Studies on the Structure of Glyceryl Ethers and the Glyceryl Ether Phospholipids of Bovine Erythrocytes*

DONALD J. HANAHAN, JANICE EKHOLM, AND CRAIG M. JACKSON

From the Department of Biochemistry, University of Washington, Seattle 5

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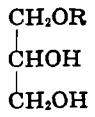
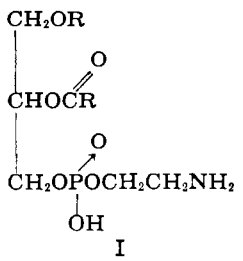
The "phosphatidyl ethanolamine" present in bovine erythrocytes has been shown to be a mixture of diacyl glycerylphosphorylethanolamine and monoalkyl, monoacyl glycerylphosphorylethanolamine. The ether-containing phospholipid, which represented nearly 75% of this fraction, was subjected to chemical degradation and was shown to be an L-1-glyceryl ether derivative. The glyceryl ether phospholipid was freed of accompanying diacyl phospholipid by a selective deacylation procedure. Although not conclusively proven, the fatty acyl group is most probably on the 2-position of the glyceryl residue. Only unsaturated fatty acids were found in this highly purified ether phospholipid. A new procedure was developed for the identification of the glyceryl ethers, wherein these ethers were converted in 95% yield to their isopropylidene derivatives by a rapid, room temperature acetonation in the presence of 10^{-2} M $HClO_4$. The 2,3-*O*-isopropylidene glyceryl ethers could be separated and identified by gas-liquid chromatography. In addition, the free glyceryl ethers, which were isolated from the phospholipid by acetolysis, were converted into their isopropylidene derivatives, and separated into saturates and unsaturates through formation of the acetoxymercurimethoxy derivatives and subsequent chromatography on aluminum oxide. The location of the olefinic bond at the 9:10 position of the side chain in a highly purified 18:1 glyceryl ether from dogfish liver and bovine erythrocytes was established by permanganate-periodate oxidation. The main products were nonanoic acid and an ether containing dicarboxylic acid, 9-(α-carboxy methoxy)-nonanoic acid.

In recent years there has been an increased interest in the biochemical behavior of the α-glyceryl ethers, which can be represented structurally as follows:



In the above formula, R most often is a hexadecyl (16:0), octadecyl (18:0), or octadecenyl (18:1) unit. These novel compounds are found in many diverse sources, e.g. pig spleen (Prelog *et al.*, 1943), bovine yellow bone marrow (Holmes *et al.*, 1941; Hallgren and Larsson, 1962), crustaceans (Karnovsky and Brumm, 1955), fish liver oil (Hallgren and Larsson, 1962), bacteria (Sehgal *et al.*, 1962), bovine erythrocytes (Hanahan and Watts, 1961), and in egg yolk (Carter *et al.*, 1958). In the latter two sources it is well established that these ethers occur as components of a phosphoglyceride molecule. At the same time attention has centered on their biological activity in erythropoiesis (Linman and Bethell, 1960), as protective agents against radiation (Brohult, 1962), and as growth stimulators (Brohult, 1960). While these latter studies have been provocative, insufficient chemical evidence precludes any definitive consideration of the biochemical role of these compounds.

In a preliminary report from this laboratory (Hanahan and Watts, 1961) the isolation of a glyceryl ether–containing phospholipid, I, from bovine erythrocytes was described.



I

The present communication documents in greater detail the chemical characteristics of I and reports observations on the isolation and identification of the glyceryl ethers.

EXPERIMENTAL

General Analysis

Many of the more common assays, such as phosphorus, nitrogen, iodine number, etc., have been described previously (Hanahan *et al.*, 1960). Thin-layer chromatography on silica gel G (Stahl, 1959) was performed with the following solvents: *Neutral lipids*, petroleum ether–diethyl ether–acetic acid (90:10:1, v/v) or (30:70:1, v/v); *phospholipids*, chloroform-methanol-water (95:35:4, v/v). The position of compounds on these chromatograms was visualized by spraying the plates with concentrated sulfuric acid and subsequently heating the plates. In certain instances, amino nitrogen–containing compounds were detected by the ninhydrin reaction. Acidic compounds were detected by their reaction to a bromothymol blue spray. Siliconized thin-layer chromatographic plates of silica gel G were prepared by gently dipping the silica gel G plates in a 5% solution of SC-87 Dri-Film (General Electric, Silicone Products Dept.) in diethyl ether and then allowing them to air dry. At best this procedure was not the most satisfactory, with only 30% of the final plates of good quality. The solvent system for reversed-phase thin-layer chromatography was acetone-water, 60:40, v/v.

Carbon and hydrogen analyses were performed by Dr. Alfred Berhardt (Micro Analytisches Laboratorium, Kaiser-Wilhelm Platz 1, Mulheim, West Germany). Fatty acid ester was determined by infrared measurement of the carbonyl absorption at 5.75 μ. Glyceryl ether could be assayed, in the absence of a phosphate, by its absorption at 9.0 μ.

Commercial samples of glyceryl ethers were obtained through the generous cooperation of Dr. William Chalmers and Mr. John Shaw of the Western Chemical Industries, Ltd., Vancouver, British Columbia. These glyceryl ether samples ranged in composition from

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those high in batyl alcohol and chimyl alcohol to those rich in selachyl alcohol (18:1) and its homolog, a 16:1 derivative. In most instances these samples were freed of contaminating sterols, free fatty acids, etc., by chromatography on silicic acid columns with hexane, hexane–diethyl ether (4:1, v/v), and 100% diethyl ether as the eluting solvents. The glyceryl ethers were found exclusively in the 100% ether eluate. Synthetic D,L-batyl alcohol was purchased from Aldrich Chemical Company and also was synthesized by the method of Gupta and Kummerow (1959).

Ethylene glycol succinate (15% on Anakrom AB 60/70) was a product of Analytical Engineering Laboratories. Silica gel G was a Merck (Darmstadt) product obtained from Brinkmann Associates. Silicic acid, Mallinckrodt, 100 mesh, suitable for chromatography, was used for column chromatography. Neutral aluminum oxide, 100–200 mesh, *p*H 7.0, Brockmann Grade 1, was purchased from Bio-Rad Laboratories. *n*-Hexane (Phillips, 95%), was redistilled over $KMnO_4$ and the fraction distilling at 67–68° was collected. All other solvents were reagent grade (A.C.S. quality). The esterification of fatty acids was effected through the use of boron trifluoride in methanol (obtained from Applied Sciences Laboratory) as described by Metcalfe and Schmitz (1961). The fatty acid methyl esters, with chain length of 12 carbons or greater, were analyzed by gas-liquid chromatography on the ethylene glycol succinate phase at 174°, while the shorter-chain fatty acid esters were chromatographed on the same phase at a temperature of 140°. Standard methyl esters of fatty acids were purchased from Applied Sciences Laboratory.

A glyceryl ether–rich phosphatidyl ethanolamine fraction was isolated from bovine erythrocytes by a previously described technique (Hanahan and Watts, 1961). The composition of this fraction has remained quite constant in twelve different preparations examined to date and can be represented in general by analyses submitted later in this report.

All infrared measurements were made on a Perkin-Elmer Model 21 infrared spectrophotometer, equipped with sodium chloride optics, and with 1-mm sodium chloride cells or 1-mm Irtran cells (Connecticut Instrument Corp.). A Barber-Colman Model 10 Argon Chromatograph, with a RaD ionization detector was used for all gas-liquid chromatographic assays.

Chemical Characteristics of the Glyceryl Ether Phospholipids

A. *General.*—The "phosphatidyl ethanolamine" fraction of bovine erythrocytes contained nearly 75% of I and 25% of the more conventional diacyl phosphoglyceride (II). The glyceryl ether phospholipid behaved in chromatography, solubility, and in certain other physical properties exactly the same as the diacyl phosphoglyceride and gave a comparable infrared spectrum. Interestingly, the infrared spectrum of the intact phospholipid did not show the absorption band at 9.0 μ expected for a glyceryl ether (Carter *et al.*, 1958), and it was evident that the deeply absorbing phosphate band in the 9.0–9.5 μ area obscured the ether band. The glyceryl ethers obtained from this phospholipid, however, showed a definite absorption at 9.0 μ. An example of the infrared spectrum of a representative glyceryl ether is shown in Figure 1.

