MG^{++} PHOSPHOLIPIDS IN CELL ENVELOPES OF A MARINE AND A TERRESTRIAL PSEUDOMONAD*

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The lipids extracted from cells and cell envelopes of a marine pseudomonad contained Mg^{++}. Fractionation of the lipid extract revealed that Mg^{++} was associated with a diphosphatidyl glycerol (cardiolipin) type compound and another compound which may have arisen from the disphosphatidyl glycerol by decomposition. Mg^{++} was also found in lipid extracts from cell envelopes of Pseudomonas aeruginosa. Fractionation showed the Mg^{++} to be associated with a fraction with chromatographic characteristics similar to those of the marine pseudomonad diphosphatidyl glycerol.

These findings are of interest since Mg^{++} and other divalent cations have been found to be components of the cell walls of gram negative bacteria (Vincent and Humphrey, 1963; Eagon, Simmons and Carson, 1965), rat liver microsomes and human red cell ghosts (Corvalho et al, 1963, 1965). That such ions are intimately involved in the maintenance of cell wall and cell membrane structure is indicated by the ability of Mg^{++} to stabilize such subcellular units as bacterial protoplasts (McQuillen, 1958; Nozolillo and Hochster, 1959), the capacity of divalent cations at low concentration to prevent the lysis of marine bacteria (MacLeod and Matula, 1961, 1962), the evidence for a Mg^{++} dependent dissociation of bacterial cytoplasmic membrane particles (Brown, 1965) and the induction of osmotically fragile rods of Pseudomonas aeruginosa by incubation with ethylene-diaminetetracetic acid and the restoration of osmotically stable forms again with multivalent cations (Asbell and Eagon,

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1966). In the case of the extreme halophile, Halobacterium cutirubrum, there is evidence that divalent cations may play their role in maintaining cell integrity through interaction with lipids. Extraction of lipids from cell envelopes of this organism markedly reduced the capacity of Mg^{++} or Ca^{++} to prevent cell envelope disintegration (Kushner and Onishi, 1966).

Experimental Methods

The marine pseudomonad (NCMB 19) was grown in a medium consisting of 0.8% nutrient broth, 0.5% yeast extract in a salt solution containing 12.7 gms NaCl, 5.32 gms $MgCl_2.6H_2O$, 0.72 gm KCl and 0.035 gms $Fe(SO_4)_2(NH_4)SO_4$ per liter. Pseudomonas aeruginosa (Mac 436) was grown in the same medium with NaCl omitted. Cells of the marine organism were harvested by centrifugation and washed three times with the salt solution used to prepare the growth medium. Cell envelopes were prepared by freezing and thawing using procedures previously described (Buckmire and MacLeod, 1965). Since preliminary experiments had established that the lipids of the marine pseudomonad could be quantitatively recovered in the cell envelope fraction, whole cells of the marine organism were used in this study. Cells of Pseudomonas aeruginosa were washed three times with distilled water and envelopes for lipid extraction prepared using a Mickle disintegrator.

Lipids were extracted from whole cells or cell envelopes with $CHCl_3$: CH_3OH 2:1 (v/v) by a slight modification of the method of Folch et al (1957) and finally precipitated with acetone. Silicic acid columns were prepared according to the procedure of Marinetti et al (1957) and developed using a combination of solvents described by the latter authors and by Vorbeck and Marinetti (1965).

Samples for glycerol determination were hydrolyzed successively with methanolic KOH and methanolic HCl. Total glycerol was determined by the periodate chromotropic acid assay in which periodate oxidation was carried out at 100° to permit the determination of glycerol in any unhydrolyzed

glycerophosphate (Defreitas and Depocas, 1964).

Carboxylic acid ester groupings were determined by the procedure of Rapport and Alonzo (1964).

Phosphorus was determined by the method of Allen (1940) and Mg^{++} by atomic absorption spectrophotometry on $HClO_4$ digests of the extracts and fractions.

