Further Studies on the Lipids of *Corynebacteria*

THE MANNOLIPIDS OF *CORYNEBACTERIUM AQUATICUM*

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1. The major free lipids of *Corynebacterium aquaticum* were characterized as dimannosyl diglyceride, monomannophosphoinositide and phosphatidylethanolamine. Bisphosphatidylglycerol and phosphatidylglycerol were also tentatively identified. 2. We regard this as the only well-documented case of an organism containing monomannophosphoinositide to the exclusion of dimannophosphoinositides and the higher homologues. 3. The co-existence of the two mannolipids in one organism is a distinctive feature. So also is the presence of phosphatidylethanolamine in a corynebacterium. 4. The monomannophosphoinositide apparently does not utilize phosphatidylinositol as a precursor, unlike the monomannophosphoinositide of *Propionibacterium shermanii*. CDP-diglyceride may be necessary for its synthesis.

The phospholipids of several corynebacteria have been the subject of recent analyses (Brennan & Lehane, 1971). All of them contained dimannophosphoinositides, as the only apparent representative of this family of lipids. In *Corynebacterium diphtheriae*, these dimannophosphoinositides occurred in two major acylated forms and are probably acylated 1-phosphatidyl-2-myoinositol 2,6-di-*O*- α -D-mannopyranoside, as in mycobacteria (Lee & Ballou, 1964), but with different types and numbers of fatty acids.

We have continued with our survey of the glycolipids of corynebacteria and related genera in a search for an organism producing largely monomannophosphoinositide instead of the ubiquitous dimannophosphoinositide. Such an organism would help considerably in solving the questions about the early biosynthetic stages of these mannolipids.

Monomannophosphoinositide has proved extremely difficult to find. First isolated from *Mycobacterium tuberculosis* (Ballou & Lee, 1964), it is present in extremely small amounts compared with the dimannophosphoinositides and the higher homologues. In *Propionibacterium shermanii*, the monomannophosphoinositide can be synthesized by a cell-free extract in the presence of phosphatidylinositol and GDP-[U-¹⁴C]mannose (Brennan & Ballou, 1968), but the natural lipid is a phosphorus-free 1-*O*-acyl-2-*O*-(6-*O*-acyl- α -D-mannopyranosyl) myoinositol (Prottey & Ballou, 1968; Shaw & Dinglinger, 1969). The claim that a monomannophosphoinositide is the sole representative of this group of lipids in an unclassified mycobacteria, *Mycobacterium P₆*, (Motomiya *et al.*, 1969) has not been confirmed by us. We recently found that this organism contained dimannophosphoinositide in addition to the higher

homologues, and monomannophosphoinositide was apparently absent (G. K. Khuller & P. J. Brennan, unpublished work).

In the present paper, we report that *Corynebacterium aquaticum* contains monomannophosphoinositide in addition to dimannosyl diglyceride as the only apparent mannolipids. The association of these two lipids is of note, but possibly of more importance was the biosynthetic implications of an organism containing only the monomannophosphoinositide. We demonstrate that this lipid is not synthesized under the conditions suitable for its synthesis in *P. shermanii* (Brennan & Ballou, 1968), and for the synthesis of the dimannophosphoinositides in *Mycobacterium phlei* (Brennan & Ballou, 1967). This implies that a precursor other than phosphatidylinositol is necessary. A preliminary account of this work has been reported (Khuller & Brennan, 1971).

Experimental

Materials

The sources and isolation of phosphatidylinositol, dimannophosphoinositides and the deacylated forms, glycerylphosphorylinositol and glycerylphosphorylinositol dimannoside, have been described by Brennan & Lehane (1971). GDP-[U-¹⁴C]mannose was synthesized by the method of Preiss & Greenberg (1967) and its purity was checked with their paper-chromatographic systems. Dimannosylglycerol was a gift from Dr. N. Shaw, Department of Organic Chemistry, University of Newcastle upon Tyne, Newcastle upon Tyne, U.K. *C. aquaticum* (no. B2252) a non-pathogenic strain (not associated with a plant or animal

disease) originated in the Center for Disease Control, Atlanta, Georgia, U.S.A., and was supplied to us by Dr. H. Lechevalier, Institute of Microbiology, Rutgers University, New Brunswick, N.J., U.S.A.

Lipid analysis

The organism was grown in shaken culture for 4–5 days in the medium described by Brennan *et al.* (1970). Harvesting of cells, extraction and washing of lipids are also described elsewhere (Khuller & Subrahmanyam, 1968). Total chloroform–methanol-soluble lipids were fractionated on silicic acid columns into neutral lipids, glycolipids and individual phospholipids (Subrahmanyam & Singhvi, 1965). Alternatively, dry total solubilized lipids were repeatedly triturated with acetone to yield the acetone-soluble neutral lipids and glycolipids, and the acetone-insoluble phospholipids.

