reduced pressure at 40°, and the residue was dried at room temperature in a vacuum of 0.1 mm. A solution of the crude cephalin in a 1:1 (v/v) mixture of benzene and chloroform was passed through a column of silicic acid (3 × 35 cm). Exhaustive elution of the column with a 1:2 mixture of benzene and chloroform (eluate 1), followed by a 1:3 mixture of benzene and chloroform (eluate 2) and evaporation of the eluates under reduced pressure from a bath at 30–35°, gave a small amount of material low in both nitrogen and phosphorus (eluate 1) and 0.76 g (eluate 2) of fairly pure cephalin (found: N, 1.81; P, 4.09). Rechromatography of the latter on silicic acid, elution of the product with a 1:3 mixture of benzene and chloroform, evaporation of the eluate under reduced pressure at 30–35°, and drying of the waxy, slightly yellowish solid for 3 days at room temperature in a vacuum of 0.02 mm gave 0.53 g (59% of theory) of chromatographically pure L-α-(dioleoyl)cephalin. $[\alpha]_D^{23}$ +5.8° in ethanol-free anhydrous chloroform (*c*, 5). Reported (Baer and Buchnea, 1959) $[\alpha]_D$ +6.0° in chloroform (*c*, 7).

Anal. Calcd for $C_{41}H_{78}O_8NP$ (744.05): N, 1.88; P, 4.18; iodine number, 68.3. Found: N, 1.82, 1.84; P, 4.15, 4.12, iodine number, 66.0, 66.3.

L-α-*(Dilinoleoyl)cephalin.*—The hydrazinolysis of dilinoleoyl L - α - glycerylphosphoryl - 2′ - hydroxyethylphthalimide (1.0 g), and the separation of the reaction products by column chromatography on silicic acid was carried out as described for the oleoyl compound. The L-α-(dilinoleoyl)cephalin, a slightly yellowish-colored waxy solid, weighed 0.44 g (51.7% of theory). On exposure to air it becomes a viscous gum. $[\alpha]_D^{23}$ +5.8° in anhydrous ethanol-free chloroform (*c*, 5). Reported by Dorofeeva *et al.* (1963): $[\alpha]_D$ +6°. The L-α-(dilinoleoyl)cephalin is soluble in all of the commonly used organic solvents.

Anal. Calcd for $C_{41}H_{74}O_8NP$ (740.0): C, 66.56; H, 10.08; N, 1.89; P, 4.18; iodine number, 137.2. Found: C, 66.12; H, 10.35; N, 1.79; P, 4.07; iodine number, 133.1, 132.9.

L-α-*(Distearoyl)cephalin.*—Hydrogenation of L-α-(dilinoleoyl)cephalin (91 mg) in ethanol with platinum dioxide as catalyst, and purification of the reaction product as described by Baer (1957) gave 63 mg (69.2% of theory) of pure L-α-(distearoyl)cephalin; mp 180–182°. Reported (Baer, 1957) mp 180–182°.

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On the Structure of Cardiolipin*

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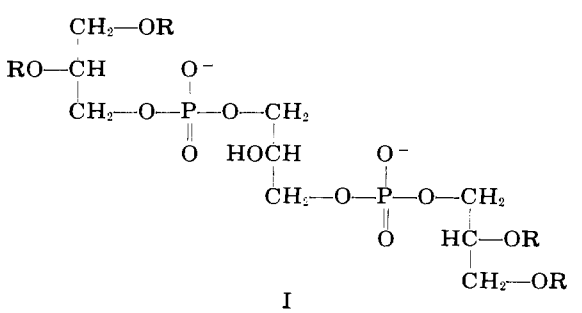
Cardiolipin (diphosphatidylglycerol) from beef heart and from *Mycobacterium phlei* has been deacylated and degraded to glycerol-1,3-diphosphate by the action of sodium metaperiodate and 1,1-dimethylhydrazine. The results establish that the lipid has the 1,3-diphosphatidylglycerol structure, a point that was still open to question. For references, crystalline cyclohexylamine salts of glycerol-1,3-diphosphate and L-glycerol-1,2-diphosphate have been prepared. The reported optical activity of the triglyceroldiphosphate obtained by deacylation of cardiolipin has been confirmed, and is found to be identical with that of synthetic 1,3-di-*O*-(L-glycerol-3′-phosphoryl)-glycerol synthesized by P. Plackett (*Australian J. Chem. 17*, 101, 1964), thus establishing the over-all absolute configuration of the cardiolipin molecule.

