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STUDIES ON CARDIOLIPIN

III. STRUCTURAL IDENTITY OF OX-HEART CARDIOLIPIN AND SYNTHETIC DIPHOSPHATIDYL GLYCEROL

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SUMMARY

Chemical synthesis of diphosphatidyl glycerol, a long-chain fatty acid ester of diphosphatidyl glycerol, phosphatidyl diglyceride and phosphatidyl glycerophosphate has stimulated a structural comparison with natural cardiolipin. Although in certain properties the various polyglycerol phospholipids are quite similar, the results of enzymic hydrolyses with phospholipase A (EC 3.1.1.4), acylation studies, optical rotation measurements and chromatography of the intact phospholipids and deacylated products indicated that beef-heart cardiolipin has a diphosphatidyl glycerol structure. Conclusive evidence was obtained by means of the breakdown of the phospholipids with phospholipase C (EC 3.1.4.3). The enzyme was found to hydrolyse both natural and synthetic diphosphatidyl glycerol into 1,2-diglyceride and 1,3-glycerol diphosphate, phosphatidyl glycerophosphate being an intermediate hydrolysis product.

INTRODUCTION

The structure of cardiolipin from beef-heart has been the subject of many investigations. FAURE AND MORELEC-COULON[1,2] as well as MACFARLANE *et al.*[3] provided strong evidence that this phospholipid is identical with diphosphatidyl glycerol. LECOCQ AND BALLOU[4] confirmed that the lipid has the 1,3-diphosphatidyl glycerol structure and established its overall configuration. However, concurrently ROSE[5], concluded that its structure is more complex, and he proposed a structure isomeric with that originally derived by PANGBORN[6].

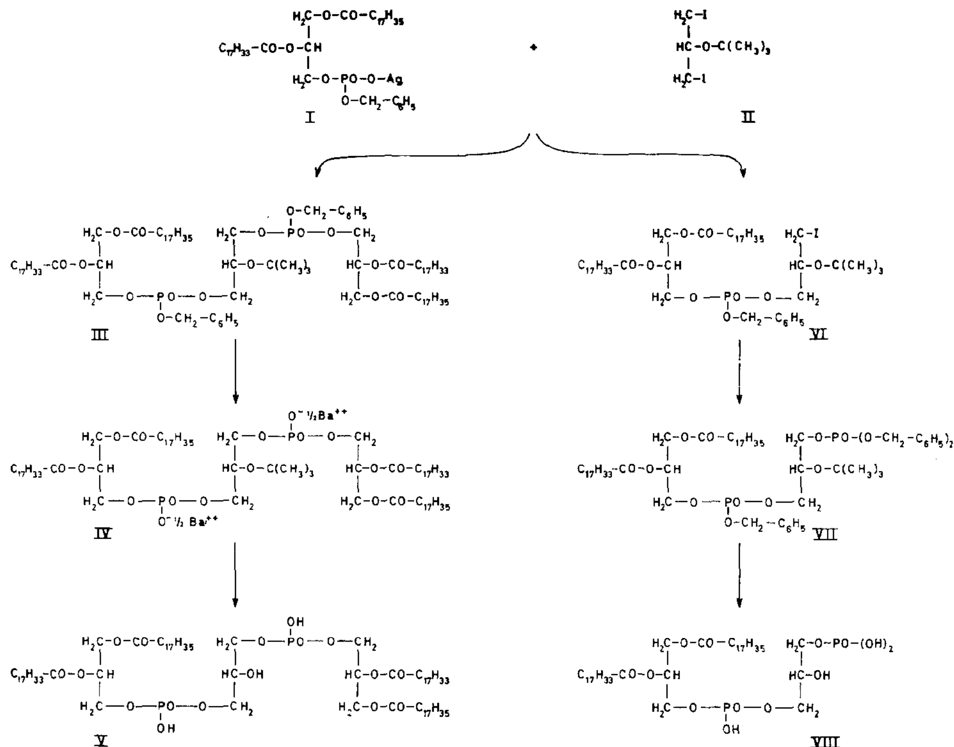
In the present study, cardiolipin has been compared with synthetic polyglycerol phospholipids such as diphosphatidyl glycerol, a long-chain fatty acid ester of diphosphatidyl glycerol, bis-phosphatidic acid (phosphatidyl diglyceride) and phosphatidyl glycerophosphate.

