Some Structural Investigations on Phospholipids from Membranes

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Abstract

A complete chemical characterization of phosphatidyl glycerol from green leaves and algae demonstrated that 1-linoleneoyl-2-trans-Δ^3-hexadeconoyl-glycero-3-phosphoryl-1′-glycerol represented the major molecular species and its occurrence appeared to be related to photosynthesis.

Beef-heart cardiolipin was demonstrated to be identical to synthetic diphosphatidyl glycerol.

Chemical structures of synthetic amino acid esters of phosphatidyl glycerol were compared with those of amino acid and glucosamine containing phospholipids from bacterial cell membranes.

The molecular species of lecithin from animal tissues were recognized and the influence of dietary fats on their composition was determined.

Physical characteristics of natural and synthetic phospholipids indicate that nature is eloquent to preserve the properties offered by particular fatty acid combinations in the phospholipid molecule.

Mammalian tissues were found to contain phospholipase A activity which produces two structurally isomeric monoacyl-phosphoglycerides.

Utilizing five isomeric lysolecithins of known structure micro methods involving enzymic hydrolysis were developed to distinguish among these isomers. Lysolecithins from different natural sources were demonstrated to consist of both 1-acyl-glycero-3-phosphorylcholine and 2-acyl-glycero-3-phosphorylcholine.

In connection with the nonrandom distribution of fatty acids of different apolarity at the two positions of phosphoglycerides, the various metabolic pathways of lysolecithin enantiomers in red cell ghosts, yeast and liver were investigated using different doubly-labeled substrates.

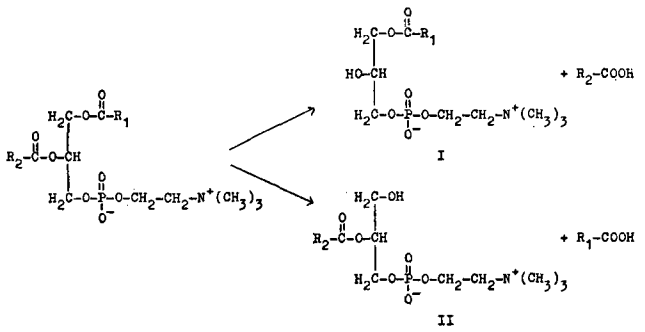
Introduction

BIOLOGICAL MEMBRANES contain a great diversity of lipid constituents which differ significantly in chemical structure. Although a number of lipid characteristics may be common to many membranes, several striking distinctions are to be noted in the makeup of lipids between various membranes (1). The possibility has to be envisaged that a particular lipid composition is of functional importance for the interface concerned and that possible variations in the molecular architecture of membranes are related with differences in the makeup of their lipids. The answer to many compelling questions about structure and function of biological membranes will require the efforts of investigators from many disciplines, the lipid (bio) chemist being responsible for donating data on the detailed composition, structure and dynamic aspects of the membrane lipids. The present contribution summarizes some recent studies from the authors' laboratory on two subjects: a) chemical structure and metabolic aspects of phosphoglyceride analogs containing only one acyl chain b) the complex phospholipids containing several glycerol moieties.

Lysolecithins

Lysolecithin Structure

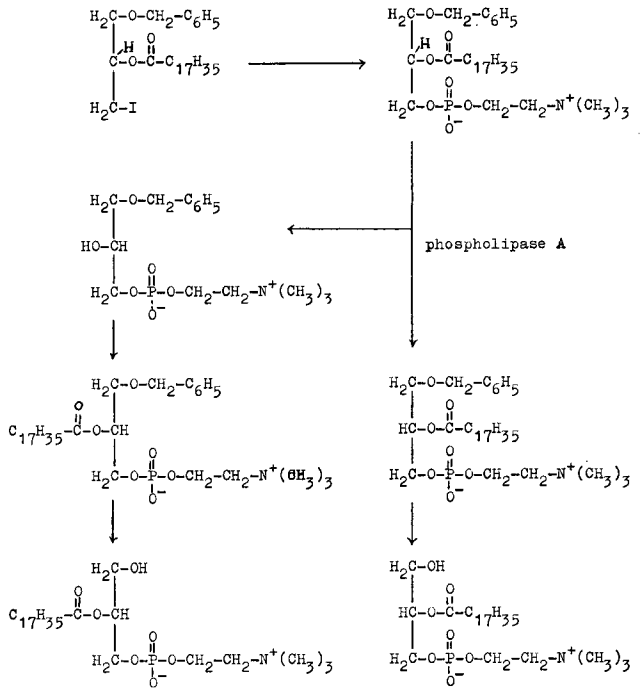
Studies carried out in this laboratory with the aid of synthetic phosphoglycerides containing two different fatty acids in defined positions unequivocally demonstrated that snake-venom (*Crotalus adamanteus*) contains a phospholipase A which exclusively acts at the 2-position of 3-phosphoglycerides (2–5). In 1963 Tattrie and Bennett (6) also concluded that this enzyme exhibits a specificity for the hydrolysis of the fatty acid ester linkage at the 2-position. The conclusion that the enzyme acts irrespective of the nature of the fatty acid constituents was endorsed by hydrolysis experiments on two synthetic lecithins containing oleic acid and butyric acid in different positions (7). The presence of unsaturated fatty acid constituents having the *trans* configuration appears not to interfere with the positional specificity of the snake-venom phospholipase A (8).



SCHEME 1. Possibilities for the mode of action of phospholipase A.

Whereas the snake-venom phospholipase A catalyses the hydrolysis of 3-phosphatidyl choline so as to give 1-acyl-glycerol-3-phosphoryl choline (I, Scheme 1), Lloveras et al. (9) and van den Bosch and van Deenen (10) obtained indications that animal tissue may contain lipolytic enzymes which in addition to I produce 2-acyl-glycerol-3-phosphoryl choline (II). The presence of these two isomeric lysolecithins would be of particular interest with respect to the reacylation process of lysophosphoglycerides in animal tissues, which according to Lands (11,12) involves a preferential transacylation of I and II with unsaturated and saturated fatty acids, respectively.