Cardiolipin was first isolated from beef heart by Pangborn (1942), who demonstrated that the substance was essential for the reactivity of beef heart antigens in the serologic test for syphilis. On hydrolysis, cardiolipin yielded fatty acids and a "phosphorylated polysaccharide" which was shown to be a polyglycerolphosphate (Pangborn, 1947). From the analytical data, she proposed a formula in which four glycerol residues were connected by three phosphate groups in diester linkage, the fatty acids being esterified to the remaining hydroxyl groups of the glycerol molecules.

McKibbin and Taylor (1952) later isolated from dog liver a polyglycerolphosphatide in which the ratio of glycerol to phosphorus was 3:2, a result in disagreement with Pangborn's formula. In 1956, Faure and Morelec-Coulon repeated the preparation of cardiolipin from cardiac muscle, and found molar ratios for glycerol–phosphorus–fatty acids of 3:2:4. This result was confirmed by MacFarlane and Gray (1957) and Gray and MacFarlane (1958), who examined preparations of cardiolipin obtained by various methods. These authors (1958), as well as Faure and Morelec-Coulon (1958a) showed that glyceroldiphosphate could be obtained as one of the degradation products of cardiolipin. Based on these results, MacFarlane and Gray



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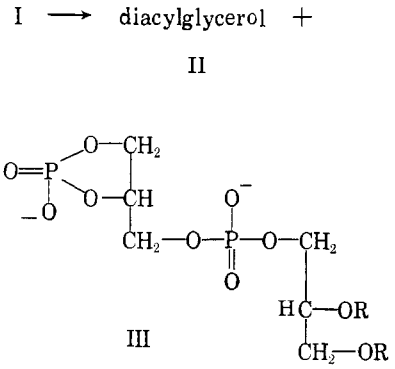
(1957) proposed formula I (R = fatty acyl group) for cardiolipin, that is, a diphosphatidylglycerol.

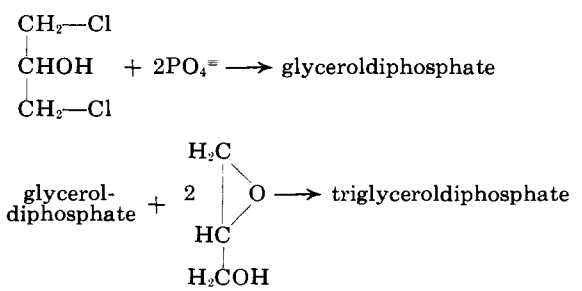
Other observations by MacFarlane (1958) confirm this structure. Thus, the polyglycerolphosphate, obtained from cardiolipin by mild alkaline hydrolysis, reacted with sodium metaperiodate to produce one mole of formaldehyde per mole of phosphate, and the product had about one aldehyde function per phosphorus atom.

The positioning of the fatty acids in the cardiolipin molecule comes from the following observations. Coulon-Morelec and Faure (1958) found that, by heating cardiolipin in anhydrous acetic acid (100° for 30 minutes), a diacylglycerol (II) was produced with no liberation of acidic functions, that is, no breakage of diester phosphate bonds. No free fatty acid was liberated. It was shown later (Coulon-Morelec *et al.*, 1960) that this was due to cyclic phosphate (III) formation with elimination of diacylglycerol (II).