B. *Isolation of Glyceryl Ether Phospholipid from Mixed Phospholipids.*—In this purification procedure the glyceryl ether phospholipid can be freed of a diacyl phospholipid component by selective deacylation in a chloroform–methanolic alkali medium with the formation of methyl esters, the monoalkyl, monoacyl glyceryl-

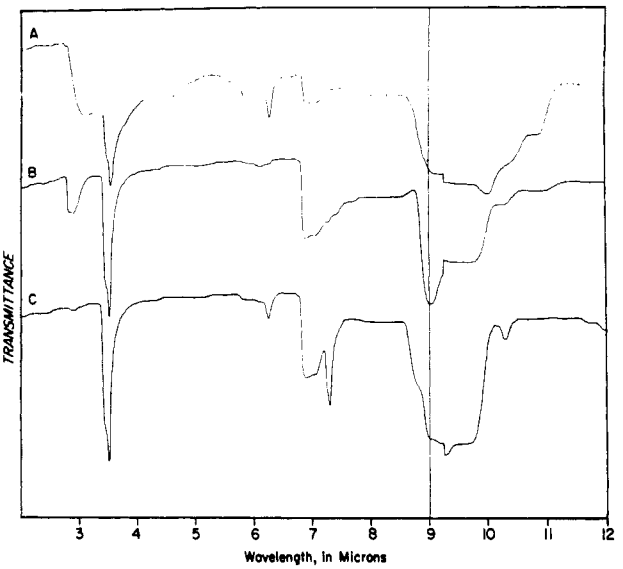


FIG. 1.—Infrared spectra of (A) glyceryl ether phosphoric acid, (B) D,L-1-*O*-(octadecyl)-glycerol (batyl alcohol) and (C) 2,3-*O*-isopropylidene-D,L-1-*O*-(octadecyl)-glycerol. Solvent, $CHCl_3$; cell length, 1 mm (Irtran cells).

phosphorylethanolamine (I) and a relatively small amount of a monoalkyl glycerylphosphorylethanolamine. The details of this procedure have been described previously (Hanahan and Watts, 1961). The yields of I, which was isolated by silicic-acid chromatography, have ranged from 80–90% of the expected value. The analytical data obtained on a phosphatidyl ethanolamine fraction from bovine erythrocyte and a purified glyceryl ether phospholipid from this same fraction are presented in Table I.

TABLE I

PURIFICATION OF GLYCERYL ETHER PHOSPHOLIPID BY SELECTIVE DEACYLATION TECHNIQUE

The "phosphatidyl ethanolamine" fraction of bovine erythrocytes was treated with a 0.5 N NaOH in methanol-chloroform mixture (10:1, v/v) for 2 minutes at 10°. After neutralization, the products, which were primarily methyl esters of long-chain fatty acids and the glyceryl ether phospholipid, I, were isolated by silicic-acid chromatography as described previously (Hanahan and Watts, 1961).

	"Phosphatidyl Ethanolamine" Fraction	
	Starting Material	After Partial Deacylation
P, per cent	3.90	3.96
N, per cent	1.79	1.87
N/P, molar ratio	1.00	1.04
Fatty acid ester/P, molar ratio	1.18	1.03
Iodine number	63.6	63.5
Plasmalogen/P, molar ratio	0.02	None detectable

In a typical experiment, the water soluble phosphorus, derived from diacyl phosphatidyl ethanolamine in this partial deacylation, was recovered in excellent yields and had the chemical properties of glyceryl-phosphorylethanolamine. As an example, 103 mg phosphorus as glyceryl ether–rich phosphoglyceride was subjected to partial deacylation. The chloroform-soluble fraction contained 78.6 mg phosphorus and the water soluble fraction contained 23.6 mg phosphorus for a total recovery of 102.2 mg. Analysis of the water-

soluble fraction showed no inorganic phosphorus, a nitrogen-phosphorus molar ratio, 1.05, and a periodate uptake of one mole per mole of phosphorus. On paper chromatography as well as on high voltage paper electrophoresis, only one phosphorus-containing spot, which migrated in a manner similar to glycerylphosphorylethanolamine, was found and only a trace amount of free ethanolamine was detected.

C. *Hydrolytic Cleavage.*—1. Alkaline. A 230-mg sample of purified glyceryl ether phospholipid (obtained as described in B above) containing 8.3 mg phosphorus and 4.0 mg nitrogen and having a fatty acid ester to phosphorus molar ratio 1.02, was dissolved in 2 ml diethyl ether, and 15 ml 1.0 N KOH in 90% ethanol was added. This mixture was refluxed for 1.5 hours, cooled, and 3 volumes of water were added. The resultant turbid mixture was extracted twice with 3 volumes of diethyl ether. The diethyl ether soluble fraction was washed 4 times with 0.1 volume of water and the water washings combined with the aqueous fraction from the initial extraction. The water-soluble fraction was acidified carefully with 6 N HCl and the resultant turbid mixture extracted twice with equal volumes of diethyl ether. This latter diethyl ether–soluble fraction, which was ninhydrin negative, was washed several times with 0.1 volume water, evaporated to dryness *in vacuo*, and dissolved in 20 ml absolute ethanol. A slight turbidity developed, and after standing overnight at 4° a small amount of insoluble material formed and was removed by filtration through Whatman No. 2 filter paper To the clear filtrate (A) a solution of 20% NaOH in absolute ethanol was added dropwise until precipitation was visually complete. The precipitate was recovered by centrifugation, and the supernatant (B) saved. The precipitate was washed several times with absolute ethanol, and after it was dried *in vacuo* at 25° a light tan powder, freely soluble in water, was obtained. In the acid form, it analyzed as follows: P, 7.22%; meq base consumed per meq phosphorus, 2.06; iodine number, 20.6 (on a comparative basis: batyl alcohol phosphate, P, 7.31%; meq base consumed per meq phosphorus, 2.00; iodine number, 0). It contained no nitrogen and showed no reaction with sodium periodate. The acid form of this compound also gave an infrared spectrum, as shown in Figure 1, with no carbonyl (e.g., carboxylic ester) absorption at 5.75–5.85 μ. It did possess a strong phosphate band at 9.25 μ but showed no ether absorption at 9.0 μ. This material was considered to be a glyceryl ether phosphoric acid. The yield of this acid was 96.5 mg (as disodium salt), and represented 42% of the starting material (theory, 57%).

The ethanol-soluble fraction B, which contained 0.2 mg phosphorus (approximately 2.5% of the starting material), was acidified and extracted with diethyl ether. The diethyl ether extract was washed well with water and dried over sodium sulfate. This latter fraction analyzed as follows: neutral equivalent, 294; iodine number, 143. On thin-layer chromatography and by infrared spectral examination, it contained only free fatty acid. Gas-liquid chromatographic analysis of the methyl esters prepared from this fraction gave the following values. *Major components:*[1] 18:1, 57 mole per cent; 18:2, 30 mole per cent; and 20:4, 13 mole per cent. *Minor components:* (trace amounts, with no more than 1% for the total), 14:0, 16:0, 16:1, 17:0, 18:0, 18:3. The calculated neutral equivalent of 286 was in reasonable agreement with the observed value of 294. Thus, only unsaturated fatty acids were present, presumably on the 2-position of the glyceryl ether phospholipid.

A different and equally satisfactory approach to separation of the free fatty acids and the glyceryl ether phosphoric acid from an alkaline hydrolysate has been used. In brief, the hydrolysate A (above) was acidified and then extracted with chloroform. This extract was placed on a silicic acid column in the same solvent and elution with chloroform continued until all the fatty acids were removed; subsequent elution with chloroform-methanol, 4:1 (v/v) removed the glyceryl ether phosphoric acid. Analyses on these fractions gave results similar to those cited above.

2. Acid. A sample of glyceryl ether phospholipid, which weighed 345 mg, contained 12.5 mg phosphorus, and had a fatty acid ester to phosphorus value of 0.98, was suspended in 15 ml of 3 N HCl and refluxed gently for 1.5 hours. The mixture was cooled, and then extracted with diethyl ether. The diethyl ether extract was washed well with water and both phases saved.

The diethyl ether soluble fraction was evaporated to dryness *in vacuo*, dissolved in absolute ethanol and treated with alkali in absolute ethanol as above (C, 1). The precipitate was washed with absolute ethanol, acidified and extracted with diethyl ether. The diethyl ether was washed well with water and dried over anhydrous sodium sulfate. The yield or material, based on phosphorus, was 7.27 mg or 60% of the expected value (additional phosphorus was found in the ethanol washes). Analysis showed that it contained 7.26% phosphorus, consumed 2.1 meq base per meq phosphorus, and had an iodine number of 25.8. An infrared spectrum of this material showed it to be identical to that obtained with the product of the alkaline cleavage experiments.

The water-soluble fraction from the acid hydrolysis contained over 95% of the added nitrogen (98% as free ethanolamine) and usually contained no more than 2% of the total added phosphorus. Significant amounts of inorganic phosphorus, however, were liberated on longer hydrolysis periods, e.g., 4–6 hours.

D. *Acetolysis.*—An expedient route to the isolation of the glyceryl ethers was through the acetolysis of the glyceryl ether–rich phosphatidyl ethanolamine fraction or the purified glyceryl ether phospholipid (II). Essentially, a modification of the technique of Bevan, *et al.* (1953) was employed. In a typical experiment, 135 mg of purified glyceryl ether phospholipid were subjected to acetolysis, and then saponification. The glyceryl ethers were isolated and dissolved in hexane (or hexane-ether, 9:1 v/v) and chromatographed on a 5-g silicic acid column. Any residual fatty acid (as the salt) was removed by elution with hexane-ether (3:1, v/v), then the glyceryl ethers were eluted with hexane-diethyl ether (1:3, v/v) or pure diethyl ether. This latter eluate, which weighed 48.5 mg, represented 36% of the starting material (calculated, 36%).