Lipids were examined by t.l.c. in the following solvent systems: *A*, chloroform–methanol–water (65:25:4, by vol.); *B*, chloroform–methanol–7M-NH₃ (6:30:5, by vol.); *C*, chloroform–methanol–acetic acid–water (30:15:4:2, by vol.). The lipids were eluted from the silica gel with chloroform–methanol (1:2, v/v) and chloroform–methanol (2:1, v/v) (Brennan & Lehane, 1971). Reagents for the detection of lipids on t.l.c. plates have been described previously (Khuller & Subrahmanyam, 1968).

The products of mild alkaline hydrolysis of lipids were chromatographed on Whatman no. 1 paper in propan-2-ol–aq. NH₃ (sp. gr. 0.88) (2:1, v/v) (solvent *D*) or in butan-1-ol–pyridine–water (6:4:3, by vol.) (solvent *E*). Lipids were also hydrolysed in 2M-HCl at 100°C for 2h and the products were chromatographed in ethyl acetate–acetic acid–formic acid–water (18:3:1:4, by vol.) (solvent *F*). Methods for analysis of polyols and their detection on paper chromatograms have been described previously (Brennan, 1968).

Assay for mannolipid synthesis

Exponential-phase cells of *C. aquaticum*, grown under shaking conditions, were harvested, washed with 0.9% NaCl and stored at –10°C for no longer than 2 weeks. Cells (3g) were suspended in 0.02M-tris–HCl buffer, pH7.4 (50ml), chilled to 1°C and subjected to sonic oscillation with a Branson probe sonifier at full power for 9min. This disrupted material was centrifuged at 3000g for 10min. The supernatant was removed and centrifuged again at 7000g for 10min. The resulting supernatant was finally centrifuged at 100000g for 60min. Sediments were suspended in 0.02M-tris–HCl buffer, pH7.4, and all cellular fractions were used immediately.

To remove the endogenous mannose acceptor from the 100000g particulate fraction, the acetone-dried-

powder technique of Brennan & Ballou (1967) was used.

Incubation conditions are described in the text. Reactions were stopped by the addition of sufficient chloroform–methanol (2:1, v/v) to form a single phase. This was washed once with 0.2vol. of 0.29% NaCl and three times with 0.1vol. of chloroform–methanol–water (3:48:47, by vol.) containing 0.29% NaCl. The chloroform layer was evaporated to dryness before counting for radioactivity and further analysis.

Results

Identification of the mannolipids and other lipids of C. aquaticum

Phospholipids were separated from other lipids on the basis of their acetone-insolubility or by elution from silicic acid columns with chloroform–methanol (1:1, v/v) and methanol. Acid hydrolysis of these and paper chromatography in the solvent containing ethyl acetate showed glycerol, mannose and inositol as the principal polyols. Ethanolamine was clearly visible on chromatograms stained with ninhydrin. Chromatography of the deacylated phospholipids in solvent *D* showed the pattern in Plate 1. The probable nature of some of the components is shown on Plate 1. We concentrated on identifying the two unknowns (X and Y, Plate 1). Unknown X was ninhydrin-positive, with chromatographic properties indicative of glycerylphosphorylethanolamine. The intact lipid yielding this derivative was eluted from a silicic acid column with 15% (v/v) methanol in chloroform. T.l.c. in solvent *A* showed a ninhydrin-positive spot with *R_F* 0.60, identical with reference phosphatidylethanolamine. Acid hydrolysis of this lipid, paper chromatography of the products and detection with ninhydrin showed only ethanolamine. Small amounts of lysophosphatidylethanolamine were eluted from silicic acid columns with 25% (v/v) methanol in chloroform and characterized in a similar manner.

The other unknown (Y in Plate 1) had a chromatographic mobility intermediate between glycerylphosphorylinositol and glycerylphosphorylinositol dimannoside, indicative of glycerylphosphorylinositol monomannoside. When this material was eluted from preparative chromatographic strips and rechromatographed in the same solvent, this chromatographic feature was confirmed. The radioactive glycerylphosphorylinositol monomannoside previously synthesized by extracts of *P. shermanii* had identical chromatographic properties in the same solvent system (Brennan & Ballou, 1968). Acid hydrolysis of the deacylated phospholipid and paper chromatography showed only glycerol, mannose and inositol. Assay of these components and of phosphorus showed that there were 0.8mol of mannose,

EXPLANATION OF PLATE I

Paper chromatography in propan-2-ol-aq. NH_3 (2:1, v/v) of the deacylated phospholipids of *C. aquaticum*. Spots on the chromatogram were detected with the AgNO_3 -NaOH reagent (Trevelyan *et al.*, 1950). 1, Deacylated phospholipid from *C. aquaticum*; 2, glycerylphosphorylinositol (major spot); 3, glycerylphosphorylinositol dimannoside; GPG, glycerylphosphorylglycerol; diGPG, diglycerylphosphorylglycerol.