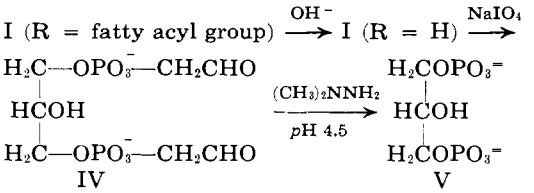
MacFarlane and Wheeldon (1959) reported that heating cardiolipin in 90% acetic acid (100°, 90 minutes) gave mainly glyceroldiphosphate and diacylglycerol in the molar ratio of 1:2. Assuming that these degradations are not preceded by acyl migration, the results show that the fatty acids are located on the terminal glycerols and there is a free hydroxyl group on the center glycerol as shown in formula I.

The glycerol-1,3-diphosphate structure of cardiolipin is generally assumed. MacFarlane and Gray (1957) oxidized cardiolipin with permanganate, hydrolyzed the product with acid, and isolated a 12% yield of methylglyoxal, which was presumed to come from an intermediate diphosphatidyldihydroxyacetone. However, this degradation, when applied to lysolecithin (Hanahan, 1954; Gray, 1958), gives results which are now known to be erroneous (Hanahan *et al.*, 1960). Proof of structure by synthesis has been attempted by Benson and Strickland (1960). They prepared a triglyceroldiphosphate by the following reaction sequence:



Although the product probably has the glycerol-1,3-diphosphate structure, this is not a necessary consequence of the presence of the halogen in the α positions, since Bailly (1916) has shown that the epoxide is an intermediate in the condensation of α-monochlorohydrin with trisodium phosphate. To establish identity of their synthetic polyglycerolphosphate with the deacylated product from plant diphosphatidylglycerol, Benson and Strickland (1960) showed that these substances could not be distinguished from each other by chromatography or electrophoresis. However, it is not certain that the isomeric glycerol-1,2-diphosphate structure would be distinguishable by these techniques.

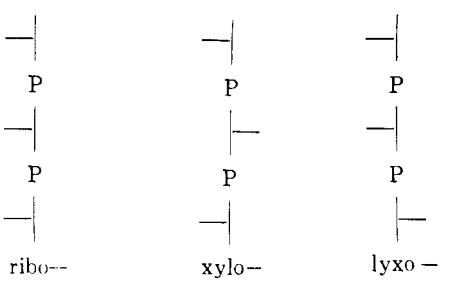
We have investigated the structure of the polyglycerolphosphate from beef heart cardiolipin and diphosphatidylglycerol of *Mycobacteria* by making use of the reaction devised by Brown *et al.* (1961) for the degradation of phosphatidylmyoinositol and based on the observations of Fleury *et al.* (1952). This allows the selective removal of α-linked glycerol moieties, by the following reaction sequence (I → IV → V):



Thus, cardiolipin with structure I should yield glycerol-1,3-diphosphate V, while the isomeric compound in which the middle glycerol is substituted on positions 1 and 2 should yield glycerol-1,2-diphosphate. Moreover, a molecule such as proposed by Pangborn would give a diglyceroltriphosphate in this degradation.

For the purpose of this investigation, we have synthesized glycerol-1,3-diphosphate and L-glycerol-1,2-diphosphate and obtained them as crystalline cyclohexylamine salts which gave characteristic infrared spectra. We have also shown that they can be distinguished by paper chromatography, and that acid-catalyzed migration of each compound produces a mixture of both isomers. When a sample of cardiolipin from beef heart was subjected to the above degradation procedure, the glyceroldiphosphate component was found by chromatography to consist entirely of the 1,3 isomer. The same reaction was carried out on a larger scale with diphosphatidylglycerol isolated from *Mycobacterium phlei* (Faure and Maréchal, 1962), and the glyceroldiphosphate was isolated as the crystalline cyclohexylamine salt. It proved to be identical with glycerol-1,3-diphosphate and was easily distinguishable from glycerol-1,2-diphosphate, which result confirms structure I.