Abbreviation: DPG, diphosphatidyl glycerol.

EXPERIMENTAL

Synthetic compounds

Details of the synthesis of diphosphatidyl glycerol (Scheme 1, V) have been reported recently[7]. Scheme 1 also shows the sequence of reactions finally leading to phosphatidyl glycerophosphate (VIII), a degradation product of diphosphatidyl glycerol.



Scheme 1. Synthesis of diphosphatidyl glycerol (V) and phosphatidyl glycerophosphate (VIII).

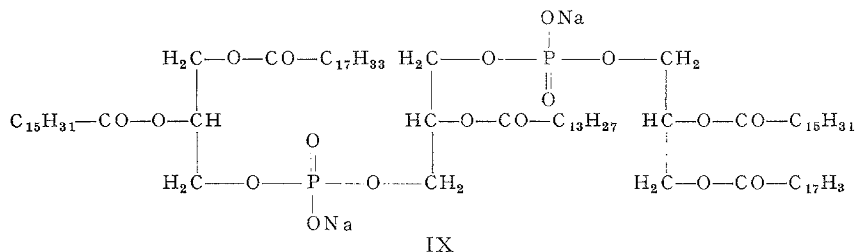
Reaction between silver benzyldiacyl-L-α-glycero-phosphate (I) and 2-*tert.*-butoxy-1,3-diiodoglycerol (II) in a molar ratio of 2:1 yielded the triester III contaminated with small amounts of the mono-condensation product (VI). After chromatographic purification, III was freed from protecting benzyl groups by a reaction with barium iodide, affording the barium salt IV. Removal of the glycerol-protecting *tert.*-butyl-ether group was carried out by anhydrous hydrogen chloride in chloroform. Diphosphatidyl glycerol (V) was obtained in a yield of about 70% based on III.

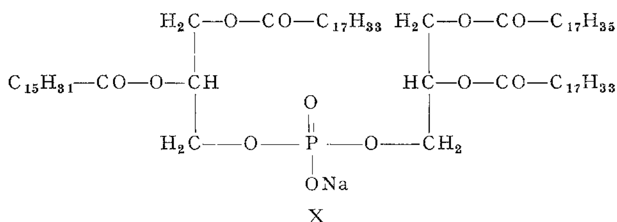
By changing the molar ratios of I and II to 1:1 (instead of 2:1) the yield of the mono-condensation product VI could be enlarged to about 50%. After chromatographic removal of small amounts of III, VI was reacted with silver dibenzyl phosphate*, yielding the triester VII. Treatment of VII with liquid hydrogen bromide at

* Afterwards reaction of VI with silver di-*tert.*-butylphosphate was found to give a triester that could be converted more easily into the triester VII.

—80° furnished phosphatidyl glycerophosphate VIII in yields of about 60% based on VII. Details of the preparation of VIII will be reported elsewhere.

For reasons of comparison, two other acidic phospholipids were synthesized, namely a long-chain acyl derivative of diphosphatidyl glycerol (IX) and a phosphatidyl diglyceride (bis-phosphatidic acid) X.





The preparation of these compounds has been reported previously[8,9].

Natural cardiolipin

Cardiolipin isolated from ox-heart and purified as described by PANGBORN[10] was obtained (as an International Reference Preparation from the World Health Organization) through the courtesy of Dr. J. H. DE BRUIJN of the Rijksinstituut voor de Volksgezondheid, Utrecht, The Netherlands.

METHODS

Melting points were determined on a Kofler hot plate and are uncorrected. Optical rotations were measured in a Lichtelektrisches Präzisions polarimeter 0.005°, Carl Zeiss. Infrared spectra were measured with a Perkin-Elmer (model 21) double beam spectrophotometer (NaCl prism), using KBr discs containing the compounds.