The glyceryl ether fraction assayed by periodate uptake for one vicinal glycol per mole of compound and had an iodine number of 19.6. On thin-layer chromatography (see below and Figure 2) and by its infrared spectrum (Figure 1A), this fraction contained only glyceryl ethers. Gas-liquid chromatographic analysis of this fraction from bovine erythrocytes gave the results presented in Table II.

[1] Abbreviations for chain length and unsaturation of fatty acids follow current convention. Thus, palmitic acid will be indicated as 16:0, oleic acid as 18:1, nonanoic acid as 9:0, etc. In certain instances, the term "alkyl" is used to describe the hydrocarbon residue attached in ether linkage to a glycerol unit. It does not fully express, however, the exact nature of this substituent, particularly with reference to the presence of any (olefinic) unsaturation.

TABLE II

COMPOSITION AND OPTICAL ACTIVITY OF GLYCERYL ETHERS FROM DOGFISH LIVER OIL AND BOVINE ERYTHROCYTES

The composition of the glyceryl ethers, in mole per cent, was determined from gas-liquid chromatography data. Optical rotation values were obtained on a Rudolph polarimeter, with the compounds in the solvent indicated in a 2-dm tube; samples were examined as the isopropylidene derivatives.

Chain Length Hydrocarbon Side Chain	"Selachyl Alcohol" I[a]	"Selachyl Alcohol" II[a]	Bovine Erythrocyte Glyceryl Ether Phospholipid	
			Sample A[e]	Sample B
15:0	—	—	—	tr
16:0	tr	tr	41.5	44.0
16:1	15.0	22.0	3.0	tr
16:2	—	—	—	6
17:0	tr	tr	2.8	3
18:0	tr	15.8	24.4	26
18:1	85.0	62.0	28.4	21
$[\alpha]^{23}_D$ in hexane	−17°[b]	Not run	−15.7°[c]	−16.2°[c]
in chloroform	− 8.1°[b]	−7.84°[d]	− 7.5°[c]	Not run

[a] These samples were obtained from dogfish liver oil (Western Chemical Industries, Ltd.). [b] Contained 34 mg/ml solvent. [c] Contained 29 mg/ml solvent. [d] Contained 11.5 mg/ml solvent. [e] After hydrogenation over PtO_2 catalyst, the following distribution in mole per cent was obtained: 16:0, 38; 17:0, 5; 18:0, 54; unknown, 3.

Chemical Behavior of the Glyceryl Ethers and Derivatives

A. *Preparation and Properties of 2, 3-O-Isopropylidene Glyceryl Ethers.*—The acetonation of glyceryl ethers could be effected at room temperature through a rapid perchloric acid–catalyzed reaction. This procedure was suggested from the observations of Fried *et al.* (1958), who reported on the acetonation of certain cortisol derivatives.

In a typical preparation 399 mg of D,L batyl alcohol, (1-*O*-[octadecyl]-glycerol) were suspended in 10 ml of acetone, and 0.05 ml of 12 N perchloric acid was added, with stirring, to this suspension. There was an almost immediate reaction and a clear solution resulted within 10 minutes. At the end of 20 minutes water was added until turbidity resulted, and the mixture was extracted with 4 volumes of diethyl ether. The diethyl ether extract was washed with water until free from traces of acid, evaporated to dryness *in vacuo*, and dissolved in hexane. This clear solution was then placed on a 50-g silicic acid column and the major component, which weighed 410 mg, was eluted with hexane-ether (9:1, v/v). This eluate yielded a component which gave a single spot on thin-layer chromatography, and was soluble in chloroform, hexane, and in acetone-water mixtures (85% or greater). It could be crystallized from an acetone-water mixture and upon filtration 350 mg of white crystals was obtained, mp 31–32° (Fisher-Johns melting-point block). Reported for 2,3-*O*-isopropylidene-D,L-,-1-*O*-(*n*-octadecyl)-glycerol, 32–33° (Baer and Fischer, 1947). This sample analyzed as follows: Calculated for $C_{24}H_{48}O_3$ (384): C, 75.0; H, 12.5. Found: C, 75.3; H, 12.5. The infrared spectra of 2,3-*O*-isopropylidene-D,L-1-*O*-(octadecyl) glycerol and D,L-1-*O*-(octadecyl)-glycerol (batyl alcohol) are recorded in Figure 1, and the obvious differences between these two substances are evident. A further differentiation was achieved by their behavior on thin-layer chromatography, where the isopropylidene derivatives migrated in a manner similar to triglycerides and the free glyceryl ethers migrated much more slowly in the solvent systems used here (Figure 2).

In a general procedure designed for preparation of isopropylidene glyceryl ethers the scheme was similar to that described above except that a 45-minute reaction period was allowed and no crystallization was attempted. In certain instances, a preparation would show, by thin-layer chromatography, the presence of a small amount of unreacted glyceryl ether. This latter contaminant could be removed by chromatography of the sample on silicic acid, wherein hexane–diethyl ether (9:1, v/v) would elute the isopropylidene component and 100% diethyl ether would remove any unreacted glyceryl ether. The acetonation procedure and subsequent silicic acid treatment, if necessary, resulted in 95–97% recovery of starting material (based on glyceryl ether content).

The isopropylidene glyceryl ethers were usually dissolved in *n*-hexane for gas-liquid chromatography (or for storage at 4°), or in methanol for conversion to the acetoxymercurimethoxy derivative (see B, 2 below). Although the naturally occurring glyceryl ethers should possess optical activity, the specific absorption values are very low. The isopropylidene glyceryl ethers however, possess significant optical activity and as measured in *n*-hexane and in $CHCl_3$, exhibit values close to those expected for an L-1-glyceryl ether derivative (Baer and Fischer, 1947). The optical activity of isopropylidene derivatives of naturally occurring glyceryl ethers from dogfish liver oil and bovine erythrocytes is presented in Table II.

B. *Separation Studies.*—1. Gas-liquid chromatography. The glyceryl ethers, per se, could not be eluted from any of the phases described below, and apparently were tightly adsorbed to the column material. On the other hand, the isopropylidene glyceryl ethers were easily chromatographed on a commonly available polar phase. An examination of many different phases and supporting media (such as neopentyl glycol succinate, SE-30, Apiezon L, etc.) showed each to possess varying ability to separate the isopropylidene derivatives by chain length only and not by (olefinic) unsaturation. The only completely satisfactory phase and support was 15% ethylene glycol succinate on Anakrom AB, 60/70 mesh. Under conditions of a column temperature of 175° and an inlet pressure of 16 psi of argon a satisfactory separation of the isopropylidene glyceryl ethers was achieved within 60 minutes. A chromatogram of a typical run in which these derivatives were applied in *n*-hexane is presented in Figure 3. In this system, as with the fatty acid methyl esters on a similar polar phase, the unsaturates follow the analogous saturates and the position of any component of an unknown was an indication of its probable chain length and unsaturation. As an added check on the chain length of the unsaturates, samples containing these components were hydrogenated at atmospheric pressure over platinum oxide and then