The triglyceroldiphosphate molecule can have one of three steric relationships, which for simplicity can be called the *ribo-*, *xylo-*, and *lyxo-* configurations, in pseudo-Fischer projections. Only the latter could exhibit optical activity. The optical rotation of

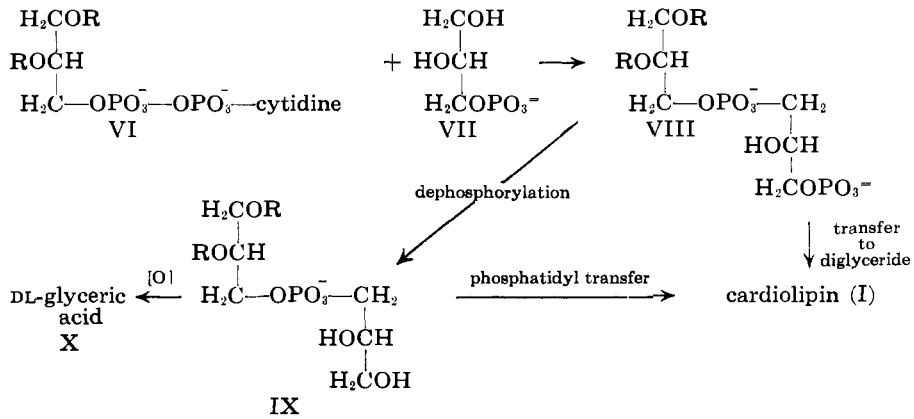


the polyglycerol phosphate from deacylated cardiolipin has been reported by Faure and Coulon-Morelec (1963), who found a value of $[M]_D$ −553°.[1] We obtained values of $[M]_{589}$ −520° at neutral *p*H, and −840° in 2 N hydrochloric acid. This small, but sig-

[1] Calculated from the specific rotation Faure and Coulon-Morelec (1963) cite for the sodium salt dissolved in water, assuming a molecular weight of 442.

nificant, optical activity is consistent only with the lyxo-configuration.

Speculations on this structural aspect of the cardiolipin molecule have been stimulated by the biosynthetic studies. The experiments of Kiyasu *et al.* (1963) show that phosphatidylglycerol (IX) can be formed by a transfer of the phosphatidyl group to L-glycerol-3-phosphate (D-glycerol-1-phosphate, VII), followed by a dephosphorylation reaction (VIII → IX).



If this is so, the two glycerolphosphate moieties must have opposite configurations, and Benson and Miyano (1961) have shown that oxidation of the diglycerolphosphate obtained by deacylating phosphatidylglycerol yields a product, which gives DL-glyceric acid (X) on hydrolysis of the phosphate diester. Kiyasu *et al.* (1963) suggest that phosphatidylglycerol may be converted to cardiolipin by a second phosphatidyl transfer from cytidinediphosphatediacylglycerol (IX → I). However, a feature of the cardiolipin molecule (formula I) is the occurrence of a 1-L-phosphatidyl-L-glycerol relationship at one end and of a 1-L-phosphatidyl-D-glycerol relationship at the other end.[2] This suggests (Benson and Miyano, 1961) a different stereospecificity for the introduction of the two phosphatidyl residues, and the possibility should be considered that cardiolipin may be formed by a transfer of the phosphatidylglycerolphosphate molecule to D-2,3-diacylglycerol (VIII → I).

Plackett (1964) has described the synthesis of 1,3-di-(L-glycerol-3'-phosphoryl)-glycerol. This substance corresponds to the polyglycerolphosphate with the D-lyxo- configuration and should be identical with the substance formed by deacylation of cardiolipin. The barium salt of the synthetic material dissolved in 2 N hydrochloric acid has a molecular rotation of $-610°$ ($\pm 60°$). This value is of the same sign and very close in magnitude to those found for the natural substance and establish that the absolute configuration of the glycerolphosphate backbone of the cardiolipin molecule is that shown in I.