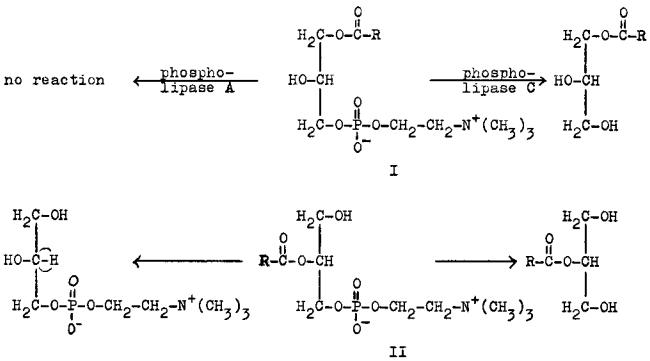
Further evidence that lysolecithins I and II are present in animal tissues was obtained by two different approaches. First, lysophosphoglycerides of defined structures were synthesized and methods were developed which enabled us to recognize these compounds in mixtures containing various structural or stereoisomeric monoacyl phosphoglycerides. Five of the six possible isomers have been prepared by Slotboom and de Haas (13,14) and the procedures utilized are illustrated by a synthesis of two stereoisomeric lysolecithins, viz., 2-stearoyl-glycerol-3-phosphoryl choline and 2-stearoyl-glycerol-1-phosphoryl choline (Scheme 2).



SCHEME 2. Synthesis of stereoisomeric (2-acyl)lysolecithins.

Starting from racemic 1-O-benzyl-2-stearyl-glycerol-3-iodohydrine a racemic "benzyl lecithin" was obtained which upon treatment with snake-venom phospholipase A was resolved into optically active 1-benzyl-glycerol-3-phosphoryl choline and 3-benzyl-2-acyl-glycerol-1-phosphoryl choline. Introduction of a fatty acyl residue in the former and removal of the protective benzyl groups furnished the two stereoisomeric lysolecithins, the structures of which were confirmed by reacylation.

In order to distinguish the five isomeric lysolecithins, use was made of lipolytic enzymes, viz., snake-venom phospholipase A and phospholipase C from *Bacillus cereus* (Fig. 1). Our previous work on the former enzyme established that only the fatty acid ester linkage located adjacent to the phosphoryl linkage is hydrolyzed, provided that a given stereochemical is realized (3,15). This positional and stereochemical specificity implies that 1-acyl-glycero-3-phosphoryl choline is not attacked, whereas from the two stereoisomeric lysolecithins 2-acyl-glycero-3-phosphoryl choline and 2-acyl-glycero-1-phosphoryl choline only the former is susceptible to hydrolysis by this enzyme. Similarly, of the two lysolecithins having the phosphoryl choline moiety attacked at the 2-position of glycerol, only one stereoisomer, viz., stearyl-glycero-2-phosphoryl choline is attacked, whereas stearoyl-glycero-2-phosphoryl choline is not susceptible at all. Phospholipase C displays a lower degree of stereospecificity, but de Haas found standard conditions which allowed one to make a distinction between the lysolecithin isomers (14). Although it cannot be precluded that (lyso)-lecithins derived from either glycero-1-phosphate or glycero-2-phosphate occur in nature, the phosphoglycerides from animal tissues are well established to be derived from glycero-3-phosphate, thus making it important to discuss in more detail the analysis applied for mixtures consisting of I and II (Scheme 3).



SCHEME 3. Analysis of positional isomeric lysolecithins.

A determination of the quantity of glyceryl-phosphoryl choline (or eventually of fatty acid released) after phospholipase A treatment gives information about the quantity of I present. Phospholipase C acts on both lysolecithins, but the isomeric monoglycerides can be separated by thinlayer chromatography (TLC) on hydroxyl-apatite according to the method of Hofmann (16). The model-experiments demonstrated that the presence of either isomer down to 10% in a mixture of both lysolecithins can be recognized.

Application of these procedures to the lysolecithin fraction from rat liver enabled van den Bosch (17)

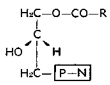
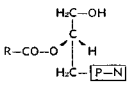
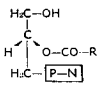
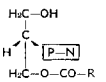
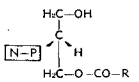
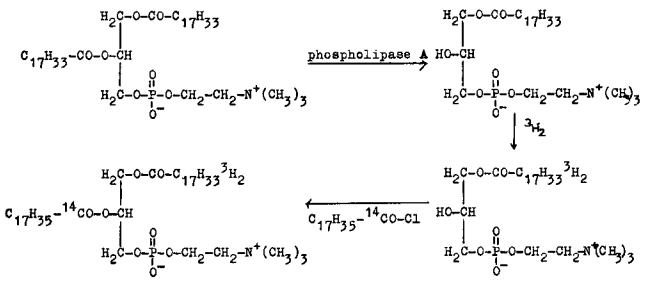
Type of lysolecithin	I A	II A	II B	III A	III B
Structure					
Degradation by phospholipase A	−	+	−	+	−
Degradation by phospholipase C	+	+	−	−	+

FIG. 1. Hydrolysis of stereoisomeric lysolecithins by phospholipase A and phospholipase C (Ref. 14).

to conclude that indeed both I and II are present in the lipid extracts obtained from this tissue. The quantitative data showed considerable scattering which had to be expected in as much as synthetic lysolecithin II was found to be subject of a migration furnishing lysolecithin I. Although precautions were taken and the liver-lysolecithin was isolated by TLC instead of by column chromatography, the migration reaction apparently was not prevented completely. Nevertheless these analysis qualitatively confirmed the conclusions already obtained by enzymatic hydrolysis experiments on biosynthetically prepared lecithins containing as labels ^{32}P and a ^{14}C-fatty acid constituent (10).

The enzymatic approach was recently extended to a synthetic lecithin substrate containing two differently labeled fatty acid constituents at the 1- and 2-positions, respectively. This substrate was prepared by a procedure (Scheme 4) previously applied to the partial synthesis of mixed-acid lecithins (18). Synthetic dioleoyl-lecithin was degraded with snake-venom phospholipase A, so as to give 1-oleoyl-glycero-3-phosphoryl choline. After introduction of tritium, the lysocompound was acylated so as to give 1-[9. 10.^{3}H]-stearoyl-2-[1. ^{14}C]-stearoyl-glycero-3-phosphoryl choline.



