34.1 ± 3.3 FB	325 ± 10 C	15.0 ± 0.8 D
	LF	412 ± 21 AC	22.1 ± 1.3 EA	354 ± 24 C	12.7 ± 1.0 CD
			Carcass		
		g	mg	g	mg
12 wk	BT[a]	48.2 ± 3.2 A[b]	429 ± 23 AB	28.8 ± 2.8 B	271 ± 11 C
	CO	48.4 ± 1.8 A	508 ± 34 B	33.1 ± 2.3 B	277 ± 12 C
	LF	35.5 ± 3.9 B	399 ± 16 A	27.6 ± 2.7 B	285 ± 14 C
18 wk	BT	77.5 ± 6.5 C	532 ± 18 B	49.8 ± 5.7 E	313 ± 10 C
	CO	61.5 ± 6.4 CA	529 ± 27 B	35.4 ± 3.6 BF	325 ± 14 C
	LF	55.1 ± 5.5 D	512 ± 24 B	41.4 ± 2.7 FE	317 ± 9 C

[a] BT = 40% beef tallow + 2% corn oil; CO = 42% corn oil; LF = 2% corn oil.

[b] Mean ± standard error of the mean. Values sharing any one letter designation are not statistically different from each other. Compare diets within sex and age, same diet between sexes, same diet between ages.

sufficiently to make the value for males on CO significantly higher than those on LF. There were no significant differences related to age. The overall pattern of serum cholesterol indicates very little effect of age, sex, or diet under the conditions studied, although there appears to be a trend toward increasing serum cholesterol of rats fed beef tallow in both sexes.

Liver. Data on cholesterol and noncholesterol lipid of liver are shown in Table I. There was little difference in noncholesterol lipid among the groups. Males, 12 weeks old, fed CO had a higher mean value than their counterpart groups for diet and sex. The only notable point in regard to liver cholesterol is the relatively high amount for males fed CO at both ages.

Carcass. Final carcass weight was closely related to food consumption. The calorie intake of the rats on LF was somewhat less than that of either group fed 42% dietary fat. The amount of noncholesterol lipid in the carcasses of males was greater when either dietary fat was fed than with LF. This was partially related to size of the rat because the per cent lipid in the carcass was only significantly greater with BT vs. LF and not with CO, and only at 12 weeks (not shown in table). Total carcass lipid increased with age in all dietary groups with significant gains by the BT group and by the LF group. These gains were associated with increased body size because the small increases in per cent carcass lipid were not significant.

Carcass cholesterol in males was directly related to body size. The only significant difference in the values is that 12-week-old CO rats had higher carcass cholesterol than similar LF rats. Concentration of cholesterol in mg/g of carcass weight varied only from 1.43 to 1.66.

There were no significant differences in carcass lipids in females at 12 weeks of age. The concentrations of noncholesterol lipid (per cent of carcass) and cholesterol (mg/g carcass weight) were very similar to those of males (not shown in table). Carcass noncholesterol lipid increased with age in the females on BT, both in amount and concentration. The increased quantity was significant and the increase from 17.2 to 22.5% was also significant. The LF group gained fat with both quantity and concentration significantly higher at 18 than at 12 weeks of age.

Acetate Incorporation into Lipids

The values reported indicate net synthesis within the 4-hr period of exposure to C^{14}-acetate. This would include some recycling of C^{14}. The total amounts of cholesterol and noncholesterol lipid in the groups studied in this experiment did not vary greatly. It is, therefore, assumed that the values reported are an estimate of net synthesis useful for comparing the several groups.

Liver. Table II shows data for biosynthesis of fatty acids as estimated by incorporation of acetate into total lipids with subtraction of cholesterol radioactivity. In males at 12 weeks of age the total activity was less with either fat in the diet than for LF but the significance was minimum; specific activity was less, however, with high significance. The difference between the two dietary fat sources was not significant. At 18 weeks of age the differences in total activity had disappeared because of a decrease in TA in the group on low-fat diet. Differences in specific activity were still significant but barely so.

Females at 12 weeks of age had significantly less incorporation of acetate into noncholesterol lipid when either fat was fed in comparison to the group fed a low-fat diet. Specific activity followed the same pattern with BT and CO groups having significantly lower SA than the LF group. The two fats did not differ significantly in SA values from each other.

TABLE II

Incorporation of C^{14}-Acetate into Liver Noncholesterol Lipids

Age and diet	Males		Females	
	TA × 10³[a]	SA[b]	TA × 10³[a]	SA[b]
12 wk BT[c]	286 ± 37 A[d]	0.71 ± 0.14 A	311 ± 54 AD	0.93 ± 0.16 AC
CO	274 ± 12 A	0.47 ± 0.04 A	439 ± 36 D	1.30 ± 0.13 C
LF	771 ± 224 B	1.93 ± 0.48 B	641 ± 79 BE	2.05 ± 0.30 BD
18 wk BT	278 ± 65 A	0.52 ± 0.12 A	390 ± 35 A	1.02 ± 0.09 C
CO	256 ± 24 A	0.49 ± 0.04 A	329 ± 71 AD	0.99 ± 0.18 C
LF	511 ± 141 BA	1.23 ± 0.34 B	626 ± 139 AB	1.98 ± 0.64 BC

[a] Total activity, % of injected dose.
[b] Specific activity, TA/g of compound.
[c] BT = 40% beef tallow plus 2% corn oil; CO = 42% corn oil; LF = 2% corn oil.
[d] Mean ± standard error of the mean. Values sharing any one letter designation are not statistically different from each other. Compare diets within sex and age, same diet between sexes, same diet between ages.

None of the differences in TA and SA was significant in the females 18 weeks of age.

There were significant differences in noncholesterol lipid between the two sexes. In the 12-week-old CO groups females had significantly higher noncholesterol lipid TA and SA than males. Neither BT nor LF groups differed in this respect between sexes. In the 18-week-old groups noncholesterol lipid TA differences were not significant, but SA was higher in females than males when either fat was fed.

Acetate incorporation into liver cholesterol is shown in Table III. Beef tallow caused an increase of approximately 3-fold in total activity in comparison to LF in male rats 12 weeks old. Corn oil caused a 30-fold increase. These marked differences were present in a situation where the total amount of cholesterol was similar for BT and LF and only 2-fold greater for CO. Specific activity of liver cholesterol was not significantly greater for BT rats than for the LF group, apparently because of a large variation among BT rats. The SA for corn oil rats was significantly greater than that for the LF group.

Combination of the effect of dietary fat in lowering fatty acid synthesis and increasing cholesterol biosynthesis is estimated by the figure for per cent of C^{14}-cholesterol in total lipid-C^{14}. That figure shows significant differences between 40 and 2% fat calories for male rats 12 weeks of age and significant differences between the two fats. Corn oil had about 7 times as much effect as beef tallow upon these combined biosynthetic processes.

In male rats 18 weeks of age the difference between BT and LF effects on TA had disappeared, while the corn oil effect was still significantly greater than LF, although decreased from the earlier age. Both TA and SA values for corn oil fed rats were significantly greater than either beef tallow or low fat fed rats. The figures indicating combined effect of dietary fat upon fatty acid and cholesterol biosynthesis also decreased from 12 to 18 weeks of age, but remained significantly higher when either fat was fed than with low fat and higher with corn oil than with beef tallow.

In female rats 12 weeks old, cholesterol TA again was significantly higher when the 42% corn oil calorie diet was fed than when BT or LF diets were fed. BT did not result in a significantly higher cholesterol TA than did LF, apparently because of wide variation among the BT rats. Specific activity followed the same patterns as TA, with the same levels of significance of differences.

The combined effect of dietary fat upon fatty acid and cholesterol biosynthesis was significant for both sources of dietary fat in comparison to low fat in 12-week-old rats. The effect of corn oil was significantly greater than that of beef tallow. In the females, in contrast to the males, there was an increase in cholesterol TA and SA with age when BT was fed; this increase, although not statistically significant, resulted in a significant difference between BT and LF at 18 weeks of age. The values of TA and SA for corn oil decreased with age, but remained significantly greater than those for LF. There was no significant difference between corn oil and beef tallow for either TA or SA at 18 weeks, no doubt because of the large variation among the corn-oil-fed rats. The combined effect of dietary fat upon fatty acid and cholesterol biosynthesis reflected the changes shown in TA and SA of the BT group, thus making all groups significantly different from each other.

For cholesterol TA the only 12-week-old groups which exhibited a sex difference were those on LF diet. Cholesterol SA in females was significantly higher than in males for CO and LF groups, but the BT groups were not significantly different. The figures for per cent of cholesterol TA in total lipid TA were similar between sexes.

TABLE III

Incorporation of C^{14}-Acetate into Liver Cholesterol and Per Cent of Lipid-C^{14} in Cholesterol

	Males			Females		
	Cholesterol			Cholesterol		
Age and diet	TA × 10^3[a]	SA[b]	TA cholesterol/ TA total lipid	TA × 10^3	SA	TA cholesterol/ TA total lipid
12 wk BT[c]	11 ± 3 A[d]	0.61 ± 0.19 A	3.8 ± 0.6 A	27 ± 13 AE	2.09 ± 0.85 AD	6.4 ± 1.4 AG
CO	120 ± 20 B	3.73 ± 0.79 B	29.3 ± 3.5 B	174 ± 28 BD	11.26 ± 1.48 C	27.6 ± 2.6 B
LF	4 ± 0.3 C	0.22 ± 0.02 A	0.8 ± 0.1 C	8 ± 1 A	0.67 ± 0.07 DF	1.2 ± 0.3 C
18 wk BT	6 ± 0.6 A	0.27 ± 0.03 A	2.7 ± 0.3 AD	41 ± 6 E	2.95 ± 0.41 D	9.6 ± 1.1 G
CO	37 ± 12 D	1.12 ± 0.38 E	11.3 ± 2.8 E	100 ± 30 ED	6.48 ± 1.81 DC	22.2 ± 2.2 B
LF	5 ± 0.5 AC	0.25 ± 0.05 A	1.3 ± 0.2 F	11 ± 3 FA	0.82 ± 0.11 F	1.9 ± 0.3 CF

[a], [b], [c], [d] Same as Table II.

Cholesterol TA in 18-week-old groups indicates that values for females were significantly higher than males when beef tallow was the dietary fat, but neither CO nor LF resulted in significant sex differences. SA values, which eliminate differences related to total quantity of the compound, were significantly higher in females than males for all dietary groups.

The combined effects of fat feeding upon cholesterol in relation to total lipid biosynthesis in 18-week-old rats indicated a sex difference when either fat was fed, but no sex difference on a low fat diet. The value for females was higher than males (9.6 vs. 2.7%) when beef tallow was fed and also higher (22.2 vs. 11.3%) when corn oil was the dietary fat.

Serum. The radioactivity found in serum lipids indicates synthesis of the lipids at another site, primarily liver, and secretion into blood. Because these values are obtainable in human subjects, whereas liver and carcass values are not readily obtainable, it is desirable to determine whether they reflect biosynthetic processes in the liver. Figure 1 shows that serum values for the combined effect upon fatty acid and cholesterol biosynthesis of diet, sex and age, did indeed, reflect values shown for liver. The figures from which those shown were derived (TA of noncholesterol lipid and cholesterol) are not given, but also paralleled liver values. A very high percentage of serum lipid C^{14} was in cholesterol of both male and female rats fed high corn oil diet—more than 70% in the younger group and 40–60% in the older groups. This compares to a maximum of 29.3% of cholesterol-C^{14} in total lipid-C^{14} of liver. These very large differences in net synthesis were associated with a very stable serum cholesterol content.

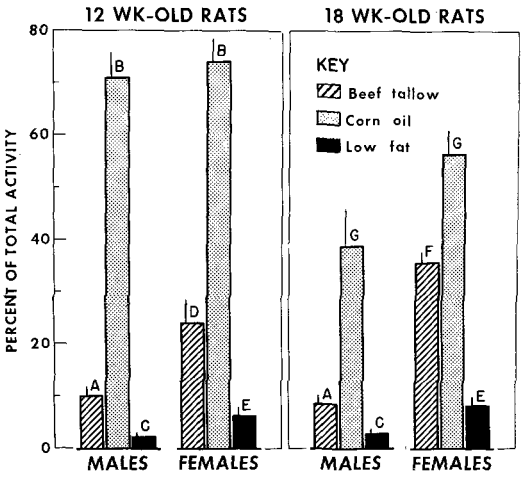

FIG. 1. Serum cholesterol-C^{14} total activity as percent of total lipid-C^{14} in sera of rats fed beef tallow, corn oil, or low-fat diets. Columns having any one letter designation in common are not statistically different from each other comparing diets within age and sex, same diet between sexes, same diet between ages. (Bar = mean; | = plus one standard error of the mean).

In 12-week-old males the value of 10.5% cholesterol-C^{14} in total lipid-C^{14} for BT rats was significantly higher than for LF (2.6%). The corn oil group grossly exceeded all others with a value of 70.7%. In females all three groups were significantly different from each other, also. Females had higher values than males at 12 weeks for BT (24.0 vs. 10.5%) and also for LF (6.2 vs. 2.6%). There was no sex difference in the corn oil groups.

Groups 18 weeks old showed decline in values for cholesterol-C^{14} in total lipid-C^{14} with age in both sexes when corn oil was fed, an increase in females when beef tallow was fed, and little change when low fat diets were fed or, in males, when beef tallow was fed. The sex differences at 18 weeks were greater than they were at 12 weeks. For CO groups there was no significant sex difference. When beef tallow was fed the sex difference at 18 weeks was very pronounced as a result of an increase in the value for females (females 36.0, males 8.6%). Females had higher values on LF than males at 18 weeks.

DISCUSSION

Data showing the effects of diet, sex, and age upon incorporation of acetate into liver fatty acids (the major part of noncholesterol lipid which incorporates acetate) and cholesterol are summarized in Table IV. When the low-fat diet is set at 1 and dietary fat effects are compared to it, it is apparent that either type of dietary fat reduced the rate of biosynthesis of fatty acids in the liver. In 12-week-old males fatty acid synthesis was reduced to about one third while in all other age and sex groups it was reduced to about one half. This difference may or may not have physiological significance. There appears to be no difference between response to the saturated compared to polyunsaturated types of fat.

Conversely, the effects of dietary fat upon cholesterol biosynthesis in the liver differed with sex and kind of fat. The effect of corn oil was extreme in both males and females. Incorporation of acetate into cholesterol was 28.6 and 21.8 times greater in 12-week-old males and females, respectively, when 40% corn oil calories were added to the low-fat diet.

TABLE IV

Ratios of Incorporation of C^{14}-Acetate into Total Lipid and Cholesterol of Liver when Diets Containing 40% Calories from Beef Tallow (BT) or Corn Oil (CO) Were Compared to a Base Line Set at 1 for Rats Fed 2% Corn Oil (LF)

Age and diet		Males			Females		
		Non-cholesterol lipid-C^{14}	Cholesterol -C^{14}	Cholesterol -C^{14}/total lipid-C^{14}	Non-cholesterol lipid-C^{14}	Cholesterol -C^{14}	Cholesterol -C^{14}/total lipid-C^{14}
12 wk	BT	0.37	2.6	5.0	0.49	3.4	5.1
	CO	0.36	28.6	38.5	0.68	21.8	22.1
	LF	1.00	1.0	1.0	1.00	1.0	1.0
18 wk	BT	0.54	1.2	2.1	0.62	3.7	4.9
	CO	0.50	7.3	8.6	0.52	9.1	11.5
	LF	1.00	1.0	1.0	1.00	1.0	1.0

Beef tallow raised cholesterol synthesis only 2.6 and 3.4 times for the same respective groups. The small quantity of cholesterol present in beef tallow may contribute to this lack of synthetic response.

Increased age had little effect upon the response of fatty acid synthesis to dietary fat. Age had a marked effect, however, upon cholesterol biosynthesis in response to dietary fat. Both sexes showed decreased response to corn oil with age. The response of males to beef tallow also decreased, in fact, to a point of practically no difference from the low-fat base line. Older females remained responsive to beef tallow in comparison to low-fat with regard to cholesterol biosynthesis.

The combination of the figures as per cent of lipid-C^{14} in cholesterol magnifies the differences apparent in the two separate figures. In essence, the results indicate that corn oil caused a very large increase in cholesterol synthesis accompanied by decreased fatty acid synthesis. Males 12 weeks old exhibited a more marked response to corn oil than did females, but by 18 weeks of age the response of the males had declined to less than that of females the same age. The response of both males and females to dietary beef tallow was less than the response to corn oil. At the age of 12 weeks the difference in response to the 2 fats was 4-fold in females and 7-fold in males, but the beef tallow effect was similar for the sexes. Response to beef tallow declined with age in males but not in females. It appears that females became adapted to beef tallow in a way different from males and different from adaptation to corn oil.

In previous publications in this area of research it has been proposed that cholesterol biosynthesis may perform a direct function in utilization of fatty acids for energy (1,3). That possibility remains and, in fact, is reinforced by the differing response of cholesterol biosynthesis to dietary fat in the two sexes and with different kinds of dietary fat. Sex and fatty acid saturation are known to be involved in onset of abnormalities of lipid metabolism. Because young females are less likely to suffer atherosclerosis than males it may be assumed that the response of females to fat in the diet is more favorable than that of males. Upon this assumption, then, rapid cholesterol biosynthesis in response to dietary fat should be a favorable condition. The fact that this parameter in males showed little response to beef tallow in the diet may be of importance in further clarification of the role of lipids in atherosclerosis.

Another proposal made in an earlier publication (3) was that the incorporation of C^{14}-acetate into serum lipids of humans might be of some use in diagnosis of state of lipid nutrition. The differences observed between sexes and type of fat are, indeed, sufficient to be applied to individuals. To determine whether such individual measurements of lipid metabolism could be related to onset of atherosclerosis would require extensive human testing. It is also necessary to investigate the effects of advancing age upon rates of lipid biosynthesis in both sexes, to find whether individual differences would be discernable as rates of lipid metabolism decrease.

ACKNOWLEDGMENTS

Technical assistance by J. E. Wilson, Jr., and Mrs. Ethel B. Hall.

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[Received March 14, 1966]

Fatty Acid Oxidation in Relation to Cholesterol Biosynthesis in Rats

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ABSTRACT

Groups of male and female rats were fed diets containing (calorie basis) 2% corn oil (low-fat, LF), 42% corn oil (CO) or 2% corn oil plus 40% beef tallow (BT) for 2 weeks. Then rats of each sex and diet group were given an intraperitoneal injection of ^{14}C-acetate, -stearate, -oleate or linoleate. Acetate incorporation into cholesterol and rate of oxidation of each fatty acid were determined. Specific activity of cholesterol was higher in females than males, higher with 40% lipid in the diet than with 2% corn oil and higher for CO than BT. Linoleate was oxidized more rapidly than oleate which exceeded stearate. An index of dietary lipid oxidation was computed based on fatty acid oxidation rate, per cent of each fatty acid in the diet and per cent of lipid calories in the diet. Serum cholesterol-^{14}C was found to be proportional to dietary lipid oxidation index.

INTRODUCTION

RECENTLY IT HAS BEEN SHOWN that feeding beef tallow to female rats in amounts of from 2 to 80% of dietary calories resulted in concomitant increments in incorporation of ^{14}C-acetate into cholesterol of liver, serum and carcass (1). Further studies showed that in female rats, corn oil fed as 42% of dietary calories caused a 22-fold increase in serum cholesterol-^{14}C from ^{14}C-acetate compared to a group receiving 2% corn oil calories (2). Male rats also exhibited increased incorporation (29-fold) of acetate into cholesterol when fed 42% compared to 2% corn oil calories. Beef tallow fed as 40% of calories caused a 2.6-fold increase in acetate incorporation into cholesterol in males and a 3.4-fold increase in females. Other investigators have reported an inverse relationship between fatty acid synthesis and cholesterol synthesis (3). The contribution of fatty acids to energy needs has been discussed in relation to the synthesis of cholesterol in an earlier publication (1).

The effects of saturation of fatty acids and individual fatty acids in the diet upon cholesterol in serum have been studied extensively. Stare has recently reviewed findings in this area (4). Studies have also been made of the rates of oxidation of individual fatty acids. Mead et al. (5) fed mice 1-^{14}C labeled stearate, oleate or linoleate and found that oleate and linoleate were more rapidly oxidized than stearate. Lynn and Brown (6) reported that ^{14}C-linoleate was more rapidly oxidized than ^{14}C-stearate when they were fed to rats. That sex may be involved in rates of fatty acid oxidation has been shown by Kritchevsky (7). Oxidation of octanoate by rat liver mitochondria was more rapid in females than in males.

It appears from the above observations that the factors which result in greater cholesterol synthesis are also factors which are involved in more rapid fatty acid oxidation. The following experiment was designed to determine whether rate of oxidation of dietary lipid and rate of synthesis of cholesterol vary in proportion to each other in rats.

PROCEDURES

Animals

Rats were obtained from Holtzman Farms in 4 groups of 18 of each sex. Upon arrival they were caged singly and fed laboratory chow (Wayne Lab Blox) until they were placed on experimental diets. Each group of 36 rats was divided into 3 subgroups containing 6 of each sex. The first group was used for study of ^{14}C-acetate incorporation into cholesterol. The other 3 groups were used for study of ^{14}C-fatty acid oxidation. The ^{14}C-fatty acid oxidation studies constituted 3 replications carried out at one-week intervals using 2 rats of each sex for each of 3 fatty acids. Mean initial weight of group 1 females was 179 g; males 266 g. Mean initial weight of groups 2, 3 and 4 females was 195 g; males 273 g.