Experimental

General Methods.—Phosphorus was determined by the method of Bartlett (1959). The determination of α-linked glycerol residues was by oxidation with sodium metaperiodate, the consumption of periodate being estimated by the arsenite-iodine method of Fleury and Lange (1933), and formaldehyde formation by the chromotropic acid method (Hanahan and Olley, 1958).

For chromatography, we used Whatman No. 1 paper and a solvent of isopropanol with various amounts of concentrated ammonia. For paper chromatographic separation of the glyceroldiphosphates, Whatman No. 1 paper washed with 1 N acetic acid was used and the chromatogram was developed descending with isopropanol-ammonia-water (7:1:2) for 1 week. Our synthetic samples gave single spots. The 1,3 and 1,2 isomers were well separated, and in a typical experiment the distance traveled by the former was 8.9 cm, by the latter 11.2 cm. The spots were detected by the phosphate reagent of Bandurski and Axelrod (1951). Polyglycerolphosphate was detected by the α-glycerol reagent of Gordon *et al.* (1956). If phosphorus was to be determined in the spots, the paper was first washed with 2 N acetic acid. Perchloric acid was used for digestion of the pieces of paper (Usher, 1963). However, sulfuric acid and hydrogen peroxide also worked well. In this case, the spots are cut out after detection by the reagent of Bandurski and Axelrod (1951) and the paper was digested in test tubes at 200° with 0.3 ml of concentrated sulfuric acid, about 1 ml of 30% hydrogen peroxide being added progressively to bring complete digestion. After addition of water and the reagents, the volume was finally adjusted to 10 ml.

Degradation of Beef Heart Cardiolipin to Glycerol-1,3-diphosphate.—A sample of beef heart cardiolipin, supplied as the sodium salt by Mlle. Faure, had been prepared according to Faure and Morelec-Coulon (1958b). The material was deacylated with ethanolic potassium hydroxide (Pangborn, 1947). The potassium salt of the polyglycerolphosphate precipitated during the reaction. It was centrifuged and thoroughly washed with absolute ethanol, then redissolved in water in the presence of a small amount of IRC-50(H^+) resin to remove any alkali still present. The resin was filtered off and the filtrate was extracted three times with a 10:1 mixture of ether and methanol. The aqueous solution was finally evaporated to dryness *in vacuo* yielding a sirup (67 mg from 217 mg of cardiolipin). On paper chromatography (isopropanol-ammonia-water, 7:1:2) only a slight impurity was detectable. No more than 2% of monoester phosphate was present, as determined by the action of prostatic phosphomonoesterase. The material consumed 0.99 mole of sodium metaperiodate per atom of phosphorus in potassium phosphate buffer at *p*H 6.6.

To 0.05 ml of a solution of this deacylated cardiolipin, which contained 195 μg of phosphorus, was added 0.2 ml of 0.1 N sodium metaperiodate. After 90 minutes at room temperature, the excess of periodate was consumed by addition of 0.2 ml of 0.025 M ethylene glycol. Then 0.3 ml of freshly prepared aqueous 1,1-dimethylhydrazine, 1% v/v, adjusted to *p*H 6 with acetic acid, was added. The mixture was kept at 37° for 16 hours, then extracted several times with chloroform. Ninety-four per cent of the phosphorus was recovered in the aqueous phase. The solution was freed of cations with

[2] The "1-L-phosphatidyl" group corresponds to 1,2-diacyl-L-glycerol-3-phosphoryl.

Dowex-50(H^+) resin, adjusted to *p*H 7 with dilute cyclohexylamine, and evaporated to dryness *in vacuo*. The residue was analyzed by paper chromatography. It showed a single phosphorus-containing spot which traveled with authentic glycerol-1,3-diphosphate.

