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SYNTHESIS OF PHOSPHOLIPIDS BY MEANS OF CYCLIC ENEDIOL PYROPHOSPHATES†

OPTICALLY ACTIVE MONOVALENT AND DIVALENT CATION SALTS OF DIPHOSPHATIDYLGLYCEROL (CARDIOLIPIN)

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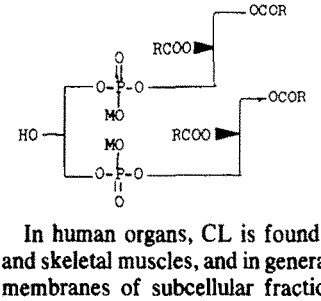
Abstract—A new method for the synthesis of the cardiolipin family of phospholipids which are characteristically located at the inner membrane of mitochondria, and particularly abundant in the heart, is described in this paper. A 1,2-diacyl-*sn*-glycerol, derived from stearic, palmitic, or myristic acid, is converted into the corresponding 1′,3′-diphosphatidylglycerol (DPG), by means of the phosphorylating reagent di(1,2-dimethylethenylene) pyrophosphate in 29%, 23% and 25% overall yields, respectively. The synthesis involves four steps, two of which are carried out in one-flask; only one intermediate and the final product, DPG·$2NH_4^+$·$2H_2O$, are purified, the former by silica gel, and the latter by DEAE-cellulose column chromatography. The ammonium salts are converted into metal ion salts, DPG·$2Na^+$·$2H_2O$, DPG·Mg^{2+}·$2H_2O$ and DPG·Ca^{2+}·$2H_2O$ by metathesis with NaCl, $MgCl_2$ and $CaCl_2$, respectively.

The isolation by Pangborn[2] of a phospholipid from beef-heart, and the demonstration that this substance was identical with the antigen for the specific antibodies previously found by Wasserman *et al.*[3] in the sera of syphilitic patients, spurred further work on the purification and structural determination of the compound.[4–10] Pangborn's[2] "cardiolipin" (CL) proved to be a member of a family of compounds[5,11] with the structure and configuration of 1′,3′ - *bis*(1,2 - diacyl - *sn* - glycero - 3 - phosphoryl)glycerol[12] (**1**), as established largely through the work of Le Cocq and Ballou,[8] and de Haas and van Deenen.[9,10] CL from mammalian tissues are rich in unsaturated fatty acids, those from bacteria contain mostly saturated acids, and those from plants have various proportions of these acid types.[11]



1a, M = H^+
1b, M = NH_4^+
1c, M = Na^+
1d, MM = Mg^{2+}
1e, MM = Ca^{2+}

In human organs, CL is found mainly in the heart[11,13] and skeletal muscles, and in general, CL is associated with membranes of subcellular fractions which display high metabolic activity, i.e. mitochondria.[14,15] There is virtually no CL in microsomes and in nuclei.[11] In rat liver mitochondria, CL is found mostly in the inner membrane, and there is disagreement[16,17] on the orientation of the molecules, as revealed by the anti-CL antibody. Lehninger *et al.*[16] concluded that the polar group of CL is buried within the membrane structure, or is shielded by binding to other membrane components. Schiefer[17] proposed that the polar group is located, at least partially, at the surface of the membrane and is free to bind the antibody.

The functional role played by CL in mitochondrial and prokaryotic membranes is not known, but some speculations have been offered.[6b,18] The specific association of mitochondrial ATPase with CL,[19] and the tight binding of cytochrome oxidase to CL,[20] have been recently reported. The distribution of CL in the membranes of malignant and normal cells has been compared by Bergelson *et al.*,[21] who reported the presence of CL in microsomes of hepatomas, but not in those of normal liver cells. Apparently the chemical structure of the CL in normal cell mitochondria, and in hepatoma (and Jensen sarcoma) microsomes, is the same, but there seem to be differences, still undetermined, in the cation content of the CL from these two sources.[21]

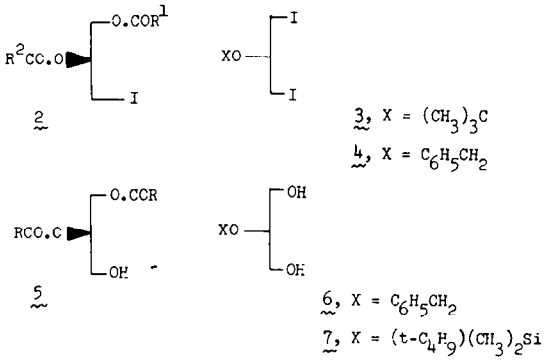
Current methods for the isolation of CL do not provide samples with a unique, and precisely known, distribution of fatty acid chains and metal ions, which is a prerequisite for a detailed study of the physicochemical and biological properties of this phospholipid, in particular of its molecular conformation. The effect of monovalent and divalent metal ions on the properties of CL is of particular interest, in view of the presence of two phosphate anions in the molecule. The present study has been aimed at the development of a practical route to DPG[22] with the configuration of natural CL, and with various types of metal ions and fatty acid residues.[1c]

The literature on the synthesis of phospholipids has been reviewed.[23,24] There are three reports on the synthesis of DPG[10,25,26] and others on DPG-derivatives.[27–29] de Haas and van Deenen[10] converted the optically active iodide **2** ($R^1 = C_{17}H_{35}$, $R^2 = C_{17}H_{33}$) into the corresponding DPG in seven steps, with an overall yield of 26%. The phosphorylating reagent in the first step was silver dibenzyl phosphate. They removed one phosphate-blocking group and used the silver salt of the resulting phosphatidic[22] benzyl ester for the second phosphorylation step in conjunction with 1,3-diiodo-2-t-butoxypropane (**3**). Finally, they removed the second blocking group and the t-Bu protecting group to obtain the DPG. Inoue and Nojima[25] transformed the *racemic* iodide **2** ($R^1 = R^2 =$

†To the memory of Prof. E. BAER.

$C_{15}H_{31}$) into the DPG in 4% yield by the same procedure[10] but using 1,3-diiodo-2-benzoxypropane (**4**).

Saunders and Schwarz[26] claimed the conversion of 1,2-diacyl-*sn*-glycerol **5** (R = $C_{17}H_{35}$) into DPG in four steps, with an overall yield of 50%. They used phosphorus oxychloride in the first phosphorylation, and the phosphatidic acid *dichloride* in conjunction with 2-benzylglycerol (**6**) in the second phosphorylation. In essence, this procedure dispenses with a phosphate-blocking group. We have made several unsuccessful attempts to duplicate these results; in our hands, the procedure afforded a complex mixture of products, which contained at most 10% of DPG, according to TLC analysis.