SCHEME 4. Synthesis of doubly-labeled lecithin.

A comparison of the [^{3}H]/[^{14}C] ratio in the lysolecithin produced from this substrate allows a precise determination of the proportions of 1-acyl-glycero-3-phosphoryl choline (I) and 2-acyl-glycero-3-phosphoryl choline (II). As demonstrated by the isotopic ratio of the lysolecithin formed by snake-venom phospholipase A (Table I), which enzyme can be expected to produce exclusively the former lysolecithin. Some interchange of fatty acids occurred during the synthesis of the doubly-labeled lecithin, which synthesis was carried out on a small scale, and for the labeling involved a transportation from Holland to England and vice versa. The deviation, however, was rather limited and can be accounted for by making an appropriate correction. Incubation of the doubly-labeled lecithin with rat liver homogenates furnished lysolecithin preparations which by virtue of their isotopic ratio can be considered to consist of nearly equal amounts of 1-acyl and 2-acyl lysolecithins (Table I). Hence, the various approaches are in good agreement and endorse the conclusion that rat liver is capable of producing the two lysolecithin isomers which are needed for the transacylation reactions described by Lands and co-workers (11,12). The evidence available allows one to formulate a monoacyl-diacyl phosphoglyceride cycle (Fig. 2).

TABLE I

Degradation of Doubly-Labeled Lecithin ([9, 10, ^{3}H$_2$] Stearoyl-2-[1. ^{14}C] Stearoyl-Glycero-3-Phosphoryl Choline) by Phospholipase A

Enzyme source	^{3}H/^{14}C ratio		2-acyl lysolecithin II formed (calc.) %
	Lecithin substrate	Lysolecithin formed	
Crotalus adamanteus	9.7	246	0
Liver homogenate		6.9	58
Pancreas homogenate		3.8	75
Pancreas heated		45.7	12
Purified pancreas phospholipase A		184	1

Before discussing a few biological aspects of this metabolic pathway, some attention will be paid to the question whether the formation of isomeric lysolecithins from the doubly-labeled lecithins is brought about by one phospholipase or by two distinct enzymes. In this respect the results obtained on the lipolytic enzymes of pancreas tissue are of interest. Previous work showed that pancreas tissue contains a heat-stable phospholipase A, which exhibits the same mode of action as the enzyme from snake-venom and acts at the 2-fatty acid ester linkage only, so as to give lysophosphoglycerides of structure I (19). Incubation of the doubly-labeled lecithin with a fresh homogenate of pancreas tissue, however, revealed that a mixture of both I and II is formed (20), the latter even being the dominant one (Table I). Heat-treatment of the tissue induced a shift in the ratio of I and II produced from doubly-labeled lecithin, while further purification of the heat-stable phospholipase A furnished a preparation which, in agreement with our previous work on this enzyme produced only 1-acyl lysolecithin. Apparently pancreatic tissue contains two lipolytic enzymes acting on the 1- and 2-fatty acid ester linkage of phosphoglycerides, respectively. We have tentatively denoted these enzymes as phospholipase A^1 and phospholipase A^2, respectively (20). However, it soon became doubtful whether the pancreatic enzyme acting at the 1-position

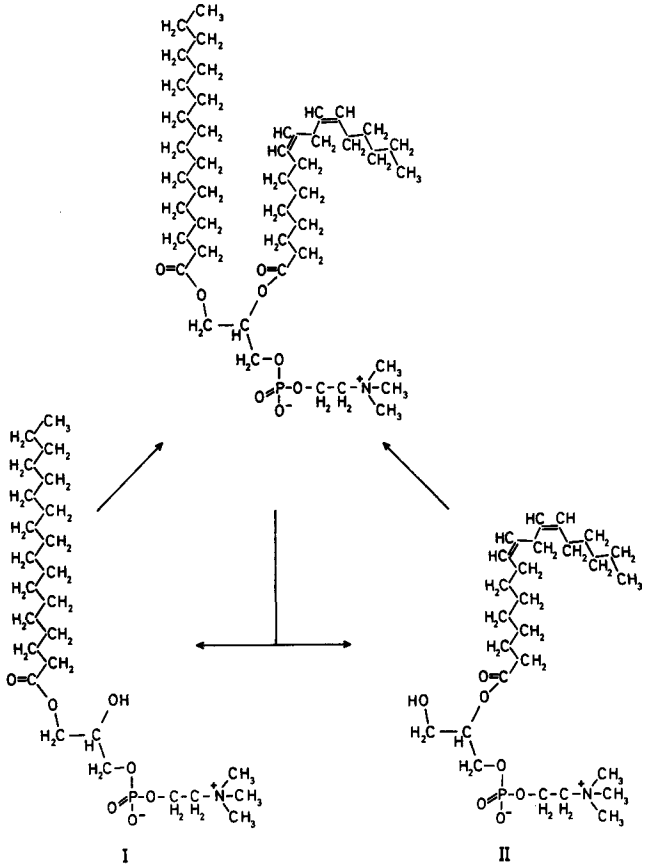


FIG. 2. Diacyl—monoacyl phosphoglyceride cycle.

of the phospholipid can be regarded as being a "true" phospholipase. Dr. de Haas, working in the laboratory of Professor Desnuelle at Marseille tested the activity of the pure lipase preparations isolated in that laboratory from pig pancreas towards phospholipids. It was found that this enzyme preparation acted on phospholipids also, though at a slower rate than on glycerides, and the fatty acids liberated from the phospholipid appeared to originate from 1-fatty acid ester linkage exclusively. Although further studies are required, it appears reasonable to assume at present that the phospholipase A^1 activity found in pancreatic tissue was due to a lipase (21). No conclusion can be made at present in regard to the nature of the lipolytic enzyme(s) from rat liver producing two isomeric lysophosphoglycerides.

The cycle formulated in Figure 2 is considered to preserve the nonrandom distribution of saturated and unsaturated fatty acids within the phosphoglyceride molecules and thus may contribute to the attainance of phospholipid molecules with the physical properties desired by biological structures. These processes involved in fatty acid renewal may contribute considerably to the dynamic character of the membrane structures concerned.