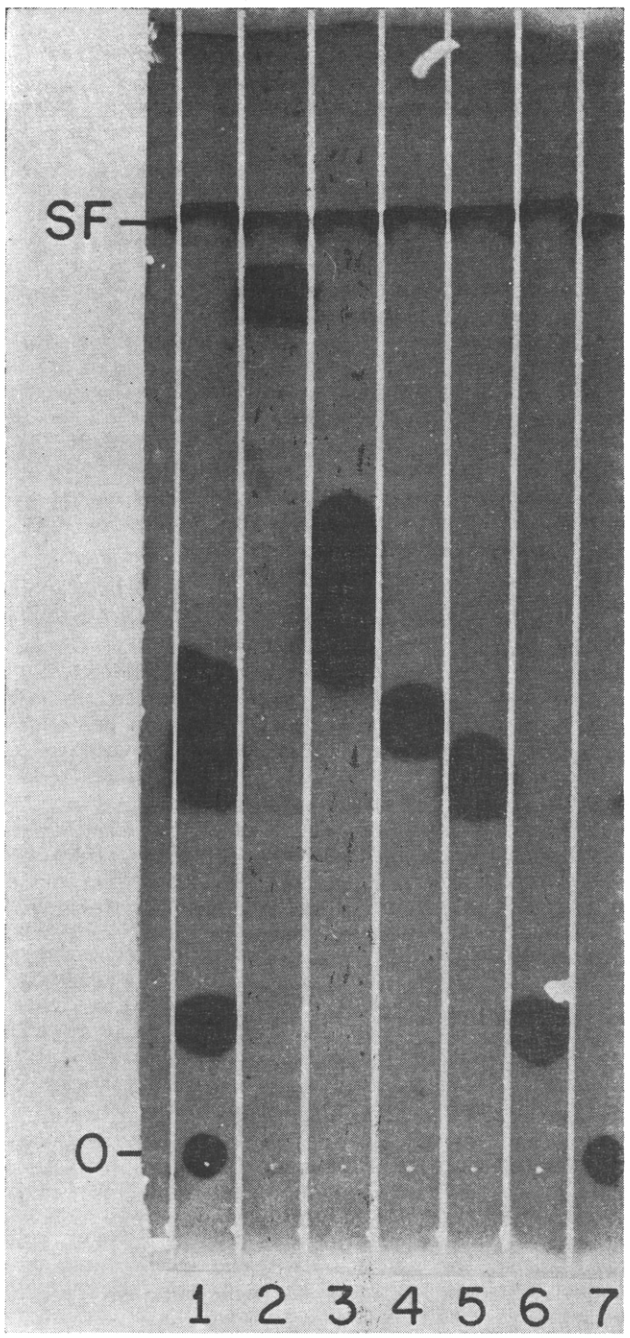


FIG. 2.—Thin-layer chromatogram of (1) mixture of 1-*O*-(octadecenyl)-glycerol (selachyl alcohol) (at origin), cholesterol, palmitic acid, and triolein; (2) methyl palmitate; (3) 2,3-isopropylidene-1-*O*-(octadecenyl)-glycerol; (4) triolein; (5) palmitic acid; (6) cholesterol; (7) 1-*O*-(octadecenyl)-glycerol (selachyl alcohol). O, origin; SF, solvent front. Adsorbent, silica gel G. Solvent system, petroleum ether–diethyl ether–acetic acid, 90:10:1 v/v). The off-center marking noted approximately one-half way up lane 7 is an artifact. The individual spots were brought up by the sulfuric acid charring reaction.

re-examined by gas-liquid chromatography. The isopropylidene derivatives could be hydrogenated as such without any evidence of degradation. Thus, after hydrogenation, the peak commonly associated with the 18:1 isopropylidene glyceryl ether, as an example, would disappear and a concomitant and expected increase in the 18:0 peak area would occur. In a few instances a component(s), with a retention time similar to the 18:1 isopropylidene derivative, remained after hydrogenation, but no further identification of this small

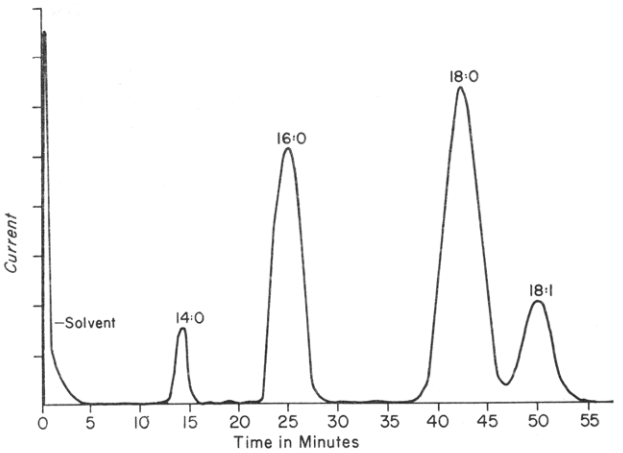


FIG. 3.—Gas-liquid chromatographic pattern of 2,3-isopropylidene glyceryl ethers. Conditions for chromatography were as follows: 15% ethylene glycol succinate on Anakrom AB 60/70 mesh; column temperature, 174°; inlet pressure, 16 psi.

component was made. These separations were reproducible, and there was no evidence of any degradation of the isopropylidene glyceryl ethers on the column. The ionization detector responded in a linear manner to various loadings of each of the major classes of glyceryl ether derivatives and each class of compounds (chain length, unsaturation) gave a comparable response.

Although the diacetates of the glyceryl ethers were easily prepared by the method of Blomstrand and Gurtler (1959) and could be chromatographed on an ethylene glycol succinate column, the separation of the saturates from the unsaturates was not as satisfactory as desired (separation factor, 1.04), and, further, a decided instability of these derivatives on this polar phase and on storage was evident.

2. Column chromatography of mercuric acetate adducts of 2,3-*O*-isopropylidene glyceryl ethers. An excellent route to the separation of the isopropylidene glyceryl ethers (but not the glyceryl ethers) differing in content of olefinic double bond, is through the acetoxymercurimethoxy derivatives (mercuric acetate adducts). In a procedure similar to that described for separation of fatty acids by Kuemmel (1962), the glyceryl ether derivatives were converted in high yields to the acetoxymercurimethoxy derivatives with subsequent chromatography on aluminum oxide. The completeness of the separation was verified by gas-liquid chromatography.

In all instances, the glyceryl ethers were converted first to the isopropylidene derivatives as detailed above, and in a typical example the following sequence was followed for adduct formation. A synthetic sample of glyceryl ether derivatives weighing 340 mg and containing 68% "saturates" and 32% "unsaturates" (as determined by gas-liquid chromatography), was dissolved in 15 ml methanol, and 1 g mercuric acetate (Baker analyzed, 99%) was added. The mixture was refluxed for 45 minutes, cooled, and evaporated to dryness. The residue was treated with petroleum ether and the soluble fraction, which contained the mercuric acetate adducts of the "unsaturates" as well as unreacted "saturates," was subjected to chromatography on aluminum oxide.

The aluminum oxide column was prepared as follows: 33 g aluminum oxide, Brockman Grade 1, *p*H 7.0 (Bio-Rad Laboratories) was added to 3.1 ml distilled water in a 250-ml Erlenmeyer flask, and the mixture was shaken until homogeneous. The reaction is exo-

thermic and slight warming of the flask is noted. This preparation is then poured through a long-stem funnel into a glass column containing petroleum ether. The final dimensions of the packed aluminum oxide column were 17 mm × 155 mm. The sample in petroleum ether was placed, with suitable washing, on the column, and elution with petroleum ether was continued at a flow rate of 5–10 ml/minute. The progress of the elution was followed qualitatively by charring of a sample of the eluate on glass-fiber paper with sulfuric acid. A total of 550 ml of petroleum ether was sufficient for elution of all the "saturates." Subsequently, elution with diethyl ether–methanol (95:5, v/v) was initiated and continued until no further elution of "monounsaturates" was indicated by the charring reaction. In this instance, a total of 220 ml of 5% methanol in ether was required. Finally, the column was eluted with methanol-HCl (12 N, 10:1, v/v), for removal of any "polyunsaturates." The glyceryl ethers were regenerated from the latter two eluates by mixing with 10% HCl in methanol for 30–45 minutes at room temperature. Extraction with diethyl ether gave a gray interfacial fluff, which was removed by filtration through Whatman No. 1 filter paper, and the free glyceryl ethers were obtained from the diethyl ether–soluble fraction and then converted to the acetonides by the procedure described above. The petroleum ether eluate contained only the isopropylidene derivatives with no indication of any degradation of this derivative on aluminum oxide.

The results of this separation and the recoveries are shown in Table III. Several other preparations with different levels of "unsaturates" and "saturates" have been separated equally well.

3. Column chromatography of glyceryl ethers. Although several different absorbents, such as factice (Hirsch, 1961), polyethylene granules, charcoal, and silicic acid were investigated for their suitability in the fractionation of glyceryl ethers, only silicic acid has proven satisfactory in initial studies. Attempts at the separation of the glyceryl ether acetonides on "wetted" silicic acid (Sahasrabudhe and Chapman, 1961) proved unsuccessful with no significant purification indicated.

In the most satisfactory procedure, glyceryl ethers were subjected to chromatography on silicic acid columns with a hexane–diethyl ether solvent system, and subsequently the eluates were treated to low-temperature fractionation. Specifically, the more unsaturated glyceryl ethers were eluted first from silicic acid and were followed, as expected, by the more saturated forms. As an example, 10 g commercial selachyl alcohol was placed in hexane on a column containing 420 g dried silicic acid in hexane, with a height-to-diameter ratio of 15. The initial solvent, hexane–diethyl ether (7:3, v/v), was allowed to run slowly without pressure overnight, during which time nearly 2,000 ml of eluate was collected. Inasmuch as there was a negative charring reaction and a negative Lieberman-Burchard reaction at this point, the solvent was changed to hexane–diethyl ether (1:1, v/v) and the column was then attached to a fraction collector. The collection time for each 25 ml of eluate was 3.5 minutes. An early, fast-running component appeared, which gave a positive Lieberman-Burchard reaction, and was followed by a slower-moving component (negative Lieberman-Burchard reaction). Essentially, 2–3 liters of eluent were collected with this particular solvent. At this point elution with hexane–diethyl ether (2:3, v/v) was initiated and was continued for 4 hours. As the final solvent, 100% ether was applied and the elution was continued until no further organic material was removed. The various fractions were tested individually on reversed-phase thin-layer chromatography (Figure 4), and on a qualitative basis those with similar composition were combined.