After 2 weeks of feeding the experimental diets the following regime was used for ^{14}C-acetate and for ^{14}C-fatty acid studies. Food was removed in the evening; 10–12 hr later it was replaced and the rat was allowed access to it for exactly 1 hr. Then the food was again removed and the radioactive compound injected intraperitoneally. Four hours later rats given ^{14}C-acetate were anesthetized with sodium amytal, and a blood sample taken by heart puncture. Immediately upon injection of a fatty acid the rat was put into a respiration chamber and the expired CO_2 was collected for 3 hr.

Diets

Diets were formulated as previously described (1) to contain on a calorie basis either 2%

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corn oil (low fat diet, LF), 42% corn oil (high corn oil diet, CO), or 2% corn oil plus 40% beef tallow (high beef tallow diet, BT). All other nutrients were supplied in adequate amounts. Throughout the feeding period food and water were allowed ad libitum.

Radioactive tracers

Group 1 was given Na-1-^{14}C-acetate (30.7 mc/mM); females received 10 μc, males 20 μc. Two rats of each sex in groups 2, 3 and 4 received Na-1-^{14}C-stearate (3.2 mc/mM), Na-1-^{14}C-oleate (22.5 mc/mM), or Na-1-^{14}C-linoleate (6.3 mc/mM). All radioactive compounds were obtained from Chem Trac Division of Baird Atomic, Inc. The fatty acids were saponified with alcoholic NaOH, neutralized with HCl, diluted with physiological saline solution and stored at −20C. The process was performed in an atmosphere of nitrogen. Actual doses were: stearate 3.81×10^6 cpm, oleate 4.89×10^6 cpm, linoleate 4.81×10^6 cpm.

Chemical and Radiochemical Analyses

Samples of the saponified ^{14}C-fatty acids were counted in a Nuclear-Chicago liquid scintillation counter (efficiency, 68%) in a toluene solution containing 0.05 g/l 1,4-di 2-(5-phenyloxazolyl) -benzene and 4 g/l 2,5-diphenyloxazole. Serum obtained from rats given ^{14}C-acetate was stored at −20C until analyzed. Lipids were extracted by the Folch procedure (8) and the chloroform-methanol extract was washed with 0.73% NaCl. Samples of the resulting solution were saponified and cholesterol obtained as the digitonide. Cholesterol digitonide was dissolved in methanol, then added to the scintillation solution for counting. Quenching was checked by internal standards. A sample of the nonsaponifiable material was analyzed for total sterol colorimetrically using an acid iron reagent (9).

Carbon dioxide was collected from rats given a ^{14}C-fatty acid. Air was freed of CO_2 by passing through NaOH absorbers, then passed through the respiration chamber by suction. Expired CO_2 was collected in standard NaOH solution. Collecting absorbers were changed at 30-min intervals. Samples of the solution were titrated with standard HCl solution to a phenolphthalein end point. From other samples $BaCO_3$ was precipitated and plated on filter paper with a suction apparatus. These samples were counted in a low-background Geiger system (Wide-Beta, Beckman Instrument Company). Counting rates were corrected to infinite thinness by use of a self-absorption curve. The efficiency of the instrument was 22% and data were corrected to equivalence with the data obtained by liquid scintillation counting.

RESULTS

Acetate Incorporation into Cholesterol

The values for total serum cholesterol and ^{14}C in serum cholesterol are shown in Table I. Total activity (TA) is per cent of injected dose in 1 ml of serum and specific activity (SA) is TA per milligram of cholesterol. In males the ratio of TA when beef tallow was the dietary fat to TA when the rats were fed low-fat diets was about 9. The same ratio for corn oil to low-fat was about 22. Specific activity values allow similar comparisons.

Females had higher TA and SA values (Table I) than males for each dietary treatment. The values for LF and BT were significantly higher than similar values for males. The TA values for males and females fed CO were not significantly different, but SA values were. The ratio of TA when CO was fed to TA when LF was fed, in females, was about 6. The same ratio for BT compared to LF was about 4.

In general, males had lower TA and SA values for serum cholesterol than comparable females. The effect upon those values of feeding fat, however, was greater in the males. In both sexes corn oil had a greater effect upon TA of serum cholesterol in comparison to a low-fat diet than did beef tallow.

Oxidation of Fatty Acids

The values for ^{14}C in CO_2 after intraperitoneal administration of carboxyl labeled fatty acids to rats are shown in Figure 1. The points indicate per cent of injected ^{14}C expired in each ½ hr interval, not cumulative totals. Cumulative totals for 3 hr are shown in Table II. The base 10 logarithm of the per cent of ^{14}C ex-

TABLE I

Cholesterol in 1 ml Serum 4 hr after Intraperitoneal Injection of Na-1-^{14}C-Acetate

Diet	Cholesterol		
	mg/100 ml	TA[a] × 10^3	SA[b] × 10^3
	Males		
LF[c]	42 ± 5.2[d]	0.14 ± 0.02	0.33 ± 0.04
BT	54 ± 5.0	1.22 ± 0.26	2.34 ± 0.52
CO	64 ± 3.9	3.17 ± 0.55	5.12 ± 1.01
	Females		
LF	45 ± 4.6	0.65 ± 0.18	1.44 ± 0.33
BT	54 ± 4.4	2.34 ± 0.21	4.34 ± 0.31
CO	42 ± 3.9	4.17 ± 0.70	10.29 ± 1.90

[a] Total activity (% of injected dose).
[b] Specific activity (TA/g).
[c] LF = low-fat, BT = beef tallow, CO = corn oil.
[d] Mean ± standard error of the mean of 6 observations.

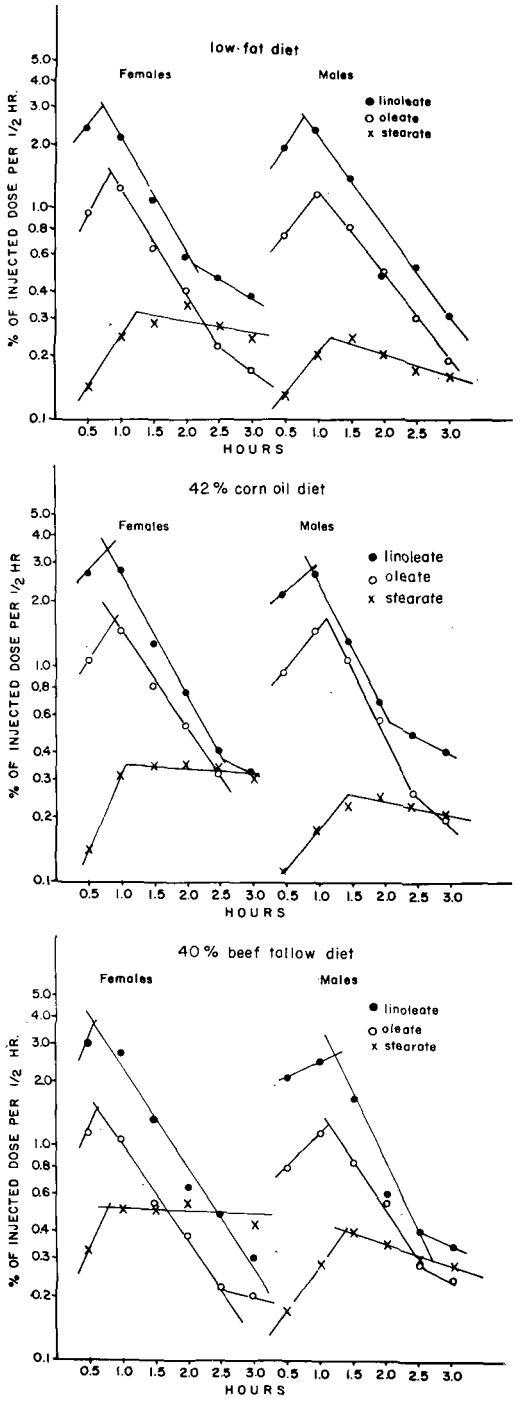

FIG. 1. $^{14}CO_2$ expired by rats fed various diets and intraperitoneally injected with Na-1-^{14}C-fatty acids.

pired per half hour was used to accentuate the differences in the rates of $^{14}CO_2$ expiration. Appearance of $^{14}CO_2$ indicated disappearance of the ^{14}C-fatty acid. Total activity was used for $^{14}CO_2$ because specific activity of CO_2 indicates only the contribution of the particular fatty acid to the fuel of respiration. The portion of the curve for the first half hour indicated absorption of the fatty acid and mixing with the liver-serum pool of fatty acids. The pool at that time reflected ingested lipid and/or biosynthesized lipid. The next 1½ hr on the curve appeared to represent metabolism of the labeled fatty acid in connection with the meal consumed just prior to administration of the label. The curve over the last hour appeared to indicate completion of the process of metabolism in relation to the meal and attainment of a constant rate of oxidation of fatty acids which was similar for all fatty acids. Presumably the source of lipid by that time was adipose tissue or other "endogenous" sources. Dilution of the label by oxidation of fatty acids over the time studied was not great enough to constitute a separate factor in interpretation.

Low-Fat Diet. There was a clear difference in amount of ^{14}C expired between linoleate and oleate, but no difference in rate of expiration in the first 2 hr (Fig. 1). The total linoleate completely oxidized in 3 hr was almost twice as great as oleate (Table II). Stearate was different from linoleate and oleate both in amount expired and rate of expiration. The amount expired was one-sixth that of linoleate in 3 hr. The rate of appearance of ^{14}C in expired CO_2 from stearate was extremely slow and apparently unrelated to ingestion of a meal. In rats on the LF diet there was no sex difference in oxidation of oleate or linoleate. The cumulative total for 3 hr of stearate oxidation was greater in females than males but the difference was not statistically significant.

TABLE II

Total Activity of $^{14}CO_2$ Expired in 3 Hours after Intraperitoneal Injection of Na-1-^{14}C-stearate, -oleate, or -linoleate

Diet	$^{14}CO_2$		
	Stearate	Oleate	Linoleate
	% of injected dose		
	Males		
LF[a]	1.10 ± 0.17[b]	3.63 ± 0.64	6.90 ± 0.91
BT	1.77 ± 0.37	3.85 ± 0.46	7.50 ± 0.97
CO	1.17 ± 0.11	4.37 ± 0.68	7.58 ± 0.62
	Females		
LF	1.52 ± 0.12	3.60 ± 0.34	7.00 ± 0.87
BT	2.72 ± 0.36	3.57 ± 0.26	8.39 ± 0.49
CO	1.76 ± 0.45	4.47 ± 0.67	8.20 ± 0.10

[a] LF = low-fat, BT = beef tallow, CO = corn oil.
[b] Mean ± standard error of the mean.

High Corn Oil Diet. The expiration of $^{14}CO_2$ from each fatty acid when rats were fed diets containing 42% corn oil was almost identical with values from rats fed only 2% corn oil (Table II and Fig. 1). It thus appears that the rate of oxidation of linoleate was not affected by the large difference in the linoleate pool brought about by ingestion of a meal containing corn oil.

High Beef Tallow Diet. Linoleate and oleate were oxidized to CO_2 at the same rate in rats fed 40% beef tallow and those fed 2% corn oil, regardless of sex (Table II and Fig. 1). The cumulative total of $^{14}CO_2$ expired from stearate was greater when the rats were fed beef tallow than when they were fed either 2 or 42% corn oil. In females the difference was statistically significant at the 5% level, comparing BT to LF. The presence of a large amount of stearate apparently caused some adaptation of the rats to more rapid stearate oxidation. In females stearate oxidation was not significantly less than oleate. Although the value for females was higher than that for males, the difference was not statistically significant. Obviously the pool size was not a factor because dilution of the label with dietary stearate should result in lowering the quantity of expired $^{14}CO_2$.

General. The observed differences in appearance of ^{14}C in respired CO_2 after administration of different fatty acids were not related to changes in total CO_2 expired. Table III shows data for total CO_2 expired and the specific activity of respired CO_2. The values for specific activity differ little in relation to each other from total activity values, because within each sex CO_2 expired was very uniform. The only difference between TA and SA comparisons is that females fed BT had significantly greater SA of $^{14}CO_2$ from oleate than from stearate, contrary to the values for TA.

The specific activity data indicated that when linoleate was available it was selectively used for energy and oleate was more readily used than was stearate. These conditions held even when the rat had just consumed a high carbohydrate meal. The contribution of those two fatty acids to respiration was unrelated to dietary supply of the fatty acids. The contribution to respiration made by stearate was increased somewhat when the diet contained stearate.

Relationship between Incorporation of Acetate into Cholesterol and Oxidation of Fatty Acids

The computation of a "dietary lipid oxidation index" was made from the above data for each diet and sex. Per cent of the injected dose of each fatty acid expired in 1 hr was multiplied by the per cent of that fatty acid in the dietary lipid (10) and by the per cent of calories supplied by dietary lipid. Palmitate was assumed to be oxidized at the same rate as stearate for the purpose of this computation. Assuming palmitate to be the same as oleate changed the figures only slightly. The resulting values were plotted against SA of serum cholesterol of comparable rats. Figure 2 shows the straight line relationship between dietary lipid oxidation index and cholesterol biosynthesis (SA of serum cholesterol) for each sex. There appeared to be a sex difference in overall rates of both parameters as shown by the different intercepts. There also appeared to be a sex difference in the relation of the two parameters to each other as shown by the different slopes.

DISCUSSION

The data for incorporation of acetate into cholesterol are in agreement with previous results obtained under similar conditions (2). In earlier reports differences in specific activity of cholesterol between treatments in the presence of constant total body cholesterol were

TABLE III

Specific Activity of $^{14}CO_2$ Expired in 3 Hours after Intraperitoneal Injection of Na-1-^{14}C-stearate, -oleate, or -linoleate

Diet	mM CO_2	Cpm/mM Co_2		
		Stearate	Oleate	Linoleate
		Males		
LF[a]	7.86 ± 0.14[b]	1.72 ± 0.31[c]	6.83 ± 1.39	13.26 ± 2.28
BT	7.83 ± 0.13	2.71 ± 0.61	7.59 ± 1.20	14.40 ± 2.02
CO	7.64 ± 0.08	1.78 ± 0.19	8.64 ± 1.36	15.08 ± 1.35
		Females		
LF	7.34 ± 0.09	2.43 ± 0.21	7.40 ± 0.84	14.36 ± 1.56
BT	7.09 ± 0.12	4.51 ± 0.70	7.68 ± 0.50	17.79 ± 1.32
CO	7.18 ± 0.10	3.00 ± 0.82	9.24 ± 1.41	17.11 ± 2.61

[a] LF = low-fat, BT = beef tallow, CO = corn oil.
[b] Mean ± standard error of the mean of 18 observations.
[c] Mean ± standard error of the mean of 6 observations.

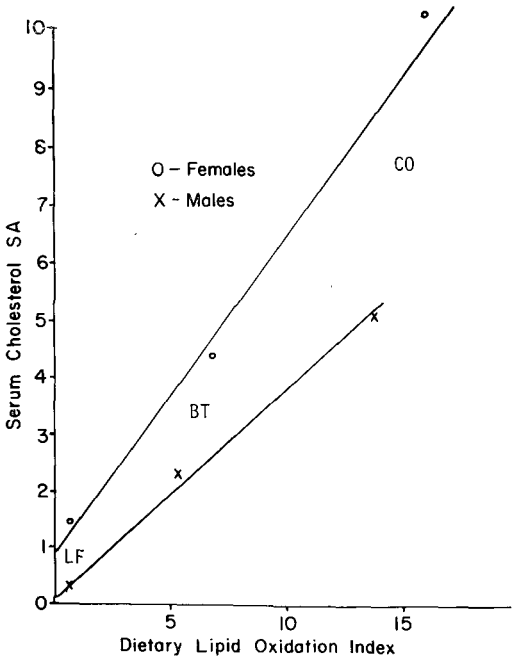

FIG. 2. Relationship between cholesterol turnover as shown by ^{14}C-acetate incorporation into cholesterol and oxidation of dietary lipid by rats. SA = % of injected dose per gram cholesterol in 1 ml serum × 10^3. Dietary lipid oxidation index = % of injected dose of each fatty acid expired in 1 hour × % of that fatty acid in the dietary lipid × % of calories supplied by dietary lipid. LF indicates values for rats fed 2% corn oil calories; BT, 40% beef tallow plus 2% corn oil calories; CO, 42% corn oil calories.

interpreted to indicate differences in excretion of total steroids. Several reports have shown that total steroid excretion does not vary consistently with the kind or amount of fat in the diet (reviewed by Miettinen et al., 11). The specific activity of fecal total steroids does vary with kind of fat in the diet (12). Serum cholesterol specific activity has been shown to be very similar to fecal steroid specific activity and has been used as the basis for determination of net steroid excretion (13,14). It is thus apparent that the differences in specific activity of serum cholesterol indicate differences in turnover rate (or half-life) rather than in excretion rate. Direct determination of biological lifetime of cholesterol when either linoleate or stearate was fed also indicated a shorter half-life with linoleate (15).

The conclusions which may be reached about the present cholesterol specific activity data are that females had a more rapid rate of cholesterol turnover than males regardless of diet; and that dietary corn oil markedly accelerated cholesterol turnover in both sexes in comparison to a low-fat diet. Dietary beef tallow slightly accelerated cholesterol turnover in both sexes.

The fatty acid oxidation data are in agreement with reports in the literature (5,6,16) showing that unsaturated fatty acids are more rapidly oxidized than are saturated fatty acids. Neither sex nor diet had a marked effect upon rate of oxidation of individual fatty acids. There is some indication that the rats, particularly females, were able to adapt to more rapid oxidation of stearate when a large quantity was in the diet. Perhaps this adaptation would become significant over a longer period of time. Although individual fatty acid oxidation was independent of diet, the fatty acid composition of the diet determined its overall rate of oxidation. The presence of a large amount of slowly oxidizable lipid would necessarily mean that dietary lipid would be more slowly oxidized than if a large amount of rapidly oxidizable lipid were present. That the rate of dietary lipid oxidation bears a linear relationship to the turnover time of cholesterol suggests that the two processes may have some physiological connection.

One possibility for a physiological connection is that the acetate formed from fatty acid oxidation is the substrate for cholesterol biosynthesis. Mead et al. (5) found no such connection in their study. De Leo and Foti (17) specifically studied this possibility and found no relationship between cholesterol labeling and fatty acid source of acetate.

Other possibilities must take in a wider range of physiological utilization of lipids than can be seen in one tissue. In tracing fatty acid and cholesterol metabolism through absorption from the gut to final oxidation and excretion, respectively, some possibilities for their interdependence emerge.

Absorption from Gut

Most of the lipid absorbed from the gut appears in the thoracic duct as chylomicrons. Chylomicrons contain 2–4% cholesterol (18) which is present even in the absence of dietary cholesterol. Intestinal wall cells have a large capacity to synthesize cholesterol (19). In the process of absorption of lipids with the formation of chylomicrons there is considerable specificity for location of fatty acids in lipid classes. The net specificity (disregarding pathways) has been studied (20,21) and there was a strong selectivity of cholesterol esters for

oleate, and of phospholipids for stearate and less pronounced for linoleate. Glycerides were not selective of fatty acids and contained 92% of all the fatty acids in chylomicrons.

Chylomicron lipids enter liver and then lipids reenter the circulation from liver in the form of lipoproteins which contain 20–60% of the lipid as cholesterol. Naidoo et al. (22) studied the disappearance of chylomicrons into liver. Cholesterol-^{14}C from labeled chylomicrons disappeared from serum at a rapid rate at first then began to increase in concentration in the serum. This indicated entrance of the chylomicrons into liver, then reappearance of the cholesterol, probably as higher density lipoproteins. The amount of cholesterol in the chylomicrons is insufficient to account for the amount found in lipoproteins secreted from the liver.

Liver is the most active organ in cholesterol biosynthesis, and is the major site of lipoprotein synthesis. In the presence of large quantities of dietary lipid, specific activities of liver and serum cholesterol have been shown to increase (1,2). There have been several reports upon the specificity of lipoproteins for certain fatty acids, particularly certain cholesterol esters. Gidez et al. (23) studied the effects of diet upon composition of liver and plasma lipoprotein cholesterol esters. In general, the lower density lipoproteins were the most similar of the lipoproteins to diet in fatty acid composition of cholesterol esters. (They also contained the most glyceride which was non-fatty acid specific in gut.) Higher density lipoprotein cholesterol esters were selective in incorporating linoleic and arachidonic acids. The highest density group contained the most cholesteryl-arachidonate. Goodman and Shiratori (24) used ^{14}C-labeled cholesterol to study turnover of cholesterol esters. Both the mass and specific activities of those esters containing linoleic and arachidonic acids were greater in plasma than in liver. Stearic and oleic acid cholesterol esters were consistently lower in plasma than in liver. Oleate ester was the most abundant in liver and had the highest specific activity of any cholesterol ester in liver. The differences between cholesterol esters in the liver and those secreted into plasma indicate some specificity in esterification in the liver. Swell and Law (25) studied cholesterol ester turnover using ^{14}C-labeled cholesterol and labeled cholesterol esters. They found more rapid turnover of linoleic and arachidonic esters than of palmitic and oleic esters.

These various observations indicate that there is specificity in formation of cholesterol esters and in content and type of cholesterol esters in lipoproteins of different density. It is proposed that this specificity is associated with the destination of the lipoproteins secreted by the liver.