Molecular Species of Lecithin

Alterations in the composition of diets are well known to be reflected by the fatty acid composition of the phospholipids from various animal tissues. Lecithin preparations from liver of rats fed on a fat-free diet or a regimen containing coconut oil or corn oil differ considerably in the makeup of the apolar side-chains. Whereas monomolecular films of synthetic phospholipids containing different fatty acid constituents revealed great differences in molecular packing (22,23), the films of the three natural lecithin preparations under discussion showed limited differences in force-area curves only (23). An explanation for this phenomenon may be obtained by analyzing the lecithin preparations in terms of the composition of their molecular species (24–27). For that purpose the following procedures were utilized: a) Subfractionation of the lecithins by TLC on silica impregnated with silver nitrate and determination of the fatty acid distribution in the fractions obtained with the aid of snake-venom phospholipase A; b) Degradation of the phospholipids with phospholipase C from *B. cereus* and subfractionation of the diglycerides formed.

The positional distribution of fatty acids in the diglyceride fractions was ascertained by means of hydrolysis with pancreatic lipase. Although it has not been possible to date to achieve a complete quantitative analysis of all molecular species of rat liver lecithin, the results (Table II) appear to indicate

TABLE II

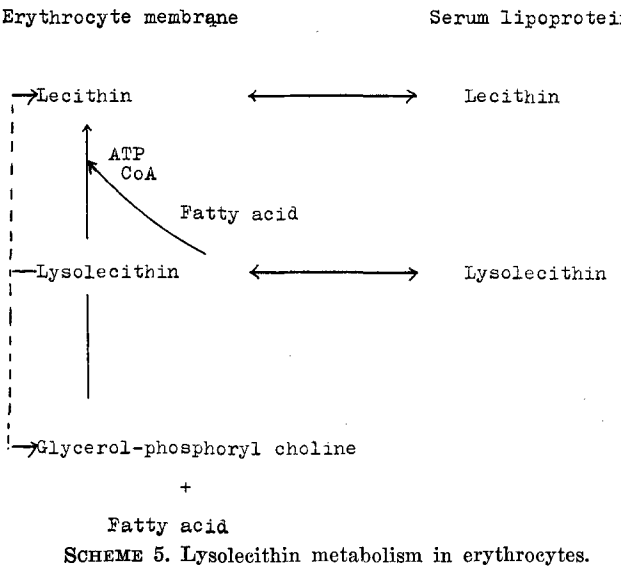
Major Molecular Species of Liver Lecithin from Rats Fed on Fat-Free Diet (A), Diet Containing Coconut-Oil (B) and Diet Containing Corn-Oil (C)

1-position	2-position	A	B	C
16:0	16:0	+	+	+
16:0	18:1	7½	11	5½
18:0	18:1	6	5	2
16:0	18:2	3	7	12½
18:0	18:2	4	13	11
16:0	20:3	11	5	....
18:0	20:3	12½	8	....
16:0	20:4	3½	9	11
18:0	20:4	4	15	23
18:1	16:1	2	....	....
18:1	18:1	7	....	....
18:1	18:2	....	2	4
18:1	20:3	3½	....	....
18:1	20:4	....	1	4

that apart from the interchange between members of the different families of unsaturated fatty acids, changes occur in the relative proportions of a number of molecular species. Apparently, animal tissues are equipped with systems attempting to maintain the physicochemical properties of the phospholipids within certain limits. Under extreme conditions, e.g., in the case of animals suffering from a deficiency of essential fatty acids, this system may not be adequate. It remains to be seen whether some of the symptoms were due to failures which involved other functions of these fatty acids.

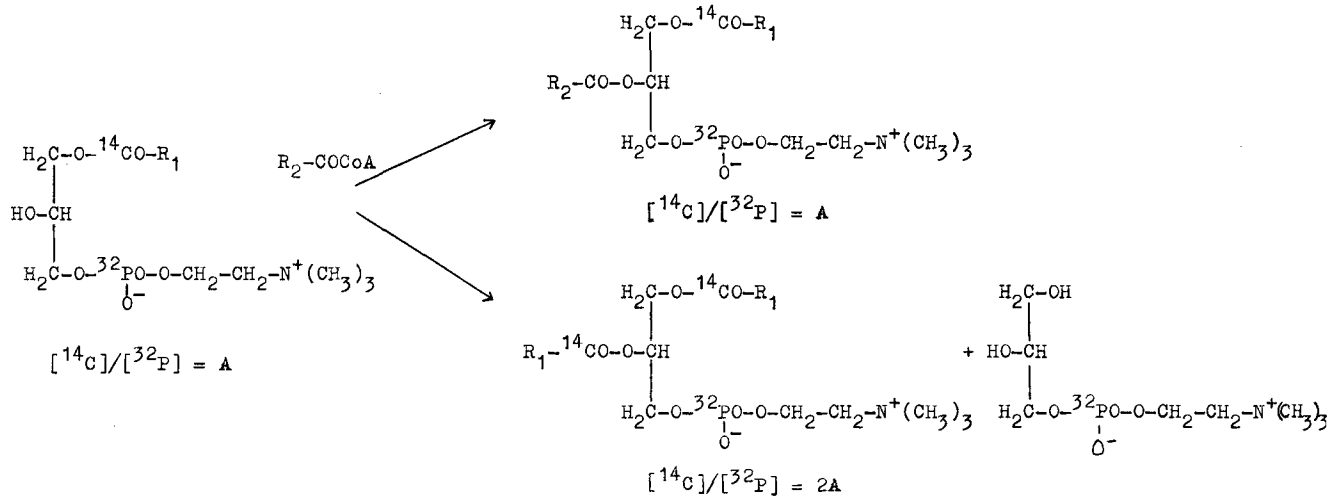
Lysolecithin Metabolism of Erythrocytes

The fatty acid composition of the phospholipids from circulating erythrocytes appears to depend on the nature of ingested fatty acids (28). Alterations in the fatty acids of e.g., lecithin may be accounted for by exchange of this lipid constituent between the lipoprotein of the erythrocyte membrane and serum (Scheme 5). Furthermore, independent studies of Oliveira and Vaughan (29,30) and van Deenen and Mulder et al. (31–33) demonstrated that erythrocytes are capable of incorporating fatty acids into phosphoglycerides. Both research groups related this fatty acid uptake with the ability of erythrocyte ghosts to convert monoacyl phosphoglycerides into the diacyl analogs. The existence of this ATP-CoA dependent