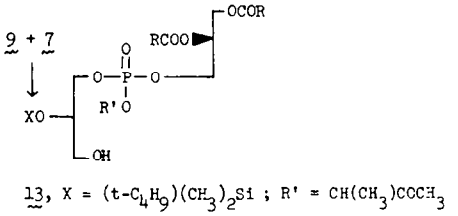
RESULTS

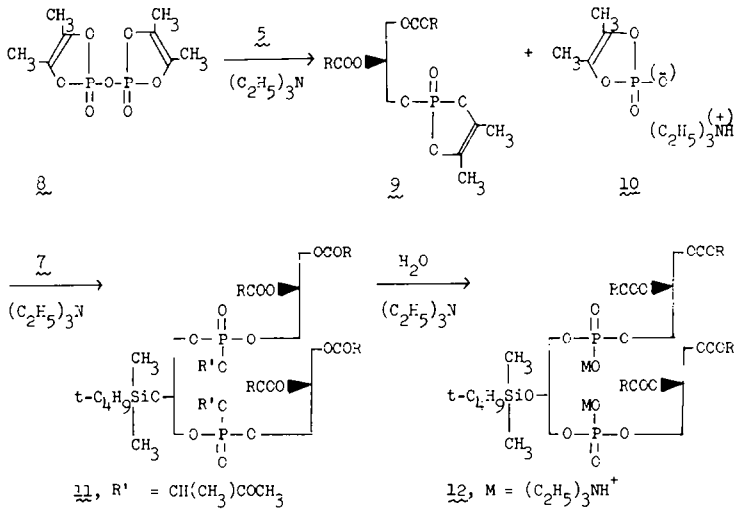
Synthesis of ammonium salts of DPG,[22] **1b**. A 1,2-diacyl-*sn*-glycerol,[30] **5**, R = $C_{17}H_{35}$ (stearoyl), $C_{15}H_{31}$ (palmitoyl), and $C_{13}H_{27}$ (myristoyl), is converted into the corresponding DPG diammonium-diaquo salt, **1b**·$2H_2O$, by means of the new phosphorylating reagent, di(1,2-dimethylethenylene) pyrophosphate (**8**),[31,32] as shown in Scheme 1.

This approach is an application of the general "Procedure 3"[32] for the synthesis of unsymmetrical phosphodiesters, $(R^IO)(R^{II}O)P(O)OH$, from two different alcohols, R^IOH, $R^{II}OH$, which was described recently.[33,34] The first alcohol in the phosphorylative coupling is R^IOH = diglyceride **5**, and the second is $R^{II}OH$ = 2-(t-butyldimethylsilyl) glycerol (**7**).[35] The first step is a displacement at phosphorus with ring-retention, which yields 1,2-diacyl-*sn*-glycerol 3-(1',2'-dimethylethenylene) phosphate (**9**) and the phosphate salt **10**. To minimize the possibility of an isomerization of the 1,2- into the 1,3-diglyceride,[36,37] the proton-acceptor, triethylamine, is introduced with the pyrophosphate **8**, and the reaction is carried out for 45 min at 25°, in 0.05 M dichloromethane solution.

The diglyceride cyclic phosphate **9** is not isolated, but is allowed to react in the same flask with the silylglycerol, **7**, since the salt **10** does not interfere. Two mols of **9** and 1 mol of **7** are kept 2 hr at 25° in 0.04 M dichloromethane solution, in the presence of 3 mol of triethylamine, which is an effective catalyst[32] for the phosphorylation. The appearance of 2' - (t - butyldimethylsilyl) - 1',3' - *bis*[(1'' - methylacetonyl)(1,2 - diacyl - *sn* - glycero - 3)phosphoryl]glycerol (**11**), is followed by TLC as described in the Experimental.[38-41] The fully blocked and protected silyl-DPG triester, **11**, is purified by silicic acid column chromatography; Tables 1, 3 and 4 summarize the analytical data for the compounds.

Two new phosphate ester bonds are generated in this step of the synthesis. The first phosphorylation yields the silyl-PG[22] triester **13**, which immediately reacts with a





Scheme 1.

Table 1. Elemental analyses and physical constants of 1',3'-diphosphatidylglycerol (DPG) derivatives

Compd. No.	R	Yield, %	Mp°, n_D^{22}	$[\alpha]_D^T$, deg.	M_D	Molecular Formula (MW)	Calcd., % C	H	P	X[a]	Found, % C	H	P	X
						Silyl-DPG Triesters								
	$C_{17}H_{35}$	37[b]	41-42	+ 1.66[c]	+ 28.5	$C_{95}H_{184}O_{19}P_2Si$ (1720)	66.3	10.8	3.6	1.6	65.0	10.6	3.5	1.2
11	$C_{15}H_{31}$	30	29-30	+ 1.48[c]	+ 23.9	$C_{87}H_{168}O_{19}P_2Si$ (1608)	65.0	10.5	3.8	1.7	64.4	10.5	3.4	1.2
	$C_{13}H_{27}$	37	1.4582	+ 1.34[d]	+ 20.0	$C_{79}H_{152}O_{19}P_2Si$ (1496)	63.4	10.2	4.1	1.9	63.6	9.9	4.4	1.8
						DPG.$2NH_4^+$.$2H_2O$								
	$C_{17}H_{35}$	78[e]	182-183[f]	+ 8.50[g,h]	+130.5	$C_{81}H_{164}O_{17}N_2P_2.2H_2O$[i] (1536)	63.3	11.0	4.0	1.8	63.2	10.9	4.2	1.7
1b. $2H_2O$	$C_{15}H_{31}$	76	177-178	+ 9.25	+128.1	$C_{73}H_{148}O_{17}N_2P_2.2H_2O$ (1424)	61.6	10.8	4.3	2.0	61.3	10.7	4.5	1.7
	$C_{13}H_{27}$	68	181-182	- 9.50	+124.6	$C_{65}H_{132}O_{17}N_2P_2.2H_2O$ (1312)	59.5	10.4	4.2	2.1	59.7	10.4	4.7	2.0
						DPG.$2Na^+$.$2H_2O$								
	$C_{17}H_{35}$	92[j]	201-202[k]	+ 7.00	+108.2	$C_{81}H_{156}O_{17}P_2Na_2.2H_2O$[l] (1546)	62.9	10.4	4.0	3.0	61.2	10.2	3.8	2.8
1c. $2H_2O$	$C_{15}H_{31}$	87	199-201	+ 8.50	+121.8	$C_{73}H_{140}O_{17}P_2Na_2.2H_2O$ (1434)	61.1	10.1	4.3	3.2	60.6	9.9	4.4	3.3
	$C_{13}H_{27}$	ca 95[m]	186-188	+ 9.50	+126.6	$C_{65}H_{124}O_{17}P_2Na_2.2H_2O$ (1333)	59.1	9.8	4.7	3.5	59.8	9.7	...	3.4
						DPG.Mg^{2+}.$2H_2O$								
	$C_{17}H_{35}$	100	190-192	+ 8.50	+129.5	$C_{81}H_{156}O_{17}P_2Mg.2H_2O$ (1524)	63.8	10.6	4.0	1.6	63.7	10.7	3.9	1.6
1d $2H_2O$	$C_{13}H_{27}$	100	185-186	+11.50	+149.3	$C_{65}H_{124}O_{17}P_2Mg.2H_2O$ (1299)	60.0	9.9	4.8	1.9	59.1	9.9	4.5	1.8
						DPG.Ca^{2+}.$2H_2O$								
1e. $2H_2O$	$C_{17}H_{35}$	94	185-187	... [n]	...	$C_{81}H_{156}O_{17}P_2Ca.2H_2O$ (1540)	63.2	10.5	4.0	2.6	62.6	10.6	3.9	2.3
	$C_{13}H_{27}$	70	190-192	+ 8.10[o]	+106.5	$C_{65}H_{124}O_{17}P_2Ca.2H_2O$ (1315)	59.3	9.8	4.7	3.0	59.2	9.5	4.5	2.9