Next, the eluates from this column were divided into five main fractions (labeled I through V) and then submitted to low-temperature fractionation. Each of the three 50%-ether-in-hexane eluates was evaporated *in vacuo* to dryness and dissolved in 10 ml *n*-hexane and placed at −20° (no precipitate at +4°) overnight. The crop of white crystals was collected by filtration at −20° and the yellow-colored filtrate saved. The crystals were dissolved in 10 ml *n*-hexane and the crystallization process was repeated twice. The crystals collected from fractions I, II, and III, and the respective combined filtrates were assayed by gas-liquid chromatography and the results are presented in Table IV. The 60%-diethyl-ether-in-hexane eluate was treated in a manner similar to the above samples, except that an initial treatment at +4° yielded a small amount of insoluble, white crystals, labeled IV (insoluble at +4°), and a light yellow filtrate. This latter filtrate was then cooled to −20° and stored overnight. The white crystals were removed by filtration at −20°, and these crystals, labeled IV (insoluble −20°), and the filtrate, labeled IV (soluble at −20°), were collected and saved. These various fractions were then analyzed as the isopropylidene derivatives by gas-liquid chromatography. The results of these assays are given in Table IV. A procedure similar to that described above for the 60% diethyl-ether-eluate was applied to the 100%-diethyl-ether-eluate. The data on the composition of the fractions from this latter eluate, labeled V (insoluble at 4°), V (insoluble at −20°) and V (soluble at −20°) are recorded in Table IV.

C. *Position of Olefinic Bond in "Selachyl Alcohol."* — An unsaturated glyceryl ether fraction, which was isolated by the technique described above (C,3) and contained over 99% of an 18:1 component as assayed

TABLE III

SEPARATION OF 2,3-*O*-ISOPROPYLIDENE GLYCERYL ETHERS BY CHROMATOGRAPHY OF ACETOXYMERCURIMETHOXY DERIVATIVE ON ALUMINUM OXIDE

Here 340 mg 2,3-*O*-isopropylidene glyceryl ethers, treated with mercuric acetate as described in text, was placed on a 33-g aluminum oxide column. Elution was carried out with the three listed solvents in sequence. The glyceryl ethers sample was a synthetic one, which was prepared from commercial samples of glyceryl ethers.

Chain Length, Hydrocarbon Side Chain	Mole Per Cent in			
	Starting Material	Petroleum Ether Eluate	Diethyl Ether–Methanol (95:5, v/v eluate)	Methanol–12 N HCl, (10:1, v/v eluate)
14:0	0.7	1.3	—	—
14:1	tr	tr	—	—
15:0	tr	0.6	—	—
16:0	27.8	34.6	—	—
16:1	3.3	—	13.5	15.7
17:0	tr	1.9	—	—
unk.	tr	tr	1.1	—
17:1	tr	—	2.3	tr
18:0	41.5	57.7	—	—
18:1	26.6	—	82.0	84.5
19:0	tr	tr	—	—
19:1	tr	—	—	tr
20:0	tr	2.7	—	—
Yield, in mg.	340	229	98	18
Per cent of theory	100	67	28	5

TABLE IV

PARTIAL FRACTIONATION OF COMMERCIAL SELACHYL ALCOHOL ON SILICIC ACID

Ten commercial selachyl alcohol was chromatographed on silicic acid and subsequently subjected to low-temperature fractionation as described in the text. The solvents were hexane (H) and diethyl ether (E).

	I		II		III		IV			V		
Eluting Solvent (in v/v)	H-E 1:1		H-E 1:1		H-E 1:1		H-E 2:3			E (100%)		
Volume Collected (in ml)	900		1000		1500		2200			1400		
	Insoluble (−20°)	Soluble (−20°)	Insoluble (−20°)	Soluble (−20°)	Insoluble (−20°)	Soluble (−20°)	Insoluble (+4°)	Insoluble (−20°)	Soluble (−20°)	Insoluble (+4°)	Insoluble (−20°)	Soluble (−20°)
Weight (in mg)	950	89	2175	79	2225	145	58	409	1612	45	102	562
Iodine number	74.2	Not run	71.5	Not run	69.8	Not run	Not run	Not run	78.5	Not run	Not run	102.5
Chain length, hydrocarbon side chain												
14:0	tr	tr					15.2	5.2	1.5	61.0	23.1	2.1
14:1							tr	1.2	tr		tr	5.7
15:0		2.4			tr	1.5	4.9	1.4	1.6	6.6	tr	2.7
15:1				tr			tr	0.8			tr	
16:0		2.8	2.6	3.8	8.3	tr	58.2	11.0	1.7	21.5	11.3	
16:1		2.8		5.5	5.0	20.5	5.1	18.5	39.7	7.2	36.7	49.0
17:0						tr						
17:1		tr		tr	2.6	6.8		3.1	2.6		tr	5.4
18:0							tr				tr	
18:1	100	64.4	97.5	80.8	84.1	65.4	16.6	52.3	50.5	3.7	25.8	31.5
19:1				tr		5.8		2.3	2.4			
20:1	tr	18.8		tr				1.4				3.3

by gas-liquid chromatography, was subjected to permanganate-periodate oxidation as described below. This procedure was essentially a modification of the method of von Rudloff (1956).

The 18:1 sample, which weighed 880 mg and was a colorless oil at room temperature, was dissolved in 250 ml of 5-butanol and to this solution was added a mixture of 4 g sodium periodate, 150 mg potassium permanganate, and 1.5 g potassium carbonate in 500 ml water. This solution was then placed on a shaker and mixed gently for 6 hours. At this time, sodium metabisulfite was added cautiously to stop the reaction, approximately 2 g of KOH was added to insure an alkaline medium, and the clear colorless solution was then placed at 4° overnight. A small precipitate of MnO_2 formed and was removed by filtration through Whatman No. 1 filter paper. The clear colorless filtrate was evaporated under reduced pressure in a 50° bath on a rotary evaporator until foaming commenced. Then 95% ethanol was added until heavy precipitation occurred, and after 3 to 4 hours at room temperature the precipitate (inorganic salts) was removed by filtration through Whatman No. 1 filter paper. The clear filtrate was evaporated under a stream of nitrogen to an approximate volume of 250 ml. This clear colorless solution was then acidified with 12 N HCl and a light-yellow turbid mixture resulted. It was extracted twice with 20 vol of diethyl ether and the ether extract was washed 8 times with 0.1 vol of distilled water. A clear colorless diethyl ether phase and a highly colored water phase resulted.

The diethyl ether phase was dried over anhydrous sodium sulphate and then evaporated under nitrogen or *in vacuo* (in either case similar results were obtained) to dryness. A white amorphous residue was obtained. This was dissolved in 5 ml acetone, and 30 ml petroleum ether was added with stirring. A turbidity developed and an oil (A) settled out. The opalescent supernatant (B) was removed and placed at 4° overnight. (A) was dissolved in 5 ml diethyl ether, and to this solution was added 35 ml petroleum ether (30–60°) and the opalescent solution was placed at 4° overnight. In both fractions A and B, crystals formed and were recovered by centrifugation at 4° for 20 minutes at 2,000 rpm. The supernatants, which were labeled *monocarboxylic acid*, were combined and saved, and the crystals were washed twice with 20 ml petroleum ether at 4°.

The above crystals were dried at room temperature under nitrogen and were labeled the *dicarboxylic acid*. The yield was 300 mg (50% of theory), melting point (Thomas-Hoover melting point apparatus) 81.5–82.0° (heating rate, 10°/minute to 70°, then 3°/minute). It analyzed as follows: $C_{11}H_{20}O_5$ (232.0); theory: C, 57.3; H, 8.62; found: C, 57.2; H, 8.5. Neutral equivalent: theory, 116; found, 120. An infrared pattern of this dicarboxylic acid is shown in Figure 5, and behavior of its methyl ester derivative on gas-liquid chromatography is illustrated in Figure 6. A thin-layer chromatograph of the free acid is presented in Figure 7. On the basis of this information, this acid was considered to be 9-(α-carboxymethoxy)-nonanoic acid.

