The Phospholipids of *Pneumococcus* I–192R, A.T.C.C. 12213

SOME STRUCTURAL REARRANGEMENTS OCCURRING UNDER MILD CONDITIONS

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1. The phospholipids from the non-capsulated strain of *Pneumococcus* I–192R, A.T.C.C. 12213, were separated into three fractions by chromatography on columns of silicic acid and DEAE-cellulose (acetate form). 2. The water-soluble phosphate esters produced by deacylation of each fraction were separated by chromatography on columns of DEAE-cellulose (HCO_3^- form). 3. Three deacylated products, diglycerol phosphate, glycerylphosphorylglycerol phosphate and bis(glyceryl-phosphoryl)glycerol, were identified by analysis, by chemical degradations and by comparison with synthetic materials. 4. From a study of freshly isolated lipids prepared and worked up under conditions where exposure to acid was minimal, it was concluded that the *Pneumococcus* contains phosphatidylglycerol and bis-phosphatidylglycerol, in the molar proportion 1:2·5–3·0, and that the deacylation product glycerylphosphorylglycerol phosphate was probably an artifact of the isolation procedure. 5. Acid-catalysed isomerization (phosphodiester migration) of diglycerol phosphate and bis(glycerylphosphoryl)glycerol and transesterification (glycerol phosphate transfer) of diglycerol phosphate were observed. The structures of the products were established by degradation. 6. A novel mechanism for the biosynthesis of bisphosphatidylglycerol is presented.

The lipids of the non-capsulated strain of *Pneumococcus* I–192R, A.T.C.C. 12213, contain a relatively large amount of a diglycosyl diglyceride 1-[*O*-α-D-galactopyranosyl-(1→2)-*O*-α-D-gluco-pyranosyl]-2,3-diacyl-D-glycerol and a smaller amount of its immediate biosynthetic precursor, a glucosyl diglyceride (Brundish, Shaw & Baddiley, 1965*a*). In view of the current interest in bacterial lipids as intermediates in the biosynthesis of lipopolysaccharides (Rothfield & Horecker, 1964) and glycosaminopeptides (Anderson, Matsuhashi, Haskin & Strominger, 1965), an investigation of the phospholipids of this organism was undertaken.

The present paper describes the separation of the deacylated products* from the phospholipids and also reports novel isomerization and transesterification reactions of phosphodiesters under mild acidic conditions. A preliminary account of these observations has been published (Brundish, Shaw & Baddiley, 1965*b*).

* In the abbreviated formulae of deacylated products in this paper G represents glycerol and P represents phosphate; unless otherwise indicated, linkages are between positions 1 and 3 (αα) on glycerol; structures containing more than one phosphate group are designated αα′ where all linkages involve positions 1 and 3, and αβ′ when position 2 may be involved (see Scheme 1).

EXPERIMENTAL AND RESULTS

Materials

Sephadex G-25 was obtained from Pharmacia (Uppsala, Sweden), DEAE-cellulose from H. Reeve Angel and Co. Ltd. (London), silicic acid 'for chromatography' was from L. Light and Co. Ltd. (Colnbrook, Bucks.), 'Mallinckrodt 100 mesh' silica was from Kodak Ltd. (Kirkby, Lancs.), intestinal alkaline phosphomonoesterase was from Sigma Chemical Co. (St Louis, Mo., U.S.A.) and fatty acid methyl esters were from British Drug Houses Ltd. (Poole, Dorset), Sigma Chemical Co. and California Corp. for Biochemical Research (Los Angeles, Calif., U.S.A.). Solvents for column chromatography were purified and dried before use.

Analytical methods

Phosphorus was determined by the method of Chen, Toribara & Warner (1956), formaldehyde was determined by the chromotropic acid procedure of Hanahan & Olley (1958), periodate was determined by the spectrophotometric method of Dixon & Lipkin (1954) and fatty acid esters were determined by the method of Rapport & Alonzo (1955).

Paper chromatography

Whatman no. 4 paper, previously washed with 2N-acetic acid and then water, was used with the following solvent systems: *A*, propan-1-ol–aq. NH_3 (sp.gr. 0·88)–water (6:3:1, by vol.) (Hanes & Isherwood, 1949); *B*, propan-2-ol–aq. NH_3 (sp.gr. 0·88)–water (7:2:1, by vol.) (LeCocq & Ballou, 1964).

