Aliphatic Diether Analogs of Glyceride-derived Lipids. II. Synthesis of Naturally Occurring L-2,3,-Di-*O*-3′,7′,11′,15′-tetramethylhexadecyl Glycerol and Its D Isomer*

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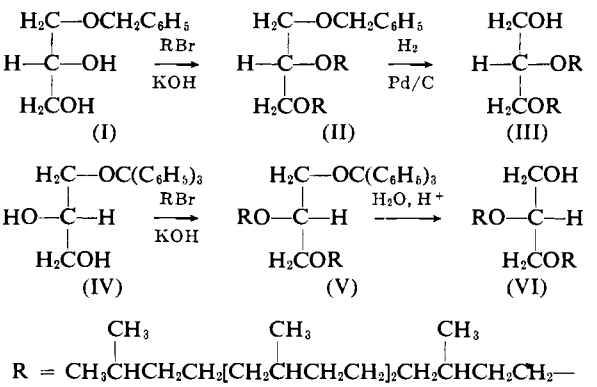
ABSTRACT: L-2,3-Di-*O*-dihydrophytyl glycerol (compound VI) was synthesized by alkylation of D-3-*O*-triphenylmethyl glycerol with dihydrophytyl bromide (1-bromo-3,7,11,15-tetramethylhexadecane) and potassium hydroxide in boiling benzene, followed by acid hydrolysis of the triphenylmethyl group. The D isomer (III) was synthesized by alkylation of L-3-*O*-benzyl glycerol with dihydrophytyl bromide and potassium hydroxide, followed by palladium-catalyzed hydrogenolysis of the benzyl group. The dialkyl glycerol derived from the lipids of *Halobacterium cutirubrum* was shown to be identical with the L-2,3-di-*O*-dihydrophytyl glycerol.

Recent studies in this laboratory have shown that the phosphatides of *Halobacterium cutirubrum* are derivatives of a 2,3-di-*O*-alkyl glycerol ether in which both alkyl chains are 3,7,11,15-tetramethylhexadecyl (dihydrophytyl) groups (Sehgal *et al.*, 1962; Kates *et al.*, 1963b, 1965). Since the diether from *H. cutirubrum* was found to be dextrorotatory ($[\alpha]_D$ +7.8° (Kates *et al.*, 1965); $[\alpha]_D$ +7.5° (Faure *et al.*, 1963)), whereas synthetic D-α,β-dialkyl glyceryl ethers are known to be levorotatory (Kates *et al.*, 1963a), the natural diether was considered to be L-2,3-di-*O*-3′,7′,11′,15′-tetramethylhexadecyl glycerol.[1]

To confirm the proposed structure and the configuration of the glycerol moiety of the natural diether, the synthesis of the D and L stereoisomers of 2,3-di-*O*-dihydrophytyl glycerol was undertaken. The synthesis of each isomer was carried out by procedures similar to those described previously for straight-chain dialkyl glycerols (Kates *et al.*, 1963a), as outlined in the following paragraphs (see Reaction Scheme).

For the D isomer, L-3-*O*-benzyl glycerol (compound I) (Sowden and Fischer, 1941) was alkylated in boiling benzene with 1-bromo-3,7,11,15-tetramethylhexadecane (dihydrophytyl bromide) and potassium hydroxide; the resulting 1-*O*-benzyl-D-2,3-di-*O*-dihydrophytyl glycerol (compound II) was then debenzylated by catalytic hydrogenolysis to the D-2,3-di-*O*-dihydrophytyl glycerol (compound III). For the L isomer, D-3-*O*-triphenylmethyl glycerol (compound IV) (Baer and Fischer, 1945) was alkylated in the same way as the L-3-*O*-benzyl glycerol, and the resulting 1-*O*-triphenylmethyl-L-2,3-di-*O*-dihydrophytyl glycerol (compound V) was converted to the desired L-2,3-di-*O*-dihydrophytyl glycerol (compound VI) by acid-catalyzed hydrolysis. Each isomer was isolated and purified by chromatography on a column of silicic acid, and was shown to be homogeneous by thin-layer and gas-liquid chromatography.