Mycobacterial Polyglycerolphosphate.—The isolation and deacylation of *Mycobacterium phlei* phospholipid has been described (Lee and Ballou, 1964). The water-soluble material was fractionated on DEAE-Sephadex with ammonium carbonate buffer, *p*H 8.6. The glycerolmyoinositol phosphates were eluted with 1 liter of 0.07 M buffer, while the polyglycerolphosphate component corresponding to deacylated cardiolipin came off the column as a single sharp peak with 0.18 M buffer. Concentration of the solution on a rotatory evaporator removed solvent and ammonium carbonate yielding the ammonium salt of triglyceroldiphosphate as a sirup. The ratio of α-linked glycerol (Hanahan and Olley, 1958) to phosphorus was 0.97:1.0. The material gave a single periodate-reacting, phosphate-containing spot when chromatographed on Whatman No. 1 paper with isopropanol-ammonia (3:1), with $R_{\text{glycerol-1-phosphate}} = 2.0$ and $R_{\text{trehalose}} = 0.75$. The substance had an R_F identical to that of the polyglycerolphosphate obtained by deacylation of beef heart cardiolipin. It showed $[\alpha]^{25}_{589} -1.3°$ and $[\alpha]^{25}_{400} -3.1°$ (5.75% concentration of the free acid) when the ammonium salt was dissolved in water. On addition of 2 moles of sodium metaperiodate, the optical activity was destroyed immediately attending the rapid oxidation of the two terminal glycerol moieties.

The molecular rotations ($[M] = [\alpha] \times$ mol wt) were $-520°$ at 589 mμ and $-1240°$ at 400 mμ. The molecular rotation at 589 mμ in 2 N hydrochloric acid was $-840°$. Faure and Coulon-Morelec (1963) report $[\alpha]_D = 1.25°$ (4% concentration of the sodium salt in water), from which $[M]_D -553°$[1] may be calculated.

Degradation of Deacylated M. phlei Diphosphatidylglycerol to Glycerol-1,3-diphosphate.—To 10 ml of a solution of deacylated *M. phlei* diphosphatidylglycerol which contained 1.5 mmoles of organic phosphate was added 400 mg (1.85 mmoles) of sodium metaperiodate. After 30 minutes the excess of periodate was consumed by addition of 0.37 mmole of ethylene glycol. The sodium iodate formed in the reaction was precipitated by adding 17.5 ml of absolute ethanol and cooling the mixture in ice water for 15 minutes. It was filtered, and the filtrate was evaporated at reduced pressure to a volume of 10 ml to remove the ethanol and formaldehyde.

To this solution was added 17 ml (2.25 mmoles) of 1%, v/v, 1,1-dimethylhydrazine (Stewart, 1963) which had been adjusted to *p*H 4.5 with formic acid. The mixture was allowed to stand at room temperature for 4 hours, during which it became cherry-red in color. About 20 ml of Dowex-50(H^+) resin (200–400 mesh) was added to remove all cations, and the mixture was then filtered by suction through Filter-Cel. Most of the color was removed by the resin, and the filtrate was evaporated to half volume at reduced pressure with a bath temperature of 30° to remove the formic acid. The strongly acidic solution was adjusted to *p*H 10 with cyclohexylamine and then evaporated to dryness. This gave a colored residue, which on trituration with ethanol yielded crystals. The product was collected by filtration, washed with absolute ethanol, and dried. It weighed 350 mg.

The crude glyceroldiphosphate was dissolved in about 75 ml of 95% ethanol, a few drops of cyclohexylamine were added, and the mixture was left to crystallize. It formed small hard plates that were identical in appearance to synthetic glycerol-1,3-diphosphate tetracyclohexylamine salt monohydrate and different from the tetracyclohexylamine salt of glycerol-1,2-diphosphate.

Anal. Calcd. for $C_{27}H_{62}O_9P_2N_4 \cdot H_2O$ (666): N, 8.4; P, 9.3. Found: N, 8.3; P, 9.4.