Utilization of Lipids

Low density lipoproteins contain fatty acids in a pattern similar to diet. Adipose tissue can gradually assume a pattern of fatty acids similar to diet (26–28). The lipids of low density lipoproteins and chylomicrons can be removed from circulation by extrahepatic tissues (29). It is proposed that the specific destination of low density lipoproteins secreted by liver is adipose tissue.

The use of fatty acids as fuel of respiration occurs in heart (30), kidney (30), and skeletal muscle (31). Human myocardium has been shown to preferentially extract oleic acid (32). Bressler and Friedberg (33) reported that heart mitochondria oxidized linoleate in preference to oleate or palmitate. Linoleate successfully competed with those two fatty acids when both were in the medium. Those results are in close accord with oxidation of fatty acids by the intact rat reported herein.

The rate of oxidation of fatty acids appears to decline with chain length for saturated fatty acids (34) and increase with unsaturation, except for arachidonate. Coots (35) ascribes the lower rate of oxidation of arachidonate than linoleate to specific use of the arachidonate for a structural role in the cell. This may also explain its specific concentration into cholesterol esters of high density lipoproteins. The graph of stearate oxidation in this report presents a more complicated picture than merely a lower rate. It appears to have a route of metabolism entirely different from that of unsaturated fatty acids. Its systematic exclusion from cholesterol esters and incorporation into phospholipids may be part of the difference. It may also be that it must go through some extra intermediate step. Elovson (36) has shown that a large percentage (30–50) of stearate injected into the rat was rapidly converted to oleate. Another small percentage (10–20) was converted to palmitate without further oxidation (37). The selective placement of stearate into phospholipids may be part of these processes.

To this point a summary of lipid metabolism indicates rapid synthesis of cholesterol in gut during absorption of lipids, and in liver during synthesis of lipoproteins. Low density lipoproteins go to adipose tissue becoming "endo-

genous" in terms of further metabolism and highest density lipoproteins take arachidonate as the cholesterol ester to its cellular site of further metabolism. High and medium density lipoproteins, then, may go to muscle, delivering their fatty acids as cholesterol esters for oxidation. During oxidation of the fatty acids, cholesterol must be released. During times of high specific activity of serum cholesterol there is concomitant high specific activity of bile sterols (12,14). The possibility arises that during oxidation of the fatty acid moiety of cholesterol esters, cholesterol is also oxidized to bile acids and thereby completes a cycle, returning to gut. This would imply that cholesterol oxidation is proportional to fatty acid oxidation in muscle mitochondria. The opposite has been reported for liver mitochondria (38). The liver mitochondria of rats fed a low fat diet oxidized cholesterol-26-^{14}C to propionate faster than those fed hydrogenated shortening which did so faster than those fed corn oil. Similar studies of muscle mitochondria have not been reported, and this question remains open.

In conclusion, an hypothesis has been offered to explain the observed graphic relationship between oxidation of dietary lipid and biosynthesis of cholesterol. Cholesterol is synthesized in gut and liver in response to lipid ingestion, some fatty acids are esterified in gut, requiring bile acids, and the lipid goes to liver as chylomicrons; more cholesterol synthesis and esterification occurs in the liver with preferential arrangement of fatty acids so that lipoproteins are synthesized with discreet cholesterol ester composition; those lipoproteins rich in oleic and linoleic acid esters of cholesterol go preferentially to muscle for fatty acid oxidation; the cholesterol ester composition conveys some specificity to acceptance of the fatty acid for oxidation in mitochondria and in the process cholesterol is oxidized (probably not 100%) to bile acids. This process is fast or slow depending upon the fatty acid composition of the diet. The rate regulating part of the cycle must be oxidation of the fatty acids. The development of this hypothesis presumes that only dietary lipid oxidation causes concomitant turnover of cholesterol. Fat-free diets, or diets mildly restricted in calories are associated with lipid metabolism, but cholesterol turnover is low and constant in those conditions (1,2). Starvation and other extreme conditions of lipid metabolism may not be explainable by this hypothesis.

ACKNOWLEDGMENT

Supported in part by the Washington Heart Association.

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[Received May 11, 1966]

Thin-Layer Chromatographic Separation of Dimethylphosphatidates Derived from Lecithins

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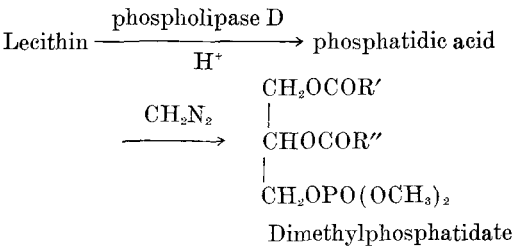
ABSTRACT

The separation of lecithin derivatives based on their fatty acid substituents has been investigated. Several synthetic and natural lecithins were converted to their corresponding dimethylphosphatidates by hydrolysis with phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) and methylation of the resulting phosphatidic acids with diazomethane. These dimethylphosphatidates were separated into fractions by reversed-phase thin-layer chromatography. Separations were dependent on the total number of methylene groups and double bonds in the two fatty acid chains. Fractionated dimethylphosphatidates were extracted from the plates and fatty acids were determined.

INTRODUCTION

RECENT PAPERS HAVE been concerned with the fractionation of lecithin mixtures according to their fatty acid substituents (1–5). These separations have generally been based on the degree of fatty acid unsaturation, and have often involved removal of the phosphorus, thereby eliminating the possibility of studying P^{32} incorporation. There is need for a method retaining the phosphorus and effecting separations according to fatty acid chain length. We have found that dimethylphosphatidate derivatives of lecithins can be separated on this basis using reversed-phase thin-layer chromatography (TLC). This paper describes the technique and applies it to lecithin mixtures of both synthetic and biological origin.

Dimethylphosphatidate preparation involved hydrolysis of the lecithins with phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) (6), followed by methylation with diazomethane to form the corresponding dimethylphosphatidates [phosphatidic acid dimethyl ester; dimethyl-(1,2-diacylglycerol-3-phosphate)] (7,8) as follows:



Dimethylphosphatidate

The dimethylphosphatidates were cleanly separated into various fractions by reversed-phase TLC. Fractions differed by the total number of methylene groups in the two fatty acid chains, with each double bond reducing the number of effective methylene groups by two. The procedure is applicable to other phospholipid classes (7,8).

EXPERIMENTAL

Materials and Analytical Methods

Reagent grade solvents were used as obtained commercially. Silica gel G and kieselguhr G were from E. Merck AG (distributed by Brinkmann Instruments Inc., Westbury, N. Y.); TLC plates were developed in closed tanks lined on all four sides by solvent-saturated filter paper. The kieselguhr G was prewashed with chloroform-methanol (2:1, v/v). Infrared spectra were run in KBr pellets containing 0.3 to 1 mg of sample, using a Perkin-Elmer Model 21 spectrophotometer. Ester analyses were performed by the method of Rapport and Alonzo (9), and phosphorus was determined as described by Bartlett (10). Phospholipid samples were stored in chloroform-methanol (2:1, v/v) solution within glass-stoppered containers at −15C.

Source and Purity of Lecithin Samples

Dimyristoyl α-lecithin was kindly supplied by Prof. E. Baer, and samples of dioleoyl, γ-stearoyl-β-oleoyl, and γ-oleoyl-β-stearoyl α-lecithins were generous gifts of Prof. L. L. M. van Deenen. Bovine brain lecithins were prepared by silicic acid chromatography (5) from a commercial sample of beef brain phospholipids (Nutritional Biochemicals Co.), while

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rabbit kidney cortex lecithins containing P^{32} were prepared by tissue slice incubation, extraction by the method of Folch et al. (11), and silicic acid chromatography as described earlier (5). TLC analysis using silica gel G plates developed with chloroform-methanol-water (65:25:4, v/v) showed all lecithin samples to be either pure or with only minor impurity (Fig. 1b), and all exhibited infrared spectra identical to those published by Rouser et al. (12) and DeHaas and Van Deenen (13). In addition, the bovine brain, rabbit kidney cortex, and dimyristoyl lecithins gave ester/phosphorus ratios approximating 2.

Lecithin Hydrolysis with Phospholipase D

The hydrolysis procedure was similar to that of Davidson and Long (6). To 5–10 mg of lecithin in a 10-ml volumetric flask equipped with a ground glass stopper was added 1.25 ml of 0.1 M sodium acetate (pH 5.6), 0.25 ml of 1 M calcium chloride, 1 ml of ethyl ether, and 2 ml of fresh enzyme solution. Enzyme solution was prepared when needed from equal weights of cabbage and water; these were homogenized in a blender for 5 min, filtered through filter paper, centrifuged for 30 min at 13,000 × g, and the supernatant was used directly. The stoppered flask was shaken vigorously by hand, then agitated in a vortex mixer at room temperature for 3 hr. The product was transferred to a 20-ml beaker and adjusted to pH 2.5 by the addition of a few ml of glacial acetic acid. In a 15-ml centrifuge tube with ground glass stopper this solution was extracted three times with 4-ml portions of chloroform-methanol (2:1, v/v), each extraction involving vigorous shaking, centrifugation, and removal of the lower phase with a Pasteur pipette. Solvent was removed from the three combined extracts with a rotary evaporator, with warming to 45C at the end until no residual odor of acetic acid remained. The residue, primarily phosphatidic acid, was dissolved in 1 ml of ethyl ether; a representative TLC analysis of this product is shown in Figure 1c.

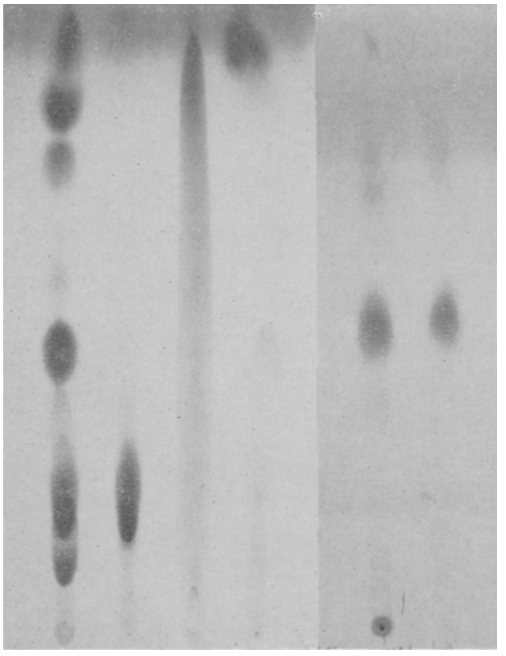

FIG. 1. Thin-layer chromatograms of phospholipids on silica gel G plates. Developing solvents: A-D, chloroform-methanol-water (65:25:4, v/v); E-F, carbon tetrachloride-chloroform-glacial acetic acid-ethanol (60:40:0.5:3, v/v). Samples: A, mixed rabbit kidney cortex phospholipids and neutral lipids; B, bovine brain lecithins; C, phosphatidic acids after rabbit kidney lecithin hydrolysis; D-E, crude dimethylphosphatidates after methylation of C with diazomethane; F, dimethylphosphatidates after purification of D-E by preparative TLC. Plates were charred after spraying with sulfuric acid saturated with potassium dichromate.

Phosphatidic Acid Methylation with Diazomethane (7)

A solution of diazomethane in ethyl ether, prepared from commercial diazomethane precursor (EXR-101 from E. I. du Pont de Nemours and Co., Wilmington, Del.), was added to the ethereal phosphatidic acid solution until a bright yellow color remained. The container was glass stoppered and allowed to stand at room conditions for 16–18 hr. Ether was removed, and the residue was dissolved in 1 ml of chloroform-methanol (2:1, v/v). Representative TLC analyses of this crude dimethylphosphatidate product are shown in Figure 1d and 1e; developing solvents of two different polarities were used.

Dimethylphosphatidate Purification Procedure

The crude dimethylphosphatidates were purified by preparative TLC, using 8 in. × 8 in. silica gel G plates and carbon tetrachloride-chloroform-glacial acetic acid-ethanol (60:40:0.5:3, v/v) as developing solvent. The product from 5–10 mg of lecithin could be purified on 2 or 3 plates. Material was applied in a line along the bottom of the plate. After development, the dimethylphosphatidates near R_f 0.4–

0.5 were detected by masking most of the plate with two panes of glass and spraying a narrow, vertical strip with 0.005% aqueous rhodamine 6G. Under ultraviolet light the desired spot was outlined, and corresponding horizontal lines were drawn across the plate. With a razor blade, all material between the lines, except the sprayed area, was scraped into a 15-ml glass-stoppered centrifuge tube and extracted twice with centrifugation, using 3–4 ml portions of chloroform-methanol (2:1, v/v). Alternatively, the material was sometimes scraped into a small fritted-glass filter and extracted with the same solvent. Solvent was removed and the dimethylphosphatidates were dissolved in 1 ml of chloroform-methanol (2:1, v/v). A representative TLC analysis of the purified dimethylphosphatidates is shown in Figure 1f. Based on phosphorus analyses, yields varied (29–81%) for different lecithins. Dimethylphosphatidates from bovine brain lecithins showed ester/phosphorus ratios approximating 2. All dimethylphosphatidates yielded infrared spectra similar or identical to that published by Crone (8).

Separation of Dimethylphosphatidates by TLC

After preheating for 1 hr, kieselguhr G plates were dipped in a 5% solution of *n*-tetradecane in ethyl ether (14) and used after evaporation of the ether. After spotting the dimethylphosphatidates (20–100 μg per spot), the plates were developed with acetonitrile-acetone-water (8:1:1, v/v) saturated with *n*-tetradecane. Spots were readily visible after spraying the plates with either water, or 0.005% rhodamine 6G when viewed under ultraviolet light. The separation of several dimethylphosphatidates is shown in Figure 2.

Extraction from Plates and Fatty Acid Determination

For fatty acid determination, plates were sprayed with water and spots were outlined. The encircled material was then scraped into a small fritted-glass filter and extracted several times with small portions of chloroform-methanol (2:1, v/v), the extract dripping into a 5-ml glass-stoppered centrifuge tube. Solvent was removed with a stream of nitrogen.

Methanolysis was essentially by the method of Morgan, Hanahan, and Ekholm (15). The sample (dimethylphosphatidate or lecithin) was dissolved in a drop of chloroform and 200 μl of 0.5 N methanolic NaOH was added. After standing at room conditions for 10 min, 20 μl of 6 N aqueous HCl, 1 ml of water, and 2 ml of chloroform were added, and the tube was shaken vigorously. The pH of the aqueous upper layer was then below 2. The lower layer was removed with a pipette, concentrated to less than a drop of solution with a stream of nitrogen, and injected directly into a Jarrell-Ash Model 28–710 gas chromatograph containing a 6 ft × ¼ in. column packed with 15% diethylene glycol succinate on 80–90 mesh Anakrom (Jarrell-Ash Co., Waltham, Mass.). Fatty acid methyl esters were identified by comparing retention times with those of known samples; no attempt was made to identify minor components consisting of a few percent. Quantitative analyses of these esters are reported in area percent as determined by triangulation, and minor components were generally ignored. Fatty acid compositions of each of the starting lecithins, their corresponding dimethylphosphatidates, and individual di-

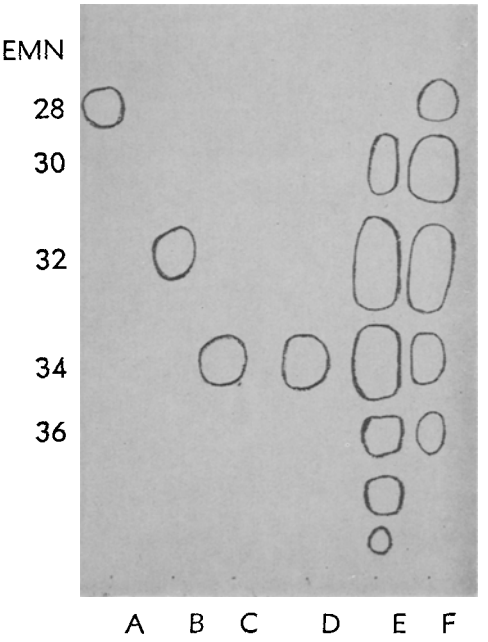

FIG. 2. The reversed-phase TLC of various dimethylphosphatidates on a kieselguhr G plate. The plate was impregnated with *n*-tetradecane by dipping in a 5% solution of *n*-tetradecane in ethyl ether. Developing solvent: acetonitrile-acetone-water (8:1:1, v/v) saturated with *n*-tetradecane. Dimethylphosphatidate samples: A, dimyristoyl; B, dioleoyl; C, γ-stearoyl-β-oleoyl; D, γ-oleoyl-β-stearoyl; E, from bovine brain lecithins; F, from rabbit kidney cortex lecithins. The effective methylene numbers (EMN) of the spots are as indicated. Spots were outlined under ultraviolet light after spraying with 0.005% aqueous rhodamine 6G.

TABLE I

Fatty Acid, Phosphorus, and ^{32}P Determinations on Lecithins, Their Corresponding Dimethylphosphatidates, and Individual Fractions after Separation of the Dimethylphosphatidates by TLC (see Fig. 2)

					Bovine brain		Rabbit kidney cortex		
	Dimyristoyl	Dioleoyl	γ-Stearoyl-β-oleoyl	γ-Oleoyl-β-stearoyl	% Phosphorus	Fatty acids	% Phosphorus	^{32}P sp. act.[a]	Fatty acids
Lecithin	M 100%	O 100%	S 52% O 46	S 48% O 50		P 31% S 15 O 52 N 1			P 44% S 12 O 15 L 26
Dimethyl-phospha-tidate	M 100	O 100	S 53 O 46	S 48 O 50		P 31 S 14 O 52 N 1			P 48 S 9 O 15 L 25
EMN of spot									
28	M 100						3.5%	59	L[b] P 54
30					4.6%	[c]	8.0	197	S <1 O 4 L 35
32		O 100			52.8	P 48 O 49	43.4	301	P 53 S 15 O 21 L 10
34			S 48 O 45	S 50 O 47	30.6	P 15 S 46 O 29 N 5	37.6	298	P 33 S 45 O 15 L 2
36					6.2	P,S,O[b,d]	7.6	281	[c]

Abbreviations: EMN, effective methylene number; M, myristate; P, palmitate; S, stearate; O, oleate; L, linoleate; N, Linolenate.

[a] Counts/min/μg phosphorus. [b] Insufficient material for quantitative analysis, but main constituent as indicated. [c] Insufficient material for meaningful analysis. [d] Two spots of lower R_f value containing 5.7% of recovered phosphorus yielded insufficient material for meaningful analysis.

methylphosphatidate spots shown in Figure 2, are presented in Table I. The table also gives phosphorus analyses to estimate ratios of the spots from biological mixtures, as well as specific activities for each spot resulting from the rabbit kidney lecithins.

DISCUSSION

The behavior of the dimethylphosphatidates on a reversed-phase TLC system is evidently similar to the fatty acid methyl esters (16) and the triglycerides (17,18), in that separations are based on chain length and number of double bonds, and "critical pairs" are not readily separated. Thus the migration rate of a lipid with one double bond is about the same as a saturated lipid with two fewer methylene groups. Therefore palmitate, with an effective methylene number (EMN) of 16, is readily separated from stearate (EMN 18), but not from oleate (EMN 16), and linoleate (EMN 14) is separated from palmitate, stearate, and oleate, but not from myristate (EMN 14). Dimethylphosphatidate separations, then, are based on the total EMN in the molecule, irrespective of γ- or β-positioning. The same fatty acid might appear in several spots, depending on the nature of the fatty acid with which it is paired in the molecule.

Given the fatty acid content of a lecithin mixture, it is therefore possible to predict the number of spots expected from separation of the corresponding dimethylphosphatidates, and the potential fatty acid content of each spot. Bovine lecithin, for example, contains three major fatty acids (Table I): palmitate (EMN 16), stearate (EMN 18), and oleate (EMN 16). Other than the relatively unlikely stearate-stearate combination (EMN 36), and ignoring minor components, two major spots with EMN 32 and 34 should be expected. The EMN 34 spot should contain palmitate, stearate, and oleate, resulting from stearoylpalmitoyl and stearoyloleoyl dimethylphosphatidates. The EMN 32 spot should contain palmitate and oleate, but no stearate, since the mixture contains no fatty acid with an EMN of 14 with which stearate could pair to produce a total EMN of 32. With the rabbit lecithins, however, linoleate (EMN 14) is a major constituent with which stearate can be paired; stearate would therefore be expected in the EMN 32 spot in this case. The results in Table I generally confirm the validity of this reasoning.

While dimethylphosphatidates can be produced directly from lecithins by reaction with diazomethane (8), our product by this method was heavily contaminated (methylene polymers?); a product of higher purity resulted from the two-step process employed. The data in Table I show that fatty acid selectivity in the nonquantitative conversion of lecithin to dimethylphosphatidate did not occur, although the possibility with other lecithins cannot be eliminated. The method was also used to investigate rates of P^{32}(phosphate) incorporation during incubation (3,5); Table I includes these results.

The combination of reversed-phase and silver nitrate TLC systems has yielded more than 50 fractions from a natural triglyceride mixture (14). A similar combination involving silver ion TLC (1,2) and the reversed-phase TLC method described in this paper should be suitable for phospholipid class analyses.

ACKNOWLEDGMENTS

Technical assistance by Miss M. C. Abell. This investigation was supported by United States Public Health Service grant HE-01639-12.