0.7 mol of glycerol and 1.2 mol of inositol per g-atom of phosphorus. The evidence therefore strongly suggested the presence of a monomannophosphoinositide in *C. aquaticum*. Efforts to isolate the intact lipid by silicic acid column chromatography and by t.l.c. were unsuccessful. Therefore the nature and number of fatty acid substituents is unknown.

Mannose was also a major component of the acetone-soluble lipids of *C. aquaticum*. It and glycerol were the only two products on paper chromatograms of the hydrolysed lipid. They did not appear to be accompanied by inositol. This lipid fraction was subjected to preparative t.l.c. in solvent *B*. The main area that reacted with iodine extended from R_F 0.70 to 0.85. This was removed from the plate, eluted, deacylated and chromatographed in solvent *D*. The only product detected with the AgNO_3 -NaOH dip reagent corresponded to dimannosylglycerol. This similarity was confirmed by chromatography in solvent *E*. The compound yielded a distinct purple colour with the periodate-Schiff reagent, typical of α -glycols (Shaw, 1968). Acid hydrolysis and paper chromatography revealed mannose and glycerol only. Determination of these showed 1.9 mol of mannose/mol of glycerol.

Attempts at biosynthesis of monomannophosphoinositide of C. aquaticum

The conditions initially used were similar to those successfully employed for the biosynthesis of monomannophosphoinositide in *P. shermanii* (Brennan & Ballou, 1968) and the dimannophosphoinositides in *M. phlei* (Hill & Ballou, 1966; Brennan & Ballou, 1967). In the presence of the 100000g pellet from *C.*

aquaticum there was high incorporation (about 25%) of $[\text{U-}^{14}\text{C}]$ mannose from $\text{GDP-}[\text{U-}^{14}\text{C}]$ mannose into chloroform-methanol-soluble lipids (Table 1). Addition of exogenous phospholipids from *C. aquaticum* did not result in increased incorporation, indicating the presence of considerable endogenous acceptor of $[\text{U-}^{14}\text{C}]$ mannose in the enzyme preparation.

The radioactive lipid products of the reaction were subjected to t.l.c. in solvent *C*. Radioactivity was close to the solvent front, well ahead of marker mannophosphoinositides and the expected position of a monomannophosphoinositide. This result suggested that the product was either a highly acylated mannophosphoinositide or a neutral mannolipid. The radioactive lipid was deacylated and chromatographed (Fig. 1). There was no evidence for the synthesis of monomannophosphoinositide or any other related lipid. Most of the radioactive material was identical with dimannosylglycerol and it co-chromatographed with dimannosylglycerol, obtained from acetone-soluble lipids of this organism, in solvent *E*. The possible nature of the minor product on these chromatograms is discussed below.

A number of variations were introduced to the assay system in an effort to demonstrate the synthesis of monomannophosphoinositide (Table 2). Surprisingly, there was considerable synthetic activity in the 5000g pellet, but this also was largely a dimannosyl diglyceride synthetase and monomannophosphoinositide synthetase could not be demonstrated in any cellular fraction. In another experiment endogenous $[\text{U-}^{14}\text{C}]$ mannose acceptor was removed from the 100000g pellet by acetone fractionation (Brennan & Ballou, 1967) and exogenous lipids (phosphatidylinositol of yeast and phospholipids of *C. aquaticum*) were added as acceptors. However,

Table 1. *Incorporation of $[\text{U-}^{14}\text{C}]$ mannose from $\text{GDP-}[\text{U-}^{14}\text{C}]$ mannose into chloroform-methanol-soluble lipids by a particulate enzyme of *C. aquaticum**

The complete system contained: tris-HCl buffer, pH 7.4, 80 μmol ; MgCl_2 , 5 μmol ; $\text{GDP-}[\text{U-}^{14}\text{C}]$ mannose, 0.087 μmol (23.3 nCi), except where indicated; phospholipids from *C. aquaticum*, 1.5 mg, where indicated; enzyme protein (100000g pellet), 0.3–0.6 mg, in a final volume of 1.0 ml. The mixture was incubated at 37°C for 2 h. Different batches of cells grown for 2 days were used as enzyme source. Washed chloroform-methanol-soluble lipid was evaporated to dryness, made up to volume and the radioactivity of a sample was counted.

Conditions	Incorporation of radioactivity (d.p.m./mg of protein)
Experiment I	
Complete system	33 332
Experiment II	
Complete system	28 664
Complete system + phospholipids	25 324
Experiment III	
Complete system ($\text{GDP-}[\text{U-}^{14}\text{C}]$ mannose; 0.435 μmol , 116.5 nCi)	71 358

Fig. 1. Distribution of radioactivity in the lipids synthesized by the particulate enzyme of *C. aquaticum*

Details of incubation conditions are described in Table 1. Radioactive lipids were deacylated and chromatographed in propan-2-ol-aq. NH_3 (2:1, v/v). GPIM₂, glycerylphosphorylinositol dimannoside; GPIM₁, glycerylphosphorylinositol monomannoside; GPI, glycerylphosphorylinositol.