Compounds were detected by the periodate–Schiff reagents for α-glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956), the ninhydrin spray for amino compounds (Consden & Gordon, 1948) and the acid molybdate spray for phosphoric esters (Hanes & Isherwood, 1949).

Growth of organism

Bacteria were grown as described by Brundish *et al.* (1965*a*).

Extraction and fractionation of lipids

Lipids were extracted from freeze-dried cells with chloroform–methanol (2:1, v/v) and fractionated on a silicic acid column by stepwise elution with chloroform–methanol mixtures (Brundish *et al.* 1965*a*). A typical elution curve is shown in Fig. 1. Fraction B contained glycosyl diglycerides in addition to phospholipids and was further fractionated on a DEAE-cellulose (acetate form) column as described by Brundish *et al.* (1965*a*). After elution of the glycolipids, phospholipids were eluted by the application of 33% (v/v) methanol in chloroform containing 4% (w/v) of ammonium acetate. The eluate was evaporated to dryness, ether (5ml.) was added and the mixture was shaken with water (2×5ml.) to remove ammonium acetate. The lipid was recovered by evaporation of the dried ethereal layer. From 1·96g. of lipid, 284mg. of fraction A, 100mg. of purified fraction B and 450mg. of fraction C were obtained. Fraction N contained 450mg. of neutral lipid and was not investigated further.

Analysis of fraction A

The lipid (284mg.) was dissolved in chloroform–methanol (1:1, v/v; 15ml.), and 0·5M-sodium methoxide in methanol (3ml.) was added (Marinetti, 1962). The mixture was kept at room temperature for 10min., water (15ml.) was added, and it was then passed through a column (10ml.) of Dowex 50 (H^+ form) resin and the aqueous and organic phases were separated. The aqueous phase was evaporated to dryness *in vacuo* at room temperature. The residue (10mg.) was applied to a column of DEAE-cellulose (HCO_3^- form) and, after washing with water (150ml.), material was eluted with a linear gradient (0–0·15M) of $(NH_4)_2CO_3$ solution (660ml. total volume). Fractions (5ml.) were collected at a flow rate of 0·8ml./min. and samples (0·2ml.) were analysed for phosphorus. The elution pattern is given in Fig. 2. The appropriate fractions were combined and the material was recovered by rotary evaporation at 35°. The yields were: fraction A1, 1·0mg.; fraction A2, 5·4mg.; fraction A3, 4·0 mg. Examination of the fractions by paper chromatography indicated that extensive degradation had occurred during work-up and the samples were rejected. The degradation was probably due to the action of the $(NH_4)_2CO_3$ during the

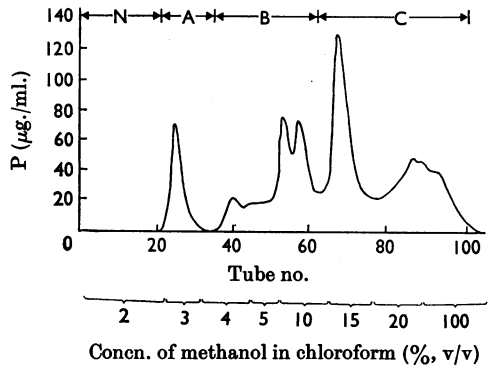


Fig. 1. Fractionation of the purified lipid extract on a column of silicic acid by stepwise elution with chloroform–methanol mixtures.