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[Received Feb. 28, 1966]

Fatty Acid Relationships in an Aquatic Food Chain

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ABSTRACT

The relationships amongst the fatty acids of the lipids from members of a model aquatic food chain were examined. The basic pattern of the fatty acids in the members, algae-brine shrimp-hydra, originated in the phytoplankton. Fatty acids in the neutral lipids of adult brine shrimp, *Artemia salina,* closely resembled dietary, or algal, fatty acids, whereas the phospholipid acids differed considerably from those in the algae. Fatty acids from the total lipids of *Hydra pseudoligactis* fed brine shimp nauplii also resembled the dietary acids, but more C_{20} polyunsaturates and fewer C_{18} unsaturated acids were present in those raised at 10C than were found at 20C.

INTRODUCTION

LOVERN (1) REPORTED that the fatty acids of freshwater and marine fish oils reflect the fatty acid patterns of the freshwater branchiopods (high in C_{18} unsaturates) and marine copepods (high in C_{20} and C_{22} polyunsaturates) on which the fish feed. In a model food chain consisting of the diatom *Chaetoceros simplex,* the brine shrimp *Artemia salina,* and the fish *Lebistes reticulates,* Kayama et al. (2) found that *Artemia* lipids contained arachidonic and eicosapentaenoic acids, *Lebistes* lipids had, in addition, docosatetraenoic, docosapentaenoic and docosahexaenoic acids, whereas *Chaetoceros* contained no highly unsaturated acids. Thus, highly unsaturated acids appeared in the food chain beginning with less unsaturated acids. When the fish were raised at 17C and 24C, the degree of unsaturation in the fatty acids was greater at the lower temperature. Farkas and Herodek (3) reported that lowering the temperature at which freshwater planktonic crustacea were grown led to an altered fatty acid pattern quite similar to that found in marine species.

Clearly, there is a continuity in the nature of fatty acids in a food chain, which is tempered by the regulatory mechanisms of members of the chain, including their response to temperature changes in the environment. These phenomena have been further explored by culturing the brine shrimp *Artemia salina* on algae, and feeding *Hydra pseudoligactis,* which were raised at 10C and 20C, on *Artemia* nauplii. The food chain was therefore not a continuous one, the size of the brine shrimp culture required for such a chain being impractical to maintain. Each of the relationships studied must therefore be considered separately. However, the specific relationships may be considered to be typical of those occurring in a continuous situation.

EXPERIMENTAL

Desiccated *Artemia salina* (Conn. Valley Biol. Supply, Southampton, Mass.) were placed in artificial sea water, the nauplii harvested 36 hr later and fed to *Hydra pseudoligactis* cultured according to Loomis and Lenhoff (4). Similar nauplii were grown to the adult stage at room temperature, with unicellular green algae from Long Island Sound as the food source. *Hydra* were removed from continuous cultures maintained at 10C and 20C and were deprived of food for 24 hr prior to use to insure the absence of undigested nauplii.

Lipids were extracted and washed by the method of Folch et al. (5). All washed lipid samples were evaporated to dryness under nitrogen in a tared container, weighed, and diluted to a volume suitable for preparation of the fatty acid esters by BF_3-catalyzed methanolysis (6). Where noted, neutral and phospholipids were separated by silicic acid chromatography (7) before transesterification.

The methyl esters were purified on silicic acid columns and analyzed in a Glowall Corp. gas chromatograph equipped with an argon ionization detector. Six foot columns (3.4 mm I.D.) packed with 15% EGS on 100/120 mesh Gas-Chrom CLZ (Applied Science Laboratories) were used at an operating temperature of 170C. Identifications of individual esters were made by comparison of their relative carbon numbers to those established for known acids under the same conditions (8) and the percentage composition of the samples were calculated by the method of Carroll (9). Identifications of the esters were confirmed by hydrogenation (10) and rechromatography of a portion of several ester preparations, an

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equivalence of the total amount of esters of given chain lengths found before and after hydrogenation being used as the criterion. Preparative thin-layer chromatography of the esters on silica gel plates impregnated with 25% silver nitrate (11) was also used to separate ester samples on the basis of unsaturation, each class then being analyzed by GLC for comparison to the total esters.

RESULTS

Fatty acids from the neutral (49.4 mg) and phospholipid (25.3 mg) fractions of nauplii lipids were similar, but the phospholipids contained less palmitic and palmitoleic and more oleic and C_{20} polyunsaturates than the neutral lipids (Table I, cols. 1 and 2). Acids from the lipid (6.4 mg) obtained from 372 mg of algae differed considerably from those in the nauplii lipids, with less palmitoleic and oleic acids and much more linolenic acid. No acids containing more than 18 carbons were found in this lipid (Table I, col. 3). The neutral lipids (3.6 mg) of adult brine shrimp (278 mg) grown on these algae had a fatty acid composition very similar to the algal lipid, with a high percentage of linolenic acid, but did contain some C_{20} acids, mainly eicosapentaenoic, as in the nauplii neutral lipids (Table I, col. 4). The phospholipids (1.9 mg) of the adults were more similar to those of the nauplii phospholipids, but with reduced oleic and eicosapentaenoic and raised stearic and linoleic content.

The fatty acids from the total lipids of *Hydra* raised at 10C and 20C (3.5 and 4.7 mg, respectively) had a pattern generally like that of the fatty acids from the brine shrimp nauplii on which they were fed (Table I, cols. 6 and 7). In both cases, the oleic acid level was lower, while in the 20C animals the linolenic acid content was higher, and in the 10C animals the C_{20} acids were increased, especially arachidonic. The total amount of unsaturation in the fat was the same in both groups, although the individual acids differed, the 10C group having 1.75 moles of double bonds per mole of fatty acid and the 20C group having 1.76.

DISCUSSION

Fatty acids in the lipids of brine shrimp are easily altered by diet, as is evident from the considerable change in the fatty acids from egg to adult in this study and by comparison with the findings of Kayama et al. (2). The fatty acids of the diatoms in Kayama's experiments were very different from those of the algae used in our studies and the difference was reflected in the fatty acids of brine shrimp fed these algae. It is clear, however, that brine shrimp can synthesize higher polyunsaturated acids, probably through the pathways of the linolenic family (12). Phospholipid fatty acids are apparently less influenced by diet than those of the neutral lipids, as has also been suggested for crayfish lipids (13). The higher percentage of the long-chain polyunsaturates found in the phospholipid fatty acids conforms to a commonly observed pattern (14), which is thought to be related to the structural role of the phospholipids. In natural and in synthetic phospholipids, the hydrocarbon chains of those

TABLE I

Gas Chromatographic Analyses of Fatty Acid Methyl Esters (as percent total esters)

Fatty acid	(1) Brine shrimp nauplii, NL[a]	(2) Brine shrimp nauplii, PL	(3) Algae	(4) Brine shrimp adults, NL	(5) Brine shrimp adults, PL	(6) Hydra, 10°C	(7) Hydra, 20°C
14:0	1.4	0.3	0.6	2.8	1.4	1.0	0.7
14:1	0.6	0.2	0.6	0.8	0.8	0.6	0.7
15:0	0.4	0.1	.....	tr	tr	0.5	0.3
15:1	0.3	tr	.....	tr	tr	0.5	0.7
16:0	15.5	10.7	14.5	10.7	11.8	11.6	10.2
16:1	16.6	10.9	5.3	4.0	12.9	8.4	8.1
17:0	tr	0.3	tr	tr	tr	0.7	0.9
17:1	1.4	1.1	2.4	2.7	1.6	1.3	1.7
18:0	4.0	6.1	1.7	4.2	11.1	6.7	5.5
18:1	30.7	37.8	20.4	13.9	24.7	23.4	24.5
18:2	4.8	6.4	6.8	8.1	17.0	5.2	6.6
18:3	15.8	13.5	43.0	45.4	16.6	16.1	23.7
18:4	1.5	2.4	.....	1.6	2.3	1.8	3.6
20:0	0.2	tr	.....	.....	3.4	1.5	tr
20:2	.....	tr	.....	.....	.....	0.2	.....
20:3	tr	tr	.....	.....	tr	0.3	0.3
20:4	0.9	3.0	.....	0.9	4.2	9.4	5.3
20:5	5.3	8.0	.....	4.4	2.3	6.2	4.0
22:4	1.1	tr	.....	.....	tr	1.2	.....

[a] NL = neutral lipids, PL = phospholipids, tr = trace.

lipids containing unsaturated fatty acids exist in an essentially liquid state at room temperature, while those with only saturated acids do not (15). This characteristic may be of considerable importance for their function in the biological system, since the liquidity determines the particular phase which can exist at any given temperature and is in turn related to permeability processes.

Hydra fed on brine shrimp nauplii display a fatty acid pattern similar to that of the nauplii but cold-adapted *Hydra* have a higher concentration of the C_{20} and C_{22} unsaturated fatty acids and smaller amounts of the less-highly unsaturated C_{18} acids than do the 20C animals. A similar increase in docosahexaenoic and other unsaturated acids has been noted in the brain lipids of goldfish exposed to lowered temperatures while maintained on a constant diet (16). Such regulations of unsaturation in homeotherms also has long been known (17). Johnston and Roots (16) suggest that the process of temperature acclimation involves the ability to control the degree of unsaturation of cellular lipids, thereby maintaining the specific liquid-crystalline state of the cell membranes. From our results, it would appear that the specific type of acids in the lipids, not just the total amount of unsaturates, may be important in this control.

The results of this study support the view that the higher members of aquatic food chains maintain a degree of control over the composition of their fatty acids, and particularly of the polyunsaturates, but that the basic pattern originates in the primary members of the chain, the phytoplankton, and is carried throughout the chain. Fatty acids of phytoplankton are known to be considerably altered by such factors as the availability of nitrate, the age of the culture and the temperature (3,18), thus allowing a great variability in the acids of any given food chain. The need for control of phospholipid liquidity could therefore be the basic cause of the higher percentage of polyunsaturated fatty acids in the lipids of animals from the colder marine waters. Neutral lipids may not be primarily affected in this way, as suggested by the present investigation and also by Van Handel (19), who has reported that the synthesis of fatty acids into the triglycerides of mosquitos is unaffected by temperature. Such variation might occur secondarily, however, as a result of the turnover of fatty acids from endogenous phospholipids.

ACKNOWLEDGMENTS

Supported in part by grants No. AI-04953 and GM-06377, US Public Health Service.

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[Received January 28, 1966]

The Behavior of Glyceride-Fatty Acid Mixtures in Bile Salt Solution: Studies by Gel Filtration

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ABSTRACT

Bile salt lipid emulsions were prepared which simulated the emulsified oil-micellar phase system of the small intestinal content during fat digestion.

Application of such emulsions to gel columns prepared and eluted with 6 mM sodium taurodeoxycholate separated an emulsion phase and a micellar phase. The distribution of lipid solutes into the two phases under these conditions was measured.

Micellar dimensions were larger as lipid concentrations were increased. Inclusion of multiple lipid classes resulted in larger micellar particles.

Monoglyceride and fatty acids were eluted completely in the micellar phase under these conditions. Minimal measurable amounts of triolein were recovered in micellar solution. This was confirmed by extraction, chromatographic separation and quantitative analysis. As diolein concentration was increased, less was recovered in the micellar phase. When monoglyceride was added, more diolein entered the micellar phase. Addition of triglyceride enhanced the distribution of diolein into the emulsion phase.

INTRODUCTION

LIPIDS IN INTESTINAL content during fat digestion coexist in an oil-in-water emulsion phase, and a micellar phase in equilibrium (1). Micelles are aggregates of amphipathic molecules in equilibrium with dispersed single molecules in solution. Micelles form spontaneously in some solutions above a certain concentration, the critical micellar concentration (CMC). The size and shape of micelles vary with temperature and concentration and depend in part on the carbon chain length (2). An emulsion is an organic nonequilibrium dispersion of particles. Particle diameters usually range from 0.1 to 10 μ, in diameter. Emulsification can be produced by mechanical methods, and emulsions can be stabilized by addition of a micellar colloid. The intestinal emulsion contains diglycerides and triglycerides. The end products of digestive lipolysis, fatty acids and monoglycerides, are highly soluble in the micellar phase and are absorbed readily. The surface-active conjugated bile salts are primarily responsible for spontaneous micellar aggregation. The distribution of lipids into micelle and emulsion might determine the selectivity of fat absorption (3).

Gel filtration separates particles according to size. Large emulsion particles are "excluded" from the gel. Such samples pass through rapidly in the elution liquid in column elution experiments. With Sephadex G 100 gel, prepared and eluted with bile salt solution, samples of lipid micelles behave like macromolecules of corresponding "molecular weight" of 40,000, partly "included" in the gel and eluted later (4).

Bile salt lipid emulsions can be prepared to simulate the fat content of the small intestine during digestion. Elution volumes obtained after gel filtration of such mixtures of glycerides, fatty acid and bile salt will provide data concerning micellar dimensions. By gel filtration of samples incorporating radioactive lipid solutes, the distribution of labeled solutes into eluents containing particles of different sizes can also be determined.

MATERIALS AND METHODS

Sephadex G-100 (TO 5218) obtained from Pharmacia was the filtering gel. Sodium taurodeoxycholate (NaTDC) was synthesized and was at least 98% homogeneous as assessed by thin-layer chromatography (TLC) (5). Triolein and oleic acid were obtained from Merck & Co. Triglyceride and fatty acid homogeneity was assessed by TLC as better than 98%. Homologue purity was examined by gas-liquid chromatography (GLC) and was better than 99% for oleic acid; triolein contained 81% oleic acid, 13% palmitoleic acid and small amounts of 14:1 and 18:2 fatty acids. 1,3-Diolein was obtained from L. W. Beck, Procter & Gamble Co. Its purity was greater than 99% by TLC and GLC. 1-Monoolein was obtained from Distillation Products Industries, purified by TLC; the fatty acid composition was 58% oleic acid, 17% palmitoleic acid, and about 5% each of 14:0, 14:1, 16:0, 18:0 and

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18:2 fatty acids. A commercial intravenous fat emulsion, Intralipid (Vitrum), containing per ml 220 mg soybean oil and 12 mg egg lecithin, was used for the preparation of mixed triglyceride emulsions incorporating other glycerides or fatty acid.

(1-^{14}C) 1-Monoolein, (9,10-$^{3}H_2$) 1,2-diolein and (1-^{14}C) triolein were synthesized as described elsewhere (6,7). The radioactive label was in the fatty acid portion of the molecule. (1-^{14}C) oleic acid, (9,10-$^{3}H_2$) oleic acid and (2-^{3}H) glycerol were obtained from The Radiochemical Centre, Amersham, England. Radiohomogeneity was verified by TLC with purification when indicated by preparative TLC. Purity was better than 98%.

Sodium taurodeoxycholate has a CMC for monoolein of 0.6 mM, in contrast to the higher CMC's of chenodeoxycholate (0.8 mM) and cholate (4.2 mM) conjugates. The CMC of a solution of mixed bile salts of composition similar to that of human intestinal content, is 1.4 mM (8). Micellar solutions and emulsions prepared from Intralipid in bile salt-NaCl solution had a pH of 6.0. The triolein emulsions prepared in the laboratory with phosphate buffer were at pH 7. The pH of intestinal content is about 6. Hofmann's previous experiments (8) indicated that monoolein solubility in sodium taurodeoxycholate was identical from pH 5.5 to 7.0. Gel filtration studies of oleic acid (4) yielded the same elution volumes despite variation of the pH of the solution from 5 to 12. Emulsions prepared with Intralipid contained lecithin (about 2.5 μmoles per ml) which also enters the bile salt micellar phase and may enhance the solubility of other lipids. 1-Monoolein and 1,3 diolein, lipids not normally occurring in intestinal content, have micellar dispersions similar to those of 2-monoolein and 1,2 diolein (3,8) normally found in intestinal content during fat digestion by pancreatic lipase.

Micellar solutions were prepared in glass ampoules. The lipids were added in organic solvent. The specimens included one solute labeled with either (^{14}C) or (^{3}H), or two solutes, each labeled with one isotope. After evaporation of solvent, 3 ml 6 mM NaTDC in 0.15 M NaCl were added, the ampoules sealed and shaken at 37C for at least 12 hr. To indicate the void volume, a few crystals of Blue Dextran 500 (op. no. 8459B, Pharmacia, Uppsala) were added to the ampoules before sealing.

Emulsions were prepared in ampoules. The lipids were added in organic solvent and the latter evaporated. Appropriate amounts of Intralipid and NaTDC were added to give the final desired concentration of triglyceride in 6 mM bile salt in 0.15 M NaCl. The ampoules were then treated as described above.

Emulsions were also prepared by combining labeled triolein, diolein, monoolein and oleic acid in organic solvent. After the solvent was evaporated and 5 to 10 ml 6 mM NaTDC in 0.15 M phosphate buffer (pH 7) added, the mixture was emulsified by ultrasonication for one minute with a Branson Sonifier. Such emulsions were stable for 14 days at room temperature.

The gel was packed in columns with a diameter of 16 mm and a total gel volume of 60 ml. The gel was equilibrated with bile salt solution before packing and washed with the same solution. Columns were run in a thermostated room at 37C. One milliliter of the sample micellar solution or emulsion was added to the top of the column. This solution was made up with the same bile salt concentration and salt composition as that used for column equilibration. The column was eluted with 60 ml of bile salt solution of the same concentration as that used for gel equilibration. Flow rates between 6 and 12 ml per hour were used. Fractions close to 1 ml were collected using a drop counter and weighed in tared test tubes. Since the drops of the emulsion phase failed to actuate the drop counter, drops from these tubes were counted visually. The concentration of Blue Dextran was measured in a spectrophotometer at 600 mμ. Radioactivity was assayed in a liquid scintillation spectrometer using dioxane-naphthalene with PPO and POPOP as scintillators (9). On the average, 96% of the activity applied to the column was eluted (range 86-110%).

The fraction of the volume of the gel available for the substance applied, K_{av}, was calculated according to Laurent and Killander (10) as follows:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

V_o, the void volume of the column, was obtained from the peak elution volume of Blue Dextran or from the peak elution volume of radioactivity in the turbid fractions. V_e, the effluent volume, was the volume of maximum radioactivity which was obtained by trisangulation of the radioactivity values plotted vs. elution volume. V_t, the total volume of the gel bed, was 60 ml. In all experiments V_t, included the dry volume of the dextran. Radioactivity (counts/min) or optical density of each fraction collected were plotted as the ordinates. The serial number of 1 ml fractions collected were the abscissas. The points plotted were connected

by triangulation. The peak volumes, given by the intersecting lines, of radioactivity or optical density were corrected for the actual volumes collected as determined by weights. As V_e approaches V_t, K_{av} increases and approaches 1 and particle size is smaller. As V_e approaches V_o, K_{av} decreases and approaches 0, indicating larger particle size.

The proportion of radioactivity eluted in each phase was calculated. The total emulsion phase radioactivity was usually recovered in 4–5 ml volume and the micellar phase radioactivity in 8–10 ml volume.

Other experiments reported earlier (4) indicated that duplicate and triplicate experiments with the same sample mixtures applied to columns gave values for K_{av} that varied by 0.02 or less; therefore, each column elution experiment was usually performed once. (With 3 mM monoolein in bile salt, K_{av} values in 3 experiments were 0.36, 0.35, 0.36.)

In one experiment, the partition of (^{3}H) diolein between emulsion and micellar phases was estimated by ultracentrifugation. A bile salt-lipid emulsion was centrifuged in a Spinco Model L preparative ultracentrifuge for 1 hr at 40,000 rpm. The rotor and solutions were equilibrated at 37C at the start and the refrigeration of the rotor housing was not used during the run. Under these conditions the emulsion "floated" to the top, whereas the micelles were evenly distributed in the centrifuge tube. The volumes of the top (emulsion + micelles) and bottom portions were measured and radioactivity determined in 1 ml portions. The ratio of micellar diolein to emulsion diglyceride was 0.45.

Radioactivity eluted from the Sephadex column was recovered in the same chemical lipid class as that applied. This was verified in two column elution experiments carried out with the same emulsion containing (^{3}H)-triolein and (^{14}C)-oleic acid. One column was treated as usual. With the second column, the fractions containing the emulsion and micellar phases were pooled separately. The pooled micellar phase, the emulsion phase and a portion of the emulsion sample applied were individually extracted with 3 times their volume of diethyl ether-heptane-ethanol (1:1:1 by vol). Ether-heptane phases were combined after re-extraction. After solvent evaporation, the lipids were dissolved in heptane and separated by thin-layer silicic acid chromatography (9). After identification, mono-, di- and triglycerides, and fatty acids were scraped off from one plate into counting vessels. Of the radioactivity applied to the plate, 92.8% was recovered in the appropriate lipid class (^{3}H as triglyceride, ^{14}C as fatty acid). Mono-, di- and triglycerides were each scraped off duplicate plates. The lipids were eluted with 5 ml chloroform after the scrapings were placed in small chromatography columns. Ester bonds were determined by the hydroxamic acid reaction (11). This permitted calculation of lipid distribution into micellar and emulsion fractions by quantitative methods for comparison with radioactivity measurements.

RESULTS

The present experiments (Fig. 1) investigated the following situations:

1) Lipids were incorporated into micellar solutions (diagram a, Fig. 1). Solute concentrations and composition were varied. The dimensions of the resultant bile salt lipid micelles were characterized.