The purity of intermediates and end-products was checked by paper chromatography and by thin-layer chromatography on silica gel as described previously[11]. Detection was carried out by established procedures. After chromatographic separation and methanolysis, quantitative analyses of the fatty acids present in the various lipids were carried out by gas–liquid chromatography as described previously[12]. Mild alkaline hydrolysis of the various phospholipids was carried out according to the procedures developed by DAWSON *et al.*[13,14]. Water-soluble phosphorus diesters were investigated by paper chromatography on Whatman No. 1 paper with propanol–ammonia–water (6:3:1, v/v) or by high voltage electrophoresis, with a pyridine–acetic acid buffer (pH 3.6).

As source of phospholipase A (EC 3.1.1.4), crude lyophilized venom of *Crotalus adamanteus* was used. Incubation experiments and determination of the action of the enzyme were performed as described previously[15].

Active preparations of phospholipase C (EC 3.1.4.3) were obtained from *Bacillus cereus*. Incubations were carried out at room-temperature by shaking ethereal solutions of the phospholipids with the buffered enzymic extract as described previously[16].

Phospholipase D (EC 3.1.4.4) from Brussels sprouts was obtained commercially (C. F. Boehringer und Söhne). Incubation conditions have been given in an earlier publication[16].

RESULTS AND DISCUSSION

A number of physical and biochemical properties of isolated ox-heart cardiolipin were compared with those of diphosphatidyl glycerol (V), the acyl derivative of diphosphatidyl glycerol (IX) and the phosphatidyl diglyceride (X). The melting points (Table I) and optical rotations do not allow us to differentiate between these

TABLE I

MELTING POINTS AND OPTICAL ROTATIONS OF SOME ACIDIC PHOSPHOLIPIDS

	Ox-heart cardiolipin	*Diphosphatidyl glycerol (V)*	*Acyl derivative of diphosphatidyl glycerol (IX)*	*Phosphatidyl diglyceride (X)*
Melting point °	206–208°	202–204°	210°	79–80°
$[\alpha]_D^{20}$ sodium salt	+5.4°	+5.8°	+4.7°	+4.9°
$[\alpha]_D^{20}$ barium salt	−6.0°	−6.2°		

acidic phospholipids. The infrared absorption spectra of all the six anionic phospholipids investigated appeared to be nearly identical. As was noted previously[8], a rather strong absorption in the region 2.8–3.2 μ (OH) in the spectrum of ox-heart cardiolipin is only due in part to a free glycerol–OH group. Probably the presence of some water, introduced during the preparation of the sample, is responsible for this strong band. Carefully dried samples of all six phospholipids showed only a weak absorption at the region of 3 μ. Since substitution of the free glycerol–OH group by a long-chain acyl ester or a *tert.*-butylether function scarcely influenced the absorption spectrum, it is evident that even conspicuous alterations in structure of these acidic phospholipids are difficult to ascertain by infrared spectrometry.

Chromatographic behaviour

Paper chromatography. With the exception of the long-chain acyl derivative of diphosphatidyl glycerol, all the acidic phospholipids which were investigated gave well-defined spots in the solvent system introduced by MARINETTI *et al.*[17]. As can be seen from Fig. 1, synthetic diphosphatidyl glycerol and ox-heart cardiolipin revealed identical R_F values, whereas the diphosphatidyl glycerol derivatives in which the free OH group was substituted moved faster. For reasons of comparison Fig. 1 shows also the relative R_F values of synthetic phosphatidic acid and phosphatidyl glycerol.