Reaction Scheme

The diethers were obtained as faintly yellow, viscous oils, which could not be induced to crystallize. Their infrared spectra showed the expected bands for OH, alcoholic C—O, ether C—O—C, CH_2, C—CH_3, and $C(CH_3)_2$ groups, and were identical with that of the natural bacterial diether (Kates *et al.*, 1965). Both stereoisomers also had the same R_F value on thin-

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[1] The configurational notation used here is that devised by Baer and Fischer (1939b) for the nomenclature of asymmetrically substituted glycerol derivatives (see also Fischer and Baer, 1941), except that the carbon atoms in glycerol are numbered 1, 2, 3 instead of α, β, α', the potential aldehyde carbon having the number 1.

TABLE I: Physical Properties of Synthetic and Natural 2,3-Di-*O*-dihydrophytyl Glycerols.

Substance	$[\alpha]_D$[a]	M_D	Thin-Layer Chromatography (R_F)[b]	Gas-Liquid Chromatography, Relative Retention[c]
D-2,3-Di-*O*-dihydrophytyl glycerol (III)	−7.03°	−45.8°	0.24	2.12
L-2,3-Di-*O*-dihydrophytyl glycerol (VI)	+7.6°	+49.6°	0.24	2.10
Dialkyl glycerol from *H. cutirubrum*	+7.8°	+50.9°	0.24	2.11

[a] In chloroform. [b] On silica gel G; solvent, chloroform (containing 0.75% ethanol). [c] On a short (0.5-m) column of silicone SE-52 at 220° and 30 psi; retentions are given relative to DL-2,3-di-*O*-hexadecyl glycerol (retention time 20 minutes).

layer chromatography and the same relative retention on gas-liquid chromatography as the bacterial diether, but only the L isomer had an optical rotation ($[\alpha]_D$ +7.6°) identical in magnitude and sign with that of the natural diether (Table I). The bacterial diether is therefore established as being L-2,3-di-*O*-3′,7′,11′,-15′-tetramethylhexadecyl glycerol.

The finding that the 2,3-dialkyl glycerol ether moiety of the phosphatides in *H. cutirubrum* has the L configuration was unexpected, since the 2,3-diglyceride moieties of naturally occurring phosphatides are known to have the D configuration (Baer and Kates, 1950; see review by Kates, 1960). There may be two possible explanations for this finding, depending upon whether or not inversion of configuration occurs during enzymatic formation of an ether linkage. If no inversion of configuration takes place, then the diether might be formed by *O*-alkylation of D-3-glycerophosphate, followed by dephosphorylation. In this case, formation of α-glycerophosphate either by enzymatic reduction of dihydroxyacetone phosphate or by phosphorylation of glycerol would be required to yield the D-3 isomer rather than the usual L-3 isomer. In the second possibility, formation of an ether linkage would involve inversion of configuration at carbon atom 2 of glycerol, so that *O*-alkylation of the usual L-3-glycerophosphate, followed by dephosphorylation, would give the desired L-2,3-dialkyl glycerol. The latter explanation has the advantage that an unusual triose phosphate metabolism in *H. cutirubrum* need not be postulated. Experiments designed to test these hypotheses are now in progress.

There still remains the question of the configuration of the asymmetric carbon atoms 3, 7, and 11 in the dihydrophytyl groups of the bacterial diether. Since straight-chain L-diethers would have a molecular rotation of +41° (Kates *et al.*, 1963a), whereas the natural dihydrophytyl diether has M_D +51° (Kates *et al.*, 1965), the net contribution of the asymmetric centers in the dihydrophytyl groups must be dextrorotatory. In the synthetic dihydrophytyl diethers, only the asymmetric centers at carbons 7 and 11 can contribute to the optical rotation, since that at carbon 3 is racemic as a result of the hydrogenation step in the preparation of the dihydrophytol from natural phytol. Thus, the fact that the synthetic L-dihydrophytyl diether has M_D +50°, whereas the D isomer has M_D −46° (Table I), indicates that the asymmetric carbons 7 and 11 in the synthetic dihydrophytyl groups also have a net dextrorotatory contribution. This line of reasoning would suggest that carbons 7 and 11 in the dihydrophytyl groups of the bacterial diether have the same configuration as the corresponding carbon atoms in phytol of plant origin, now known to have the *RR* configuration (Burrell *et al.*, 1959; Crabbe *et al.*, 1959). Furthermore, the fact that the rotations of the synthetic L isomer and the natural diether are identical within experimental error suggests that the contribution of the asymmetric carbon atom 3 in the natural diether is probably very small. Work is now in progress on the determination of the absolute configuration of carbon atoms 3, 7, and 11 in the dihydrophytyl groups of the bacterial diether.