The infrared spectrum in potassium bromide pellet of the glyceroldiphosphate from deacylated diphosphatidylglycerol was identical with that of the synthetic 1,3-diphosphate (peaks at 785, 810, 850, 895, and 965 cm^{-1}), but differed, in the region of 700–1000 cm^{-1}, from the synthetic 1,2-diphosphate (peaks at 770, 800, 850, 880, 900, 950, 970, and 985 cm^{-1}).

Finally, chromatography on Whatman No. 1 paper with the solvent isopropanol-ammonia (2:1), developed for 6 days, confirmed that the natural glyceroldiphosphate had the 1,3- structure.

Glycerol-1,3-diphosphate.—This compound was prepared by Mr. E. Wagner by reaction between epichlorohydrin and sodium phosphate (Bailly, 1922). In a 1-liter stoppered bottle, 35.5 g (0.25 mole) of disodium hydrogen phosphate and 95 g (0.25 mole) of trisodium phosphate ($Na_3PO_4 \cdot 12H_2O$) were dissolved in 500 ml of water. To this solution was added 23.1 g (0.25 mole) of epichlorohydrin and the mixture was shaken for about 5 hours. It was left at room temperature for 48 hours, then heated on a steam bath for about 4 hours. The inorganic phosphate, which precipitated upon cooling to 5°, was filtered off, and the remaining inorganic phosphate was determined in the filtrate and quantitatively precipitated with the calculated amount of barium chloride at alkaline *p*H. The glyceroldiphosphate was then precipitated as a calcium salt by addition of calcium chloride and ammonia. It was filtered off and washed with dilute ammonia and ethanol. The yield was 9 g. For purification, it was redissolved in water in the presence of some Dowex-50(H^+), the solution was filtered, and the calcium salt was reprecipitated as above with calcium chloride and ammonia.

Two g of the calcium salt, 20 ml of water, and an excess of Dowex-50(H^+) were mixed together for 5 minutes. The resin was filtered off, and the filtrate was adjusted to *p*H 10 with cyclohexylamine and then concentrated to dryness. The product was crystallized from 95% ethanol with the addition of a few drops of cyclohexylamine. The yield was 1.5 g of small, hard, glasslike crystals.

Anal. Calcd. for $C_{27}H_{62}O_9P_2N_4 \cdot H_2O$ (666): C, 48.6; H, 9.6; N, 8.4; P, 9.3. Found: C, 48.5, H, 9.4; N, 8.5; P, 9.6.

Glyceroldiphosphate prepared according to Michelson (1959) was found by paper chromatography to contain more than one phosphate-reacting component.

L-*Glycerol-1,2-diphosphate.*—To a cold solution of 40 g of sodium metaperiodate in 500 ml of water was added 34.2 g of 1,2,5,6-di-*O*-isopropylidene-D-mannitol (Baer, 1952). The oxidation to isopropylideneglyceraldehyde was complete in a few minutes. One liter of ethanol was added to precipitate the sodium iodate, which was removed by filtration. A solution of 10 g of sodium borohydride in water was added cautiously (foaming) to the cold filtrate. After 2 hours the solution was adjusted to *p*H 7 with acetic acid and the ethanol was removed by evaporation *in vacuo*. The resulting water solution was extracted six times with chloroform, and the combined chloroform extracts were dried over sodium sulfate and then concentrated to a sirup that weighed 28.5 g. The *1,2-O-isopropylidene-L-glycerol* had $[\alpha]_D +14.5°$ (in substance) and was obtained in 85% yield. The reported rotation is +14° (in substance) (Baer, 1952).

To a solution of 28.5 g of isopropylideneglycerol in 50 ml of dry pyridine was added 48 ml of benzoyl chloride, and the mixture was left at room temperature for 2 hours. The *3-benzoyl-1,2-isopropylidene-L-glycerol* was isolated according to the published procedure (Baer and Fischer, 1939) and purified by distillation at 0.2 mm pressure. The major fraction, 52 g, distilled at 155° bath temperature and had $[\alpha]_D$ +14.6 (in substance), the yield being quantitative. The reported rotation is +13.9° (in substance) (Baer and Fischer, 1939).