SCHEME 5. Lysolecithin metabolism in erythrocytes.

pathway in erythrocyte ghosts was confirmed by Robertson and Lands (34). Considerable attention has been given by these three research groups to the question whether erythrocytes are capable of producing lysolecithin. To date, no phospholipase A-like activity could be demonstrated in these corpuscles, which is in contrast to the activity found in serum. Polonovski and Paysant (35) and later Mulder and van Deenen (36) demonstrated that lysolecithin is subject to exchange between erythrocytes and serum lipoproteins. The latter authors reported that erythrocytes may convert the lysolecithin derived from its environment into lecithin. These observations appear to support the cyclic pathway outlined in Scheme 5. The reactions concerned were detected in erythrocytes from different mammalian species, although notable quantitative differences may occur. The incorporation of saturated and unsaturated fatty acids was found to be directed preferentially towards the 1- and 2-position of lecithin, respectively (33). A



SCHEME 6. Use of doubly-labeled lysolecithin in studies on the pathways involved in its conversion to lecithin.

transacylation of lysolecithin and lysophosphatidyl ethanolamine appeared to be mainly responsible for the uptake of fatty acid into the erythrocyte lipids (36). Structural investigations on the lysolecithin fraction from erythrocytes and serum carried out with the aid of methods described above revealed that both 1-acyl- and 2-acyl-lysolecithin were present.

Under conditions unfavorable for fatty acid incorporation a limited conversion of lysolecithin into lecithin by erythrocyte ghosts still could be observed (37). The possibility was envisaged that this formation of lecithin was due to the following reaction: 2 lysolecithin → lecithin + glycerophosphoryl choline. Erbland and Marinetti (38) and later Kokke et al. (39) claimed this pathway to occur in a supernatant fraction of liver and yeast, respectively. Conclusive evidence for this reaction was recently given by both research groups utilizing lysolecithin labeled in the acyl chain and the phosphoryl group (40,41). As indicated in Scheme 6, the occurrence of a transacylase reaction will give rise to the formation of a lecithin having an isotopic ratio identical to that of the lysolecithin, whereas the intertransacylation will produce a lecithin having a ratio of $[^{14}C]/[^{32}P]$ twice that of the lysolecithin substrate. Mulder et al. (42) found that erythrocyte ghosts indeed are capable of carrying out the second reaction, but with ATP and CoA present the transacylase reaction appeared to be the dominant one. Apart from the reactions discussed so far, lysolecithin was hydrolyzed by a lysophospholipase which enzyme Heemskerk and van Deenen (43) and Mulder et al. (42) demonstrated to be present in erythrocytes. The fact that erythrocytes are equipped with enzymes catalysing at least 3 different reactions of lysolecithin may stimulate investigations on the implications of these processes with regard to membrane function.

Polyglycerol Phospholipids

Phosphatidyl Glycerol

Determinations of the fatty acid distribution in natural phosphoglycerides based on hydrolysis with snake-venom phospholipase A demonstrated a preference of saturated and polyunsaturated fatty acids for the 1- and 2-position, respectively. However, not all phosphoglycerides appear to obey this rule, and another striking exception became clear when phosphatidyl glycerol from photosynthetic tissues was analyzed in terms of its molecular species (26,44). Both subfractionation of the intact phospholipids and separation of diglycerides formed by phospholipase C hydrolysis furnished good results and allowed one to derive 9 molecular species accounting for about 80% of the phospholipid (Table III). The major molecular species was found to be 1-linolenoyl-2-*trans*-Δ^3-hexadecenoyl-glycero-3-phosphoryl-1′-glycerol. The *trans*-fatty acid was also found to occupy the 2-position of phosphatidyl glycerol from *Chlorella vulgaris,* whereas α-linolenic acid was located for the greater part at the 1-position (45). The specific association between *trans*-Δ^3-hexadecenoic acid and phosphatidyl glycerol was found to be absent from photosynthetic bacteria (46,47). Experiments of Haverkate and van Deenen (45) on *Euglena gracilis* showed that phosphatidyl glycerol was virtually absent from etiolated cells grown in the dark, but green cells were found to produce phosphatidyl glycerol containing *trans*-Δ^3-hexadecenoic acid esterified at the 2-position. This result suggests that the presence of this lipid combination may be related with the occurrence of chloroplast structure. Nichols (48) observed that the content of *trans*-fatty acid in phosphatidyl glycerol from *Chlorella vulgaris* is related to photosynthetic activity, but a role in the Hill reaction was considered unlikely (49). Apart from the question which particular function (1-linolenoyl-2-*trans*-Δ^3-hexadecenoyl) phosphatidyl glycerol displays in membrane structures of photosynthetic tissues of the type occurring in the green plant, it will be of interest to elucidate which biochemical mechanisms are responsible for the formation of the intriguing lipid combination.

TABLE III

Major Molecular Species of Phosphatidyl Glycerol Isolated from Spinach Leaves

Position 1	16:0	16:0	16:0	16:0	16:0	18:1	18:1	18:2	18:2	18:3	18:3	Total
Position 2	16:0	Δ^3 trans 16:1	18:2	18:3	18:1	16:0	18:2	16:0	Δ^3 trans 16:1	16:0	Δ^3 trans 16:1	
	1%	4%	12%	10%	1%	1%	+	1½%	+	7%	47%	84.5%