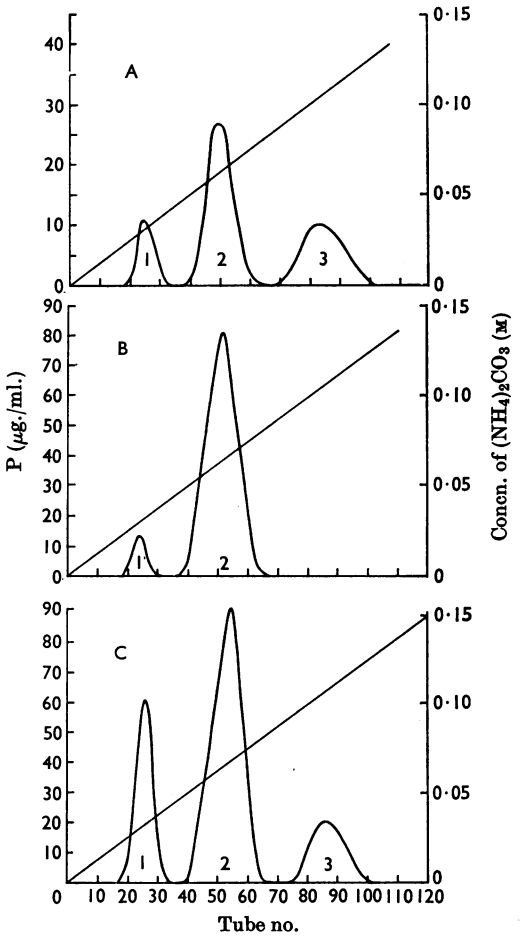


Fig. 2. Separation of the phosphates produced by deacylation of lipid fractions A, B and C on columns of DEAE-cellulose (HCO_3^- form).

Table 1. *Percentage composition of fatty acid methyl ester mixture from fraction B*

The chromatography of methyl esters is described by Brundish *et al.* (1965*a*). The esters are designated *x*:*y*, where *x* is the no. of carbon atoms/fatty acid mol. and *y* is the no. of double bonds/fatty acid mol.

Methyl ester	Composition (moles/100 moles)
12:0	14·1
14:0	14·2
14:1	4·7
16:0	34·3
16:1	21·4
18:0	2·9
18:1	8·4

rotary evaporation. This step was carried out under conditions that should not lead to degradation (cf. LeCocq & Ballou, 1964), and it is possible that some laboratory grades of $(NH_4)_2CO_3$ contain substantial amounts of harmful impurities. The elution curve is similar to that obtained for fraction C, and it is reasonable to assume that the three fractions A1, A2 and A3 correspond to the fractions C1, C2 and C3 (see below).

Analysis of fraction B

The lipid (100 mg.) was dissolved in chloroform–methanol (1:1, v/v) and deacylated as described for fraction A. The fatty acid methyl esters were investigated by gas–liquid chromatography on a column (150 cm.) of polyethylene glycol adipate by using a Pye Panchromatograph as described by Brundish *et al.* (1965*a*). The fatty acid composition of fraction B (Table 1) is very similar to that previously described for the glycolipids of this organism.

The water-soluble phosphate esters produced by deacylation were fractionated on a DEAE-cellulose (HCO_3^- form) column as described above for fraction A. The elution pattern is given in Fig. 2. The fractions were combined as indicated and the solutions reduced to small volumes by rotary evaporation at 20°. Dowex 50 (H^+ form) resin was then added to destroy excess of $(NH_4)_2CO_3$ and the resin was removed by filtration. The filtrates were carefully dried by rotary evaporation.

*Fraction B*1. This sample was lost after preliminary paper-chromatographic studies had suggested the presence in it of diglycerol phosphate (GPG) (I).

*Fraction B*2. The material (23 mg.) was dissolved in water (1 ml.) and examined by paper chromatography; it behaved as a single compound, R_F 0·40 in solvent *A*, corresponding to bis(glycerylphosphoryl)glycerol (GPGPG) (III). This was confirmed by the following degradations.

(1) Hydrolysis by acid and alkali. Samples (0·05 ml.) of the solution were heated for 1 hr. with N-HCl and N-KOH respectively (0·2 ml. of each). The acid hydrolysate was evaporated to dryness over KOH *in vacuo*; the alkali hydrolysate was deionized by passage through a column (1 ml.) of Dowex 50 (H^+ form) resin and evaporated to dryness. The products were examined by paper chromatography in solvent *A*. In each case glycerol and its mono- and di-phosphates were detected.

(2) Oxidation with periodate. A sample (0·2 ml.) of the solution was added to 20 mM-$NaIO_4$ (2·5 ml.) in a graduated flask and diluted to 5 ml. The mixture was kept in the dark at room temperature. Samples (0·1 ml.) were withdrawn periodically and diluted to 10 ml. with water for analysis of periodate. The first sample was taken after 16 hr., at which time reaction was complete. A further sample (1 ml.) of the oxidation mixture was diluted to 10 ml. and samples of this solution were analysed for total phosphorus (0·2 ml.) and formaldehyde (1 ml.). The material gave the molar proportions periodate reduced:formaldehyde formed:phosphorus as 0·99:0·94:1·00.