2) Lipids were incorporated in quantities exceeding that which could undergo micellar dispersion. Excess monoglyceride yielded a liquid crystalline phase; excess diolein was emulsified. These phases were in equilibrium with a micellar phase.

3) Lipids were dispersed in buffer without bile salt, yielding an emulsion (diagram b, Fig. 1).

4) Lipids were incorporated in bile salt-triglyceride emulsions, yielding an emulsified oil phase in equilibrium with a micellar phase (diagram c, Fig. 1). The relative distribution of lipid solutes into the two phases was measured.

The determinations of K_{av} in column elution experiments are summarized in Table I and Figs. 2 and 3. Three mM solutions of oleic acid or monoolein, and 0.1 mM diolein solutions in 6 mM NaTDC were clear and were eluted in one peak from Sephadex columns. Oleic acid bile salt micelles had the highest K_{av}. Monoolein bile salt micelles were intermediate. Diolein bile salt micelles had the lowest K_{av}.

Increasing the solute concentration of oleic acid, monoolein or diolein in 6 mM NaTDC resulted in variable lowering of micellar K_{av} (Table I, Figs. 2, 3). Despite the turbidity of 9 mM solutions of oleic acid and 30 mM monoolein (the excess forming liquid crystals or emulsions), these lipids were still eluted in one peak of clear solution (volume 8–10 ml). "Solutions" of 2 mM and 6 mM diolein applied to the column were turbid (the excess forming an emulsion) and were eluted in two or more peaks, one totally excluded from the gel and turbid. The lowering of K_{av} was more pro-

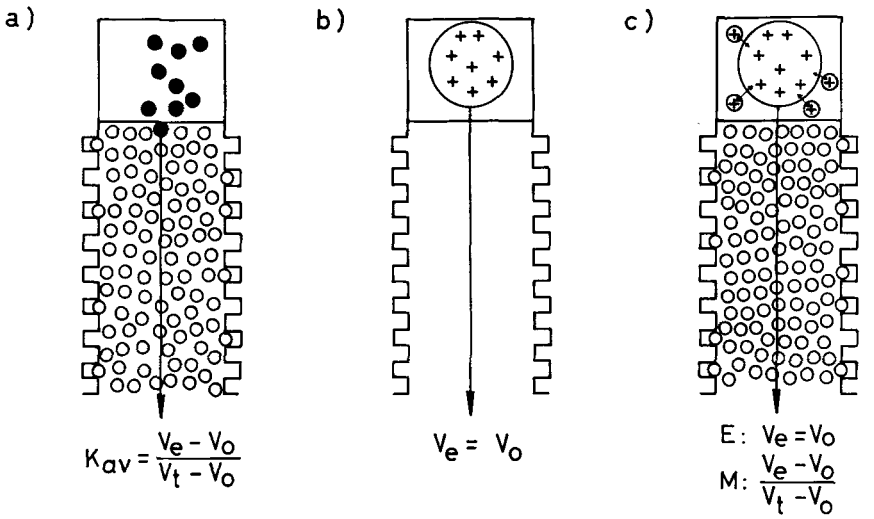

FIG. 1. Design of column elution experiments. The specimens contain lipid labeled with (^{14}C) and/or (^{3}H). Applications to the column are depicted in the boxes at the top of each diagram. The columns are drawn as porous structures containing the equilibration solution. The particles depicted include: ⊕-radioactive-labeled emulsion, diameter 5000Å; ○-pure bile salt micelle, diameter 48Å; ●-bile salt-lipid micelle, radioactive-labeled, K_{av} 0.43–0.33, diameter 48–54Å; ⊕, bile salt-lipid micelle, radioactive-labeled, in equilibrium with emulsion, K_{av} 0.33–0.24, diameter 54–66Å. V_o, V_e, V_t and K_{av} defined in Materials and Methods. E, emulsion phase; M, micellar phase. In *a*, a micellar solution of lipid and bile salt is applied to a column equilibrated with the same concentration bile salt. *b*, a lipid emulsion in buffer is applied to a column equilibrated with buffer. *c*, a lipid emulsion in bile salt in equilibrium with a micellar phase, is applied to a column equilibrated with the same concentration bile salt solution.

nounced with solute increments at low concentrations, and tapered off at higher concentrations.

When a second lipid solute was added to bile salt lipid micelles K_{av} was lower in 12 of 14 experiments (Table I). Addition of monoolein did not change K_{av}. Solution of two additional lipids into bile salt-lipid micelles gave still lower K_{av} values than addition of either solute alone. The lowest K_{av} value was observed when three solutes were included in the bile salt-lipid micelles. As the total amount of lipid incorporated into the micelle increased, the K_{av} decreased (Fig. 3). The approximate amount of lipid in the micelle was derived by assuming that 98–100% of the oleic acid or monoolein applied was recovered in micellar solution, calculating the diolein distribution from the experimental data in Table I and assuming a value of 0.4 μEq for micellar triolein.

The corresponding molecular radii, assuming the particles to be spherical, have been interpolated for the K_{av} values obtained by Laurent and Killander (10) for various proteins using Sephadex G-100. The radii transposed from their data to Figure 3 ranged from 24Å for the "dilute" bile salt micelle containing one lipid solute to 33Å for the bile salt-lipid micelle containing three lipid solutes. It was assumed, without calibration, that the elution properties of proteins would be similar in columns prepared in either laboratory using Sephadex G-100.

In one experiment, 10.8 mM labeled oleic acid was incorporated into an emulsion containing 10.8 mM triolein, 5.4 mM diolein and 5.4 mM monoolein in 0.15 M phosphate buffer brought to pH 9 by addition of 6N NaOH. NaTDC was not included in this emulsion. After gel filtration all the radioactivity was recovered in the emulsion phase.

Distribution experiments are summarized in Figure 4. Oleic acid or monoolein in bile salt solution in various mixtures with other lipids were always recovered in the micellar peak (99%). Results were identical, when measured by recovery of activity in individual fractions eluted from the column, or by pooling the

TABLE I

Column Elution Experiments[a]

Applied to Column			Labeled lipid recovered in micellar solution, μmoles	K_{av}
Labeled lipid	Concn, mM	Other lipids, concn mM		
Diolein	0.1	.....	0.1	0.42
"	1.0	TG, 32	0.4	0.32
"	1.0	TG, 32 MO, 3	0.3	0.28
"	2.0	.....	1.6	0.29
"	3.0	MO, 3	2.7	0.33
"	3.0	OA, 9	2.3	0.27
"	3.0	MO, 3 OA, 9	2.5	0.24
"	3.0	MO, 3 OA, 9 TG, 15	1.0	0.25
"	6.0	.....	3.1	0.28
Oleic acid	3.0	.....	3.0	0.42
"	3.0	TG, 32	2.9	0.33
"	9.0	.....	9.0	0.34
"	9.0	MO, 3	9.0	0.34
"	9.0	DO, 3	9.0	0.31
"	9.0	TG, 32	8.8	0.27
"	9.0	MO, 3 DO, 3	9.0	0.27
"	9.0	MO, 3 DO, 3 TG, 15	8.9	0.25
Monoolein	3.0	.....	3.0	0.36
"	3.0	TG, 32	2.9	0.32
"	3.0	DO, 1 TG, 32	3.0	0.34
"	30.0	.....	30.0	0.31
Triolein	10.8	MO, 5.4 OA, 10.8 DO, 5.4	0.41	0.26
"	21.6	MO, 5.4 OA, 10.8 DO, 5.4	0.37	.....

TG = triglyceride, MO = monolein, OA = oleic acid, DO = diolein.

[a] All solutions were prepared in 6 mM NaTDC. 1 ml solution or emulsion was applied to columns of Sephadex G-100, total volume 60 ml. Columns were prepared and eluted with 6 mM NaTDC.

eluted fractions, extracting the lipids and determining activity after class separation by TLC.

As the concentration of diolein in NaTDC was increased, the solution applied became progressively more turbid. The portion recovered in the micellar phase decreased to about 55% of the total activity eluted (ratio micellar diolein:emulsified diolein, 1.2). Addition of oleic acid (alone or with monoolein) to the diolein-bile salt mixture did not alter the distribution of diolein into the micellar phase. Addition of monoolein alone enhanced the recovery of diolein in micellar dispersion. Addition of triglyceride markedly reduced the recovery of diolein in the micellar phase, so that diolein distribution favored the emulsion. Inclusion of fatty acid or monoglyceride in the diolein-triolein-bile salt emulsion produced no change in the distribution of diolein.

A small, but measurable quantity of labeled triolein was persistently recovered in the micellar phase (Table I). It was verified that the radioactivity was eluted as triglyceride by extracting the lipids from the pooled micellar phase, and determining the radioactivity in triglyceride isolated by TLC. Ninety-six per cent of the triglyceride activity in the emulsion applied to the column was recovered in triglyceride of the pooled emulsion phase plus triglyceride of the pooled micellar phase. Chemical analysis yielded a small (although higher) value for triglyceride in the micellar phase. From 0.2 to 0.4 μmoles total of triolein were

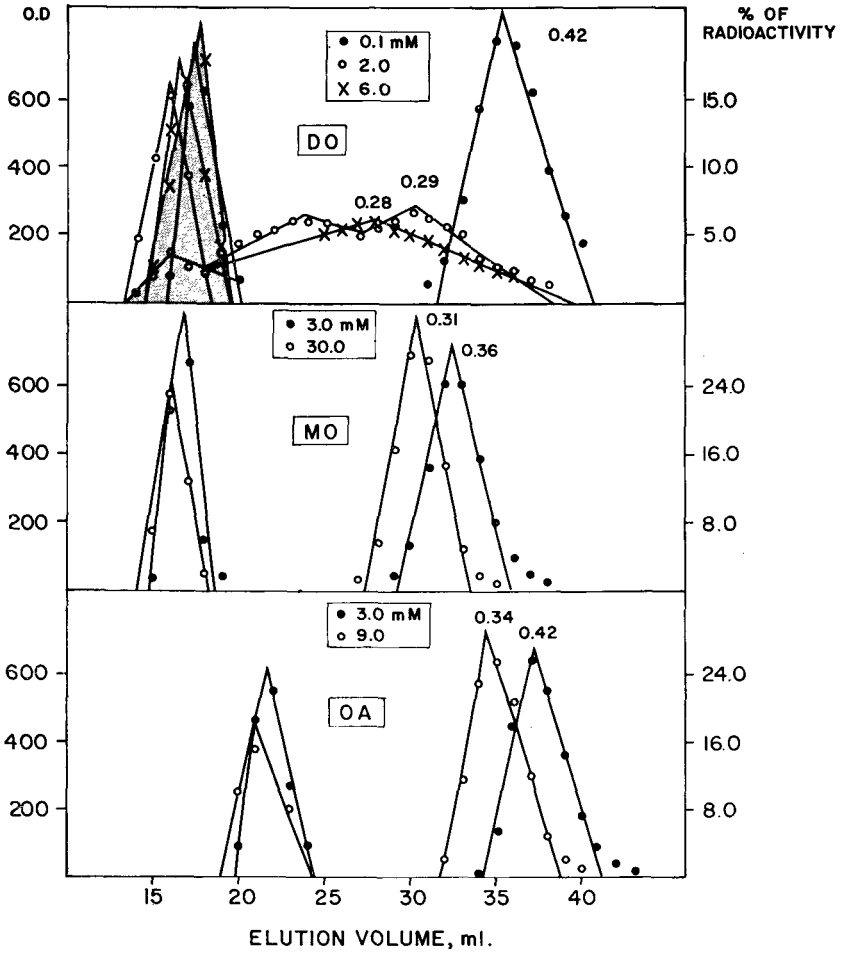

FIG. 2. Elution curves of mixed micelles of 6 mM NaTDC and varying concentrations of (^{14}C) 1-monoolein, (^{3}H) 1,3-diolein or (^{14}C) oleic acid with Sephadex G-100. Sixty milliliter columns were equilibrated and eluted with 6mM NaTDC in 0.15M NaCl. A 1-ml "solution" of lipid in concentrations varying from 0.1 to 30 mM was applied to the column. The elution volumes (number of 1 ml fractions collected) are the abscissas. Radioactivity per fraction, expressed as per cent of total activity recovered, and optical density of blue Dextran at 600 mμ are the ordinates. The void volume is indicated by the peak at the left. Shaded curves at left in top graph represent radioactivity. The numbers to the right of the peaks are the K_{av}. DO, diolein; MO, monoolein; OA, oleic acid.

recovered in micellar solution under the experimental conditions. When the same triolein-labeled emulsion was prepared in buffer (pH 9) without bile salt, and eluted from gel columns with phosphate buffer (pH 7) all the radioactivity was recovered in the emulsion phase.

In one experiment, 48.6 μmoles (^{3}H) glycerol was included in the glycerides-fatty acid-bile salt emulsion. All the activity was eluted from the column in one peak with K_{av} 0.85. This indicated molecular solution of glycerol in the bile salt water phase, with V_e approximating V_t.

DISCUSSION

Bile salt solutions form micelles at concentrations from about 0.6 to 2 mM for NaTDC (8). The bile salt micelle is negatively charged. Polar water molecules are bound through dipole attraction and hydrogen bonds, resulting in a protecting layer of tightly bound water in addition to counter-ions. Micelles of NaTDC, studied by ultracentrifugation, were found to have an anhydrous micellar weight of 11,900 (12). These data were obtained from sedimenta-

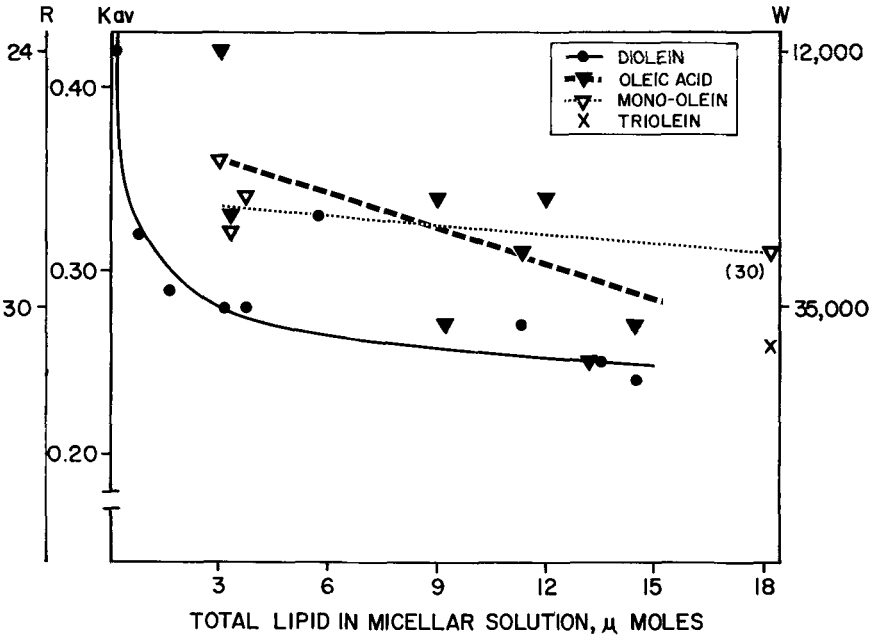

FIG. 3. K_{av} values of bile salt micelles of 6 mM NaTDC containing labeled lipid solutes (see Table I for composition) vs. the total lipid content of the micellar phase (see text). The lines were fitted visually. R, radii in Å of equivalent spheres corresponding to the K_{av} (derived from Laurent and Killander, 10); W, analogous molecular weight (derived from Whitaker, 16).

tion and diffusion experiments in 0.15 M phosphate buffer, by extrapolation to infinite dilution. The frictional ratio of 1.58 indicated a high degree of hydration, confirmed by data obtained in 0.15 M buffer. Measurements by light scattering gave values of 11,000 for NaTDC micelles (13). Gel filtration data gave a molecular radius of the bile salt micelle of about 20Å (see ref. 12).

The addition of an amphiphilic substance like monoolein is thought to expand the bile salt micelle. By increasing the effective liquid hydrocarbon "interior," solubilization of relatively nonpolar hydrocarbons is enhanced. A change in micellar structure as well as a simple increase in size may occur when added hydrocarbon and amphiphile are present (8).

The behavior of glycerides and fatty acids in bile salt solution has recently been studied in some detail. At 37C, monoglycerides of low melting point showed a remarkably high solubility in bile salt solution forming mixed micelles in isotropic solutions (8, 14). Excess of low melting point monoglycerides formed a liquid crystalline phase. Di- and triglycerides showed a low solubility in bile salt solution. The excess was emulsified or crystalline, depending on the melting point. The behavior of long chain fatty acids in bile salt solution was complex, depending on pH. At pH values around 7, excess fatty acid was emulsified or crystalline depending on the melting point. If a mixture of glycerides and fatty acids was dispersed in a bile salt solution at a concentration above the CMC, an emulsified oil phase was believed to coexist with a micellar phase (15).

The micelle behaves like a macromolecule on gel filtration when columns are equilibrated and eluted at bile salt concentrations at or above the CMC (4). In the column elution experiments, designed to measure the size of micelles, it was assumed that the labeled solutes were not in molecular solution but existed only in the micellar and/or emulsion phases. It was also assumed that the micelles in any one experiment had the same dimensions, and that the maximum dimension indicated by K_{av} represented one population of micelles (Figs. 1a, 1c). The values of K_{av} represent dilution of the micellar solute concentration 8 to 10-fold since the sample volume was 1 ml compared to the eluted micellar phase volume of 8 to 10 ml. These conditions were constant in all experiments.

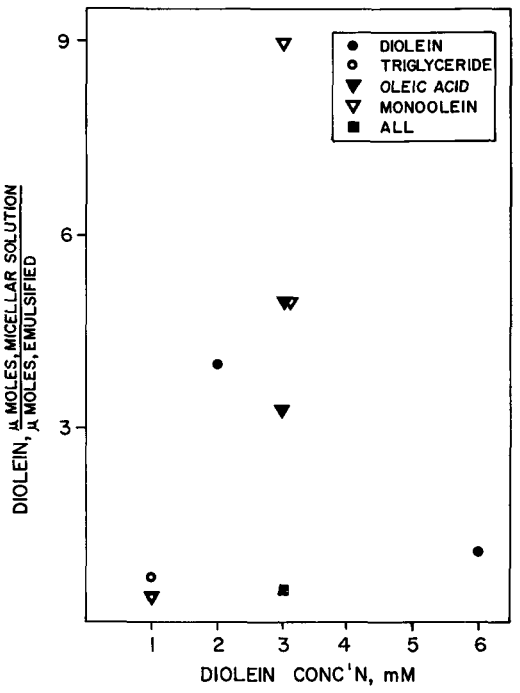

FIG. 4. Distribution of diolein into micellar and emulsified oil phases. The amount of diolein in each phase was calculated from the portion of radioactivity recovered, assuming constant specific activity. Values above 1 indicate distribution primarily in the micellar phase. Each point in the diagram represents one of the eight experiments with diolein (see Table I) in 1.0 mM concentration or higher. The open circles, open and closed triangles indicate that one or more of the appropriate lipid class (triglyceride, monoglyceride, fatty acid) was also included in the sample.

By plotting the K_{av} of various proteins vs. the radius of an equivalent sphere (10), a radius of 24Å, corresponding to a K_{av} of 0.43 in Sephadex G-100, can be calculated for pure micelles of NaTDC. This agrees exactly with the radius calculated by Laurent and Persson (12) from the diffusion coefficient of NaTDC. Similar K_{av} values were obtained with dilute diolein and with 3 mM oleic acid in 6 mM NaTDC. K_{av} was lower with monoolein, corresponding to an increase in molecular radius of 3Å. The increase in elution volumes to yield lower K_{av} values corresponds to a doubling of the analogous anhydrous "molecular weight" (Fig. 3) as determined by Whitaker (16) in gel filtration experiments of proteins of known molecular weight. Micellar dispersion of additional lipids resulted in further increase in the radius and analogous molecular weight, indicating that such micelles were bigger. The maximum size observed of mixed micelles in 6 mM NaTDC corresponded to a radius of 33Å. These values are somewhat higher than those obtained in earlier column elution experiments in this laboratory using different batches of Sephadex G-100 (see ref. 4). The increase in "size" and "weight" of micelles may be related to the steric configuration and polarity of the dissolved lipids as well as to the actual molecular weight and carbon chain length of the molecules. Changes in hydration of the micelle may also occur. The micellar sizes interpolated from experimental values of K_{av} appear independent of the "pore size" of the gel. Other experiments gave identical micellar radii for cholesterol bile salt lipid micelles when a cholesterol lipid bile salt emulsion was applied to 60 ml gel columns of Sephadex G-100 and G-200 (17).

Interpretation of data derived from column elution experiments was based on the assumption that labeled solutes were distributed into two phases. Micelles of radius less than 55Å would be detected by partial inclusion in the gel. Any particle with a larger radius (weight greater than 100,000) would be eluted with the void volume and ascribed to the emulsion (excluded) phase. Relative distributions of lipid solutes into micellar and emulsion phases appear independent of the gel column total volume. Similar distribution values for cholesterol were obtained using Sephadex G-100 columns of 30 ml and 60 ml total volume with sample size 1 ml (17).

Acker's (18) experiments demonstrated that equilibrium distribution ratios closely paralleled column distribution coefficients with Sephadex G-100. His theoretical calculations are applicable only when all of the molecular species under investigation is present in one phase. The present experiments are somewhat analogous to those of Herries, Bishop and Richards (19) who studied partitioning of solutes between a totally excluded micellar and an aqueous detergent phase using Sephadex G-25. The systems would be similar if, in the present experiments, a bile salt-lipid emulsion rather than a bile salt solution had been used for equilibration and elution.