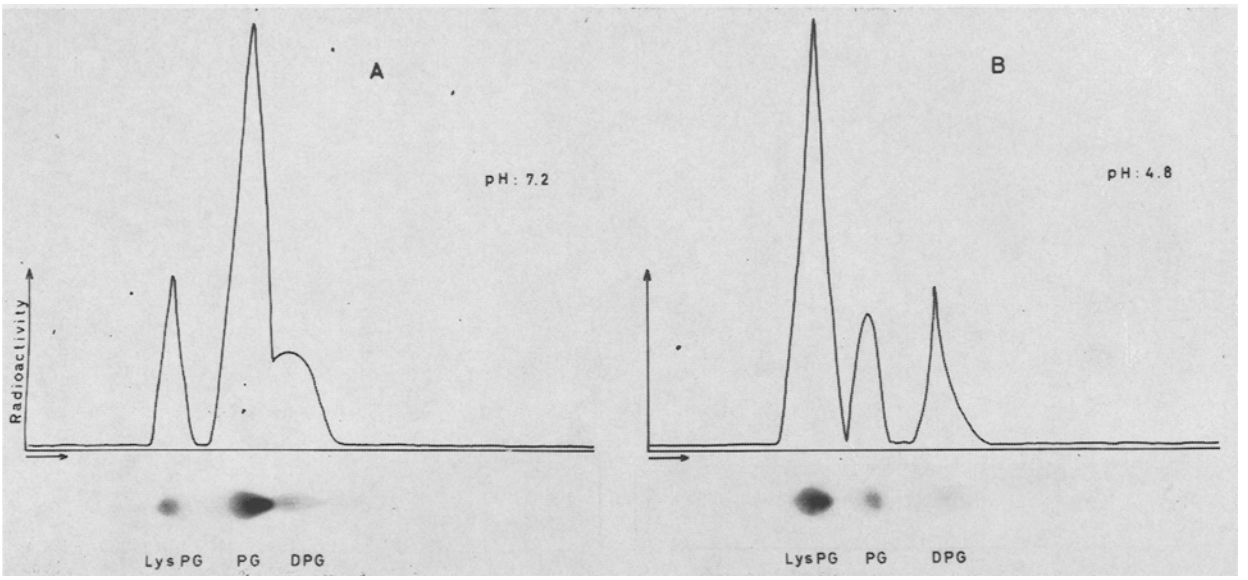
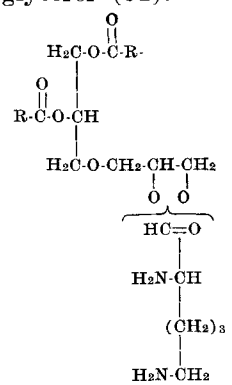


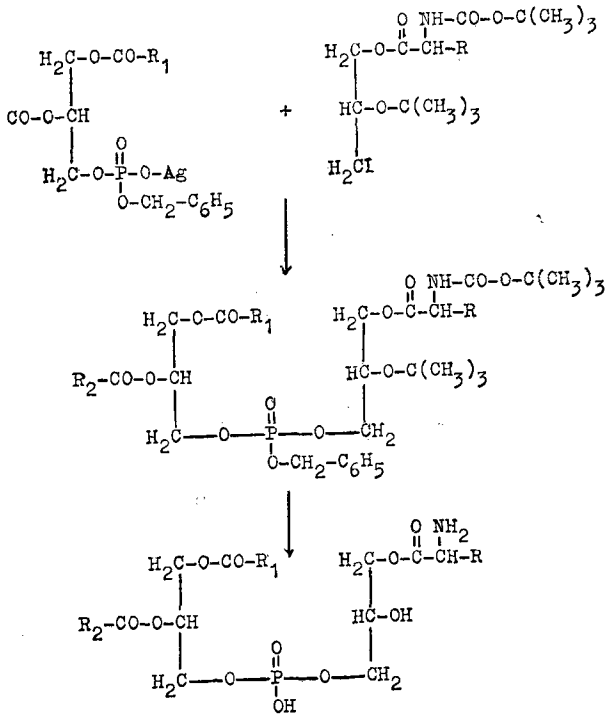
FIG. 3. Autoradiograms and radioactivity tracings of paper chromatograms showing the influence of pH of the culture medium on the phospholipid pattern of *S. aureus*. After reaching the stationary phase at pH 7.2 part of the cells was harvested and analyzed (A). The pH of the remaining culture was brought to 4.8 and incubated for another 2 hr (B). DPG, diphosphatidyl glycerol or cardiolipin; PG, phosphatidyl glycerol; Lys PG, lysyl-phosphatidyl glycerol (Ref. 54).

Amino Esters of Phosphatidyl Glycerol

The nature of the polar headgroup of phospholipids may be of particular interest with regard to lipid-protein interaction. Although hydrophobic attraction forces between fatty acid residues and apolar regions of proteins are involved in lipoprotein structures, considerable attraction forces may result from interaction between oppositely charged groups between phospholipids and sidegroups of proteins. In animal membranes the lipid micelles in general will bear a net negative charge, although considerable quantities of choline-containing phospholipids are present. These phospholipids are "neutral" in the physiological pH region. Although zwitterionic phospholipids are not absent from bacteria, in a number of cases, the membrane structures appear to be composed for the greater part of anionic phospholipids. In *Staphylococcus aureus* phosphatidyl glycerol is the prominent phospholipid at least when cells were harvested at pH 7.0 (Fig. 3). However, in this bacterium a nitrogen-containing phospholipid was detected which was found to accumulate under certain conditions of growth. The studies of Macfarlane (50,51) and Houtsmuller and van Deenen (52,53) showed that this phospholipid is a major representative class of phospholipids denoted as O-amino acid esters of phosphatidyl glycerol. The compound isolated in a pure state from *Staph. aureus* was demonstrated to contain one equivalent of L-lysine esterified to (either the primary or secondary hydroxyl group) of 3-phosphatidyl-1′-glycerol (54).



In the past numerous reports dealt with so-termed peptido-phospholipids, but it has not become clear whether covalently linked amino acids or peptides were involved. Although the work of both research groups on these bacterial phospholipids has been in good agreement, the case history made it highly desirable to support the structural work in this area by chemical synthesis. The procedure applied by Bonsen et al. (55) for the preparation of these phospholipids is outlined in an abbreviated from in Scheme 7. Use was made of a reaction between a silver salt of monobenzyl phosphatidic acid and an amino acid ester of iodoglycerol containing appropriate protecting groups. Chemical degradation, enzymic hydrolysis, chromatographic behavior and other criteria demonstrated the structural similarity between the



SCHEME 7. Chemical synthesis of amino acid esters of phosphatidyl glycerol.