(3) Action of periodate followed by dimethylhydrazine. A sample (1 ml.) of the oxidation mixture from the above experiment was treated with a solution of ethylene glycol (1%, v/v; 1 ml.) for 30 min. to destroy excess of periodate. A solution of 1,1-dimethylhydrazine (1%, v/v; 1 ml.) at pH 6 in dilute acetic acid was added and the mixture was kept at 37° for 16 hr. The mixture was shaken several times with portions of chloroform (1 ml. each), passed through a small column (1 ml.) of Dowex 50 (H^+ form) resin, neutralized with dilute aq. NH_3, evaporated to dryness and examined in solvent *B*. For reference, samples (0·5 mg.) of starting material were heated with N-HCl and N-KOH respectively for 1 hr. at 100° to give mixtures of the isomeric mono- and di-phosphates of glycerol. The product from the oxidation mixture corresponded to glycerol 1,3-diphosphate (cf. LeCocq & Ballou, 1964).

Analysis of fraction C

The lipid (297 mg.) was deacylated and the water-soluble phosphates were separated on a DEAE-cellulose (HCO_3^- form) column as described above. The elution pattern is given in Fig. 2. The weights of material recovered were: fraction C1, 15·7 mg.; fraction C2, 31·1 mg.; fraction C3, 6·0 mg. These were dissolved in water to give solutions of concentration 20 mg./ml. and stored at −14°.

*Fraction C*2. This component had R_F 0·41 in solvent *A*, corresponding to GPGPG.

*Fraction C*3. This component, which had R_F 0·25 in solvent *A*, was shown to be glycerylphosphorylglycerol phosphate (GPGP) (V) by the following degradations.

(1) Hydrolysis by acid and alkali. Samples (0·1 ml.) of the diluted fractions were examined by paper chromatography after hydrolysis in acid and alkali. In both cases glycerol and its mono- and di-phosphates were observed.

(2) Action of periodate followed by dimethylhydrazine. Treatment of a sample (0·1 ml.) of fraction C3 as described above yielded mainly glycerol diphosphate.

(3) Action of alkaline phosphomonoesterase. A sample (0·05 ml.) of fraction C3 was diluted to 0·4 ml. with water and phosphorus was determined in a portion (0·1 ml.) of this. Another sample (0·1 ml.) was treated with a 1% (w/v) solution of alkaline phosphomonoesterase (1 ml.) at pH 9·3 for 16 hr. at 37°. The resulting inorganic phosphate represented 44% of the total phosphorus. The remaining portion (0·2 ml.) of the solution of fraction C3 was treated with the enzyme solution (0·2 ml.) and the products were examined by paper chromatography. Compounds detected were inorganic phosphate and GPG.

*Fraction C*1. Paper chromatography in solvent *A* showed that this contained two components, R_F 0·61 and 0·65, the slower component corresponding to GPG. Since glycerol

Table 2. *Analysis of the four components of fraction C1 after separation by paper chromatography*

Chromatographic conditions are given in the text.

R_F in solvent *A*	HCHO produced/P	Product of periodate–dimethylhydrazine degradation	Structure
0·65	1·16	β-Glycerol phosphate	αβ′-GPG (II)
0·60	1·93	Inorganic phosphate	αα′-GPG (I)
0·48	0·51	Glycerylphosphoryl-glycerol phosphate	αβ′-GPGPG (IV)
0·40	0·99	Glycerol diphosphate	αα′-GPGPG (III)

2-phosphate has a slightly higher R_F value than does the 1-phosphate it was probable that the faster-running component, which also gave a positive periodate–Schiff reaction, could be represented as αβ′-diglycerol phosphate (II, αβ′-GPG). The fraction was kept for 2 months at −14°. Chromatography then revealed, in addition to the two components presumed to be αα′-GPG and αβ′-GPG, two components, R_F 0·40 and 0·48, together with glycerol. The sample was applied as a band (8 cm.) to a Whatman no. 4 paper and the chromatogram was run overnight in solvent *A*. The four phosphates were eluted from the paper with water and the volumes were adjusted to 5 ml. each. Samples (0·5 ml.) were analysed for total phosphorus and terminal glycol groups. The remaining samples were each concentrated to 0·5 ml., oxidized with periodate and treated with dimethylhydrazine as described for fraction B2. The results are given in Table 2.

The product obtained from the periodate–dimethylhydrazine degradation of the compound with R_F 0·48 was further treated with alkaline phosphomonoesterase and examined by paper chromatography. Inorganic phosphate and a compound with R_F 0·64, corresponding to αβ′-GPG, were detected, but insufficient of the latter product was obtained for further study.

These results establish the structure of the four components as αα′-GPG, αβ′-GPG (II), αα′-GPGPG and αβ′-GPGPG (IV).