[a] X = Si for **11**, N for **1b**, Na for **1c**, Mg for **1d**, Ca for **1e**. [b] Based on diglyceride **5** charged; *ca.* 23% of **5** is recovered in all cases. [c] c, 5.4 ($CHCl_3$); T = 24°. [d] c, 6.7 ($CHCl_3$); T = 20°. [e] Based on triester **11**. [f] The salts shrink and become translucent at 87°; melt to a clear, brownish oil, all in capillary tubes. [g] For all salts, c, 2.0 ($CHCl_3/CH_3OH/H_2O$ 4/4/1), except as noted; T = 21°. [h] The ammonium salts are soluble in pure $CHCl_3$; $[\alpha]_D^{24}$, M_D: + 5.82, + 89.4 (R = $C_{17}H_{35}$): + 6.35, + 90.4 (R = $C_{15}H_{31}$); + 6.90, + 90.5 (R = $C_{13}H_{27}$); c, 2.75 ($CHCl_3$). [i] % H_2O (Karl Fischer): *found*, 2.4, 2.4, 2.5; *calcd.*, 2.3, 2.5, 2.7 for R = $C_{17}H_{35}$, $C_{15}H_{31}$, $C_{13}H_{27}$, respectively. [j] Based on the ammonium salt, in all cases. [k] The salts become slightly translucent at *ca.* 120°; melt to clear, colorless oils; all in capillary tubes. [l] Metal ion salts are insoluble in the K. Fischer reagent. [m] May contain traces of NaCl; see Experimental. [n] Insoluble. [o] Sparingly soluble; c, 1.35 ($CHCl_3/CH_3OH/H_2O$ 4/4/1).

second mol of the cyclic phosphate **9** to produce the silyl-DPG triester **11**. In both reactions there is displacement at phosphorus with ring-opening.

The third step of the synthesis is the removal of the 1-methylacetonyl groups from **11**, which is carried out for 2.5 hr at 25°, in 0.035 M water/pyridine 1/1 v/v, in the presence of four mol equivalents of triethylamine. This reaction affords silyl-DPG as its *bis*-triethyl ammonium salt **12**. TLC analysis shows that virtually no starting material, **11**, remains, while no diglyceride, **5**, nor products of carboxylic ester hydrolysis, are produced under these conditions. The crude salt, **12**, is isolated, but is not purified prior to the subsequent deprotection step.

The final step is the removal of the silyl group from **12**, which is accomplished in about 5 hr at 25°, in a 1 mM chloroform/methanol/water 1.5/3/1 medium, in the presence of 2.5 mol equivalents of hydrochloric acid. This reaction is monitored by TLC until no starting material **12** is detected, at which point the DPG free acid **1a** is neutralized with aqueous ammonia. The desired 1',3'-*bis*(1,2-diacyl-*sn*-glycero-3-phosphoryl)glycerol (**1**) is purified as its ammonium salt, **1b**, by column chromatography on DEAE-cellulose (acetate form). The salt is isolated as its dihydrate, **1b**·$2H_2O$; see Tables 1, 3 and 4, and Fig. 1.

The technique to purify the DPG ammonium salts, **1b**, is a modification of the procedure introduced by Rouser, Kritchevsky *et al.*[7,42,43] These authors had observed some decomposition during the purification of natural CL on DEAE-cellulose; however, in their experiments,[42] the CL remained on the column for about 2 days, and the eluting solvent was basic: $CHCl_3/CH_3OH$/conc.NH_3 4/1/0.02. In the present procedure, faster elution with the acidic solvent, $CHCl_3/CH_3COOH/CH_3COONH_4$, avoids DPG decomposition.

Attempts to purify DPG salts by silica gel column chromatography proved to be unsatisfactory. For example, a DPG (**1**, R = $C_{13}H_{27}$), applied as its sodium salt (**1c**, see next Section) to such a column was recovered in two fractions: (i) pure *calcium salt* (according to elemental analysis) eluted by $CHCl_3$ and $CHCl_3/CH_3OH$ 4/1; (ii) impure sodium salt, with relatively high sodium content by elemental analyses, eluted with $CHCl_3/CH_3OH$ 1.5/1. These observations confirm results by Nielsen,[44] who found an exchange between the cations of acidic phospholipids and those in the silicic acid adsorbent. Nielsen[44] noted a correlation between elution sequence and cation preference, with the relatively mobile CL showing a higher affinity for divalent cations: 80% Ca^{2+}: 15% Mg^{2+}: 5% Na^+. In general, in silica gel TLC of acidic phospholipids, reproducible results are obtained by the use of basic solvents, e.g. those containing a large excess of NH_4^+ ions.[44]