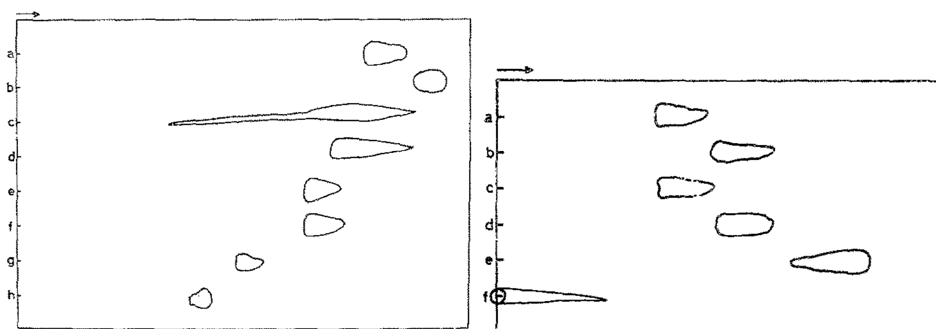


Fig. 1. Silica-impregnated paper chromatogram developed with the solvent system of MARINETTI[17]. Detection of the spots: tricomplex- and molybdate staining. a, bis-phosphatidic acid (X); b, synthetic phosphatidic acid; c, acyl derivative of diphosphatidyl glycerol (IX); d, *tert.*-butylether of diphosphatidyl glycerol (IV); e, ox-heart cardiolipin; f, synthetic diphosphatidyl glycerol (V); g, synthetic phosphatidyl glycerol; h, phosphatidyl glycerophosphate (VIII).

Fig. 2. Thin-layer chromatogram developed in chloroform–methanol–concentrated aqueous ammonia (70:20:1.5, v/v). Detection of the spots was carried out by charring (30% H_2SO_4). a, synthetic diphosphatidyl glycerol (V); b, *tert.*-butylether of diphosphatidyl glycerol (IV); c, ox-heart cardiolipin; d, acyl derivative of diphosphatidyl glycerol; e, bis-phosphatidic acid (X); f, phosphatidic acid.

Thin-layer chromatography. Sharp spots of most of the acidic phospholipids could be obtained when alkaline solvent systems were used. With acidic solvents phosphatidic acid and phosphatidyl glycerophosphate run as a compact spot only on silica that contains oxalic acid. Fig. 2 shows that also on chromatoplates identical R_F values were found for synthetic DPG and ox-heart cardiolipin. The substituted diphosphatidyl glycerol derivatives such as IX again reveal higher R_F values.

Column chromatography. As was pointed out earlier, most of the acidic phospholipids are stable only in the form of salts. Also in the present study ox-heart cardiolipin as well as synthetic DPG in their free acid form appeared to be highly labile. We could completely confirm the findings of FAURE AND MORELEC-COULON[18]: that rapid degradations occur involving loss of fatty acids and diglycerides. Therefore chromatographic separation on silica columns of acidic phospholipids was carried out after a conversion into neutral salts. It has to be emphasized, however, that silica columns may act as ion-exchangers and that acidic phospholipids show a different elution pattern, dependent on the cations present. For example, the barium salt of synthetic DPG is rapidly eluted from silica columns with 5% methanol in chloroform, whereas the corresponding sodium salt required as much as 15% of methanol in chloroform. Whereas part of the sodium salt of DPG was converted into the free acid form during chromatography, no ion-exchange was found for the corresponding barium compound. Apparently the linkage between the phospholipid and barium ions is much stronger than in the case of monovalent cations, resulting in a less ionization of the former. In this connection, it is worth noting the large differences in optical rotation of DPG (or ox-heart cardiolipin) between sodium and barium salts. Furthermore, an abnormal behaviour of the barium salt of DPG was found during mild alkaline hydrolysis.

Mild alkaline hydrolysis

The chromatographic behaviour of de-acylated phospholipids is reproduced

in Fig. 3. The long-chain acyl derivative of DPG (IX, sodium salt) gave a single spot in the expected region, but the behaviour of the barium salts of ox-heart cardiolipin and synthetic DPG was rather strange. Both latter phospholipids revealed, upon mild alkaline hydrolysis, a spot with an R_F value of 0.72, when sprayed with the molybdate and Schiff reagent. This R_F value, when compared with that of glyceryl phosphoryl glycerol derived from phosphatidyl glycerol (R_F 0.67), seems to be much too high. Moreover, both deacylation mixtures contain a slower-moving product (R_F 0.61) which contains phosphorus, but which did not reveal the presence of vicinal hydroxyl groups. Upon electrophoresis the spot of R_F value 0.54, which was derived