Deacylation of phospholipids and fractionation of products under conditions avoiding isomerization

The lipid from a batch (6 l.) of organisms was extracted and contaminants were removed by chromatography on Sephadex as described by Brundish *et al.* (1965*a*). The material (301 mg.) was dried *in vacuo* over P_2O_5 for 16 hr. and then dissolved in ethanol (15 ml.). Ethanolic KOH solution (2%, w/v; 15 ml.) was added and deacylation was allowed to proceed for 4 hr. at 20° (Macfarlane, 1958).

The precipitate was collected by centrifugation and washed twice with ethanol. The supernatant and washings were diluted to 100 ml. with ethanol and portions (1 ml.) were analysed for phosphorus. The precipitate was dissolved in water (5 ml.), the strongly alkaline solution was passed through a column (1 ml.) of Dowex 50 (H^+ form) resin and the eluate was introduced immediately on to a column (15 cm. × 1·5 cm.) of DEAE-cellulose (HCO_3^- form); material was then eluted as described above. The elution pattern is given in Fig. 3. The appropriate fractions, which contained 75% of the phosphorus of the original lipid fraction, were combined and freeze-dried. Components D1 and D2 were identified as αα′-GPG and αα′-GPGPG respectively by the methods described above. Component D3, which corresponded in elution position to fraction C3 (GPGP), was not obtained in sufficient amount for analysis.

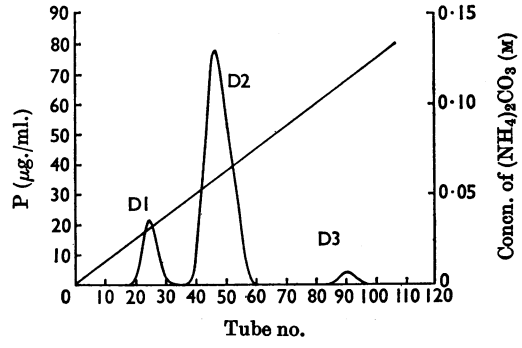


Fig. 3. Separation of the phosphates produced by the deacylation procedure of Macfarlane (1958) from a freshly isolated lipid extract on a column of DEAE-cellulose (HCO_3^- form).

Isomerization of ox-heart cardiolipin

A sample (1 mg.) of ox-heart cardiolipin was deacylated and the water-soluble products were examined by paper chromatography as described above. Two components were observed, corresponding to αα′-GPGPG and a smaller amount of αα′-GPG. A further sample (1·25 mg.) was dissolved in chloroform (1 ml.; reagent grade) and kept for 6 days at room temperature. Deacylation and examination of the products by paper chromatography showed the presence of αα′-GPG, αβ′-GPG, αα′-GPGPG and αβ′-GPGPG.

Synthesis of chromatographic standards

Diglycerol phosphate (GPG). Disodium α-glycerol phosphate hexahydrate (0·48 g.) and 2,3-epoxypropanol (4 ml.) were heated under reflux in water (100 ml.) at pH 8·0 for

8hr. (cf. Brown, Hall & Higson, 1958). After cooling, the solution was passed through a column (5cm.×1cm.) of Dowex 50 (H^+ form) resin and the eluate was introduced immediately on to a column (30cm.×2cm.) of DEAE-cellulose (HCO_3^- form).

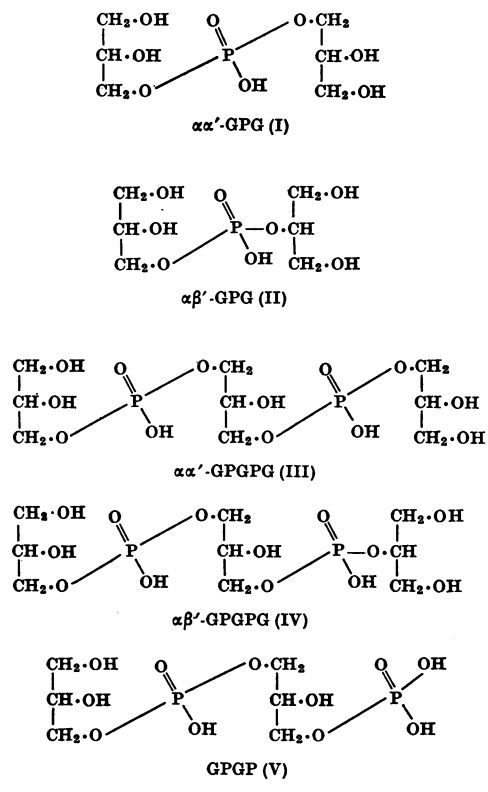
The column was washed with 3 bed-volumes of water and products were eluted with 3l. of a linear gradient (0–0·1M) of $(NH_4)_2CO_3$. Fractions (50ml.) were collected at 2ml./min. and samples (0·1ml.) were analysed for phosphorus; 54·8% of the starting material was converted into diglycerol phosphate. Appropriate fractions were combined and the desired product was recovered by rotary evaporation. It was homogeneous (R_F 0·60) on paper chromatography in solvent *A*, and gave the ratio α-glycol:phosphorus as 1·86:1·0.