Results

The phospholipid extract from the marine pseudomonad had a molar ratio of P:Mg of 7.3:1. Thin layer chromatography of the extract revealed two major and three minor components, Fig. 1A, when sprayed for phosphate. Spot 1 was the origin. Spot 2 appeared only when the chromatogram was very heavily loaded and ran with a phosphatidyl choline standard. Spot 3, which was ninhydrin positive ran with a phosphatidyl ethanolamine standard and was subsequently characterized as the latter by identification of its hydrolysis products. It accounted for over 70% of the phospholipid P. Component 4 was ninhydrin negative and its position on the chromatogram relative to phosphatidyl ethanolamine was similar to that of the "phosphatidic acid" fraction of Skidmore and Entenman (1962), who used the same solvent system. Chromatograms of the water soluble hydrolysis products of this component revealed glycerol, glycerol phosphate and some as yet unidentified ninhydrin positive spots. Subsequent analysis of this component recovered from a silicic acid column indicated a molar ratio of glycerol:P:ester of 3.1:2:3.9 indicating that this phospholipid has a diphosphatidyl glycerol (GPGPG) type structure (Macfarlane, 1964). Components 1 and 5 have not been identified but appear to be decomposition products of component 4 since they increased in amount as component 4 decreased with length of storage in $CHCl_3$ solution even when the latter was held at -20° in an atmosphere of N_2.

The phospholipid extract was fractionated using a silicic acid column. The distribution of Mg^{++} and P in the fractions collected is shown in Fig. 2.

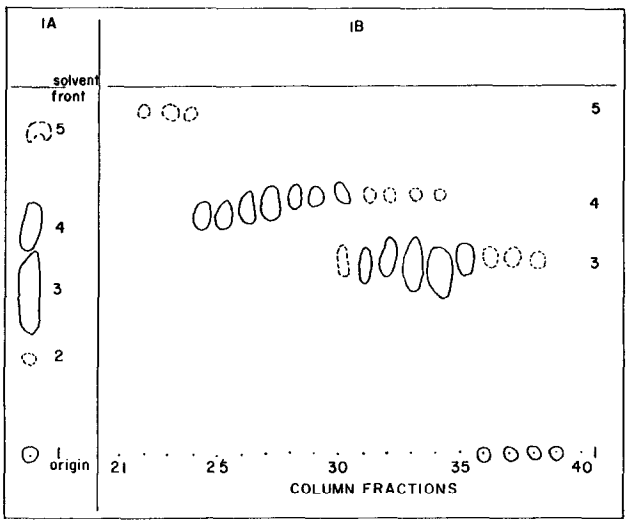

Fig.1A. Tracing of a thin layer chromatogram of the lipid extract from whole cells of marine pseudomonad B-16. Fig.1B. Tracing of a thin layer chromatogram of the silicic acid column fractions collected in the experiment illustrated in Fig. 2. Chromatograms were run using silica Gel G with $CHCl_3$:CH_3OH:7N NH_4OH 60:35:5 (v/v) as the developing solvent (Skidmore and Entenman, 1962). Spots were detected using the phosphate spray reagent of Dittmer and Lester (1964).

Three P peaks appeared, two of them associated with Mg^{++}. When the individual fractions were examined by thin layer chromatography, Fig. 1B, fractions 24 to 29 corresponding to the first Mg^{++} peak were found to contain component 4, the diphosphatidyl glycerol. Fractions 30 to 36 contained component 3 together with a small amount of component 4, the latter apparently accounting for the very small amount of Mg^{++} associated with this fraction. Fractions 36 to 39, corresponding to the second Mg^{++} peak, contained the unidentified component 1. Fractions 24 to 29 when pooled were found to contain P and Mg^{++} in a molar ratio of 2.2:1 indicating 1 mole of Mg^{++} bound per mole of diphosphatidyl glycerol present. The P:Mg^{++} ratio of fractions 38 and 39 when pooled was 1.7:1.

The phospholipid extract from the cell envelope fraction of Pseudomonas aeruginosa had a molar ratio of P:Mg^{++} of 7.4:1. Three components were revealed by thin layer chromatography, Fig. 3a. Spot 1 ran with a phosphatidyl choline standard and was positive to the Dragendorf reagent. Spot 2, the