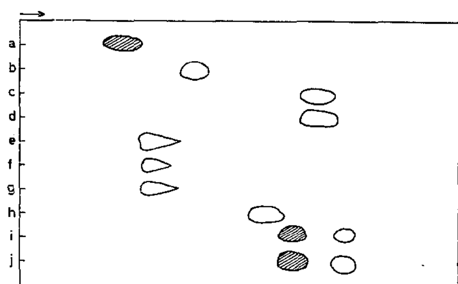


Fig. 3. Paper chromatogram of deacylated phospholipids developed in propanol–ammonia–water (6:3:1, v/v). Detection of the spots: Open areas, by the Schiff-reagent and the molybdate spray; hatched areas, by the molybdate spray only. a, inorganic phosphate; b, glycero-3-phosphoric acid; c, deacylation product of synthetic phosphatidyl glycerol; d, deacylation product of bis-phosphatidic acid (X); e, deacylation product of synthetic phosphatidyl glycerophosphate (VIII); f, deacylation product of phosphatidyl glycerophosphate obtained by phospholipase C hydrolysis of synthetic diphosphatidyl glycerol; g, deacylation product of phosphatidyl glycerophosphate obtained by phospholipase C hydrolysis of ox-heart cardiolipin; h, deacylation product of the acyl derivative of diphosphatidyl glycerol (IX, sodium salt); i, deacylation product of ox-heart cardiolipin (Ba-salt); j, deacylation product of synthetic diphosphatidyl glycerol (V, Ba-salt).

from the alkaline hydrolysis of the acyl derivative of DPG (sodium salt) exactly coincided with the spots of R_F value 0.72 of both ox-heart cardiolipin and synthetic DPG (barium salt). The acyl derivative of DPG was deacylated to produce the sodium salt and ox-heart cardiolipin and synthetic DPG to produce the barium salts, the abnormal chromatographic behaviour of both latter hydrolysis products was thought to be due to the presence of barium ions. After the addition of sodium sulphate to the deacylation solutions of ox-heart cardiolipin and synthetic DPG, however, the same abnormal chromatographic pattern was observed. However, when the intact barium salts of ox-heart cardiolipin and synthetic DPG were converted firstly into the corresponding sodium salts and then deacylated, both phospholipids yielded one defined spot (R_F 0.54) exactly coinciding with the hydrolysis product obtained from the acyl derivative of DPG (IX, sodium salt). Apparently when the sodium salts of ox-heart cardiolipin and synthetic DPG are used no formation of the R_F 0.61 compound (negative Schiff-reaction) occurred. Although the cause of failure, with barium salts in the mild alkaline hydrolysis procedure, could not be elucidated, it seems advisable to investigate naturally occurring acidic phospholipids in the presence of monovalent cations. Experiments are in progress to investigate the behaviour of various salts of phosphatidic acid upon mild alkaline hydrolysis.

Acylation of synthetic DPG and ox-heart cardiolipin

Opinions differ on the possibility of acylating the free glycerol–OH group in cardiolipin. FAURE AND MORELEC-COULON[1,2], using acetic acid and dicyclohexylcarbodiimide, were able to convert ox-heart cardiolipin into its mono-acetyl derivative; however, they found only very little esterification to occur with long-chain fatty acids such as oleic acid. On the other hand, ROSE[5] did not succeed in converting natural cardiolipin into its acetylderivative, and he concluded that no free hydroxylgroup was likely to be present. We investigated the acylation of synthetic DPG and ox-heart cardiolipin (sodium and barium salt) using myristoylchloride (3–5 moles) in dry chloroform. It appeared, from thin-layer chromatograms that with both phospholipids a very slow esterification took place, especially in the absence of acid binders like pyridine (see Fig. 2).