The *monocarboxylic acid* fraction, which was a mixture of monocarboxylic acid and some dicarboxylic acid, was placed on a column of silicic acid in *n*-hexane. A solvent mixture of hexane–diethyl ether (4:1 v/v) removed the monocarboxylic acid, whereas a mixture of hexane–diethyl ether (1:3 v/v) eluted the dicarboxylic acid. Only trace amounts of material were eluted with 100% diethyl ether. The hexane–diethyl ether (4:1) eluate was evaporated to dryness under nitrogen

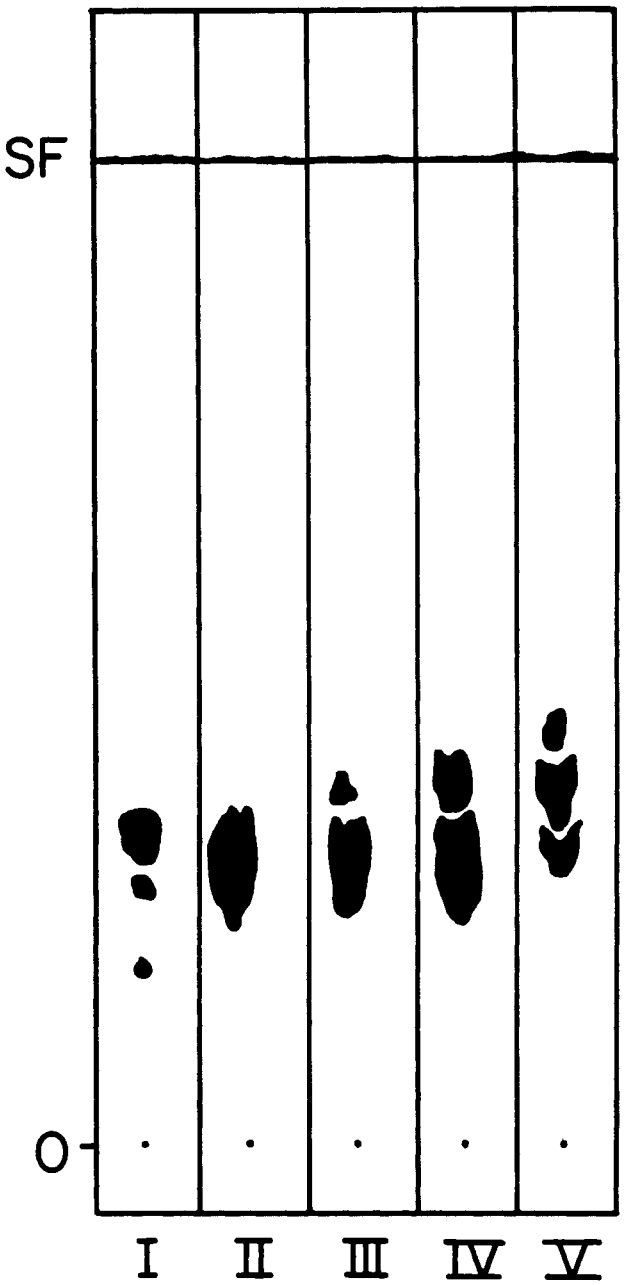


FIG. 4.—Behavior of glyceryl ethers on reversed-phase thin-layer chromatography. These samples were obtained from the silicic-acid chromatography and low-temperature fractionation of glyceryl ethers and are the same as those listed in Table IV. The various fractions are: I, soluble at −20°; II, insoluble at −20°; III, insoluble at −20°; IV, soluble at 4°; V, soluble at 4°. Thin-layer chromatographic plates of silica gel G were silane treated as described in text; solvent system, acetone-water, 65:35 (v/v). The individual spots were brought up by the sulfuric acid-charring reaction.

at room temperature and a clear colorless oil, which weighed 250 mg (60% of theory), was obtained. This material analyzed as follows: neutral equivalent, 160; calculated for nonanoic acid, 158. Its infrared spectrum is presented in Figure 5 and is very similar to that for known nonanoic and octanoic acid. Only one component was evident on gas-liquid chromatography (Figure 6) and had the same retention time as pure methyl nonanoate. In addition, as shown in Figure 7, only one component was evident on thin-layer chromatography of this sample. It was evident that this monocarboxylic acid was nonanoic acid. The

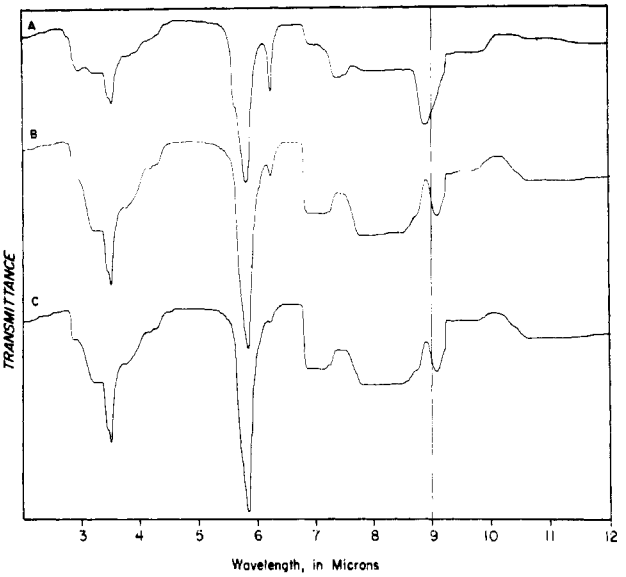


FIG. 5.—Infrared spectra of the dicarboxylic acid (A) and monocarboxylic acid (B) fractions obtained from periodate-permanganate oxidation of an 18:1 glyceryl ether. The spectrum of octanoic acid (C) is included for comparison. Solvent, $CHCl_3$; cell length, 1 mm (Irtran cells).

hexane–diethyl ether (1:3, v/v) eluate contained only dicarboxylic acid, which had characteristics the same as those described above. The yield was 75 mg, and, combined with the yield of dicarboxylic acid indicated above, gave a combined total of 375 mg, or 75% of theory.

In other experiments with the same glyceryl ether fraction, only nonanoic acid and the ether dicarboxylic acid were obtained in yields varying from 40–65% and 50–60% theory, respectively. In a sample of unsaturated glyceryl ether, which assayed as 80 mole per cent of an 18:1 component and 20% of a 16:1 component, the monocarboxylic acid fraction which was obtained in 50% yield and possessed a neutral equivalent of 161, assayed on gas-liquid chromatography of its methyl esters as follows (in mole per cent): 7:0, 17; 8:0, 3; 9:0, 80. Thus, only slight overoxidation occurred in this reaction. The dicarboxylic acid, which was isolated in 60% yield, had exactly the same properties as that described above, and was considered also to be 9-(α-carboxymethoxy)-nonanoic acid.

The same procedure essentially as described above was used for establishing the position of the olefinic bond in the 18:1 glyceryl ether fractions of bovine erythrocytes. Inasmuch as the quantities of material were small (approximately 100 mg per reaction) the products were assayed by thin-layer chromatography, gas-liquid chromatography, and infrared spectrophotometry. The major monocarboxylic acid was nonanoic acid, which comprised over 98 mole per cent of this fraction with the remainder as an 8:0 acid. The ether containing dicarboxylic acid contained over 85 mole per cent 9-(α-carboxymethoxy)-nonanoic acid with two other components, both as yet unidentified, but one of which may be an overoxidation product.

DISCUSSION

This investigation proves conclusively that a glyceryl ether phospholipid, I, with one mole of a long-chain fatty ether group and one mole of a long-chain fatty acyl group on a glyceryl phosphoryl ethanolamine, is present in bovine erythrocytes. These observations would substantiate the suspicions of Carter *et al.*