The molecular rotation[45] values of the silyl-DPG

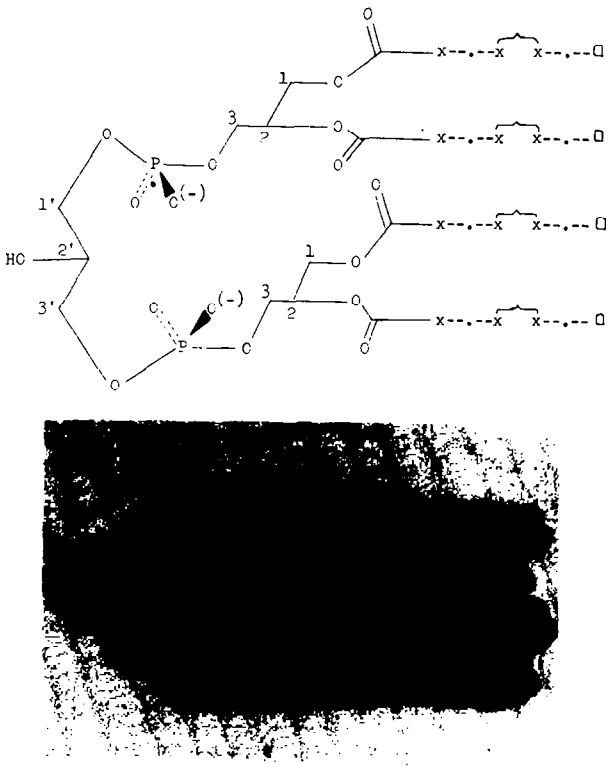


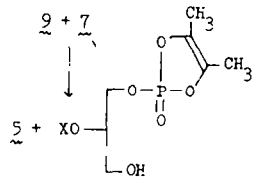
Fig. 1. Scalar model of 1',3'-*bis*(1,2-distearoyl-*sn*-glycero-3-phosphoryl) glycerol dianion in a conformation that brings the two phosphated and the four aliphatic chains close to each other. There are eight CH_2(×) and one CH_3 (□) above the plane and eight CH_2(·) below the plane, in each chain.

triesters, **11**, are close to $+24° \pm 4$ in chloroform solution, although there is, possibly, a slight trend toward less positive values with a decrease in chain length. The degree of crystallinity of one of the triesters, **11** ($R = C_{17}H_{35}$) was examined by X-ray powder photography, which reveals that, at least this particular homolog, is essentially amorphous. The corresponding data for the ammonium salts, **1b**, will be considered in the next Section on metal ion salts.

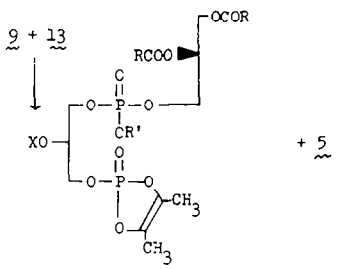
In summary, the four steps in the synthesis are carried out in three laboratory operations and consume about 10 hr of actual reaction time, although the isolation and purification steps lengthen the procedure according to the scale of the synthesis. Only one intermediate and the final product are purified, and the overall yields from diglycerides to purified DPG·$2NH_4^+$·$2H_2O$ salts are 29%, 23% and 25%, for the stearic, palmitic and myristic acid derivatives, respectively. In addition to the relatively short reaction time, and the high purity of the final product, the attractive features of the procedure are: (i) The troublesome 1,2→1,3 diglyceride isomerization[36,37] is totally prevented. (ii) There is practically no hydrolysis of carboxylic or phosphoric ester bonds. (iii) Stereospecificity is preserved.

Each of the individual steps of the synthesis was studied separately, utilizing the more accessible *racemic* diglycerides, *rac*-**5**, in order to establish the optimum conditions for the reactions, and to shed some light on their mechanisms.

The main drawback of the synthesis is associated with the transesterification that can occur during the reactions of the diglyceride cyclic phosphate, **9**, with the silylglycerol, **7**, and the silyl-PG triester **13**. These displacements at phosphorus with ring-retention produce the diglyceride, **5**, and the corresponding silylglycerol cyclic phosphate, **14**, and silyl-PG cyclic phosphate **15**.

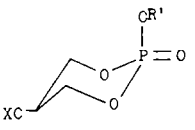


14, X = $(t\text{-}C_4H_9)(CH_3)_2Si$



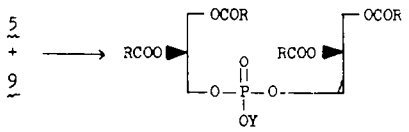
15, X = $(t\text{-}C_4H_9)(CH_3)_2Si$; R' = $CH(CH_3)COCH_3$

The by-products **14** and **15** can undergo further transformations, e.g. cyclization of **14** to the silylphosphate, **16**, or reaction of **14** with more silylglycerol, **7**, to give a symmetrical phosphotriester (not shown).



16, X = $(t\text{-}C_4H_9)(CH_3)_2Si$; R' = $CH(CH_3)COCH_3$

The by-products, **14**, **15** and **16**, have not been isolated. However, the regeneration of the diglyceride, **5**, during the reaction of cyclic phosphate **9** with silylglycerol **7** has been verified in independent studies. The diglyceride, **5**, is actually recovered in about 23% at the stage of the purification of the silyl-DPG triester **11**, in the synthesis. Some **5** reacts further with the cyclic phosphate, **9**, and produces a symmetrical phosphotriester, 3' - [(1" - methylacetonyl)(1,2 - diacyl - *sn* - glycero - 3)phosphoryl -] - 1',2' - diacyl - *sn* - glycerol (**17**). The phosphate-blocking group is easily removed from the PD[22] triester **17**, and the resulting 3' - (1,2 - diacyl - *sn* - glycero - 3 - phosphoryl) - 1',2' - diacyl - *sn* - glycerol is isolated as its monohydrated sodium salt, **18c**·H_2O. The properties of the three members of this family of phospholipids are given in Tables 2–4, and will be discussed below. The ratios of desired silyl-DPG triester, **11**, to PD triester, **17**, are estimated to be 2.35, 2.45 and 3.00 for C_{18}, C_{16} and C_{14} derivatives, respectively. Independent studies confirm that the by-product, **17**, is not formed in the first step of the synthesis, i.e. during the reaction of **5** with the pyrophosphate **8**.



17, Y = $CH(CH_3)COCH_3$

18, Y = $(C_2H_5)_3NH^+$

18a, Y = H^+

18c, Y = Na^+

An important feature of the present synthetic approach to DPG is the newly discovered[46] neighboring group effect which permits the facile removal of a t-butyldimethylsilyl group,[47-49] X, from a glycerophosphate, **19**, *after removal* of the 1-methylacetonyl blocking group. The deblocking step occurs under slightly basic conditions, **19** → **20**, which do not affect carboxylic esters. The deprotection step takes place on the phosphodiester, **20**, under relatively

Table 2. Elemental analyses and physical constants of 3'-phosphatidyldiglyceride (PD) sodium salts

Compd. No.	R	M_p°	$[\alpha]_D^{20}$	M_D	Molecular Formula (MW)	Calcd., % C	H	P	Na	Found, % C	H	P	Na
18c·H_2O	$C_{17}H_{35}$	67-69	+3.25[a]	+43.9	$C_{78}H_{150}O_{12}PNa.H_2O$[b] (1352)	69.3	11.3	2.3	1.7	69.6	11.2	2.5	1.6
	$C_{15}H_{31}$	51-56	+3.50	+43.4	$C_{70}H_{134}O_{12}PNa.H_2O$ (1240)	67.8	11.0	2.5	1.8	68.1	11.2	2.7	1.4
	$C_{13}H_{27}$	45-47	+3.90	+43.9	$C_{62}H_{118}O_{12}PNa.H_2O$ (1127)	66.0	10.7	2.7	2.0	66.4	10.7	2.9	1.4

[a] c, 4.00 ($CHCl_3$). [b] % H_2O (Karl Fischer): found, 1.2, 2.2; calcd., 1.3, 1.5, for R = $C_{17}H_{35}$, $C_{15}H_{31}$, respectively.