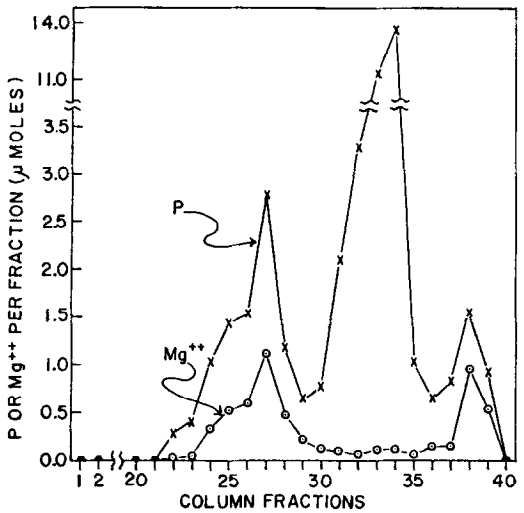

Fig. 2. P and Mg^{++} content of fractions obtained by silicic acid column chromatography of the lipid extract of whole cells of marine pseudomonad B-16. Five ml fractions were collected on elution with the following solvents in the order given: 25 ml of $CHCl_3$:acetone 1:1 (v/v), 25 ml of acetone, 25 ml of $CHCl_3$:CH_3OH 98:2 (v/v), 35 ml of $CHCl_3$:CH_3OH 94:6 (v/v), 25 ml of $CHCl_3$:CH_3OH 90:10 (v/v), 35 ml $CHCl_3$:CH_3OH 80:20 (v/v), 25 ml of CHCl:CH_3OH 1:1 (v/v), 5 ml of CH_3OH.

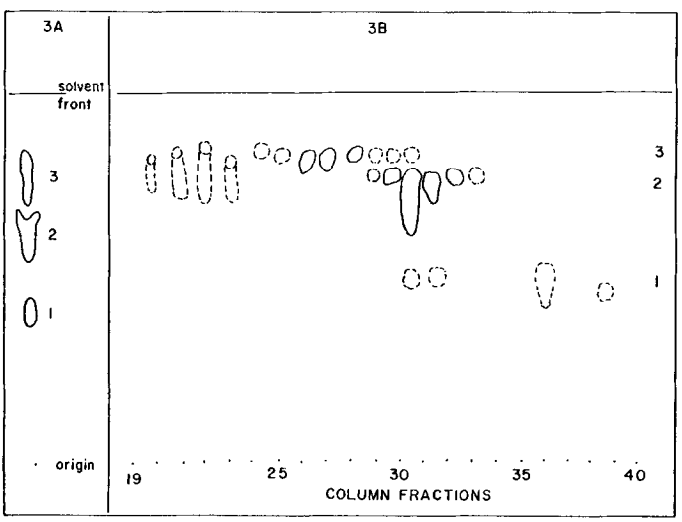

Fig. 3A. Tracing of a thin layer chromatogram of the lipid extract from cell envelopes of Pseudomonas aeruginosa. Fig. 3B. Tracing of a thin layer chromatogram of the fractions obtained when the lipid extracted was fractionated on a silicic acid column using the conditions described in Fig. 2. Chromatograms developed and spots detected as indicated in Fig. 1.

major phospholipid, proved to be phosphatidyl ethanolamine. Spot 3 was phosphate positive, ninhydrin negative and though not otherwise characterized had an R_f similar to the diphosphatidyl glycerol of the marine pseudomonad. Fractionation of the extract on a silicic acid column, Fig. 3B, showed that component 3 probably consisted of more than one molecular species. Fractions 20 to 23 had a $P:Mg^{++}$ ratio of 4.4:1 and fractions 24 to 28, 2.6:1. The remaining fractions contained only traces of Mg^{++}. It would therefore appear that in Pseudomonas aeruginosa, the Mg^{++} associated with the phospholipids is bound by acidic phospholipid components which may be diphosphatidyl glycerols.

Discussion

During the early part of this century a number of workers reported the isolation of phosphatides which seemed to contain a metal ion instead of the usual organic base (see Baer and Buchnea, 1958, for references). Chibnall and Channon (1927) obtained two ether soluble calcium containing compounds from the cytoplasm of cabbage leaves which they identified as the calcium salts of mono and diglyceridephosphoric acids. So far as we are aware the present report is the first direct evidence of a diphosphatidyl glycerol (cardiolipin) type compound being associated with a divalent cation in nature In in vitro studies Shah and Schulman (1965) showed that cardiolipin films contracted 10 to 13% in the presence of divalent metal ions.

Phosphatidyl glycerols appear to be ubiquitous and characteristically are associated with subcellular membranous particles displaying high metabolic activity (Macfarlane, 1964). The possibility that they exist naturally as chelate complexes with divalent cations has a number of interesting physiological implications.

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