The recovery of oleic acid and monoolein included in bile salt emulsions was almost exclusively in the micellar phase with all the concentrations used. Conversely triolein was recovered only slightly in the micellar phase, although it was clear that some triolein could enter the bile salt micelle. In the absence of triglyceride, diolein was readily dissolved by bile salt micelles. The presence of triglyceride

markedly altered the distribution of diglyceride in favor of the emulsion phase. In contrast, the addition of triglyceride had no effect on the micellar solubility of fatty acid and monoglyceride.

The recovery of diolein and triolein in micellar solution indicates that these lipids may reach the intestinal mucosa in the form of small aggregates and suggests the possibility that these lipids may be able to penetrate the intestinal mucosa in this form. Experimental data in support of this hypothesis have been reported (20). The effect of triolein to enhance diolein recovery in the emulsion phase may aid in the retention of the diglyceride substrate in the oil phase where pancreatic lipase is most active. This enzyme induces hydrolysis of primary ester bonds at a rapid rate (7). The 2-monoglyceride and fatty acid products of lipolysis are solubilized into the micellar phase (1) and taken up by the intestinal mucosa (20).

Gel filtration of lipid micellar solutions and emulsions provided information about the dimensions of bile salt lipid micelles. The recovery as micellar and emulsified oil phases of lipid incorporated into bile salt emulsions, simulating small intestinal content may be indications of their relative behavior in vivo. Data provided by this model system in vitro may aid in the interpretation of studies of fat digestion and absorption.

ACKNOWLEDGMENTS

A number of compounds used in this investigation synthesized by L. Krabisch. Technical assistance by Mrs. G. Björklund and Miss U. Hansson. This work was carried out during the tenure by one of us (E.B.F.) of a United States Public Health Service Special Fellowship. Additional financial support was provided by United States Public Health Grant No. H-5302, The Swedish Medical Research Council and the "Svenska Margarinindustrins Förening för Näringsfysiologisk Forskning."

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[Received October 27, 1965]

The Total Synthesis of Dilinoleoylphosphatidylserine and Its Activity in Blood Clotting Systems[1]

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ABSTRACT

A total synthesis of DL-phosphatidyl-(dilinoleoyl)-L-serine was achieved by the acylation of the barium salt of the phthalimidomethyl ester of glycerophosphoryl-N-anisyloxycarbonyl-L-serine. The dilinoleoyl intermediate was treated with hydrazine to remove the phthalimidomethyl group and with hydrogen chloride to remove the anisyloxycarbonyl protecting group. The resulting phosphatidylserine was purified by Rouser's methods, solubilized, and tested for biological activity in the antithromboplastin, recalcification, and Hicks-Pitney tests. It was found to have about the same anticoagulant activity as beef brain phosphatidylserine and hence was more active than the less unsaturated phosphatidylserine synthesized earlier.

INTRODUCTION

THE TOTAL SYNTHESIS of unsaturated phosphatides increases in difficulty as the number of double bonds increases because of the elaborate precautions necessary to prevent oxidation. The recent innovation of Baer, in which the barium salt of a glycerophosphorylphthalimidoethanol was acylated (1,2), reduces the number of steps in phosphatide synthesis in which the unsaturated fatty acid is available for oxidation and hence has important advantages. In addition, highly unsaturated diglycerides are difficult to make (3,4), but dilinolein would be required for a synthesis by our earlier technique (5,6). Accordingly, we decided to explore the acylation of the barium salt of a protected glycerophosphorylserine and chose the phthalimidomethyl ester of anisyloxycarbonyl-L-serine for conversion to the glycerophosphoryl derivative. The protected serine combined with phosphorus oxychloride and acetone glycerol readily. The resulting product was stripped of its acetone protecting group and the water soluble product was converted to a barium salt. This salt was acylated with linoleoyl chloride and the protecting groups were stripped from the product to give phosphatidyl(dilinoleoyl)-L-serine (PS). This substance was tested in several blood clotting systems as described below.

EXPERIMENTAL

Materials and Methods

Linoleic acid of 99.5% purity by gas-liquid chromatography (GLC) was purchased from Lachat Chemicals, Inc., Chicago Heights, Illinois; Amberlite XAD-2 was a gift of the Rohm and Haas Co., Philadelphia, Pa. All dry solvents were dried over molecular sieve 4A of the Linde Products Division, Union Carbide, Inc. Pyridine and dimethylformamide were allowed to stand over one third of their volume of the sieve for weeks. Chloroform was dried by shaking with the sieve for 1 hr on a shaking machine. It was not stored because of its tendency to form phosgene when dry. Anisylazidoformate was made from phenylthiolchloroformate according to the general methods of Carpino (7). In the preparation of anisyl *S*-phenylthiolformate, it is not necessary to reflux the reaction mixture. After the addition of the phenylthiolchloroformate to the solution of anisyl alcohol in pyridine chloroform, the mixture is stirred at room temperature for several hours, left overnight, and then worked up. Anisyl *S*-phenylthiolformate is a crystalline compound, from methanol, mp 50C. (*Anal.* Calcd. for $C_{15}H_{14}O_3S$: C, 65.67; H, 5.14; S, 11.70. Found: C, 66.10; H, 5.35; S, 11.71). Anisyl carbazate can be prepared from the phenylthiol ester by treatment with hydrazine in the usual manner (7).

The other methods and materials were similar to those used in our earlier work (6) including the paper chromatography of Marinetti (8) and the DEAE cellulose (acetate) column chromatography of Rouser and co-workers (9,10). All operations involving unsaturated linoleate derivatives were carried out under oxygen-free nitrogen. Melting points were determined with a Kofler hot bench and infrared (IR) spectra were determined with an Infracord spectrophotometer of Perkin-Elmer, Inc.

Phthalimidomethyl Ester of Anisyloxycarbonyl-L-serine. Anisyloxycarbonyl-L-serine (19.8 g) prepared according to Weygand and Hunger

[1] An abstract of the material in this paper appeared in Fed. Proc. *24*, No. 727 (1965).

(11) was dissolved in 200 ml of dry dimethylformamide and treated with equimolar quantities of dicyclohexylamine and phthalimidomethyl chloride (12). After standing overnight at 40C, the mixture was diluted with water to precipitate an oil which soon crystallized. The collected crystals were washed well to remove dicyclohexylammonium chloride, and recrystallized from ethanol, mp 124C, yield 20.8 g (66%), $a_D^{28} = +1.03$ (chloroform).

Anal. Calcd. for $C_{21}H_{20}N_2O_8$ (428.39): C, 58.87; H, 4.71; N, 6.54. Found: C, 59.25; H, 4.92; N, 6.61.

*DL-α-glycerophosphoryl-anisyloxycarbonyl-*L*-serine Phthalimidomethyl Ester.* To a vigorously stirred solution of 5.86 ml of phosphorus oxychloride in 50 ml of dry chloroform at −10C was added a solution of 8.4 g of acetone glycerol and 9.0 ml of quinoline in 250 ml of chloroform following the conditions described by Baer, Suzuki and Blackwell (1) in an analogous preparation from phthalimidoethanol. The conditions of Baer were also followed in the reaction of the product with a mixture of 27.4 g of anisyloxycarbonyl-L-serine phthalimidomethyl ester in 300 ml of chloroform and 20.6 ml of pyridine. The product was treated in a manner similar to that described by the earlier authors except that the product here could not be dissolved in benzene. The dried oil was deacetonated with Amberlite IR 120 (H^+) essentially as described (1). However, the lead salt purification must be omitted here because of the greater sensitivity of the serine derivative to hydrogen sulfide. The water-soluble product was converted directly to the barium salt, which was obtained as a colorless glass in a yield of 20 g. The IR spectrum (KBr disc) showed the characteristic carbonyl bands of the serine derivative together with the bands characteristic of phosphate esters. A better sample for analysis was obtained by chromatography of an aliquot of the aqueous solution from the Amberlite IR 120 column on a column of Amberlite XAD-2 resin. The material was applied to the column and eluted with water which removed acidic material. When the eluate became neutral, after a short wash with water, the organic acidic fraction was eluted with methanol, and the methanol solution was evaporated to give a product which was converted to barium salt with barium carbonate. The dried barium salt was analyzed.

Anal. Calcd. for $C_{48}H_{52}N_4O_{26}P_2Ba$: N, 4.31; P, 4.76. Found: N, 4.45; P, 5.20.

The product was examined by thin-layer chromatography (TLC) using silica gel H (E. Merck, Darmstadt) with chloroform-methanol-acetic acid-water (25:15:4:2) and appeared as a spot staining with the Pataki procedure (13) for carbobenzoxyamino acids and also with the Zinzadze stain (14) for phosphate.

DL-*Phosphatidyl(dilinoleoyl)-*L*-serine.* A portion of the crude barium salt (16 g) from the preceding preparation was acylated in dimethylformamide with linoleoyl chloride using the technique of Baer and Blackwell (2) except that the temperature was 37C instead of 60C. The crude oily product, after concentration of the ether extract, was dissolved in ethanol and centrifuged. The solution in ethanol (500 ml) was cooled to −15C and treated with 50 ml of 0.95 M hydrazine in ethanol prepared from 95% hydrazine (Distillation Products Industries, Inc.). The mixture was stored at 37C for 2 days. The pH was 7.5. Evaporation in vacuo gave an oil which was dissolved in 150 ml of dry chloroform. This solution was treated in portions in the next step in order to permit immediate column chromatography of the product. A portion of one third was cooled to 0C and dry hydrogen chloride was passed through for 5 min. The chloroform was then evaporated in vacuo. DEAE cellulose column chromatography (6,9,10) of this material yielded 980 mg of crude PS, which was purified further by chromatography on silicic acid as described previously (6) to give 580 mg of phosphatidylserine from one third of the hydrazinolysis product. The product showed only one spot when applied to silicic acid impregnated paper in amounts of 250 μg, using the solvent system diisobutylketone-acetic acid-water (40:25:5). The diisobutylketone was first freed of peroxides by distilling over triphenyl phosphine and contained 0.05% butylated hydroxytoluene as antioxidant (8,15). The synthetic PS also showed a single spot by TLC on silica gel H using the solvent recommended by Grisdale and Okany (16) as well as those recommended by Skipski and co-workers (17,18). It had the same mobility as natural phosphatidylserine and gave the same staining reactions. GLC of the fatty acid methyl esters obtained by methanolysis of the phosphatide showed 99.7% linoleic acid and 0.2% oleic acid. The oleic acid was an impurity in the linoleic acid used as starting material. The IR spectrum of the synthetic phosphatide (KBr disc) was also that of PS.

Anal. Calcd. for $C_{42}H_{74}NO_{10}P$: C, 64.3; H, 9.5; N, 1.79; P, 3.95. Found: C, 64.3; H, 9.7; N, 2.03; P, 4.07.

Activity of DL-*Phosphatidyl(dilinoleoyl)*-L-*serine in Clotting Tests.* The technique of these tests has been described elsewhere (6,19). The results of the antithromboplastin and recalcification tests are shown in Table I. Results from the Hicks-Pitney (20) test are presented in Table II. In each table, the corresponding data are given for pure beef brain PS. In the Hicks-Pitney test it was found that as little as 5 μg of the PS was able to overcome the potent acceleratory activity of the crude brain phosphatides of Bell and Alton (21). The activity of this mixture is shown for comparison in Table II. In all experiments, solubilization was effected with sodium desoxycholate exactly as described before (6,19). For reasons discussed below, the clotting tests were performed in a 0.05 M veronal buffer at pH 7.4 instead of the imidazole buffer used in our earlier work (19). The buffer was prepared with 0.85% sodium chloride solution, and dilutions were made with the buffered saline.

Similar experiments were carried out with solutions of PS solubilized with albumin (19,22). The clarification of solutions of PS by albumin is complete when PS is in the salt form (19). The acidic PS was converted to the sodium salt of PS according to the method of Abramson, Katzman and Gregor (23). After this treatment the synthetic PS was almost completely solubilized and had activity similar to the solutions in sodium desoxycholate.

Simple *suspensions* of the synthetic dilinoleoyl PS had weak to moderate activity in tests designed to detect acceleratory activity of phospholipid suspensions. Such data for other phosphatidylserines have been published elsewhere (6,19) and the data are essentially the same for this synthetic PS.

TABLE I

Anticoagulant Activities of Solubilized[a] Dilinoleoylphosphatidylserine and Beef Brain Phosphatidylserine in the Antithromboplastin and Recalcification Tests

Substance tested[a]	μg[b] Clotting test	Antithromboplastin[c] test clotting time (sec)	Recalcification test[d] clotting time (sec)
BB	100	185	.....
	50	86	.....
	25	50	1070
	5	34	770
	1	21	430
	0.5	20	275
LL	100	150	.....
	50	72	830
	25	50	750
	5	33	618
	1	24	240
	0.5	20	275
Sodium desoxycholate control	100	21	445
Buffered saline control		20	400

[a] Abbreviations: BB = Beef Brain Phosphatidylserine. LL = Dilinoleoylphosphatidylserine. Each preparation of phosphatidylserine was solubilized in a solution of sodium desoxycholate in 0.05 M Veronal buffer, pH 7.4 (19).

[b] This column shows the actual quantity in micrograms in the test mixture.

[c] 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 phosphatidylserine 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.

[d] The recalcification test is the same as the test described in (b) except that thromboplastin is omitted.

TABLE II

Anticoagulant Activities of Solubilized[a] Dilinoleoylphosphatidylserine and Beef Brain Phosphatidylserine in the Hicks-Pitney Test[b]

Incubation time (min)		4	6	8	10
Substance tested[a]	μg, Incubation mixture	Substrate clotting time, (sec)			
LL	5	>100	>100	>100	91
BB	5	>100	>100	>100	64
LL	2.5	>100	>100	62	24
BB	2.5	>100	>100	93	28
BA + LL	15 + 50	>100	>100	>100	100
BA + LL	15 + 5	>100	>100	90	30
Controls					
BA	15	45	9	9	9
BA + Desoxycholate	15 + 25	9	9	9	9
Desoxycholate	25	>100	85	33	26

[a] Abbreviations: LL = dilinoleoylphosphatidylserine. BB = Beef Brain Phosphatidylserine. BA = crude phosphatides of Bell and Alton (21). Each preparation of phosphatidylserine was solubilized in a solution of sodium desoxycholate in 0.05 M Veronal buffer, ph 7.4 (19).

[b] The modified Hicks-Pitney test is described in reference 19. Short clotting times (9 sec) indicate that the phosphatide preparation accelerates the generation of plasma thromboplastin. Long clotting times, especially when challenging an acceleratory preparation, indicate anticoagulant activity.

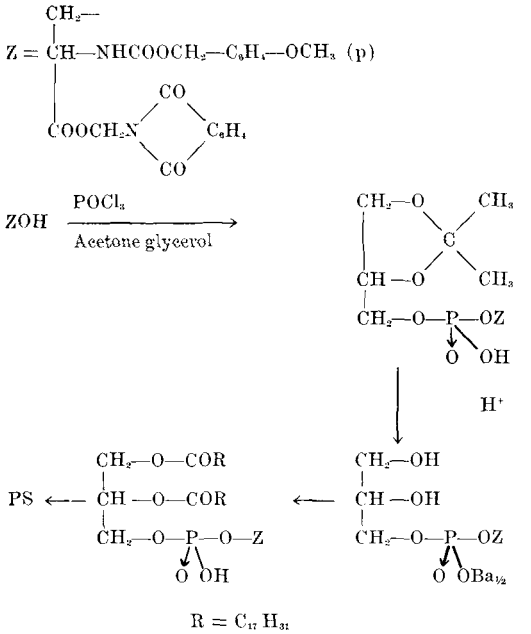
DISCUSSION

In the synthesis of unsaturated phosphatidylserines, two protecting groups must be employed to safeguard the amino and carboxyl of the serine, and these groups must be removable without destruction of the unsaturated glyceride. The phthalimidomethyl group serves well for the carboxyl (5,6).

We chose the anisyloxycarbonyl protecting group for the amino function after having discouraging results with various alternative groups in the acetone glycerol type of synthesis.

The anisyloxycarbonyl protecting group was introduced by Weygand and Hunger (11) who removed it with trifluoracetic acid, which is undesirable for unsaturated glycerides (24). We found it to be removable with dry hydrogen chloride under mild conditions. For phosphatide synthesis it appears to have advantages over the tert.-butyloxycarbonyl group in that the Carpino reagents used to introduce the group are crystalline and the serine derivative is easier to make. The phthalimidomethyl ester of anisyloxycarbonyl-L-serine was accordingly used in the phosphatide synthesis which proceeded as given in the accompanying flow-sheet (Fig. 1).

The synthetic phosphatidylserine was first tested in the antithromboplastin, recalcification, and Hicks-Pitney tests using the imidazole buffer of Mertz and Owen (25) commonly employed in blood clotting work. However, after the tests, the test preparations were examined by paper chromatography. The phosphatide was found to have undergone progessive hydrolysis apparent even after 1 hr, so that material being tested in a protracted Hicks-Pitney experiment became a mixture contaminated with such degradation products as lyso PS and phosphatidic acid. This effect was traced to the imidazole buffer, since the dilinoleoyl PS was not altered in a barbiturate buffer. Either buffer serves for testing beef brain PS or egg phosphatides which retain their integrity in imidazole as in other buffer systems for 12 hr.



It is evident that very unsaturated phosphatides like the one studied here cannot be expected to remain unaltered in aqueous dispersions. The effect of imidazole as a general base catalyst (26) has been extensively studied, but the literature does not offer a specific clue to the enhanced sensitivity of the dilinoleate as compared to natural phosphatides. Oxidative changes may play a part in this (27). It is possible that other phospholipids may be degraded in a manner similar to that of dilinoleoyl PS during the preparation of a suspension or while the aqueous dispersion is standing prior to a biological test. In this event, the preparation being studied would not have the assumed chemical composition, and its biological activity may reflect the activity of hydrolytic products, or the influence of these products on the activity of the phospholipid. Such an explanation might account for some surprising results in the literature such as the report that a combination of dilinoleoyllecithin and a phosphatidyl (linoleoyl palmitoyl) serine was completely inactive in a clot accelerating test while similar combination of less unsaturated phosphatidylserine and lecithin had strong activity (28).

Solubilization of unsaturated phosphatidylserines is easily effected in either sodium desoxycholate solution or albumin solution. Such solubilization is necessary for the demonstration of anticoagulant activity (6,19), and solubilization becomes more easily achieved with greater unsaturation in the phosphatide. In addition, solubilization in albumin requires PS as a salt rather than as a free acid (19,22). When properly solubilized, the highly unsaturated phosphatide of this paper has anticoagulant activity essentially similar to that of beef brain PS.

ACKNOWLEDGMENT

S. F. Herb and Francis E. Luddy (USDA Eastern Regional Research Laboratories) determined the fatty acid composition of the phosphatidylserine by GLC using the techniques described by them in the references quoted in our earlier papers (6,19).

Aided by a grant No. A-533 from the USPHS.

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[Received May 31, 1966]

A Micromethod for the Stereospecific Determination of Triglyceride Structure[1]

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ABSTRACT

Triglyceride lipase and diglyceride kinase can be used in a sensitive stereospecific analysis of the separate fatty acid compositions at the 1, 2 and 3 positions of a triglyceride.

Diglyceride kinase from *Escherichia coli* selectively catalyzes the phosphorylation of 1,2-diglycerides but not the 2,3-diglycerides.

The composition of the 3-position in rat liver triglycerides is clearly different from that at the 1-position.

INTRODUCTION

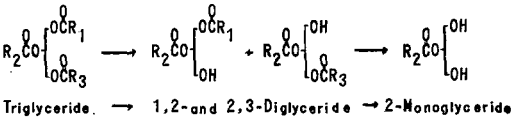
ANALYSES ON HUNDREDS of triglyceride mixtures led Hilditch to propose rules of "even distribution" and "random distribution" for vegetable and animal fats (2). These rules were designed to indicate the manner in which the various acids were combined to form the triglyceride mixtures. A considerable modification of these rules was needed after pancreatic lipase was used to produce 2-monoglycerides from triglycerides (3). The fatty acids were clearly recognized to be distributed between the primary and secondary positions in a non-random manner. This new reagent showed no preference for one of the primary ester groups over the other, but hydrolyzed both at the same rate (4). In interpreting the data obtained from studies using pancreatic lipase, Vander Wal (5) and Coleman and Fulton (6) assumed that fatty acids are distributed in the same proportion in the 1- and 3-positions. They suggested that the relative amounts of triglyceride species in a naturally occurring mixture could be predicted assuming a 1,3-random -2-random distribution of the fatty acids. Recently Vander Wal (7) properly indicated that the calculated percentage compositions of the triglycerides in a mixture are barely affected by large deviations from the 1,3-random distribution. Thus, the currently available data on the percentages of the individual triglycerides in a mixture (e.g., 8) neither support nor deny the 1,3-random hypothesis.