Table 3. Spectral data of phospholipids[a]

^1H nmr Assignments	Silyl-DPG Triesters (11)[b] τ, ppm	Ir bands, cm^{-1}[d]	DPG Ammonium Salts (1b.$2H_2O$) τ, ppm	Ir bands, cm^{-1}[d]	DPG Metal Ion Salts (1c-e.$2H_2O$) Ir bands, cm^{-1}[e]	PD Sodium Salts (18c.H_2O)[c] τ, ppm	Ir bands, cm^{-1}[d]
$(t\text{-}C_4H_9)(CH_3)_2Si$	9.89, 9.10		...			...	
$CH(\underline{CH_3})CO\underline{CH_3}$	8.56, 7.78	2930(s),	...	3300-3150(s centered at	3700-3100(s centered at 3450),	...	2930(s), 2860(s),
$\underline{CH_3}$-	9.00	2860(s), 1740(s),	9.10	3250), 2930(s),	2960(s), 2920(s), 2860(s), 1740(s),	9.14	1738(s), 1468(m),
-$\underline{CH_2}$-	8.47	1465(m),	8.75	2858(s),	1630(vw), 1740(m),	8.76	1380(w),
R'$\underline{CH_2}$COO-	7.63	1362(w), 1260(m),	7.70	1735(s), 1467(w),	1420, 1380(vw), 1250(s), 1200(s),	7.75	1255(m), 1170(m),
RCOO$\underline{CH_2}$, >P(O)O$\underline{CH_2}$, XO$\underline{CH}$	6.00, 5.23	1155(m).	6.09	1378(w), 1220(ms),	1180(s), 1140, 1110(s), 1060(s).	5.90	1110(m), 1070(m).
RCOO$\underline{CH}$	4.77		4.71	1060(s).		4.77	
Other ^1H	5.3(Acn,CH)		2.75(H_2O)			...	

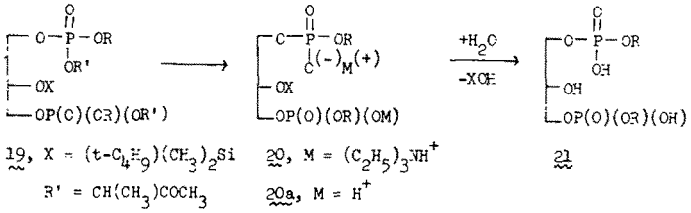
[a] The three members of the homologous series have similar spectra. ^{31}P nmr signals (in $CDCl_3$) in ppm vs H_3PO_4 = 0 (at 36.4 MHz). ^1H nmr signals (in $CDCl_3$) in ppm vs TMS = 10 (at 60 and 90 MHz); integrated intensities are in agreement with expected values. Nmr signals for corresponding non-identical protons in both phosphatidyl and diglyceryl moieties in these molecules were not resolved. [b] $\delta^{31}P$ = -1.5 ± 0.5 ppm. [c] $\delta^{31}P$ = 0.0 ± 0.5 ppm. [d] In $CHCl_3$ solution. [e] 1.5 mg in 129 mg of KBr (disc).

Table 4. TLC of diglycerides and phospholipids[a]

Compound	R	Rf
Silyl-DPG Triesters (11)	$C_{17}H_{35}$	0.19(E,A); 0.42(H,C)[b]
		0.10(H,A); 0.30(H,C)
	$C_{15}H_{31}$	0.27(H,B)
	$C_{13}H_{27}$	0.26(H,B)
Silyl-DPG Salts (12)	All homologs	0.00(N,C); 0.78(N,D)
DPG Salts (1)	All homologs	0.00(N,C); 0.37(N,D)
PD Triesters (17)	All homologs	0.33(H,A); 0.36(H,B) 0.78(H,C)
PD Salts (18)	All homologs	0.00(N,E); 0.63(N,F)
Diglycerides (5)	All homologs	0.46(H,A); 0.51(H,B); 0.85(H,C); 0.67(N,C); 0.50(H,N,E)

[a] Silica gel H (Type 60, cat. no. 7736, Merck); silica gel N (Macherey, Nagel and Co.). Solvent: A = hexane/diethyl ether 3/1; B = ditto 1/3; C = diethyl ether; D = $CHCl_3/CH_3OH$/conc-NH_3 13/5/1; E = $CHCl_3/CH_3COCH_3$ 24/1; F = $CHCl_3/CH_3OH$ 8/1. Spots developed by charring after spraying with 35% H_2SO_4, or Zinzadze Reagent: (Ref. 39); binder-free silica gel on glass plates; cf. ref. 38-41. [b] Detectable diastereomers (upper and lower values).

mild acidic conditions: **20a**→**21**; these conditions do not bring about the deprotection of the triester **19**. The rate of desilylation of the diester, **20**, is not increased at much higher hydrogen ion concentration, and the effect may be a consequence of a relatively high local acid concentration.



19, X = $(t\text{-}C_4H_9)(CH_3)_2Si$; R' = $CH(CH_3)COCH_3$

20, M = $(C_2H_5)_3NH^+$

20a, M = H^+

21

Conversion of ammonium salts of DPG, **1b**, *into metal ion salts,* **1c–e**. A simple method to prepare pure DPG monovalent and divalent metal ion salts from the ammonium salts involves the treatment of the latter with an aqueous solution of the metal ion chloride in a suitable mixture of chloroform, methanol and water:[50]

$$DPG{\cdot}2NH_4^+ + 2MCl \longrightarrow DPG{\cdot}2M^+ + 2NH_4Cl$$

$$DPG{\cdot}2NH_4^+ + MCl_2 \longrightarrow DPG{\cdot}M^{2+} + 2NH_4Cl.$$

The sodium,[51] magnesium and calcium salts, **1c–e**, prepared from NaCl, $MgCl_2$ and $CaCl_2$, respectively, are described in Tables 1, 3 and 4.

This procedure is an adaptation of the method used to isolate the ammonium salts in the synthesis of DPG. The metal ion salts are much less soluble in chloroform than the corresponding ammonium salts. A satisfactory distribution of the metal salts into the organic phase is achieved, without formation of emulsions, by increasing, both, the chloroform/water and the methanol/water ratios, while decreasing the chloroform/methanol ratio, relative to the proportions of these solvents used for the distribution of the ammonium salts between the organic and the aqueous phases. The procedure is quite sensitive to the presence of even traces of metal ion chlorides in the aqueous phase.[50] This point becomes significant, in particular among sodium salts, as the aliphatic chain decreases in size, e.g. in the myristoyl series, when there is a tendency toward emulsification, and loss of $DPG{\cdot}2Na^+$ into the aqueous phase, after practically all metal ion chloride is removed from the system.