1-Benzoyl-D-glycerol was prepared from the acetone derivative by hydrolysis on 0.5 N hydrochloric acid at 75°. It was obtained as a sirup with $[\alpha]_D$ −17.5° (10% in pyridine). The reported rotation is $[\alpha]_D$ −15.3° in the same solvent (Baer *et al.* 1943).

To a solution of 11 g of 1-benzoyl-D-glycerol in 50 ml of dry pyridine, cooled in ice water, was added 60 g of diphenylphosphorochloridate. The mixture was left at room temperature for 3 days, when water was added to decompose the excess acid chloride. The phosphorylated product was extracted into benzene, and this solution was washed with water, 1 N hydrochloric acid, 1 M sodium bicarbonate, and finally with water. The benzene extract was dried with sodium sulfate and concentrated to a sirup that weighed 37 g and represented a quantitative yield of *1-benzoyl-2,3-bis-diphenylphosphoryl-D-glycerol.*

Thirty g of the phosphorylation product was hydrogenated in 500 ml of absolute ethanol with 5 g of platinum oxide and hydrogen at atmospheric pressure. The reaction consumed 20.4 liters of gas in 5 hours, the calculated amount being 20.3 liters. The solution was decanted from the spent catalyst, 200 ml of 1 N sodium hydroxide was added to saponify the acyl ester, and the ethanol was distilled off. The aqueous solution was left overnight, then a solution of 22 g of barium chloride in water was added. A heavy precipitate formed. The mixture was digested on the steam bath for 1 hour, and the solid was collected by filtration. The air-dried barium salt of glycerol-1,2-diphosphate weighed 18.5 g.

One g of the barium salt was shaken in water suspension with excess Dowex-50(H^+) to remove the cations, the resin was filtered off, and the filtrate was adjusted to *p*H 10 with cyclohexylamine. The solution was concentrated to dryness, the residue was dissolved in 20 ml of hot 95% ethanol, and, after filtration to remove a little insoluble material, the alcohol solution was allowed to stand at room temperature overnight. The crystals that had formed were collected and dried. The weight was 0.7 g, and the product analyzed for the trihydrate of a tetraamine salt.

Anal. Calcd. for $C_{27}H_{62}O_9P_2N_4 \cdot 3\,H_2O$ (702): C, 46.1; H, 9.4; N, 7.9; P, 8.8. Found: C, 45.7; H, 9.3; N, 8.1; P, 8.9.

The following measurements were made of optical activity: $[\alpha]_{589}$ −3.5°, $[\alpha]_{400}$ −8.5° (5% concentration of the cyclohexylamine salt in water); $[\alpha]_{589}$ −1.4°, $[\alpha]_{400}$ −3.9° [3% concentration of the cyclohexylamine salt converted to the free acid with Dowex-50(H^+)].

Acid Migration of Glyceroldiphosphates.—A sample of each of the diphosphates in 1 N hydrochloric acid was heated at 100° for 15 minutes. At the end of this time, the solution of 1,2-diphosphate had lost its optical activity. The mixture of diphosphates in each sample was separated by paper chromatography, the spot corresponding to each isomer was cut out, and the phosphate was determined after digestion of the paper with perchloric acid. A ratio of 1 part 1,2-diphosphate to 2.5 parts of 1,3-diphosphate was found for each sample.

Acknowledgment

We wish to thank Mlle. M. Faure of the Pasteur Institute for a sample of beef heart cardiolipin, Dr. Y. C. Lee of this laboratory for the isolation of the polyglycerolphosphate from the deacylated phospholipids of *M. phlei*, Dr. M. Ikawa for a sample of glyceroldiphosphate prepared according to Michelson, and Mr. E. Wagner for the preparation of glycerol-1,3-diphosphate according to Bailly.

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