The discussions of stereochemistry by Schwartz and Carter (9) and Hirschmann (10) have emphasized the fact that regardless of fatty acid substituents, the 1- and 3-positions of glycerol are not interchangeable. This concept led us to attempt a stereospecific analysis of the separate acid compositions at the 1, 2 and 3 positions of a triglyceride. We felt that the new method needed to be sufficiently sensitive to characterize the small amounts of triglycerides generally obtained in metabolic studies. The method developed uses the diglyceride kinase of *Escherichia coli* to selectively phosphorylate the 1,2-diglycerides from the mixture of diglycerides formed by pancreatic lipase. Thus a comparison of the fatty acids in the intact triglyceride, the monoglyceride produced by lipase, and the phosphatidate formed in the kinase reaction indicates the acids esterified at each position of the triglyceride. Brockerhoff has described a different procedure to achieve the same goal (11). The present paper presents evidence that diglyceride kinase is a suitable reagent for use in a sensitive stereospecific determination of triglyceride structure.

MATERIALS AND METHODS

Stereoisomers of Dipalmitin

1-O-Benzylglycerol and 3-O-benzylglycerol were prepared as described by Lands and Zschocke (12). The benzylglycerols were acylated with palmitic anhydride essentially as described by Mattson et al. (13). Benzylglycerol (10 mmoles) and palmitic anhydride (22 mmoles) were dissolved in dry chloroform and 0.055 ml (0.66 mmoles) of 70% $HClO_4$ were added. The reaction was stirred at room temperature for 1.5 hr, stopped by the addition of water, and shaken with ether. The ether layer was washed three times with water, then washed with 0.1 *N* NaOH in 50% ethanol until the washings were basic. The ether solution was washed once with 50% ethanol, dried over



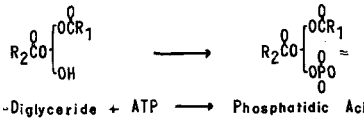


FIG. 1. Stereospecific analysis of triglycerides.

[1] A preliminary report of this work has been presented (1).

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sodium sulfate, and then evaporated. The product was recrystallized from acetone to give 4.6 g of 1,2-dipalmitoyl-3-benzylglycerol, mp 34–36C, α_D + 5.5° in chloroform. The yield of 2,3-dipalmitoyl-1-benzylglycerol was 4.8 g, mp 35–37C, α_D − 6.25° in chloroform.

Hydrogenolysis of the benzyldipalmitoylglycerol to dipalmitin was carried out in acetic acid using Pd/C catalyst. The sample was shaken at room temperature under 16 psi of hydrogen for 7 hr. The insoluble dipalmitin was taken up in ether and the catalyst removed by filtration. The ether was evaporated and the product recrystallized once from ether. The amount of 0.11 g of dipalmitin mp 57–59C was obtained from 1.5 g benzyldipalmitoylglycerol. The α_D in chloroform for the 1,2-dipalmitin was −2.15°, and for the 2,3-dipalmitin, +2.6°.

Preparation of Enzymes

Triglyceride Lipase. Steapsin (Nutritional Biochemicals Co.) was extracted with 10% saturated NaCl (200 mg steapsin per ml), adjusted to pH 7.0. Diisopropyl fluorophosphate (5×10^{-5} M) was added to inhibit proteolytic enzymes. The lipase was measured by a microadaptation of the procedure of Desnuelle (14). One unit of activity is one microequivalent of ester hydrolyzed/ml/min. The enzyme was precipitated with 60% saturated $(NH_4)_2SO_4$, dissolved in 0.05 M sodium acetate −0.005 M calcium chloride, pH 5.3 and dialyzed against the same buffer. The solution was then chromatographed on Sephadex G-200. The most active fractions were combined and the enzyme was precipitated as above and rechromatographed. The pooled active fractions from the second chromatography (specific activity, 270 units/mg; protein concentration, 0.1 mg/ml) were used for the routine triglyceride analyses described below.

Diglyceride Kinase. Diglyceride kinase was prepared as described by Pieringer and Kunnes (15). It was stored after heat treatment in cysteine phosphate buffer at −10C. No loss of activity was observed for 1–2 months.

Chromatographic Methods

Thin-layer chromatography was done on plates coated 0.25 mm thick with Mallinckrodt reagent grade silicic acid, 200 mesh. The plates were dried in air for 1 hr after spreading, activated for 1 hr at 105C, and stored in air until use within one or two days.

Gas chromatography of the methyl esters was done using a Barber-Colman instrument equipped with a hydrogen flame detector and a ¼ in. × 8 ft column packed with 10% ethylene glycol succinate on Gas-Chrom P. The column temperature was 195C.

Stereospecificity of Diglyceride Kinase

The specificity of the diglyceride kinase of *Escherichia coli* for either 1,2- or 2,3-diglycerides was determined by measuring the amount of radioactivity incorporated from ^{32}P-ATP (labeled in the γ-phosphate) into phosphatidic acid in the presence of either stereoisomer of dipalmitin under conditions described by Pieringer and Kunnes (15).

Triglyceride Analysis

The triglyceride (2 to 5 mg), dissolved in ether or hexane, was pipetted into a 13 × 100 mm test tube and the solvent was removed with a stream of nitrogen. The amounts of 0.15 ml of 1.0 M NaCl, 0.10 ml of 1.0 M tris HCl (pH 8.05) and 0.05 ml of lipase (2.7 units/ml) were added. The reaction was mixed on a Vortex mixer at room temperature for approximately 3 min, then stopped by the addition of 0.20 ml of 1 *N* HCl. The lipids were extracted first with 1.3 ml of $CHCl_3$:MeOH, 2:1 (v/v) and then with 0.80 ml of $CHCl_3$. The combined chloroform extracts were evaporated and the lipid taken up in 0.10–0.20 ml of $CHCl_3$:MeOH, 2:1 and put on a TLC plate. The plate was developed with 60% diethyl ether in petroleum ether (30–60C) to about 4 cm from the origin, dried in air for at least 15 min, then developed to the top in 12% diethyl ether in petroleum ether. The bands were visualized by spraying with 1% I_2 in methanol. Dichlorofluorescein should not be used to visualize the bands because it is carried along with the diglyceride and inhibits the kinase. The diglyceride and monoglyceride bands were scraped into small columns and the lipid eluted with 10 ml of 5% methanol in ether.

The solvent was evaporated from the diglyceride, and the following reagents were added: 10 μl of 200 mg/ml of mixed bile salts (Difco Laboratory, Detroit, Mich.); 0.10 ml of 0.05 M ATP; 0.05 ml of 1.0 M $MgCl_2$; 0.05 ml of 0.50 M sodium phosphate buffer, pH 7.95; 0.10 ml of crude diglyceride kinase, ca 8 mg/ml. (The final pH of the reaction mixture was 7.0). The reaction was incubated with constant shaking at 37C. At the end of one hour 0.20 ml 1 *N* HCl was added and the lipids extracted with 2.0 ml of $CHCl_3$:MeOH, 2:1, followed by 1.3 ml of $CHCl_3$. One drop of triethylamine was added to the combined chloroform extracts, the solvent evaporated, and the residue taken up in 0.10–0.20 ml $CHCl_3$:MeOH, 2:1, and ap-

plied to a TLC plate. The plate was developed to the top with 80% diethyl ether in petroleum ether (30–60C), allowed to dry at least 15 min, then developed to a distance of 12 cm with chloroform:ethanol:formic acid, 100:10:5. The bands were visualized with dichlorofluorescein. The phosphatidic acid band was scraped and eluted with 10% methanol in ethanol. Two components of the bile salt mixture run just above and just below the phosphatidic acid; however, contamination from these bands does not interfere with the analysis.

A known amount (ca 150 mμmoles) of methyl pentadecanoate was added as an internal standard to each sample. The solvent was evaporated and 2 ml of 0.5 *N* sodium methoxide in methanol added. The reaction was stopped after 10 min with 0.2 ml of 6 *N* HCl. 5 ml of petroleum ether and 5 ml of water were added and mixed on the Vortex mixer. The petroleum ether layer was dried over Na_2SO_4:$NaHCO_3$, 2:1 (w/w) for at least 10 min, then decanted from the drying agent. The solvent was removed under a stream of nitrogen, with gentle warming, and the methyl esters were taken up in 20–30 μl of CS_2. Portions of this solution (1–4 μl) were used for injection into the gas chromatograph.

The amount of each ester present was calculated from the product of the peak height and the retention time, measured in centimeters from the point of injection. The latter was directly proportional to the peak width at half-height, and can be measured more precisely. This product, P, was used to calculate the fatty acid content as shown below.

$$\text{m}\mu\text{moles of acid} = (P_{acid}/P_{15:0})\,(M.W._{15:0}/M.W._{acid})\,(\text{m}\mu\text{moles } 15:0)$$

The mole per cent compositions of acids in the triglycerides, the monoglycerides, and the phosphatidic acids were calculated and the distribution of the acids was calculated as shown in the sample calculation in Table III.

RESULTS

To determine that the hydrolysis of the triglycerides was independent of the fatty acid composition under the conditions used, the unreacted triglycerides and products formed at different extents of reaction were recovered and analyzed. The results in Table I show that throughout the course of the reaction, the fatty acid compositions were constant within the limits of experimental error. The triglycerides were almost completely hydrolyzed by the lipase by the time that 58% of the total esters was cleaved.

A similar check was made of the fatty acid composition of the product and unreacted substrate during the phosphorylation of mixed 1,2-diglycerides with diglyceride kinase. For this experiment a mixture of 1,2-diglycerides was prepared from the lecithins of pig liver using phospholipase C from *Clostridium welchii* as described by Lands and Hart (16). This diglyceride mixture, which contained a variety of commonly encountered types, was incubated with diglyceride kinase as described above. The results in Table II show that the relative amounts of the component acids in the substrate and product are essentially constant throughout the course of the reaction. The small differences in 20:4ω6 and 22:6ω3 could indicate a difference in phosphorylation rates, but when 45% of the diglyceride was reacted, the phosphatidate composition was

TABLE I
Composition of Lipase Hydrolysis Products

Product[a]	Acid	Composition (moles %) 0 min	3 min	6 min	12 min	Average
TG	14:0	3.1	2.4	2.2	2.4	2.5
	16:0	25	27	29	29	27
	16:1	6.9	5.3	5.8	6.1	6.0
	18:0	4.5	4.9	4.8	5.3	4.9
	18:1	27	27	29	25	27
	18:2	33	33	30	31	32
DG	14:0		2.4	2.3	2.1	2.3
	16:0		29	26	26	27
	16:1		4.7	5.2	6.3	5.4
	18:0		4.7	4.4	4.1	4.4
	18:1		26	27	26	27
	18:2		33	35	34	34
MG	14:0		2.4	1.1	1.3	1.6
	16:0		11	8.6	10	10
	16:1		5.7	5.7	6.6	6.3
	18:0			0.9	1.2	0.7
	18:1		30	29	30	30
	18:2		51	54	50	51
Total ester hydrolyzed			27%	42%	58%	

[a] TG, triglycerides; DG, diglycerides; MG, monoglycerides.

TABLE II
Diglyceride Kinase:
Composition of Substrate and Product[a]

Acid	0 min	15 min		30 min		45 min		60 min	
	DG	DG	PA	DG	PA	DG	PA	DG	PA
16:0	12	12	15	12	12	11	15	12	12
16:1	<1	<1	2	1	<1	<1	<1	1	<1
18:0	30	32	32	32	32	33	31	33	31
18:1	19	18	19	18	19	18	19	18	19
18:2	13	12	12	13	13	12	13	12	13
18:3ω3	3.4	3.1	2.9	3.3	3.3	4.1	3.3	2.8	3.5
20:3ω6	4.7	4.8	4.2	4.8	4.3	4.9	4.6	4.8	5.0
20:4ω6	5.5	5.4	3.7	5.3	4.7	5.3	5.2	5.3	5.5
22:5ω6	1.6	1.6	1.7	1.3	3.3	1.0	0.7	1.7	1.2
22:6ω3	7.5	7.4	5.8	7.3	6.3	7.5	6.7	7.0	7.2
24:4ω6	1.3	1.6	0.8	1.3	1.3	1.4	0.8	2.2	1.4
Extent of reaction	0	15%		19%		38%		45%	

[a] DG, diglycerides; PA, phosphatidic acid; acid compositions, mole percent.

identical to that of the initial diglycerides. The closer the phosphorylation reaction approaches completion, the less the differences in reaction rate are likely to influence the composition of the product.

The stereospecificity of the kinase reaction is indicated in Figure 2. The rapid leveling off of the reaction mixture containing 2,3-dipalmitin suggests that the reaction observed may have been due to some impurity in the reaction system.

The data obtained in analyzing the fatty acid distributions in a sample of rat liver triglycerides are given in Table III. The table is arranged to indicate the method of calculating the distribution of acids between the three positions of the triglycerides. The per cent of total acids at each position is, of course, 33%, so that palmitate at the 1-position is 19% of the total acids or 57% of those acids at that position in the triglycerides. The 1-position clearly contained most of the saturated fatty acids and thus differed markedly from the 3-position which contained principally unsaturated acids. Oleate and linoleate constituted over 70% of the acids at the 3-position and over 90% of those at the 2-position.

DISCUSSION

The present method for calculating the acid composition at 1- and 3-positions involves differences between separate gas chromatographic analyses. These analyses must be determined with reasonable precision to avoid large percentage errors in the final calculated values. In addition, the conditions for the enzyme-catalyzed reactions were designed to produce monoglycerides, diglycerides and phosphatidates that would be truly representative of the acids in the corresponding positions in original triglyceride.

Pancreatic lipase catalyzed hydrolysis of triglycerides has been shown to proceed independent of the long chain fatty acid composition of the glycerides (3,17). This generalization may not hold for glycerides containing short-chain acids (18), but the results in this and earlier (3) work indicate that the lipase was not influenced by the slight differences between palmitate, oleate, linoleate and stearate. Recently Bottino et al. (19) showed that certain long chain esters (20:5 and 22:6) in triglycerides of marine origin were resistant to hydrolysis with pancreatic lipase. On the other hand, Mattson and Volpenhein (20) found that the acids of chain length greater than 18 carbons were esterified almost exclusively at the 1- and 3-positions in vegetable fats, and were thus cleaved with

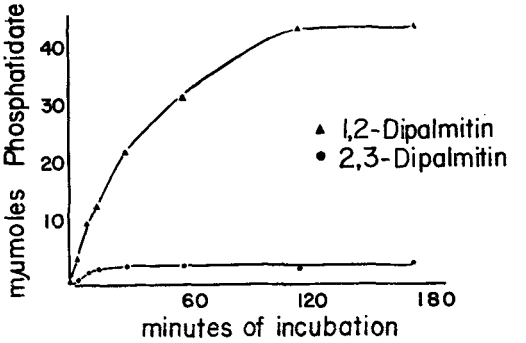

FIG. 2. Stereospecificity of diglyceride kinase. The incubation system contained 2.4 mM ^{32}P-ATP; 93 mM magnesium chloride; 0.93% (v/v) Cutscum (Fisher Scientific Co., Fairlawn, N.J.); 46.5 mM sodium phosphate buffer, pH 7.5; 0.58 mg of protein of a heat-treated particulate preparation of *E. coli* suspended in 0.05 ml of 0.1% cysteine hydrochloride 0.01 M sodium phosphate, pH 7.0; and either 0.47 mM 1,2- or 2,3-dipalmitin. The reagents in a final volume of 0.215 ml were incubated at 37C for various times as indicated.

TABLE III
Distribution of Acids Among the Three Positions of Rat Liver Triglycerides

Acid	Composition (mole percent) (I) TG[a]	(II) PA[b]	(III) MG[b]	(IV) PA × 2/3	(V) MG × 1/3	Distribution (percent) position 1 (IV)-(V)	2 (V)	3 (I)-(IV)
16:0	26.2 (28.6–29.6)[c]	30.3 (26.1–37.9)	3.7 (3.0–4.0)	20.2	1.2	19	1.2	6.0
16:1	4.0 (3.3–4.4)	1.7 (1.3–2.5)	2.4 (2.0–2.7)	1.2	0.8	0.4	0.8	2.8
18:0	3.1 (2.8–3.6)	4.1 (2.8–6.8)	0.7 (0.5–0.8)	2.7	0.2	2.5	0.2	0.8
18:1	33.8 (33.1–35.6)	28.9 (25.3–32.4)	39.3 (38.9–39.6)	19.3	13.1	6.2	13	14
18:2	32.9 (31.0–34.5)	34.9 (31.4–37.4)	53.9 (52.9–55.1)	23.3	18.0	5.3	18	10

[a] Average of 4 experiments. TG, triglyceride.
[b] Average of 3 experiments. PA, phosphatidic acid; MG, monoglyceride.
[c] Numbers in parentheses indicate range.

lipase. Several conflicting reports in the literative [e.g. Coleman (21)] make it difficult to say that all triglycerides tested will be satisfactorily analyzed by this procedure. The ease of analyzing the products and unreacted triglycerides makes it desirable to check any new triglyceride mixture during the stereospecific analysis to establish that the lipase reaction rate was independent of the component acids under the conditions used.

The observed relative distribution of oleate (Table III) between the 2- and 3-positions requires a modification of the generalization that the unsaturated fatty acids are esterified preferentially at the 2-position (22). In the past, only the average ester compositions of the primary positions could be measured and the differences between the 1- and 3-positions shown in Table III were not recognized. The recent work of Brockerhoff et al. has also shown that these two positions have different compositions in some tissues (23,24). We found a greater degree of asymmetry in the triglycerides of rat liver than has been reported for adipose tissue of rats (24).

Diglycerides, which are undoubtedly the precursors of triglycerides, may be formed either from phospholipids or preexisting triglycerides. If triglyceride lipase produced both 1,2- and 2,3-diglycerides, a nonstereoselective reacylation (25) to triglycerides could lead to a randomization of the acids between the two primary esters. On the other hand, acylation of 1,2-diglycerides derived from phospholipids would lead to differences in composition between the primary positions. This would occur if the diglyceride:acyl-CoA acyltransferases had specificities different from those of the enzymes which acylate the 1-position of the various phospholipids, or if these enzymes operated on different pools of acyl-CoAs. Our analyses indicate that in rat liver, the control of the composition of the 3-position is clearly different from that at the 1-position.

ACKNOWLEDGMENT

Supported in part by grant AM 05310 from the USPHS. The triglyceride lipase was purified for us by John Champe.

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[Received May 26, 1966]

The Very Long Chain Fatty Acids of Human Brain Sphingolipids

SPHINGOLIPIDS have long been known to contain fatty acids with comparatively long chains (centering around C_{24}) including α-hydroxy saturated and unsaturated members. With the application of gas-liquid chromatographic technique to this problem, the true nature of this fatty acid mixture has been determined and has been found to comprise almost the entire series, both odd and even chain unsubstituted and α-hydroxy, of fatty acids from relatively short chain lengths to acids with 24, 25 and even 26 carbons (1–3). O'Brien and Rouser (3) reported a trace of 27:1, but, in general, analysis of brain fatty acids has not been carried out past C_{26}. In this laboratory, in connection with a study of the lipid composition of the aging human brain, analysis of the component fatty acids of the sphingolipids was carried to the limit of the presently available equipment. The results demonstrate the existence of significant quantities of fatty acids with much greater chain-lengths than had heretofore been detected.