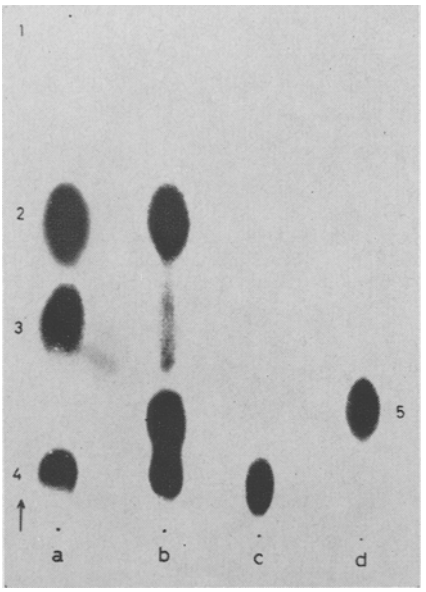


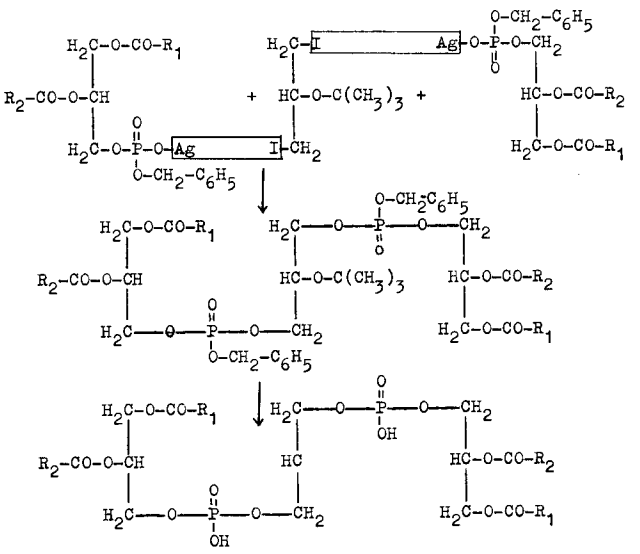
FIG. 4. Autoradiograms of phospholipids of protoplasma membranes of *B. megaterium*. Paper chromatograms were developed on silica-impregnated paper with diisobutyl ketone-acetic acid-water (40:25:5, v/v): (a) phospholipids of cells harvested from a medium without glucose; (b) phospholipids of cells harvested from a medium with glucose; (c) compound 4 isolated by column chromatography and TLC from the mixture shown under b; (d) compound 5 isolated from the mixture shown under b. The compounds are: (1) unidentified polyglycerol phospholipid; (2) phosphatidyl ethanolamine; (3) phosphatidyl glycerol; (4) L-lysine ester of phosphatidyl glycerol; (5) a glucosamine derivative of phosphatidyl glycerol of unidentified structure (Ref. 56).

synthetic and natural phosphoglycerides, although the position of the amino acid ester linkage in the natural compound still is subject to further investigations. With regard to the physiological significance of the amino acid esters in the bacterial cell membrane, one can speculate whether they function as a carrier for the amino acids which are needed for cell wall formation or serve as a stabilizing function for these NH_2-groups containing lipids and satisfy the charge requirements of the membrane.

The high degree of flexibility encountered in bacteria may be useful to study the functional aspects of membrane phospholipids. Protoplasts of *Bacillus megatherium* were prepared by Op den Kamp et al. (56) from cells cultivated under different conditions and an unknown phospholipid was found to vary in concentration (Fig. 4). This compound was tentatively identified as a glucosamine derivative of phosphatidyl glycerol. The protoplasts exhibited different osmotic properties, but it remains to be established whether this feature is directly related with the differences in phospholipid composition of the membranes.

Diphosphatidyl Glycerol

The extensive studies of Faure (57,58) and Macfarlane and co-workers (59,60) indicated that beef-heart cardiolipin is identical to diphosphatidyl glycerol. This view was supported by LeCocq and Ballou (61), but Rose (62) favored a more complex structure isomeric to that originally proposed by Pangborn. During the past few years several polyglycerol phospholipids, e.g., phosphatidyl glycerol, phosphatidyl glycerophosphate, phosphatidyl diglyceride, diphosphatidyl glycerol and diphosphatidyl monoglyceride have been synthesized in this laboratory (4), thus enabling us to obtain new information on the structure of cardiolipin. As an example of this synthetic work the sequence of reactions used for the preparation of diphosphatidyl glycerol (63) is shown in Scheme 8.