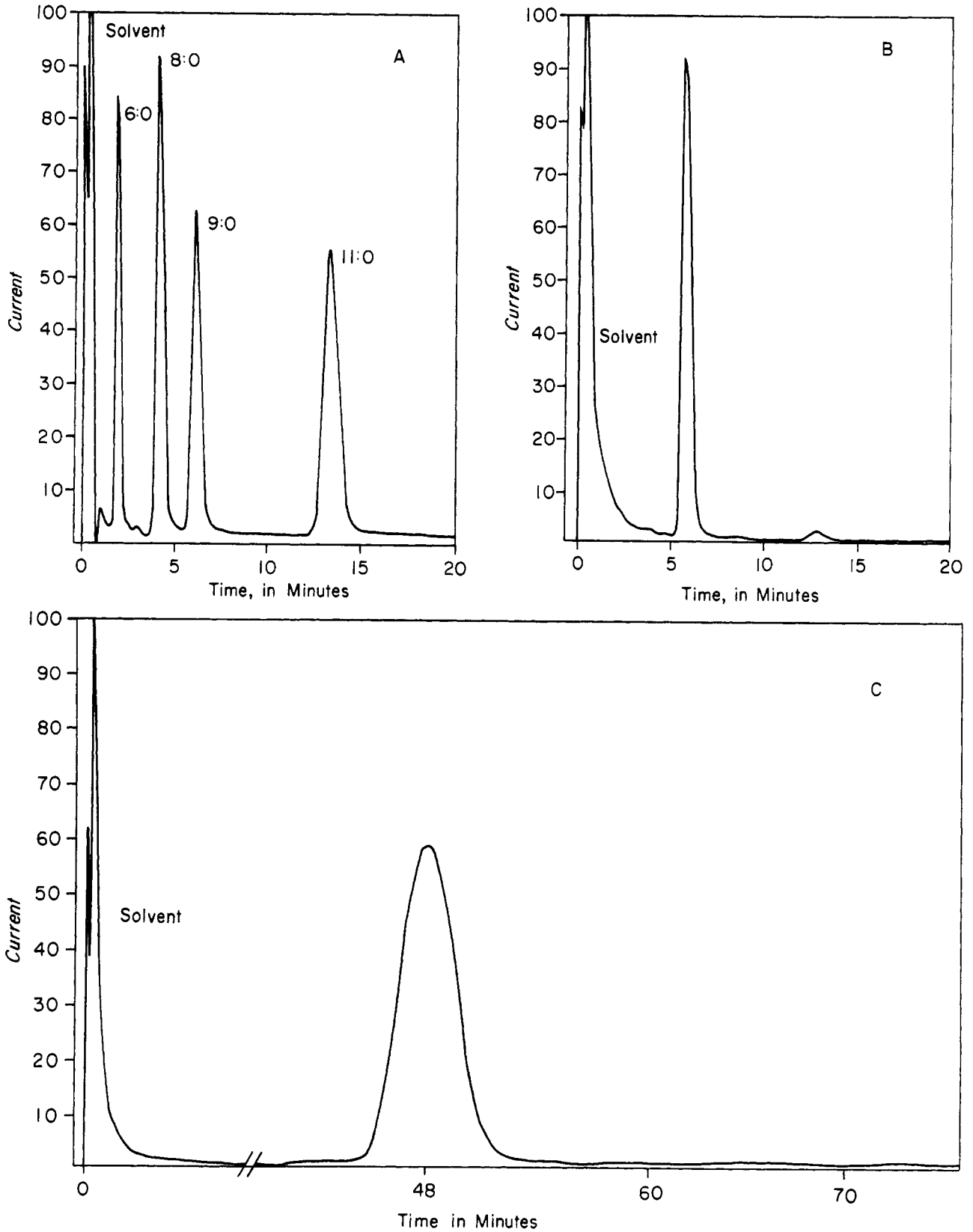


FIG. 6.—Gas-liquid chromatographic patterns of the methyl esters of the monocarboxylic acid (B) and dicarboxylic acid (C) fractions obtained from periodate-permanganate oxidation of an 18:1 glyceryl ether. (Fraction I [Insoluble at −20°] Table IV). The pattern presented in (A) represents a standard mixture of the following fatty acid methyl esters: 6:0, 8:0, 9:0, and 11:0. In each instance the chromatographic separation was run on 15% ethylene glycol succinate on Anakrom AB, 60/70 mesh; column temperature, 140° in (A) and (B), and 174° in (C); inlet pressure, 6-8 psi and 16 psi, respectively.

(1958) who isolated a fatty acyl-free glyceryl ether phospholipid from egg yolk, but surmised that their isolation procedure may have caused a release of any fatty acid esterified to this molecule. I, as obtained from bovine erythrocytes, was accompanied by a diacyl phospholipid and could be freed of this latter component through the use of a selective deacylation (methanolysis) procedure, in which the attack on the diacyl de-

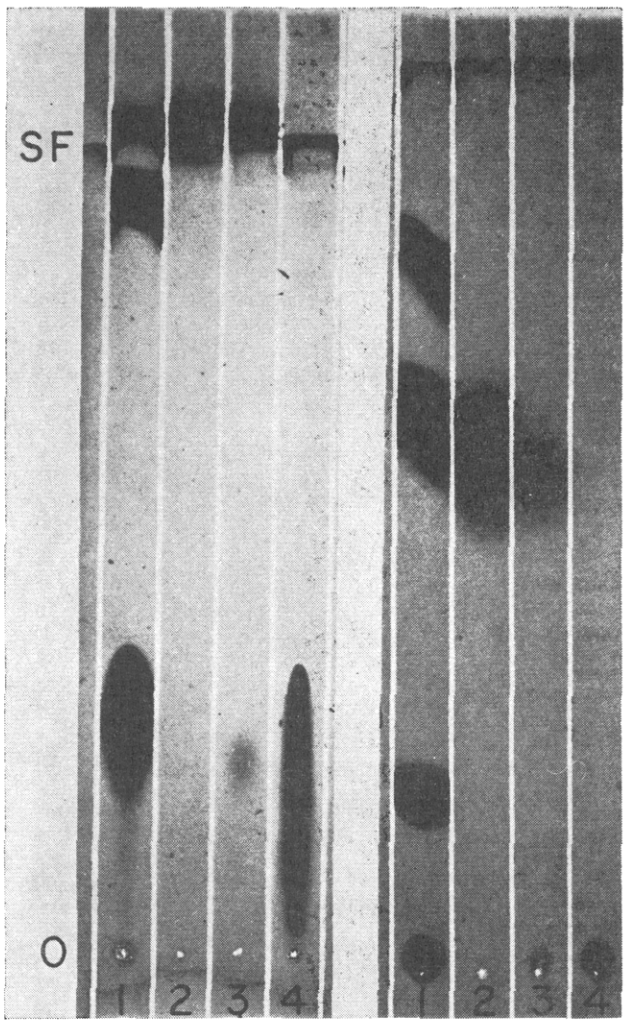


FIG. 7.—Thin-layer chromatography on silica gel G of products of periodate-permanganate oxidation of pure selachyl alcohol (1-*O*-[Octadecenyl]-glycerol). The following solvent systems were used: chromatogram on left, petroleum ether–diethyl ether–glacial acetic acid, 30:70:1 (v/v); chromatogram on right, petroleum ether–diethyl ether–glacial acid, 90:10:1 (v/v). The individual lanes contained: (1) standard mixture (in ascending order), selachyl alcohol, cholesterol, palmitic acid, and triolein; (2) pure nonanoic acid; (3) monocarboxylic acid from oxidation reaction; (4) dicarboxylic acid from oxidation reaction.

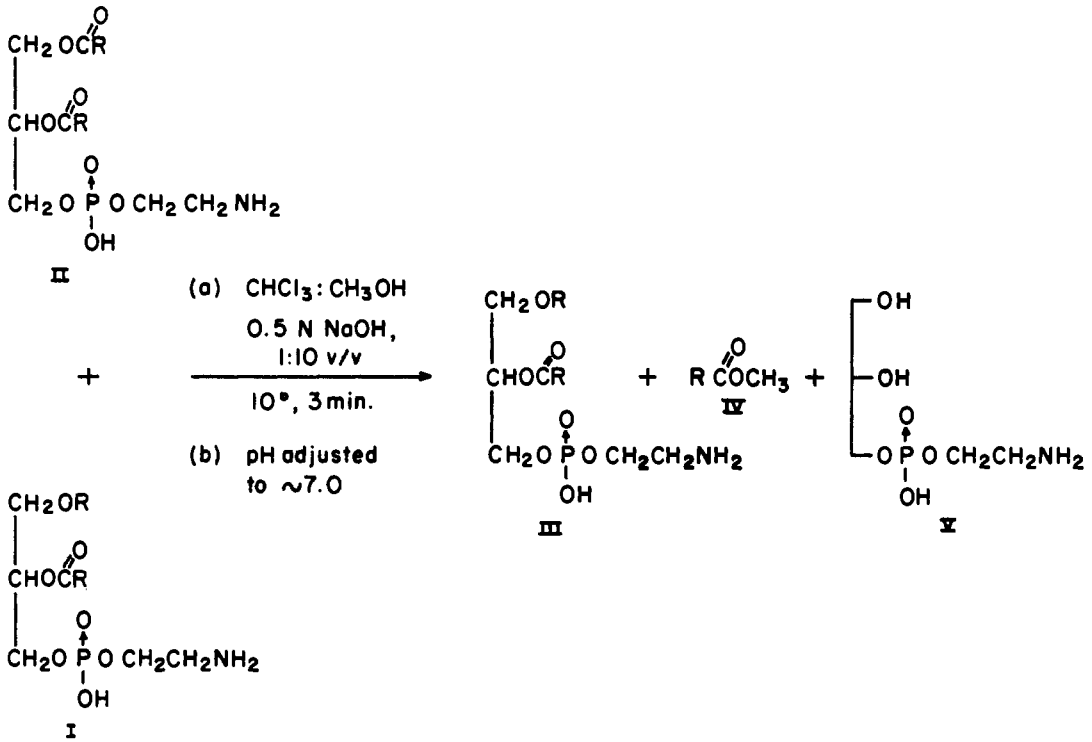
rivative by alkali in methanol is apparently faster than on the monoacyl glyceryl ether component (Figure 8). The desired product, I, could be isolated in 85–90% yield by silicic-acid chromatography. Subsequently, the structure of this purified material was more firmly established through a study of hydrolysis and acetolysis products, wherein liberated glyceryl ether phosphoric acid and glyceryl ethers, respectively, were examined in detail. An examination of the fatty acids released by alkaline or acid hydrolysis or by methanolysis of I all showed that only unsaturated fatty acids were present in this purified glyceryl ether phospholipid. Although not proven in this study, it is assumed that these fatty acids occupy only the 2-position in the original molecule.

A new and reliable technique is described for the isolation and identification of glyceryl ethers in naturally occurring materials. Thus, the glyceryl ethers can be obtained in a nearly quantitative manner from the glyceryl ether phospholipid by acetolysis and subsequent hydrolysis and assay of the material as the isopropylidene derivative. This latter compound is an excellent derivative for analytical purposes and also allows a facile recovery of the glyceryl ether in unchanged stereochemical form by gentle acid treatment. This procedure is in contradistinction to the fine technique of Hallgren and Larsson (1962), wherein dimethoxy derivatives were used for identification by gas-liquid chromatography and did not allow recovery of the ether. Interestingly, McInnes *et al.* (1960), had described the acetonation of monoglycerides, with subsequent gas-liquid chromatography, but no further study of this system has been reported. The classes of glyceryl ethers, e.g., the saturates, monounsaturates, and polyunsaturates, were separated through the mercuric acetate adduct and as such this latter technique allowed a highly suitable route to isolation of these particular components. It has been shown that selachyl alcohol, a monounsaturated 18-carbon glyceryl ether, can be obtained in high yields and purity by selective elution from a silicic acid column.