After a reaction time of 48 h at room temp., about 5% of the phospholipid was converted into a compound which was chromatographically indistinguishable from the reference product, *viz.* the synthetic long-chain acyl derivative of DPG (IX). Longer reaction times resulted in extensive decomposition of the starting material into lyso products and phosphatidyl glycerophosphate, probably because of the acidic medium. That the extent of esterification is only very limited has to be attributed to the steric hindrance and/or to the low reactivity of the secondary hydroxylgroup. Acylation of synthetic phosphatidyl glycerol containing two unesterified vicinal hydroxyl groups under similar conditions was found to give in a high yield an α-substituted mono-acyl derivative of phosphatidyl glycerol only; apparently esterfication of the secondary hydroxyl group was hindered just as happens in synthetic diphosphatidyl glycerol and ox-heart cardiolipin.

Degradation with phospholipases

Phospholipase D. This enzyme which is known to act also on certain negatively charged phospholipid molecules, *e.g.* phosphatidyl glycerol[16], was unable to degrade synthetic DPG or isolated cardiolipin. Although high enzymic concentrations and incubation periods up to 24 h were applied, no trace of phosphatidyl glycerol or phosphatidic acid could be detected by thin-layer chromatography. Addition of less negatively charged activators such as stearyl phosphorylcholine resulted in a quantitative degradation of the activator molecule (into free choline and stearyl phosphate), but no hydrolysis of the substrate was observed. Neither the addition of lysolecithin nor of lauroylcholine chloride gave rise to any detectable breakdown of diphosphatidyl glycerol or of natural cardiolipin. The enzyme was also found to be inactive towards phosphatidyl glycerophosphate, the acyl derivative of DPG and bis-phosphatidic acid (phosphatidyl diglyceride).

Phospholipase A. As reported previously[8,19] both synthetic DPG and ox-heart cardiolipin[20] are susceptible to the action of this enzyme; however, the hydrolysis was found to proceed rather slowly. Addition of stearylphosphorylcholine resulted in a greatly accelerated hydrolysis (Fig. 4). Synthetic DPG as well as ox-heart cardiolipin (R_F 0.62), upon short-time incubation with phospholipase A, yielded two lysoderivatives (one spot of R_F 0.53 and an elongated spot of R_F about 0.25). These derivatives, when analysed by gas–liquid chromatography (for the synthetic mixed-acid phosphatide) or by the hydroxamate method (for the ox-heart cardiolipin), appeared to contain 3 and 2 acyl chains per molecule, respectively. The results obtained

on the mixed-acid synthetic DPG made it clear that from both diglyceride parts, only the β-attached fatty acids were released by this enzyme. In confirmation of this, the hydrolysis products did not stain with the Schiff reagent.

For reasons of comparison also the following synthetic compounds were investigated as possible substrates for the phospholipase A of snake venom: *tert.*-butylether of DPG (IV); long-chain acyl derivative of DPG (IX); bisphosphatidic acid (X); phosphatidyl glycerophosphate (VIII). The phospholipids were incubated with snake venom in the presence of stearyl-phosphorylcholine. Phosphatidyl glycerophosphate appeared to be rapidly and quantitatively degraded to its lyso derivative, whereas probably for steric reasons the other phosphatides were hydrolysed only slowly. The DPG derivatives in which the middle glycerol hydroxyl group was substituted by a *tert.*-butylether (III) or a fatty acyl chain (IX), slowly loose upon prolonged hydrolysis, their β-attached fatty acids, yielding again 2 lyso compounds (Fig. 5, R_F