The mobility of the three homologous DPG, **1**, in silica gel TLC is virtually the same, regardless of the cation present in the salt that is applied, when the eluting solvent is basic (Table 4). The synthetic salts are considerably less mobile ($R_f = 0.37$) than a sample of beef-heart CL ($R_f = 0.55$), and this is presumably due to the presence of unsaturated fatty acid residues in the latter. Similar observations have been reported in natural CL.[25]

All the DPG salts, **1b–e**, are obtained with 2 molecules of water, which cannot be removed in 24 hr at 20° and 0.1 mm. X-ray powder photographs of the three ammonium salts, **1b**, reveal no significant degree of crystallinity in the samples. The result of similar studies on the metal ion salts, **1c–e**, will be described elsewhere in connection with studies on the ionophoretic properties of

the cardiolipins.[52] The metal ion salts, **1c–e**, are insoluble in methanol, as well as in chloroform, or mixtures of these two solvents.[53,54]

Among DPG ammonium salts, **1b**, there is a significant increase in the positive M_D values in the aqueous-organic solvent relative to the pure organic solvent: $+90.0° \pm 0.5$ in chloroform vs $+127° \pm 3$ in chloroform/methanol/water 4/4/1, at comparable concentrations. The M_D values remain virtually constant as the chain length decreases. The metal ion salts, **1c–e**, can be examined only in the aqueous-organic solvent. Among the sodium, **1c**, and the magnesium, **1d**, salts, there is a slight trend toward more positive M_D values as the chain length decreases, the figures centering around $+117° \pm 9$ and $+139° \pm 10$, respectively. Substitution of the cations of the salts have the following effects: $NH_4^+ \rightarrow Na^+$ is accompanied, in all cases, by a slight decrease in the positive M_D values, while the change $Na^+ \rightarrow Mg^{2+}$ results in a somewhat larger change in the *opposite* direction. The calcium salt has the least positive M_D value among all the salts examined. It is noteworthy that de Haas and van Deenen[10] have observed a dramatic change from $+87.5°$ to $-99.8°$ in the M_D values of the sodium vs the barium salts of their synthetic 1-stearoyl-2-oleoyl-DPG, in chloroform (the presence of unsaturation increases the solubility of the salts in the organic solvent).

It is conceivable that the changes in M_D values with the nature of the cation may reflect changes in the conformation of the molecules in solution. As illustrated in Fig. 1, the approach of the two phosphate anions to each other, and the packing of the aliphatic chains could vary significantly depending on the charge-type, the size, and the degree of hydration of the metal cation.

Characterization of PD[22] derivatives, **17** *and* **18**. The PD triester, **17**, formed as by-product of the synthesis of DPG, **1**, is easily converted into the PD sodium salt, **18c**, by removal of the 1-methylacetonyl group. This treatment permits the facile separation of this phospholipid from the diglyceride, **5**. The purification of PD is achieved by silicic acid column chromatography.[44] Homogeneity in the cation content[44] of the eluted material is insured by submitting the latter to a metathetical reaction in $CHCl_3/CH_3OH$/water containing a large excess of sodium chloride. This technique[50] is analogous to that used in the conversion of the DPG ammonium salts into their metal ion salts, **1b** → **1c–e**.

The PD free acids, **18a**, have been prepared by Baer,[55] and it is interesting that they melt above the corresponding sodium salts.

The three PD sodium salts, **18c**, are obtained with one molecule of water, which cannot be removed in 24 hr at 20° and 0.1 mm. X-ray powder photographs of two of the salts disclose that the C_{16}-salt has a significant degree of crystallinity, while the C_{18}-homolog is essentially amorphous. These sodium salts are significantly soluble in chloroform.

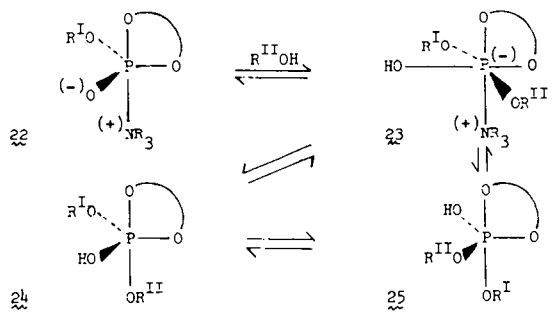
The consistency of the presence of one molecule of water per phosphate anion, regardless of the charge type of the cation, in both types of phospholipids, the DPG salts, **1c–e**, and the PD sodium salts, **18c**, is noteworthy.

The M_D values of the three PD sodium salts, **18c**, are virtually the same, $+43.3° \pm 0.3$ in chloroform solution.

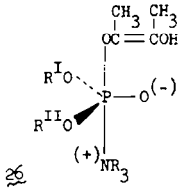
DISCUSSION

There is a substantial amount of evidence in support of the hypothesis that intermediates with 5-coordinate phosphorus are formed in nucleophilic displacements of compounds with 4-coordinate phosphorus.[56,57] Recently, one of these oxyphosphorane intermediates has been trapped in a reaction of a 5-membered cyclic phosphotriester.[58] Additional evidence on this question has been obtained from studies on the imidazole,[32] tertiary amine[33] and phenoxide ion[59] catalysis of the reaction of alkyl cyclic enediol phosphates, analogous to the diglyceride cyclic phosphate, **9**, with alcohols in aprotic solvents of low polarity. These studies have led to the further hypothesis[32,59] that the catalyzed phosphorylations proceed via intermediates with hexacoordinate phosphorus, which are formed from the oxyphosphoranes. These considerations proved to be useful in the present synthetic approach, as follows.