Experimental and Results

Thin-Layer Chromatography. For analytical purposes and for monitoring column chromatography, thin-layer chromatography was carried out on a 0.25-mm layer of silica gel G (Research Specialties Co., Richmond, Calif.) on 20- × 20-cm or 10- × 20-cm glass plates, using chloroform (commercial reagent grade containing 0.75% ethanol) as solvent. The components were visualized by charring of the plates after they were sprayed with 40% sulfuric acid. For preparative purposes, a 0.6-mm layer of silica gel G was used.

Gas-Liquid Chromatography. Dihydrophytol and its derivatives were chromatographed on a 1.2-m column of butanediol succinate polyester (10% on Gas-Chrom A) at 184°, or Apiezon L (10% on Gas-Chrom P) at 197° with argon as carrier gas (18–20 psi). Glyceryl diethers were chromatographed on a 0.5 m column of 2% GE silicone (SE-52) at 220° as described elsewhere (Kates *et al.*, 1963a).

Column Chromatography. Columns of silicic acid were prepared with Bio-Rad silicic acid, minus 325

mesh (Bio-Rad Laboratories, Richmond, Calif.), activated at 110° for several hours and suspended in petroleum ether (bp 30–60°); elution was carried out with the same solvent, followed by stepwise increasingly polar solvents (*vide infra*), and the eluates were monitored by thin-layer or gas-liquid chromatography.

Physical Measurements. Infrared spectra were taken either on thin films of the substances or on their solutions in carbon tetrachloride (0.5- or 1.0-mm cells), using a Perkin-Elmer Model 21 double-beam spectrophotometer with sodium chloride optics. Melting points are uncorrected and were taken in Pyrex glass capillaries by means of an electrically heated melting-point apparatus (Culatti, Switzerland).

L-*3-O-Benzyl Glycerol* (*Compound I*). This compound was prepared from D-2,3-*O*-isopropylidene glycerol (Baer and Fischer, 1939a; Baer, 1952) by a modification of the procedures of Sowden and Fischer (1941) and of Howe and Malkin (1951), as described elsewhere (Kates *et al.*, 1963a).

D-*3-O-Triphenylmethyl Glycerol* (*Trityl Glycerol*) (*Compound IV*). This compound was synthesized from D-2,3-*O*-isopropylidene glycerol as described by Baer and Fischer (1945). The product obtained had mp 98°, and $[\alpha]_D^{21}$ −16.8° in anhydrous pyridine (Baer and Fischer (1945) reported mp 97°, and $[\alpha]_D$ − 17.7°).

1-Bromo-3,7,11,15-tetramethylhexadecane (*Dihydrophytyl bromide*). Commercial phytol (20 g; derived from chlorophyll; C.P. grade; Mann Research Laboratories, N.Y.) was hydrogenated in 300 ml of methanol with 0.5 g of platinum catalyst, as described elsewhere (Kates *et al.*, 1965). The crude dihydrophytol obtained was heated overnight under reflux in a mixture of 200 ml of 48% hydrobromic acid and 20 ml of concentrated sulfuric acid, with magnetic stirring. The cooled reaction mixture was diluted with water and extracted with ethyl ether, and the ether extract was washed thoroughly with water. The filtered ether solution was dried over anhydrous calcium chloride and the solvent was evaporated under reduced pressure. The residual brown oil was dissolved in 50 ml of petroleum ether (bp 30–60°) and the solution was passed through a short column of 70 g of silicic acid, with 300 ml of petroleum ether (bp 30–60°) used to wash the column. The combined eluates were concentrated under reduced pressure, yielding 20 g (86% yield) of chromatographically pure 1-bromo-3,7,11,15-tetramethylhexadecane, as a yellow oil. The substance had a retention, relative to 1-bromooctadecane, of 0.804 (carbon no. 17.32) on butanediol succinate polyester at 184°.