Table 2. Distribution of radioactivity from GDP-[U- ^{14}C]mannose in the lipids synthesized by different fractions of disrupted *C. aquaticum*

Experimental details are described in Table 1. The radioactivity was estimated on the basis of that recovered from paper chromatograms.

Cell fractions	Incorporation of radioactivity			
	(d.p.m./mg of protein)	(% incorporation into mannophosphoinositides)	(% incorporation into dimannosylglycerol)	(% incorporation into unidentified lipid*)
Whole cell sonicate	882	0	59-60	23-24
3000g Supernatant	1009	0	53-55	23-24
3000g Pellet	276	0	63-64	12-13
5000g Supernatant	937	0	71-72	16-17
5000g Pellet	15037	0	75-76	14-15
100000g Supernatant	90	0	—	—
100000g Pellet	10618	0	68-69	17-18
100000g Pellet + phospholipid	10926	0	73-74	12-13

* Faster moving component in Fig. 1.

there was no synthesis of the mannophosphoinositides. The products of the reaction were also examined after 5, 10, 20 and 40 min of incubation but there was no evidence of mannophosphoinositide at any incubation period. In all of these assays dimannosylglycerol accounted for 60-70% of the lipid radio-

activity. The remainder of this radioactivity did not correspond to any major chemically detectable component and remains unidentified. Its chromatographic properties are intermediate between dimannosylglycerol and mannose, and it may be monomannosylglycerol.

Discussion

There are a number of noteworthy features about the lipid composition of *C. aquaticum*. It is different from that of previously examined corynebacteria (Brennan & Lehane, 1971) in that it contains phosphatidylethanolamine. Therefore, according to Ikawa (1967), it is less typical of the Corynebacteriaceae than are *C. diphtheriae* and *Corynebacterium xerosis*. The complete absence from *C. aquaticum* of the acylglucose present in *C. diphtheriae* and other corynebacteria (Brennan *et al.*, 1970) may also be significant, although Shaw & Stead (1970) pointed out that there was as yet insufficient information on the distribution of acylglucoses to draw meaningful conclusions. In *C. aquaticum*, acylglucoses are replaced by dimannosyl diglyceride. This is apparently the first report of a glycosyl diglyceride in corynebacteria, although dimannosyl diglyceride has been tentatively identified in a member of the Corynebacteriaceae, *Microbacterium thermosphactum* (Shaw & Stead, 1970). On the basis of the glycosyl diglyceride content of a large number of Gram-positive bacteria Shaw & Baddiley (1968) concluded that members of the same genus or closely related genera contain identical glycolipids. By this parameter *C. aquaticum* should be classified with the closely related *Micrococcus lysodeikticus*, *Arthrobacter globiformis*, *Microbacterium lacticum* and *M. thermosphactum*. However, *C. aquaticum* is also unique in having both a dimannosyl diglyceride and a monomannophosphoinositide, and the presence of this latter lipid places it among the Actinomycetales (Brennan & Ballou, 1968). A direct metabolic relationship between these two mannolipids appears unlikely. More probable is some common functional role. Possible functions for these lipids have been discussed in recent papers (Shaw, 1970; Brennan & Lehane, 1971). The significance of the co-existence of phosphatidylethanolamine and diglycosyl diglyceride in the one organism has also been mentioned (Minnikin *et al.*, 1971). It is believed that these two neutral polar lipids are interrelated as essentially complementary components with similar or identical functions.

Our inability to demonstrate the biosynthesis of the monomannophosphoinositide, in view of our previous success with other systems, may be significant. The possibility that the monomannophosphoinositide synthetase was inactivated during the preparation of the cell-free extracts was ruled out, since this enzyme in *P. shermanii* and the related di-

mannophosphoinositide synthetase in *M. phlei* were relatively stable. Moreover, the dimannosyl diglyceride synthetase activity of *C. aquaticum* was retained throughout the preparation steps. It seems more likely that the diglyceride moiety of the monomannophosphoinositide did not arise from phosphatidylinositol. The other possible source of diglyceride is CDP-diglyceride. Shaw & Dinglinger (1969) have in fact suggested that the 1-*O*-acyl-2-*O*-(6-*O*-acyl- α -D-mannopyranosyl) myoinositol from *P. shermanii* could act as an acceptor of diglyceride from CDP-diglyceride, resulting in the synthesis of a tetra-acyl monomannophosphoinositide. This may be the sequence in *C. aquaticum*.

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