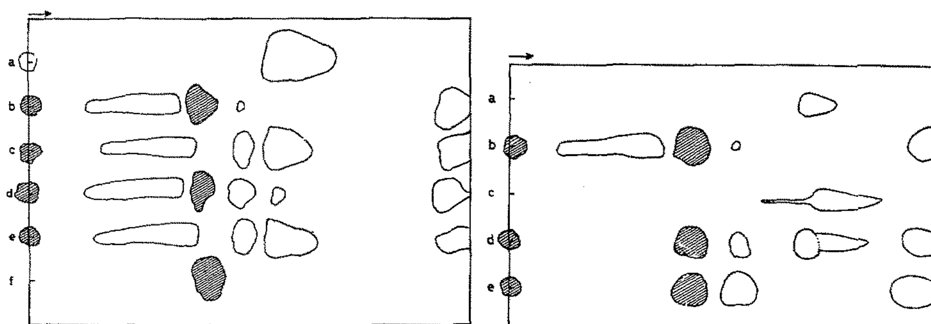


Fig. 4. Silica-impregnated paper chromatogram showing the phospholipase A degradation of synthetic diphosphatidyl glycerol (or ox-heart cardiolipin). Incubation with or without stearylphosphoryl choline. Developing system: Diisobutylketon–acetic acid–water (40:25:5, v/v). Detection of the spots: Tricomplex staining method: red spots (hatched areas) are given by stearylphosphorylcholine and enzyme (at the origin); green spots (open areas) correspond to phospholipids and fatty acids (the latter located at the front). a, synthetic diphosphatidyl glycerol (V) or ox-heart cardiolipin; b and d, incubation of synthetic diphosphatidyl glycerol with phospholipase A in the presence of stearylphosphoryl choline for 7 h and 3 h; c and e, incubation of synthetic diphosphatidyl glycerol with phospholipase A without stearylphosphorylcholine for 7 h and 3 h; f, stearylphosphoryl choline.

Fig. 5. Silica-impregnated paper chromatogram of incubation mixtures of synthetic diphosphatidyl glycerol (V) and the acylderivative of diphosphatidyl glycerol (IX) with phospholipase A. Developing system: Diisobutylketone–acetic acid–water (40:25:5, v/v). Staining method compare Fig. 4. a, synthetic diphosphatidyl glycerol (V); b, phospholipase A hydrolysis of synthetic diphosphatidyl glycerol in the presence of stearylphosphorylcholine; c, acyl derivative of diphosphatidyl glycerol (IX); d, phospholipase A hydrolysis of c) in the presence of stearylphosphorylcholine (7 h); e, prolonged phospholipase A hydrolysis of c) in the presence of stearylphosphorylcholine (43 h).

values 0.62 and 0.53). In this respect it is remarkable that in the myristoyl derivative of DPG (IX) only the 2-fatty acids of the diglyceride moieties are susceptible to the action of the enzyme. Even after incubation for 43 h (during which the 2-fatty acids of the diglyceride moieties were completely released) no hydrolysis of the myristic acid ester bond could be observed (Fig. 5).

The enzymic hydrolysis of the bisphosphatidic acid (phosphatidyl diglyceride X) proceeded even more slowly. After an incubation time of 48 h, (no release of fatty acids in the blank) about 30% of the substrate was converted into lyso derivatives, one

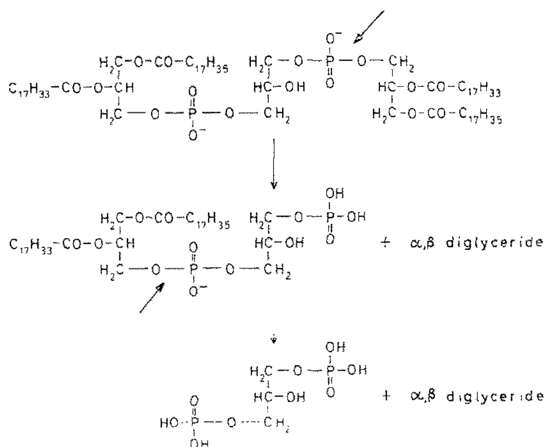
of these being a three-acyl derivative of glycerylphosphoryl glycerol (about 10%), the other a diacylglycerylphosphoryl glycerol (about 90%). Upon paper chromatography, the latter substance being a positional isomer of phosphatidyl glycerol just separated from phosphatidyl glycerol, but did not react with the Schiff reagent.

Gas–liquid chromatographic analysis of the lyso compounds and liberated fatty acids showed again that only the β-attached fatty acids were released by the enzyme used.

An indication that perhaps steric factors play a role in the slow breakdown of this compound may be found in the molar ratio of both lyso derivatives formed. The enzymic release of the first fatty acid was followed by a more rapid hydrolysis of the second β-ester bond. Although the intact bisphosphatidic acid contained three different fatty acids, one could not observe hydrolysis to favour one of the two 2-fatty acid ester bonds.