D-*2,3-Di-O-3',7',11',15'-tetramethylhexadecyl Glycerol* (*Compound III*). A mixture of 0.4 g (2.2 mmole) of L-3-*O*-benzyl glycerol, 1.8 g (5.0 mmole) of 1-bromo-3,7,11,15-tetramethylhexadecane, and 1.12 g (20 mmole) of finely powdered potassium hydroxide in 20 ml of anhydrous benzene was heated under reflux with magnetic stirring for 28 hours, the water formed being removed by means of a phase-separating head. The cooled mixture was then diluted with 50 ml of ethyl ether and washed successively with water, 1 N hydrochloric acid, 2.5% potassium bicarbonate solution, and finally with water. The crude oil (1.8 g) obtained after evaporation of the solvent *in vacuo* was chromatographed on a column of 59 g of silicic acid. The column was eluted successively with petroleum ether (bp 30–60°; 150 ml), chloroform (150 ml), and finally methanol (50 ml); the eluates were monitored by thin-layer chromatography.

The petroleum ether eluates contained mostly 3,7,11,15-tetramethyl-1-hexadecene (0.2 g), and also traces of unreacted dihydrophytyl bromide. The olefin probably arose by dehydrobromination of the dihydrophytyl bromide during the reaction with KOH; it was characterized by gas-liquid chromatography (retention relative to octadecane, 1.14 on butanediol succinate; 0.863 on Apiezon L), and infrared spectrum (absorption for vinyl group at 3060, 1638, 1420, 990, and 907 cm^{-1}); $[\alpha]_D^{20}$ −0.49 ± 0.06° (in chloroform, *c* 6.3); $[\alpha]_D^{20}$ −0.08 ± 0.04° (neat). The methanol eluates contained small amounts of material having properties of a monoalkylated benzyl glycerol (R_F on thin-layer chromatography, 0.15; strong OH, CH_2 + CH_3, ether C—O—C, alcoholic C—O, and aryl absorption in the infrared).

The desired 1-*O*-benzyl-D-2,3-di-*O*-dihydrophytyl glycerol (compound II) appeared in the chloroform eluates, and was recovered after evaporation of the solvent; yield 0.75 g (46%). Its R_F value on thin-layer chromatography was 0.48 and its infrared spectrum (thin film) showed no OH absorption at 3600–3400 cm^{-1}, and had strong CH_2 and CH_3 bands at 2940, 2880, 1465, and 1380 cm^{-1}, a doublet characteristic of the *gem*-dimethyl group at 1380–1370 cm^{-1}, a strong C—O—C ether band at 1113 cm^{-1}, and aryl absorption at 3020, 1500, 730, and 695 cm^{-1}.

A solution of 1.2 g (1.62 mmoles) of the crude benzyl ether (compound II) in 20 ml of ethyl acetate was debenzylated by stirring with freshly prepared palladium-charcoal catalyst (from 50 mg of palladium chloride; Hessel *et al.*, 1954) in an atmosphere of hydrogen at room temperature and an initial pressure of 40 cm of water. Consumption of hydrogen ceased after 2.5 hours, with uptake of 44 ml (calcd for 1 mole, 40 ml). The catalyst was removed by centrifugation and washed with ethyl acetate, and the combined supernatants were concentrated under reduced pressure. The oily residue (1 g) was chromatographed on a column of silicic acid (30 g); elution was carried out with 150 ml each of petroleum ether–chloroform (4:1) and petroleum ether–chloroform (1:1), and 50-ml fractions were collected. Fractions 2–4 (0.7 g) contained the desired D-2,3,-di-*O*-dihydrophytyl glycerol (compound III) contaminated with small amounts of benzyl ether (compound II) and unidentified fast-moving material. Fractions 5 and 6 (0.2 g) contained the pure diether (compound III) virtually uncontaminated with other products. Rechromatography of combined fractions 2–4 on silicic acid with the same solvents gave a further 0.5 g of chromatographically pure diether (compound III); total yield, 0.7 g (67% from benzyl ether [compound II]; 31% overall yield); $[\alpha]_D^{21}$ −7.03° (*c* 2.28, in chloroform); M_D −45.8°.

Anal. Calcd for $C_{43}H_{88}O_3$ (653.1): C, 79.07; H, 13.58. Found: C, 78.90; H, 13.28.