Bis(glycerylphosphoryl)glycerol (GPGPG) and glycerylphosphorylglycerol phosphate (GPGP). Cyclohexylammonium glycerol 1,3-diphosphate monohydrate (LeCocq & Ballou, 1964) (0·5g.) and 2,3-epoxypropanol (2ml.) were heated under reflux in water (50ml.) at pH8 for 8hr., and the reaction products were separated by column chromatography as described above. Analysis showed that conversion into GPGPG was 93·3% and into GPGP was 7·6%. The products were recovered by combination of the relevant fractions, rotary evaporation to a small volume and freeze-drying. The minor product (GPGP) behaved as a single compound (R_F 0·25) on paper chromatography in solvent *A* and gave the ratio α-glycol:phosphorus as 0·44:1·0, whereas the major product gave one spot (R_F 0·49) in solvent *A* with the ratio α-glycol:phosphorus as 1·03:1·0.

DISCUSSION

The phospholipids of *Pneumococcus* I–192R, A.T.C.C. 12213, were resolved into three fractions, A, B and C, by a combination of silicic acid and DEAE-cellulose chromatography. The water-soluble phosphates obtained by deacylation of each fraction were separated by gradient elution from a DEAE-cellulose (HCO_3^- form) column. Fractions A and C each yielded three components, whereas fraction B gave only two.

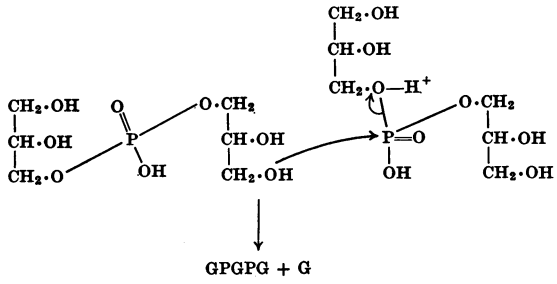
The three components of fraction A were degraded during subsequent handling, but it is reasonable to suppose that they corresponded to the three components of fraction C, since they were eluted from the column in identical positions. Fractions B2 and C2 were identified by chemical degradation as GPGPG and fraction C3 was identified as GPGP; fraction B1 was chromatographically identified as GPG. Fraction C1 was resolved into two closely related compounds on paper chromatography, and chemical studies indicated that these were αα′-GPG and αβ′-GPG. At this stage the investigation was interrupted for 2 months, during which time the solution of fraction C1, which contained the phosphates in the free acid forms, was stored at −14°. On re-examination, two additional phosphates were observed, together with glycerol. These four components were identified as αα′-GPG, αβ′-GPG, αα′-GPGPG and αβ′-GPGPG (see Scheme 1) by analysis and unambiguous chemical degradations; the periodate–dimethylhydrazine degradation (LeCocq & Ballou, 1964) was particularly useful in these studies.



Scheme 1. Glycerol phosphate derivatives obtained by deacylation of phospholipids.

The αβ-isomers in fraction C1 had presumably arisen from αα-isomers at an unidentified stage of the isolation, purification or storage of material. Acid-catalysed migration of phosphodiesters has been described by Brown, Magrath, Neilson & Todd (1956), who showed that the dinucleoside phosphate adenylyl-(2′-5′)-uridine was readily isomerized under acidic conditions to a mixture of adenylyl-(2′-5′)-uridine and adenylyl-(3′-5′) uridine, presumably through the intermediate transient formation of a phosphotriester. The acid-catalysed isomerization observed in the present case is analogous to the nucleotide example.