Phospholipase C. Although most of the foregoing results point to a great similarity between synthetic diphosphatidyl glycerol and ox-heart cardiolipin, conclusive evidence that both phospholipids are identical was obtained by degradation studies with phospholipase C from *B. cereus*.

Preliminary incubation experiments with the bacterial enzyme in an aqueous–ether system, using as substrate synthetic DPG, showed a slow release of 1,2-diglyceride. On account of the known structure of synthetic DPG and the well-established mode of action of this enzyme, such a release of 1,2-diglyceride has to be accompanied by the formation either of phosphatidyl glycerophosphate or of 1,3-diphosphoglycerol (Scheme 2).



Scheme 2. The arrows show the sites of action of phospholipase C from *B. cereus* on synthetic diphosphatidyl glycerol.

Paper chromatographic examination of the incubation mixture with the method of MARINETTI[17] showed that part of the DPG (R_F 0.62) had indeed been converted into a slower-moving phosphorus-containing spot, chromatographically indistinguishable from synthetic phosphatidyl glycerophosphate (compare also Fig. 1). The same behaviour was observed when ox-heart cardiolipin was used as a substrate*.

* Also after deacylation the three products exhibited the same chromatographic behaviour (Fig. 3).

Paperchromatograms on non-impregnated paper with propanol–ammonia–water (6:3:1, v/v) demonstrated the absence of glycerol diphosphate in the incubation mixture. Apparently, under the experimental conditions used phosphatidyl glycerophosphate did not serve as a substrate for the bacterial enzyme. In accordance with these findings, phospholipase C appeared not to degrade synthetic phosphatidyl glycerophosphate.

Recently, however, OTTOLENGHI[21] showed that zinc ions exert an activating influence on the phospholipase from *B. cereus*. Incubation of synthetic DPG as barium salt together with one equivalent of zinc sulphate, indeed showed an enhanced hydrolysis of the phospholipid without a concomittant increase in the amount of phosphatidyl glycerophosphate. From the ethereal phase, 1,2-diglyceride was isolated by chromatography on silicagel. Gas–liquid chromatographic analysis showed the presence of equimolar amounts of stearic- and oleic acid; melting point 38–40°, (previously reported[22] to be about 40°): $[\alpha]_D^{20}$—2.6° in $CHCl_3$ (previously reported[22] to be —2.6° in $CHCl_3$). The natural product exhibited the same behaviour, the diglyceride formed having $[\alpha]_D^{20}$—2.44° in $CHCl_3$.

After elution of the diglyceride, phosphatidyl glycerophosphate was obtained from the column. Its structure could be confirmed by incubation with a cell-free extract of *Escherichia coli*[23] yielding phosphatidyl glycerol and inorganic phosphate.

Investigations of the aqueous phase of the incubation mixture showed that part of the lipid phosphorus had become water-soluble. Paper chromatographic analysis revealed the presence of a substance which co-chromatographed with glycerol diphosphate. By using reference mixtures of synthetic 1,2- and 1,3-glycerol diphosphate in the solvent system applied by LECOCQ AND BALLOU[4], it appeared that the enzymic degradation of DPG as well as of ox-heart cardiolipin gave rise to the formation of the 1,3-isomer only. These findings are in excellent agreement with those of LECOCQ AND BALLOU[4] obtained by a chemical degradation of ox-heart cardiolipin.

Phospholipase C hydrolysis of synthetic phosphatidyl glycerophosphate in the presence of zinc ions yielded in the same way, besides 1,2-diglyceride, exclusively 1,3-glycerol diphosphate. Notwithstanding high concentrations of enzyme and very long incubation times in the presence of zinc ions, no hydrolysis of the bisphosphatidic acid (X) or the acyl derivative of DPG (IX) was observed.

Hence, these results confirm that cardiolipin as isolated from ox-heart by the procedure of PANGBORN is structurally identical to diphosphatidyl glycerol. Synthetic diphosphatidyl glycerol and natural cardiolipin appeared also to be indistinguishable in the Kolmer complement fixation test and the Venereal Disease Research Laboratory slide test[24].

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