The branched-chain D-2,3-diether (compound III) had solubilities similar to those of the saturated straight-chain diethers reported previously (Kates *et al.*, 1963a); soluble in acetone, ethyl ether, chloroform, benzene, petroleum ether, or ethyl acetate, sparingly soluble in cold methanol. It could not be induced to crystallize from any combination of solvents tried. Its infrared spectrum (thin film) was identical with that of the glyceryl diether obtained from the phosphatides of *H. cutirubrum* (Sehgal *et al.*, 1962; Kates *et al.*, 1965), and showed absorption bands characteristic of the following groups: OH (3450 cm^{-1}, broad), CH_2 and CH_3 (2930, 2860, and 1460 cm^{-1}), $-(CH_2)_3-$ (735 cm^{-1}), C—CH_3 and C—$(CH_3)_2$ (1380–1365 cm^{-1}, doublet), ether C—O—C (1110 cm^{-1}), and primary alcoholic C—O (1045 cm^{-1}). It also had the same chromatographic properties as the natural diether and the synthetic L isomer (Table I).

L-2,3-Di-O-3',7',11',15'-tetramethylhexadecyl-1-O-triphenymethyl Glycerol (Compound V). A mixture of 2.7 g (8 mmoles) of D-3-*O*-triphenylmethyl glycerol (compound IV), 8.0 g (22 mmoles) of 1-bromo-3,7,11,-15-tetramethylhexadecane, and 2.0 g (36 mmoles) of powdered potassium hydroxide in 120 ml of anhydrous benzene was heated under reflux with magnetic stirring for 24 hours, the water formed being removed by means of a phase-separating head. To ensure complete alkylation of the trityl glycerol a further 2.0-g portion of powdered potassium hydroxide was added, refluxing was continued for 6 hours, and finally for a further 15 hours after adding another 2.0-g portion of potassium hydroxide. The cooled reaction mixture was diluted with 120 ml of ethyl ether and neutralized with 6 N hydrochloric acid; the ethyl ether solution was washed with 0.2 N hydrochloric acid followed by water, dried over sodium sulfate, and concentrated under reduced pressure.

For analytical purposes, a small portion of the residual crude reaction product was fractionated by preparative thin-layer chromatography using petroleum ether–benzene (1:2) as solvent. The band corresponding to L-2,3-di-*O*-dihydrophytyl-1-*O*-trityl glycerol (compound V) (R_F *ca.* 0.55) was eluted with ethyl ether and finally purified by thin-layer chromatography in the same way. The product obtained was a colorless oil having $[\alpha]_D$ −4.3° (*c* 6.9, in chloroform); M_D −38.5°.

Anal. Calcd for $C_{62}H_{102}O_3$ (895.4): C, 83.16; H, 11.48. Found: C, 82.76; H, 10.94.

The infrared spectrum of the trityl ether (compound V) showed absorption bands characteristic of the following groups: CH_2 and CH_3 (2930, 2860, 1465, and 1450 cm^{-1}), trityl (3060, 1600, 1490, 755, and 700 cm^{-1}), C—CH_3 and C—$(CH_3)_2$ (1380–1365 cm^{-1} doublet), ether C—O—C (1100 cm^{-1}, broad); no OH band present; unidentified band at 1215 cm^{-1}.

L-2,3-Di-O-3',7',11',15'-tetramethylhexadecyl Glycerol (Compound VI). The crude trityl ether (compound V) from the above-mentioned reaction, without purification, was detritylated as follows: a solution of compound V in 100 ml of ethyl ether and 40 ml of methanol was acidified with 6 ml of concentrated hydrochloric acid and saturated with HCl gas, and the mixture was refluxed for 15 hours. The clear ether solution was concentrated under reduced pressure and the residue was extracted with ethyl ether; the ether extract was washed with water and dried over sodium sulfate, and the solvent was evaporated under reduced pressure.