Brain specimens were obtained from apparently normal adults (ages 26–53 years) without gross evidence of neurological disease. Five- to ten-gram samples from the frontal lobes were removed at autopsy and were kept at −20C for different periods of time before separation of gray and white matter and extraction. Extraction and separation of the sphingolipids, methanolysis to obtain the fatty acid methyl esters and chromatographic procedures based on the methods of Rouser et al. have been described in detail previously (4). Ceramide was isolated from a silicic acid-silicate-water column with chloroform/methanol 9/1 or from a Florisil column with chloroform-methanol 19/1. Cerebrosides plus sulfatides were eluted from Florisil with chloroform-methanol 2/1 and were separated by DEAE cellulose column chromatography. The cerebrosides were eluted with chloroform-methanol 2/1 and the sulfatides with chloroform-methanol-ammonium hydroxide 4/1/0.1. Sphingomyelin was eluted from a silicic acid-silicate-water column using chloroform-methanol-water 4/1/0.025. Gas-liquid chromatography (GLC) was carried out on samples of methyl esters purified by elution from a Florisil column with

TABLE I

Fatty Acid Composition of Human Brain Sphingolipids
(Expressed as area per cent of total methyl esters of each class)

Fatty Acids[a]	Cerebroside Gray 53 yrs	Cerebroside White 38	Cerbroside sulfate Gray 53 yrs	Cerbroside sulfate White 28	Ceramide Gray 53 yrs	Ceramide White 26	Sphingomyelin Gray 28 yrs	Sphingomyelin White 38
C_{14}-C_{23}	24.9	27.8	24.4	28.9	26.2	28.5	31.7	32.0
24:1	18.8	22.2	11.4	9.3	16.8	14.4	18.7	12.7
24:0	14.1	17.4	10.7	13.0	7.4	6.2	12.9	19.4
25:1	13.5	8.3	6.9	5.4	4.6[b]	17.6[b]	8.0[b]	6.9[b]
25:0	7.5	6.2	8.3	10.9	6.9	5.6	5.6	12.0
26:1	15.7[b]	11.1[b]	12.9[b]	14.4[b]	3.0[b]	17.2[b]	10.2[b]	3.5
26:0	2.3	2.2	5.5	6.7	6.7	5.6	3.2	4.7
27:1	0.4	0.1	0.6[b]	0.4	0.5	0.5	0.5[b]	1.3
27:0	0.1	[d]	3.4	2.2	5.2	0.3	1.9	[d]
28:1	0.3	0.9	0.4[b]	0.2	0.9[b]	0.1	0.4	0.6
28:0	0.4	0.5	4.2	2.5	5.1	0.5	2.1	0.5
29:1	[d]	[d]	0.4	0.2	0.8[b]	0.2	0.2	[d]
29:0	0.2	0.3	3.3	1.6	4.1	0.4	1.5	0.2
30:1	0.3	[d]	0.4[b]	0.3[b]	0.7[b]	[d]	0.1	[d]
30:0	0.4	0.3	3.1	1.6	3.9	0.4	1.5	0.4
31:1	[d]	[d]	0.3	0.2	0.6	0.1	[d]	[d]
31:0	0.5	0.3	2.0	0.9	2.3	0.3	0.8	[d]
32:1	0.6	[d]	[d]	0.1	0.3	[d]	[d]	[d]
32:0	[d]	0.4	1.8	0.8	1.8	0.3	0.7	1.8
33:1	[d]	0.7	[e]	[d]	0.2	0.1	[d]	[d]
33:0	[e]	0.6	[e]	0.3	0.8	0.2	0.2	[d]
34:1	[e]	[e]	[e]	[e]	[d]	[d]	[e]	[d]
34:0	[e]	[e]	[e]	[e]	0.6	[d]	[e]	1.1
35:0	[e]	[e]	[e]	[e]	0.5	[e]	[e]	[e]
Other[c]	0.1	0.7	0.1	0.0	0.2	1.4	0.0	2.9

[a] Fatty acids are designated by the chain-length and number of double bonds.
[b] Many of the unsaturated fatty acids consisted of isomers that were not well separated and are combined.
[c] This group includes dienoic and branched chain acids and other poorly identified acids.
[d] None seen.
[e] Analysis not continued to this point.

pentane-ether (95:5, v/v). Thin-layer chromatography and infrared absorption spectrophotometry revealed no components other than methyl esters. Two types of procedures and apparatus were used: a Barber-Colman model 20 equipped with an argon ionization micro detector and a 100-ft Apiezon L Golay column (0.020 in. I.D. stainless steel capillary) operated at 235C, and a Barber-Colman model 10 apparatus with an argon ionization detector and a 6-ft glass column (6 mm I.D.) and 12% stabilized diethylene glycol succinate on Anakrom 60/70 mesh operated at 190C. The usual sample injected was 4 μl of a 10% solution of the mixed methyl esters and 30-fold changes in detector sensitivity were employed, thus permitting easy visualization of peaks with high signal-to-noise ratios. Fatty acids were identified by comparison of their retention times with those of synthetic standards obtained from Applied Science Laboratories, State College, Pa. (for 14:0, 16:0, 18:0, 20:0, 22:0, 24:0) and from Fluka Laboratories, Buchs, Switzerland (for 30:0) or from a log retention time vs. carbon number plot which included the retention times of many known fatty acid esters.

The examples of fatty acid composition in Table I are typical for the lipids shown. They are from several individuals, indicating that the observations are not of an isolated phenomenon. Although samples were not available for all lipids from one individual, a comparison can be made of gray matter cerebroside, cerebroside sulfate and ceramide. More detailed comparisons will be the subject of future research. Only the unsubstituted fatty acids have been included. Acetoxy methyl esters with similar chain lengths have been detected, but retention times are correspondingly longer and fewer examples are presently available. Fatty acids from C_{14} through C_{23} were grouped together since they are not under consideration in this report. The data tabulated are entirely from the capillary column. The packed column was used only to check these results.

Fatty acids with chain-lengths greater than C_{26} represent a substantial fraction of the total of the brain sphingolipids. The amounts varying from an average of around 4% of the total in the cerebrosides to as high as 28% for the unsubtituted fatty acids of ceramide. It may be significant that ceramide and cerebroside sulfate (particularly of gray matter) contain similar quantities of these fatty acids and, in this respect, are quite different from cerebroside.

There are probably several reasons for the neglect of these possibly important constituents. First, particularly in the GLC apparatus with packed columns operated at fairly low temperatures, traces of very long chain fatty acids are difficult to distinguish from variations in the baseline. Second, in packed columns with a polyester liquid phase, fatty acids with very long retention times may simply not emerge at all. (In the present study, however, the DEGS column at 190C did not appear to retain the long-chain methyl esters.) Third, despite the obvious possibility that such fatty acids should exist, it has not been customary to wait the long periods necessary for their emergence from the column, particularly since the available information in the literature stops at C_{26}. It should also be pointed out that in order to visualize the recorded peaks for the very long chain esters, relatively large samples were injected and extreme changes in detector sensitivity were employed.

In any event, it seems evident that a true picture of fatty acid composition cannot afford to neglect these compounds. The stability of the myelin membrane appears to depend in part on its content of lipids with such long chains affording the possibility of "interdigitation" and increased hydrophobic bonding. It is also evident that they must play an important part in formulation of models of membrane structure.

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ACKNOWLEDGMENTS

Studies supported by contract (AT(04-1)GEN-12) between the US Atomic Energy Commission and the University of California.

Research of one of us (J.F.M.) supported by USPHS Research Career Award (GM-K6-19, 177) from the Division of General Medical Sciences.

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[Received Aug. 30, 1966]

Purification of Triglycerides With an Alumina Column[1]

SYNTHETIC MIXED ACID triglycerides (TG's) are usually prepared by acylating mono- (MG's) or diglycerides (DG's) with the requisite fatty acid chloride. If acylation is incomplete, a mixture of varying quantities of tri-, di- and monoglycerides and fatty acid results. In our hands, the TG content of acylation mixtures has ranged from about 55–90%. Purification of these acylation mixtures can be difficult and laborious as silicic acid column chromatography will handle relatively small quantities and crystallization is often wasteful with accompanying reduction in yield. Chapman et al. (1) avoided these difficulties to a large extent by using a column of neutral alumina to remove the impurities (DG's) from their preparations of synthetic TG's.

We purified preparations of glyceryl-1-palmitate-2,3-dibutyrate (PBB) with the column as this compound cannot be crystallized except at very low temperatures (−96C). We have since purified many synthetic and natural TG's with the alumina column. The data accumulated indicates that the technique will clean up relatively large quantities, with good recovery and without structural alterations. We believe the procedure will be useful to others, hence this report.

Neutral alumina, Brockman activity I (Fisher) Alcoa alumina (F-20 for chromatography) and heated Alcoa alumina (260C, 12 hr) all performed equally. We use the Alcoa alumina because it is relatively inexpensive. The alumina is routinely heated at 260C for 12 hr in batches after removal from the drum. It is then kept in a tightly sealed jar. Any standard chromatographic tube will suffice to hold the alumina which is usually added at the rate of 2 g per gram of material to be purified. The dry alumina is wetted with petroleum ether (30–60C) or a mixture of petroleum ether-ethyl (9:1), the lipid material dissolved in a minimum amount of ether solvent and placed on the column. The TG's are eluted with 200 ml of solvent per 10 g of material and 20 g of alumina. Methyl esters or other compounds less polar than TG's are also eluted, while diglycerides, free fatty acids, etc., remain on the column. The whole process requires about 15 min and eluted TG's have always been colorless. The process can be scaled up. We added 81.6 g of alumina and 106.8 g of impure synthetic glyceryl 1-palmitate-2-oleate-3-stearate (POS) to a column 3 cm in diameter and recovered 100.4 g of TG, pure by thin-layer chromatography (TLC), with 400 ml of petroleum ether-ethyl ether 9:1. The POS had been crystallized to 95% purity as estimated by TLC and contained DG's and fatty acid. Ninety nine grams of TG was recovered in the first 200 ml of solvent, 1.4 g in the second 200 ml.

Diglycerides can be eluted with 1:1 petroleum ether-ethyl ether, but they are recovered as a mixture of isomers. We have used this to produce mixtures of 1,2- and 1,3-isomers from pure 1,3-diglycerides.

Recoveries of purified olive oil and triolein (Hormel) all TG by TLC, ranged from 85–90%. Other recoveries based on the original weight of the impure mixture with quantity (%) of TG in the mixture given in parentheses were: tributyrin, 75 (80); glyceryl-1-oleate-2,3-dicaproate, 63.3 (75); glyceryl-1-oleate-2,3-dibutyrate, 66.0 (75); glyceryl-1-oleate-2,3-dipalmitate, 56.0 (70); glyceryl-2-oleate-1,3-dipalmitate, 80.0 (93); glyceryl-1-palmitate-2,3-dioleate, 75.3 (89); glyceryl-2-palmitate 1,3-dioleate, 75.0 (85.0); SOP, 94.0 (95); glyceryl-1-oleate-2,3-dimyristate, 88.7 (96); and glyceryl-1-stearate-2,3-dioleate, 92.5 (95). In general, the TG's were synthesized as described by Mattson and Volpenhein (2). Purity before and after column treatment was ascertained with TLC. Use of the column to purify acylation mixtures eliminates washing with dilute base to remove fatty acids and consequently eliminates the troublesome emulsions that often

TABLE I

Fatty Acid Composition of Triglycerides Purified with an Alumina Column and of the Free Fatty Acids and Monoglycerides Derived Therefrom by Pancreatic Lipolysis

Triglyceride[a]	Fraction	Fatty acid (M%) 16:0	18:0	18:1
SOO	Intact TG[b]	.....	33.7	66.3
	MG	...	0.3	99.7
POP	TG	66.6	.....	33.4
	FFA[b]	99.1	.....	0.9
OPP	TG	66.7	....	33.3
	FFA	50.0	.....	50.0
	MG	98.8	.....	1.2
POO	TG	33.3	.....	66.7
	FFA	49.8	.....	50.2
	MG	....	.....	>98.5
OPO	TG	33.0	.....	67.0
	FFA	....	.....	>99.0
	MG	99.0	....	....
SOP	TG	33.8	33.1	33.1
	FFA	50.3	49.7	....
	MG	...	....	>99.0

[a] S- stearate, O- oleate, P- palmitate.
[b] TG- triglyceride, MG- monoglyceride, FFA- free fatty acids.

[1] Scientific contribution No. 204, Agricultural Experiment Station, University of Connecticut, Storrs.

TABLE II

Effect of Purification with an Alumina Column on Fatty Acid Position in Olive Oil

Fatty acid	MG[a]		FFA[b]	
	Pre	Post	Pre	Post
	M%			
16:0	0.6	0.5	19.8	20.4
16:1	0.6	0.5	0.5	1.1
18:0	0.4	0.1	4.1	4.6
18:1	85.5	88.3	69.4	69.4
18:2	11.9	9.9	5.2	3.6
19:0	....	....	0.4	0.5
18:3	1.0	0.7	0.6	0.3

[a] Olive oil eluted from column of alumina with 9:1 petroleum ether-ethyl ether.
[b] MG- monoglyceride, FFA- free fatty acids.

arise. If the TG content is less than about 50% then absolute recoveries are depressed.

Exposure of double bonds to alumina has been reported to result in positional isomerism (2). To test this, triolein (Hormel) was passed through the alumina column, converted to methyl esters, examined for *trans* isomers by infrared spectrophotometry and for positional isomers by oxidation as described by Tinoco and Miljanich (3). *Trans* isomers were not detected. Further there was no difference between the oxidation patterns as determined by gas-liquid chromatography (GLC) of the original and that after passage through alumina. The major peaks obtained corresponded to retention times of authentic methyl azeleate, oleate, and pelargonate. Dicarboxylic acids other than pelargonic were not detected. We concluded that the alumina column did not cause isomerization. This experiment was repeated on methyl linoleate (Hormel) with similar results and the same conclusion.

To determine if alumina affected positional integrity we examined some of the synthetic TGs above with pancreatic lipolysis. In addition, purified olive oil was similarly checked before and after column treatment. The fatty acid composition (GLC) of the resulting free fatty acids and monoglycerides from the synthetic TG's in Table I and from the olive oil in Table II indicates that the alumina column did not cause disproportionation of the fatty acids. Many other synthetic TG's containing a large variety of acids; short-chain, *trans* isomers, etc., have been tested with pancreatic lipolysis after purification and positional purity was maintained.

The virtues of the column are apparent. Purification of TG's from acylation mixtures, particularly those that are difficult to crystallize, is rapidly achieved without alterations in structure. Recoveries of TG's from these mixtures are close to 90%. Further, no difficulties are caused if the column runs dry. If however, the mixture to be purified is not readily soluble in the eluting solvent, flow is delayed and purification does not occur. Nevertheless, the advantages of speed, good recovery, lack of structural alterations and large column load should recommend its widespread application.

ACKNOWLEDGMENT

Study supported by US Department of Agriculture Grant No. 12-14-100-7660(73) administered by the Eastern Utilization Research and Development Division, Philadelphia, Pa.

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[Received June 27, 1966]

A Simple Device For Preparative TLC

A "SANDWICH" TYPE plate holder has been developed for preparative TLC using plates of 0.25 and 0.50 mm thickness of silicic acid. This device requires only readily available materials and can be made up easily in any sheet metal shop.

The materials used were No. 18 gauge stainless steel sheet, type 316 satin 2B finish, and 3/32 in. diameter type 316 stainless steel wire which should be obtained in straight lengths rather than coiled. The holder (F) was slightly larger than the plates used, with enough clearance to prevent jamming, e.g., 8 1/8 in. × 8 1/8 in for 8 in. × 8 in. plates. The handle (A) was "safe edged" and spot welded to (F) at a height above (F) sufficient for finger clearance. The sides of (F) were turned out 3/16 in. to hold the cover, and cut so as to allow a small tab (E) to be turned down to prevent the cover sliding off. To accommodate 6 plates and 5 spacers, the

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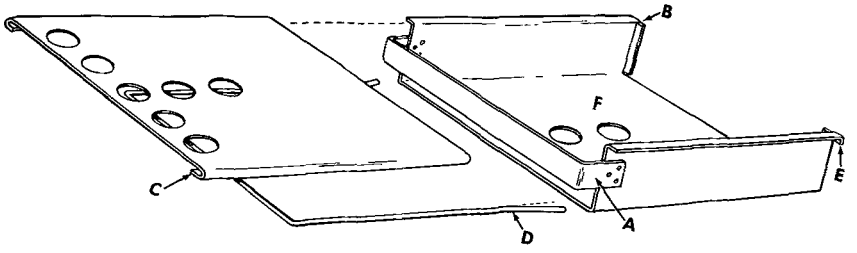

height of the side was 1 1/16 in. A 5/16 in. ledge (B) turned in on each side retained the plates in (F). The sides of the cover (C) were folded to fit over the holder edge (B) and allow perfectly free sliding on and off. The wire spacers had a 2° or 3° kick outward about half way along the length of each leg (D) to provide pressure for holding them in place.

Three pairs of plates were used. On each plate, a strip of silica gel 3/8 in. wide was removed from 3 sides of the plate. The plates were spotted uniformly and the first plate laid silica gel side up in (F). A wire spacer was compressed, put in place and the second plate, prepared in the same way as the first, was laid face down. Two more pairs of plates, with spacers between, were placed similarly in (F). The cover was then slid in place and the unit stood handle up in a chromatographic tank containing the desired solvent system.

This unit has been used for different types of lipid separations. Generally the plates developed more quickly than single plates. With most of the solvent systems used, the resolution was good so long as the spots were 1 cm or more from the sides of the silica gel. The "sandwich" unit gave satisfactory lipid separation with the following solvent systems: hexane-diethyl ether used both with silica gel and silver nitrate-silica gel; hexane-diethyl ether-acetic acid; chloroform; disobutyl ketone-acetic acid-water; benzene-methanol; butanol-acetic acid-water. However, when solvent systems containing both chloroform and methanol were used, it was found necessary to use in each pair one silica gel coated plate facing a plate lined with solvent-moistened paper (Eaton-Dikeman 320) as in a "saturation" tank. For the separation of sugar derivatives, it has performed well with solvent systems containing methyl acetate-petroleum ether; methanol-benzene; chloroform-acetone; acetone-petroleum ether.

Although developed to permit preparative TLC using plates coated with a standard thickness of silicic acid, this holder has proved equally useful, over the past 18 months, for nonpreparative purposes.

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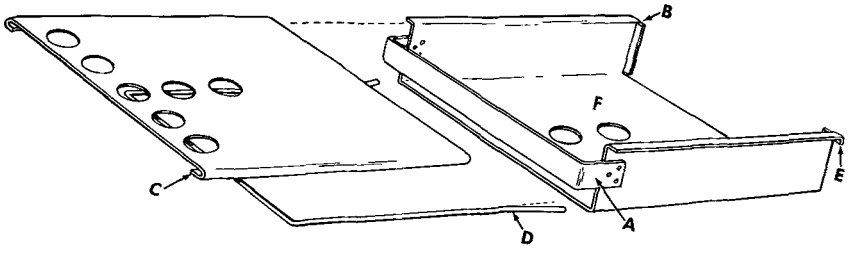
[Received Sept. 6, 1966]

LETTER TO THE EDITOR

Separation of Methyl Eicosatetraenoate from Methyl Docosenoate by Gas-Liquid Chromatography

SEVERAL PHASES have been used for the separation and estimation of fatty acids by gas-liquid chromatography, diethylene glycol succinate (DEGS) and Apiezon L being among the most widely used. Despite the fact that separation of 20:4 and 22:1 acids has been reported with DEGS, we have not been able to obtain satisfactory separation with this phase. The acids are readily separated on Apiezon L and use of this phase in combination with DEGS is strongly urged. A further demonstration of the variability of separations with DEGS is our ability to separate some methyl esters reported as inseparable by others. Our conditions and results are described.

An analytical procedure utilizing DEGS and



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Contribution No. 45 of the Food Research Institute.

[Received Sept. 6, 1966]

LETTER TO THE EDITOR

Separation of Methyl Eicosatetraenoate from Methyl Docosenoate by Gas-Liquid Chromatography

SEVERAL PHASES have been used for the separation and estimation of fatty acids by gas-liquid chromatography, diethylene glycol succinate (DEGS) and Apiezon L being among the most widely used. Despite the fact that separation of 20:4 and 22:1 acids has been reported with DEGS, we have not been able to obtain satisfactory separation with this phase. The acids are readily separated on Apiezon L and use of this phase in combination with DEGS is strongly urged. A further demonstration of the variability of separations with DEGS is our ability to separate some methyl esters reported as inseparable by others. Our conditions and results are described.

An analytical procedure utilizing DEGS and

TABLE I

Carbon Number of Methyl Esters of Fatty Acids on Diethylene Glycol Succinate and Apiezon L Columns

Methyl esters	Diethylene glycol succinate		Apiezon L	
	A	B	C	D
18:0	18.00		18.00	18.00
18:1	18.55		17.64	17.70
18:2	19.42		17.64	17.66
18:3	20.50		17.64	17.66
20:0	20.00		20.00	
20:4	22.48	22.56	19.00	19.05
20:5	23.55		19.00	19.05
22:0	22.00		22.00	
22:1	22.48	22.56	21.61	

A column temperature 180C, gas pressure 14 psi, flow rate 75 ml/min.
B column temperature 165C, gas pressure 10 psi, flow rate 50 ml/min.
C column temperature 165C, gas pressure 10 psi, flow rate 300 ml/min.
D column temperature 170C, gas pressure 10 psi, flow rate 120 ml/min.
The methyl esters were run on (1) Barber-Colman Model 10, (2) EIR gas chromatograph Model AU 8 and (3) EIR clinigraph. U-shaped columns were used for the Barber-Colman and EIR AU 8 gas chromatographs and coiled columns for the EIR clinigraph.

Apiezon L that is suitable for separation of 20:4 and 22:1 acids is as follows. Gas Chrom P, 80–100 mesh (Applied Science Laboratories, Inc.) is acid washed and siliconized with 2% dimethyldichlorosilane in toluene, 100 ml being used for every 15 g of support. Separate batches of the washed and siliconized support are coated with the two phases; 200 ml of an 8% solution of DEGS in acetone and 200 ml of a 4% solution of Apiezon L in chloroform were taken for every 15 g of support. Siliconized glass columns are used, 8 ft × 4 mm I.D. for the DEGS coated support and 6 ft × 5 mm I.D. for Apiezon L.

The methyl esters of standard 20:4 and 22:1 acids were not separated on DEGS under the conditions used in our laboratory (Table I). The carbon number of the methyl esters of these two acids, determined according to the method of Woodford and van Gent (J. Lipid Res. *1*, 188, 1960), remained the same even at a considerably lower temperature and flow rate (165C, 50 ml/min). Supina (in "Biomedical Applications of Gas Chromatography," H. A. Szymanski, ed., Plenum Press, N.Y., 1964, p. 271) reported that under the conditions usually employed there was an overlap of the methyl linolenate and methyl arachidonate peaks, while Woodford (in "Fatty Acids," K. Markley, ed., 2nd edition, Vol. 2, Interscience Publishers, Inc., New York, 1964, p. 2260) noted that methyl linolenate and methyl arachidonate could be confused with methyl behenate. However, we have been able to separate these three pairs of fatty acids compeletly (Table I).

A clear separation of the methyl esters of the 20:4 and 22:1 acids was obtained on Apiezon L columns, but in this case the methyl esters of 20:4 and 20:5 acids appeared as one peak. The methyl esters of the 18:2 and 18:3 acids counld not be separated; the methyl ester of the 18:1 acid could be partially separated when a lower temperature and flow rate were employed (175C, 120 ml/min).

ACKNOWLEDGMENT

This work was supported by USPHS Grants HE-05283 and HE-05399.

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[Received June 3, 1966]