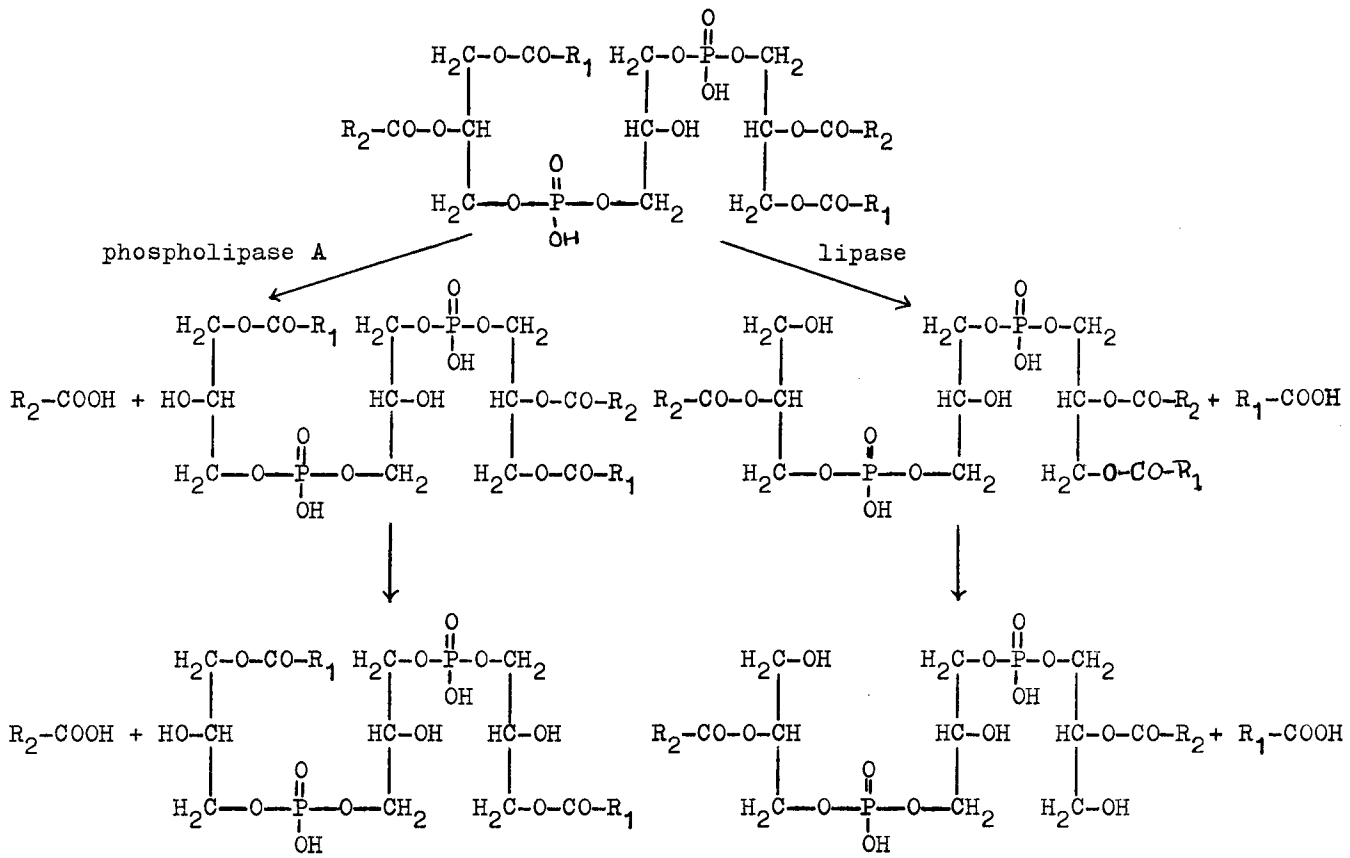


SCHEME 8. Chemical synthesis of diphosphatidyl glycerol.

A comparison of physical characteristics and chemical properties indicated that cardiolipin from beef-heart was identical with synthetic diphosphatidyl glycerol. However, most conclusive evidence was obtained by enzymic hydrolysis experiments on the natural compound and the synthetic products. Treatment of natural cardiolipin with snake-venom phospholipase A gave rise to the production of two lyso compounds. One compound contained 3-fatty acid ester linkages per two phosphorus; this compound served as the precursor of the end-product which had only 1-fatty acid ester linkage per phosphorus (Scheme 9). Synthetic diphosphatidyl glycerol exhibited similar behavior whereas all other polyglycerol phospholipids revealed a different pattern of breakdown or produced lyso compounds of different R_f values. The snake-venom phospholipase A was found to attack the fatty acid ester linkages at the 2-position of diphosphatidyl glycerol only. More recently de Haas extended this approach to pancreatic lipase, which was found to act exclusively at the 1-ester-position of synthetic diphosphatidyl glycerol. With this enzyme 2 lyso compounds were produced from both the synthetic substrate and beef-heart cardiolipin (Scheme 9).

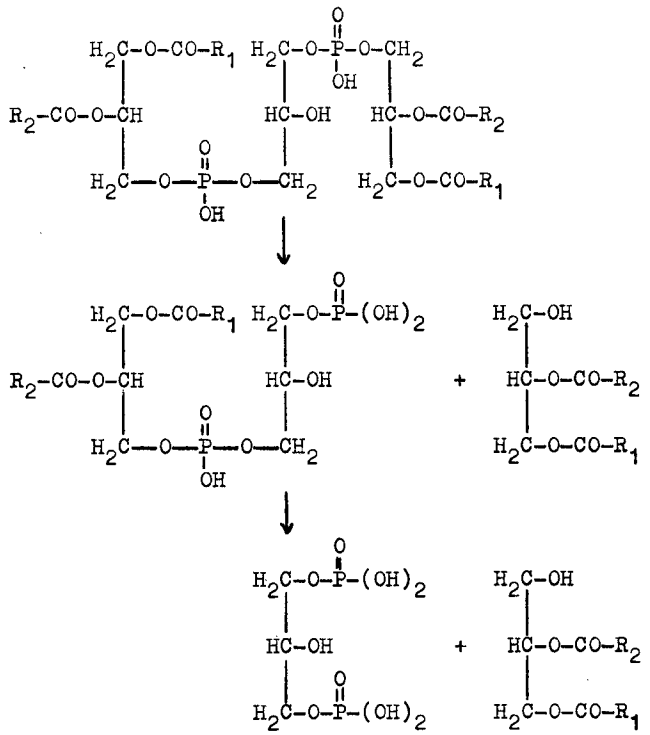
Confirmative evidence was obtained through the use of phospholipase C from *B. cereus* (64,65). This enzyme under suitable conditions appears to hydrolyse diphosphatidyl glycerol into 1,2-diglyceride and 1,3-diphosphoryl glycerol. The intermediate product which was produced during this hydrolysis reaction was found to be identical to synthetic phosphatidyl glycerolphosphate, thus allowing one to formulate the pathway given in Scheme 10. Beef-heart cardiolipin appeared to be degraded in a similar way, thus presenting an unambiguous proof of its identity with diphosphatidyl glycerol.

The serological activity of synthetic diphosphatidyl glycerol was found by de Bruijn (66) to be indistinguishable from the reactivity of beef-heart cardiolipin, this being the first time that a synthetic product was reported to be qualified as a substitute for natural cardiolipin in syphilis serodiagnosis. It should be noted that in beef-heart cardiolipin the



SCHEME 9. Action of snake-venom phospholipase A and pancreatic lipase on synthetic diphosphatidyl glycerol and beef-heart cardiolipin.

fatty acids consist of about 80% linoleic acid, whereas the synthetic product contained equimolar amounts of stearic and oleic acid. Apparently the degree of unsaturation of the fatty acid chains is not of primary importance for serological activity. Further physicochemical studies with various defined polyglycerol phospholipids may contribute to the understanding of the nature of interaction between lipid and protein in the formation of antigen-antibody complex.



SCHEME 10. Action of phospholipase C on synthetic diphosphatidyl glycerol and beef-heart cardiolipin.

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