The residual oil (8.5 g) was dissolved in petroleum ether (bp 30–60°), freed from crystals of triphenylmethanol by centrifugation, and fractionated on a column of 135 g of silicic acid. The column was eluted with the following solvents, 300-ml fractions being collected: petroleum ether (fractions 1, 2), petroleum ether–benzene (1:1; fractions 3–15), benzene (fractions 16–21), and benzene–ethyl ether (3:2; fractions 22–25). Fractions 1 and 2 contained mainly 3,7,11,15-tetramethy-1-hexadecene; fractions 3–15 had mainly triphenylmethanol with small amounts of dihydrophytyl bromide and triphenylchloromethane. The desired glyceryl diether appeared in fractions 16–25, but fractions 16–18 and 23 were contaminated with triphenylmethanol, and fractions 24–25 contained a slow-moving component identified as L-3-*O*-3',7',11',15'-tetramethylhexadecyl glycerol.[2] The purest fractions (19–22) were combined and, after evaporation of the solvent *in vacuo*, yielded 1.4 g of L-α,β-di-*O*-dihydrophytyl glycerol (compound VI). Fractions 17, 18, and 23 were also combined and yielded 1.0 g of the diether, which contained traces of triphenylmethanol; overall yield, 2.4 g (46%). For analytical purposes a small sample was purified by thin-layer chromatography using chloroform as solvent. The diether recovered from the plate showed only one spot on thin-layer chromatography, and had $[\alpha]_D^{21}$ +7.6° (*c*, 2.95 in chloroform); M_D +49.6°; reported (Kates *et al.*, 1965) for the natural diether: $[\alpha]_D$ +7.8° (*c* 1.96, in chloroform); M_D +50.9°.

Anal. Calcd for $C_{43}H_{88}O_3$ (653.1); C, 79.07; H, 13.58. Found: C, 78.73; H, 13.42.

The L-2,3-diether (compound VI) had solubilities, chromatographic properties (Table I), and infrared spectrum identical with those of the D isomer and the natural glyceryl diether.

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[2] After purification by preparative thin-layer chromatography using chloroform-ether (20:1), this product was obtained as a colorless oil having $[\alpha]_D$ −2.0° (*c*, 3.5 in chloroform); R_F on thin-layer chromatography: 0.38 in ethyl ether; 0.53 in chloroform-methanol-water (90:10:1). *Anal.* Calcd for $C_{23}H_{48}O_3$ (372.6): C, 74.14; H, 12.99. Found: C, 73.85; H, 13.04. The infrared spectrum showed the expected bands for OH, CH_2 and CH_3, C—CH_3 and C$(CH_3)_2$, ether C—O—C, and alcoholic C—O groups.

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Stabilities of Metal Complexes of Phospholipids: Ca(II), Mg(II), and Ni(II) Complexes of Phosphatidylserine and Triphosphoinositide*

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ABSTRACT: Stability constants for Ca(II), Mg(II), and Ni(II) complexes of phosphatidylserine, triphosphoinositide, *O*-phosphoserine, *O*-phosphoethanolamine, and glycerylphosphorylinositol diphosphate were determined by a *p*H titration method. The lipids were studied in aqueous micellar dispersions. Apparent stability constants (K^{M}_{ML} and K^{M}_{MHL}) for the intact lipids were 10 to 100 times greater than those for the deacylated models. These constants include a free-energy factor due to the electrostatic field of the micelle surface and are not a true measure of complexing ability. Displacement constants, which represent the equilibrium $H_2L + M^{2+} \rightleftharpoons ML + 2\ H^+$, are a more valid measure of complexing ability since this reaction involves no net change in charge on the ligand and the electrostatic free energy terms disappear. Comparison of displacement constants indicates that triphosphoinositide and glycerylphosphorylinositol diphosphate differ very little in complexing ability. Similar comparisons with phosphatidylserine and phosphoserine indicate greater complexing ability for the lipid. Phosphoserine, however, may not be a suitable model for comparison with phosphatidylserine. The former is a phosphate monoester and the latter a diester.

The binding of alkali and alkaline earth metals by phosphatides was suggested by some of the earliest investigators of lipid chemistry (Thudichum, 1901; Koch and Pike, 1910; Koch and Todd, 1911). The first quantitative study of sodium and potassium binding by cephalin was reported by Christensen and Hastings (1940). Since then very little work has been reported on phospholipid metal binding. Recently, however, many investigators have again emphasized the importance of this type of metal binding in biological systems. There are indications that such metal chelation is quite important in lipoprotein formation, cation transport, and other biochemical processes. Abramson *et al.* (1964) reported studies on the ionic properties of phosphatidylserine which indicate strong Ca(II) binding by that lipid. Hakamori *et al.* (1963) reported the isolation of a metal-bound lipid-peptide complex from ox brain, and Carter *et al.* (1962) postulated that phytoglycolipid occurs in plants as a mixed Ca(II) and Mg(II) chelate. Ca(II) and Mg(II) dramatically affect the solubilities and ionic properties of brain phosphoinositides (Hendrickson and Ballou, 1964). Papahadjopoulos and Hanahan (1964) demonstrated the requirement of

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