The amine-catalyzed reaction of the silylglycerol, **7**, with the cyclic phosphate, **9**, may proceed via the phosphorane **22**, which adds the alcohol (**7** = $R^{II}OH$) in the presence of the base to give the P(6) intermediate. The latter, **23**, can collapse to one or both of the isomeric phosphoranes, **24** or **25**, and these may, in turn, equilibrate. Isomer **24** is the phosphorane that results from the apical addition of $R^{II}OH$ to the phosphate **9** in the absence of catalyst. It has been suggested that the P(4) ⇌ P(5) step is rate-controlling and the catalysis results from the higher nucleophilicity of the amine vs the alcohol $R^{II}OH$. The P(5) ⇌ P(6) step is assumed to be relatively rapid. If ring-opening of **24** occurs prior to equilibration with isomer **25** the desired acyclic triester **13** (or **11** in the later step) is produced exclusively. Equilibration of **24** and **25**, followed by apical-departure of diglyceride **5** from **25** (OR^I) yields the cyclic phosphate **14** (or **15** in the later step). This represents transesterification and leads to by-products, e.g. the PD triester **17**. In general a phosphorane with a relatively small ligand at the apex and a bulkier ligand in the equator should be favored over its isomer with the reverse ligand-distribution.[32b] If the thermodynamically favored P(5) isomer, **24**, is initially formed by apical attack of $R^{II}OH$ on **9** (uncatalyzed reaction), or is produced exclusively by collapse of **23** (catalyzed reaction), the tendency for isomerization to **25** should be lower, and this would facilitate ring-opening resulting in less transesterification and a smaller amount of by-product **17**. The silylglycerol, **7** $R^{II}OH$, and the silyl-PG triester, **13**, are relatively bulky ligands, since they have a t-butyldimethylsilyloxy substituent in the near vicinity of the oxygen atom that must be attached to the 5-coordinate phosphorus, i.e. C2'- of glycerol. Figure 1 gives an idea of the complexity of the ligands involved in these oxyphosphoranes. It is, perhaps, not surprising that there is a significant amount of transesterification in the second step of the synthesis. In simpler systems, amine catalysis reduces the amount of symmetrical prosphotriester by-products as compared to the uncatalyzed reactions,[32b]



and this has been attributed to the formation of the acyclic phosphorane **26** by ring-opening of the P(6) intermediate, **23**. To the extent that the P(6) collapses to **26**, and not to **24** or **25**, there should be no transesterification, since **26** gives the desired products **13** (and **11**) by apical-departure of the catalyst.



EXPERIMENTAL

Analyses by Galbraith Laboratories, Knoxville, Tenn. All samples were dried for 24 hr at 20° (0.1 mm) prior to elemental analysis. M.ps were observed in open capillaries and are corrected. Data for new compounds are listed in Tables 1–4. Reactions involving cyclic enediol phosphate derivatives must be performed under strictly anhydrous conditions, preferably under N_2 or Ar.

Synthesis of ammonium salts of DPG, **1b**

First and second steps. A soln of **5**[30] ($R = C_{17}H_{35}$; 10.44 g, 16.8 mmol) in dichloromethane (350 ml) was added, over a 3-min period, to a soln of **8**[31,32] (4.75 g, 16.8 mmol) and triethylamine (2.45 ml, 17.6 mmol) in dichloromethane (25 ml), at 25°, with stirring. The soln was stirred for 45 min, and was treated with a soln of **7**[35] (i.74 g, 8.4 mmol), and triethylamine (3.5 ml, 25.2 mmol) in dichloromethane (30 ml), added at once, with stirring, at 25°. After 2 hr, the soln was evaporated (rotoevaporator, 25°, 20 mm), and the solid residue was analyzed by TLC[38–41] on silica gel H (solvent B), showing the presence of **11**, **17** and **5**; (see Table 4 for R_f values).

Purification of silyl-DPG triesters, **11**. The crude residue obtained above was dissolved in the minimum volume of dichloromethane. Three aliquots were chromatographed on separate columns of silica gel 60 (70–230 mesh; cat. no. 7734, Merck), eluting with solvents B and C (Table 4), successively. The fractions were analyzed by silica gel H microslide TLC, and the corresponding fractions from the 3 columns were pooled, evaporated, and dried in vacuum (over P_2O_5) to constant weight. Approximately 300 g of silica gel was used in each column for 6.7 g of crude residue, and 50 ml-fractions were collected at a 1.8–2.0 ml/min flow rate. The first 9 fractions (solvent B), plus 7 fractions (C) contained **5** and **17**. Fractions no. 17 and 18 (C) contained these two substances and traces of **11**. The PD-triester **17** was converted into **18c** for characterization as described below; 2.4 g of **5**, and 1.78 g of **18c** were recovered from this treatment.

The pure **11** (5.39 g) was isolated from fractions 19–38 (C) of the chromatogram; this sample was submitted for analysis. The homologs, **11**, $R = C_{15}H_{31}$ and $C_{13}H_{27}$, were obtained by essentially the same procedure.

Third step. Water (6.8 ml) was added to a soln of purified **11** ($R = C_{17}H_{35}$; 774 mg, 0.45 mmol) in pyridine (6.8 ml). Triethylamine (0.25 ml, 1.8 mmol) was added to the heterogeneous mixture, which was stirred for 2.5 hr at 25°. A gel appeared within *ca.* 30 min, and the necessary fluidity was maintained by occasional application of a warm air stream. The water and most of the pyridine was evaporated by co-distillation with benzene at 20° under reduced pressure (*ca.* 10 mm). The semi-solid residue was kept under vacuum (0.1 mm) to constant weight, to remove the last traces of pyridine. The colorless solid residue (805 mg) was analyzed by silica gel N TLC, in the solvents C, D and E; the analysis revealed the presence of the desired **12** contaminated by *ca.* 5% of **1**. No starting material, **11** nor **5** were detectable.

A similar procedure afforded the homologous **12**, $R = C_{15}H_{31}$ and $C_{13}H_{27}$.

Fourth step. Chloroform (60 ml), MeOH (120 ml), and water (41.5 ml), were successively added, with stirring, to the crude **12** ($R = C_{17}H_{35}$; 393 mg, 0.22 mmol) to produce a homogeneous soln (1 mM concentration of **12** in $CHCl_3/CH_3OH/H_2O$ 1.5/3/1). To this soln was added 550 μl of 1N HCl (2.5 molequivs of HCl relative to **12**), and the mixture was stirred for 5 hr at 25°. Silica gel N TLC (solvent D) showed only traces of starting material, **12**. The soln, which contained the DPG free acid, **1a**, was neutralized by the addition of 2 ml of 10% NH_4OH. The solvent composition was adjusted[50] to $CHCl_3/CH_3OH/H_2O$ 2.7/1.3/1 in order to achieve an efficient distribution of DPG·$2NH_4^+$, **1b**, between the organic and the aqueous phases, without the formation of emulsions. The two layers were separated, and were evaporated and dried in vacuum (0.1 mm). The lower organic layer yielded 297 mg (theory: 330 mg) of residue; silica gel N TLC in solvents D and E showed that this material contained *ca.* 95% of desired DPG·$2NH_4^+$, **1b**, *ca.*, 5% of **12** (as ammonium salt), and no significant amounts of **5**.

The upper aqueous layer yielded 88 mg of residue, of which 29 mg was presumably NH_4Cl. This residue was taken up in $CHCl_3$, filtered through cotton to remove inorganic material, evaporated, and chromatographed as described below to retrieve additional **1b**.

The removal of the silyl group: **12** → **1b** in the other homologous series was carried out by the same procedure.