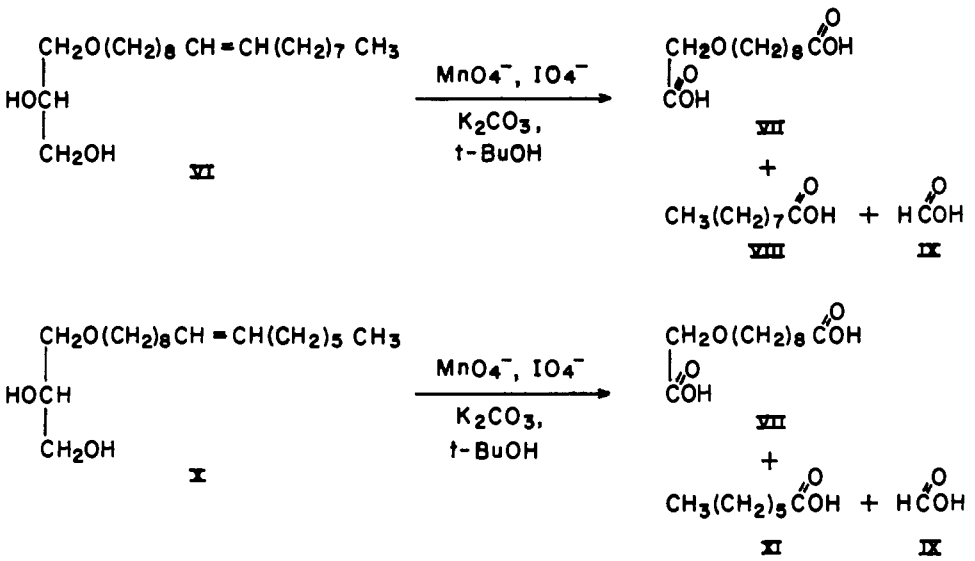
There is no doubt from the results presented in this investigation that the naturally occurring glyceryl ethers possess optical asymmetry and have an L-1-configuration. The ideal derivative for study of the optical properties of these compounds was the isopropylidene one which showed a greater optical rotation than did the free glyceryl ether. This was in agreement with the observations of Baer and Fischer (1947). Although it was not possible to ascertain the optical activity of the intact ether-containing phospholipid I, it is expected that it would possess optical asymmetry and would have an L configuration.

It is interesting to note that the glyceryl ethers present in this phospholipid possess certain characteristics in common with the plasmalogen or vinyl ether–containing phospholipids. Perhaps the most provocative characteristic is that of the chain length and unsaturation of the hydrocarbon attached in ether linkage. In the case of the glyceryl ethers, these hydrocarbon chains are predominantly saturates of 16- and 18-carbon chain lengths, e.g., 70% saturates and 30% unsaturates, with the latter as a single olefinic bond type. A more striking observation has been on the composition of bovine heart plasmalogen where the hydrocarbon unit attached to the vinyl ether is almost completely saturated (Gottfried and Rapport, 1962). Further, as reported in this current study, an examination of the fatty acids (present in ester form) in a highly purified glyceryl ether phospholipid showed only unsaturates to be present. Similarly, Gottfried, and Rapport (1962) reported that the fatty acids of a highly purified vinyl ether phospholipid (plasmalogen) were also only of the unsaturated type. Thus, on this basis alone it might be expected that these two ether-containing phospholipids may bear a close biosynthetic and/or metabolic interrelation.

Although it has been tacitly assumed in the literature that naturally occurring selachyl alcohol possesses an olefinic bond at the 9:10 position in the hydrocarbon side chain, no adequate proof of this proposal had been advanced. Toyama (1924) reported isolation of nonanoic acid, in rather low yield, from permanganate oxidation of selachyl acetate. The nature of the remainder of the products from this oxidation was not explored by this author. Accordingly, it was important to study in some detail the products of oxidative scission of this glyceryl ether. Although ozonolysis plus reductive cleavage of the ozonide allowed a possible means of proof, it was not as reliable as desired and most attention was directed to a periodate-permanganate oxidation system. The final route of choice is depicted in Figure 9. Inasmuch as the two most common



8. Scheme for selective deacylation of ethanolamine-containing phosphoglycerides of bovine erythrocytes. See text for additional details.



VI 1-O-Octadecenyl glyceral (Selachyl alcohol)
VII 9-(α carboxymethoxy)-Nonanoic acid
VIII Nonanoic acid
IX Formic acid
X 1-O-Hexadecenyl glycerol
XI Heptanoic acid

FIG. 9.—Scheme for the periodate-permanganate oxidation of 1-*O*-(octadecenyl)-glycerol (selachyl alcohol) and 1-*O*-(hexadecenyl)-glycerol. See text for further details.

unsaturates are the 16:1 and 18:1 types, the expected products are given for each. As is evident, both these compounds should give the dicarboxylic acid, 9-(α-carboxymethoxy)-nonanoic acid. In all the oxidative experiments on the monounsaturated glyceryl ethers of dogfish liver oil and bovine erythrocytes, only this dicarboxylic acid was isolated in good yields. Similarly, the only monocarboxylic acid(s) found in the products from the oxidation of pure 18:1 glyceryl ethers was nonanoic acid; or, in mixtures of 18:1 and 16:1 glyceryl ethers, nonanoic acid and heptanoic acid. Consequently, in these two sources, the glyceryl ethers of the 16:1 and 18:1 types contain an olefinic bond at the 9:10 position.

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Studies on the Nature and Formation of α-Glyceryl Ether Lipids in Bovine Bone Marrow*

GUY A. THOMPSON, JR., AND DONALD J. HANAHAN

From the Department of Biochemistry, University of Washington, Seattle 5

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The distribution and biosynthesis of α-glyceryl ether phospholipids in bovine hematopoietic bone marrow have been studied. The two major phospholipid fractions, phosphatidyl ethanolamine and phosphatidyl choline, contain, in addition to the diacyl compounds, significant levels of vinyl ether derivatives (plasmalogens) and of glyceryl ether (chimyl, batyl, and selachyl alcohols) derivatives. In incubation experiments, radioactivity from glucose-6-C^{14}, sodium palmitate-1-C^{14}, and tritiated water were incorporated into glyceryl ether phospholipids by extracts of bone marrow. At the end of the incubation times studied, radioactivity of the glyceryl ether phospholipids was less than that of the nonglyceryl ether phospholipids. Possible relationships with plasmalogens are discussed.

Until recently most evidence on naturally occurring α-glyceryl ethers came from analyses of either the total lipid or neutral lipid fraction of the tissue examined (Bodman and Maisen, 1958). In 1958 a glyceryl ether–containing phospholipid, an analog of phosphatidyl ethanolamine, was reported for the first time (Carter *et al.*, 1958). Since that time glyceryl ethers have been found associated with the phospholipids from numerous sources (Ansell and Spanner, 1961; Pietruszko and Gray, 1962; Renkonen, 1962; Pietruszko, 1962). In at least two tissues, bovine erythrocytes (Hanahan and Watts, 1961) and red bone marrow (Thompson and Hanahan, 1962), glyceryl ether phospholipids account for over 10 mole per cent of the total.

Using an *in vitro* preparation of bovine hematopoietic marrow, Thompson and Hanahan (1962) have reported preliminary experiments showing that the glyceryl moiety of glyceryl ethers can be synthesized from glucose and that when glucose-6-C^{14} is employed, the glyceryl ethers isolated from the phosphatidyl ethanolamine fraction contain radioactivity almost exclusively in the 3-position (that bearing the primary alcohol substituent) of the glyceryl moiety. These results would suggest that the glucose-derived intermediate is α-glycerophosphate, as in the case of diacyl phospholipids.

In the present paper the details of these and further experiments using glucose-6-C^{14}, palmitic acid-1-C^{14}, and tritiated water are reported. In all cases the incorporation into the glyceryl ether phospholipids is much lower than that found for their diacyl counterparts.

Experimental

Materials

Bovine red sternal marrow and yellow marrow from femurs and tibiae were obtained through the cooperation of the Seattle Packing Company. The sternal bones were removed within 30 minutes after death of the animal and were preserved at 0° until the marrow could be removed and prepared for incubation. Incubation was initiated within 3 hours after the animal's death.

Calf marrow was obtained from the femurs and tibiae of 3- to 5-week-old calves under nitrous oxide and anectine chloride anesthesia.[1] These preparations

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[1] We appreciate the donation of calf bone marrow by Dr. Patrick Goldsworthy, Department of Medicine, University of Washington.