It is unlikely that αβ-isomers of GPG and GPGPG were present as acyl derivatives in the original organisms but had not been separated from other components during the preliminary fractionation of the phospholipids. This would not agree with the finding that greater care in the isolation and



Scheme 2. Mechanism for the intermolecular transesterification of diglycerol phosphate to yield bis(glycerylphosphoryl)-glycerol.

preparation of materials from the phospholipid fraction, and the avoidance of delay, resulted in products containing no β-isomers (see Fig. 3). Moreover, the presence of glycerol in the stored material would not be explained in this way, as the original purification procedure would have removed glycerol and other neutral compounds. It therefore appears that the transesterification reaction 2GPG→GPGPG+G had occurred in the acidic solution; a possible mechanism for this reaction is outlined in Scheme 2. An analogous transesterification between two molecules of phosphatidylglycerol, under enzymic catalysis, provides a possible route to the biosynthesis of bisphosphatidylglycerol (cf. LeCocq & Ballou, 1964).

The isolation of GPGP suggests that the original lipid extract might have contained phosphatidylglycerol phosphate. This compound has not been isolated from bacteria, although the diether analogue is present in the lipids of *Halobacterium cutirubrum* (Kates, Yengoyan & Sastry, 1965), and Kanfer & Kennedy (1964) have shown that phosphatidylglycerol phosphate can act as a biosynthetic precursor of phosphatidylglycerol in *Escherichia coli* B. However, phosphatidylglycerol phosphate is a possible degradation product of bisphosphatidylglycerol (cf. Coulon-Morelec, Faure & Maréchal, 1960, 1962), and the GPGP could have arisen during isolation. In support of this view it is significant that the components of fraction B, which after silicic acid chromatography were not kept in chloroform–methanol but were refractionated on DEAE-cellulose and kept as ammonium salts, contained no detectable amount of this material. Wells & Dittmer (1966) have identified GPGP as a deacylation product of brain phospholipids, but they were unable to isolate the intact lipid, and it is possible that their GPGP is an artifact of isolation.

Macfarlane (1958) has described a deacylation procedure for phospholipids in which the phosphates are precipitated as their potassium salts. Accordingly, a freshly prepared lipid extract was deacylated by this procedure and the resulting phosphates were fractionated on a DEAE-cellulose (HCO_3^- form) column as before (Fig. 3). Components D1 and D2 were identified as αα′-GPG and αα′-GPGPG and no αβ-isomers could be detected. The elution position of component D3 corresponded to that of GPGP, but the material was not present in sufficient amounts to permit identification; the quantity obtained was considerably less than had been observed previously. Although the possibility that traces of phosphatidylglycerol phosphate occur in this organism cannot be excluded, it is certain that some of the components of the material prepared earlier were produced by acidic degradation. The absence of the isomers αβ′-GPG and αβ′-GPGPG from later preparations also shows that these are artifacts of the earlier isolation and purification procedures. It is not known when they were produced as acidic conditions could have been encountered at several stages.

Schwarz, Driesbach, Polis, Polis & Soffer (1965), investigating the lipids of rat-liver particulate fractions, reported the formation of an unidentified isomer of GPG arising during deacylation of the phospholipids. In view of our results it is probable that the isomer is αβ′-GPG, arising from acid-catalysed phosphate migration during drying of the acidic solution after passage through Amberlite IRC-50 (H^+ form) resin. These authors also reported that similar treatment of an authentic sample of a GPG derivative yielded, in addition to the unidentified isomer, a small amount (3%) of GPGPG, thus confirming our observation of the transesterification reaction. We have observed isomerization in a sample of ox-heart cardiolipin, kindly supplied by Dr G. M. Gray. The original mixture on deacylation yielded αα′-GPG and αα′-GPGPG (III); after storage of the lipid in chloroform for 6 days at room temperature deacylation yielded a mixture of αα′-GPG, αβ′-GPG, αα′-GPGPG and αβ′-GPGPG. The compounds GPG, GPGP and GPGPG were synthesized for use as chromatographic standards by using the glycidol condensation (Brown *et al.* 1958).

This investigation has shown that the phospholipids of the *Pneumococcus* type I are typical of Gram-positive organisms in general, consisting mainly of phosphatidylglycerol and bisphosphatidylglycerol. It has also revealed the ease with which isomerization and transesterification reactions can occur in phospholipids containing phosphodiester groups, and it is necessary to emphasize the dangers of misinterpretation that could arise by the use of mild acidic conditions during structural studies on phospholipids containing such groups.

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