Purification of ammonium salts of DPG, **1b**. Whatman (DE 23) advanced fibrous DEAE-cellulose was pre-cycled as described.[7,42,43] It was successively washed *prior to* sample application with equal volumes of CH_3OH, $CHCl_3/CH_3OH$ 1/1, and $CHCl_3$ (14 ml/g). The column was regenerated after use by successive washings with: (a) $CHCl_3/CH_3OH$/conc-NH_3/CH_3COONH_4 4/1/(20 ml/l)/50 mM, v/v; (b) CH_3OH; (c) glacial CH_3COOH, 14 ml/g and was stored under glacial CH_3COOH for several months.

In a typical experiment, the crude DPG·$2NH_4^+$ **1b** ($R = C_{17}H_{35}$; 350 mg) was dissolved in $CHCl_3/CH_3OH$ 3/1 (30 ml), and was applied to a 22.5 × 2.5 cm column containing 15 g of DEAE-cellulose. Elution was carried out as follows (50 ml-fractions at *ca.* 1.5–2.0 ml/min flow rate). The first 3 fractions ($CHCl_3$) removed diglycerides (36 mg). Fractions 4–10 ($CHCl_3/CH_3COOH/CH_3COONH_4$ 3/1/5 mM) contained mostly **12**, and some **1**; (*ca.* 70 mg combined weight). Fraction Nos. 11 and 12 (same solvent, but with 10 mM acetate), and 13–15 (*ditto*, but with 25 mM acetate), contained pure **1b**, according to TLC; these fractions were combined and were evaporated in vacuum. The residue was dissolved in $CHCl_3/CH_3OH$ 2/1 (45 ml), and the soln was mixed with 1 ml of water, and *carefully* (spattering may occur) concentrated at 20° and 10 mm until opalescence or a slight precipitation was noted. The mixture was freeze-dried, and was kept at 0.1 mm for 24 hr. The residue was redissolved in $CHCl_3/CH_3OH$ 2/1 (30 ml), and was evaporated and dried to constant weight at 0.1 mm, to yield 256 mg of pure **1b**·$2H_2O$, $R = C_{17}H_{35}$, which was submitted for elemental analysis.

The homologous ammonium salts, **1b**·$2H_2O$, $R = C_{15}H_{31}$ and $C_{13}H_{27}$ were obtained by the same procedure.

Conversion of ammonium salts of DPG, **1b**, *into metal ion salts*, **1c–e**

Sodium salts. A soln of **1b**·$2H_2O$ ($R = C_{17}H_{35}$; 172 mg) in $CHCl_3/CH_3OH$, 2/1 (48 ml) was mixed with $CHCl_3/CH_3OH$/4M aqueous NaCl, 1/16/16 (20 ml), and the two-phase system was vigorously stirred for 5 min at 20° (*overall solvent composition*: $CHCl_3/CH_3OH/H_2O$, 3.3/2.6/1). The mixture was allowed to stand, and the sharply separated upper aqueous layer was withdrawn. The lower organic layer was submitted twice more to the same procedure. This treatment allowed **1c** to distribute itself favorably into the organic layer without the formation of emulsions. The organic layer was washed once with $CHCl_3/CH_3OH/H_2O$, 1/16/16, was allowed to separate, and was drawn and evaporated under reduced pressure (rotoevaporator at 20°; there was some frothing, but the evaporation could be carried out without loss of material due to spattering). The residue was cooled in a dry-ice bath, and was freeze-dried and kept under high vacuum to constant weight. The properties of **1c**·$2H_2O$ ($R = C_{17}H_{35}$), are given in Table 1. The same prodecure afforded the salts with $R = C_{15}H_{31}$ and $C_{13}H_{27}$, respectively.

In the myristoyl series, $R = C_{13}H_{27}$, the partition of **1c** between the organic and the aqueous phases becomes particularly sensitive

to the presence of even traces of NaCl. As the size of the aliphatic chain decreases, the tendency toward water solubility of the salt increases. This results in some emulsification, and in loss of DPG·2Na⁺ into the upper aqueous phase, as the last traces of NaCl are removed during the washing of the organic layer with $CHCl_3/CH_3OH/H_2O$ 1/16/16, i.e. in the absence of NaCl. If emulsions are formed, a few drops of methanol should be added.

Magnesium salts. **1d**·$2H_2O$ (Table 1) were prepared from the corresponding ammonium salts, **1b**, following the procedure described for the preparation of **1c**, except that $CHCl_3/CH_3OH$/1.92 M aqueous $MgCl_2$ was used as reagent.

Calcium salts. **1e**·$2H_2O$ (Table 1) were prepared from **1b** as for the Na salts, except that $CHCl_3/CH_3OH$/1.14 M aqueous $CaCl_2$ was used as reagent.

Control experiments, without the phospholipid, show that no metal ion chloride goes into the lower organic phase in this procedure. Chlorine analysis on some of the DPG metal ion salts demonstrate no contamination by this element (*cf.* Ref. 52).

Characterization of PD derivatives, **17** *and* **18**. The mixture (4.5 g) of **17** ($R = C_{17}H_{35}$) and **5** ($R = C_{17}H_{35}$), obtained during the purification of the crude **11** ($R = C_{17}H_{35}$) by silicic acid column chromatography, was dissolved in pyridine (110 ml). Addition of water (110 ml) and triethylamine (5 ml) to this soln produced a gel, and the mixture was stirred for 4 hr at 25°; the necessary fluidity was maintained by occasional warming with air. Most of the pyridine and the water were removed by co-distillation with benzene (30°, 10 mm), and the residue was dried at 0.1 mm. The solid residue was chromatographed on a column of silica gel (Merck No. 7754). Elution with ether (200 ml), followed by chloroform (50 ml) removed **5** and only traces of PD.M⁻. Elution with $CHCl_3/CH_3OH$ 5.7/1 (600 ml) gave the pure PD.M', analyzed by silica gel N TLC (see Table 4). This sample of PD.M' (1 g) was dissolved in $CHCl_3/CH_3OH$ 2/1 (45 ml), and was mixed with 4N aq-NaCl (12 ml); overall solvent composition: $CHCl_3/CH_3OH/H_2O$, 2.7/1.3/1. The heterogeneous system was stirred for 10 min at 25°. The sharply separated lower organic phase was treated with $CHCl_3/CH_3OH$/4N aq-NaCl. The lower layer was again washed with this solvent-system, and finally with CH_3OH/H_2O 1/1. The organic solvent was evaporated, and the residue was freeze-dried and kept under vacuum (P_2O_5) to constant weight. The sample of **18c**·H_2O (936 mg) was submitted to elemental analysis.

The corresponding **18c**·H_2O ($R = C_{15}H_{31}$ and $C_{13}H_{27}$) were obtained by the same procedure.

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