ment of the cytochrome c concentration was carried out spectrophotometrically by employing the absorption coefficients for reduced and oxidized cytochrome c as described by Potter and DuBois (14). The product was further purified by electrophoresis at the isoelectric point of the cytochrome c to remove any contaminating synthetic porphyrin apoprotein c.

Properties of Synthetic Porphyrin Aporoprotein c—Crude synthetic porphyrin apoprotein c was purified as follows. The protein was dissolved in distilled water, adsorbed on Amberlite CG-50 in the NH₄⁺ form, and eluted under nitrogen in the dark first with 0.1 M phosphate buffer, pH 7.4, and then with 0.25 M phosphate buffer, pH 7.4. The 0.25 M phosphate buffer eluate was dialyzed against water under nitrogen and then lyophilized. Synthetic porphyrin apoprotein c was compared with natural porphyrin apoprotein c which was prepared by reduction of cytochrome c with platinum black in the presence of formic acid and oxalic acid according to the method of Neillands (15). The preparations were found to be identical in the following characteristics. (a) They were soluble in water over a wide range of pH. (b) They were not extractable into ether or ethyl acetate at pH 3.5. (c) Their absorption maxima were at 408, 553, and 560 μm in 1 N HCl and 408, 504, 540, 570, and 623 μm in 0.25 M phosphate buffer, pH 7.4. (d) They were identical on electrophoresis. (e) Treatment of synthetic porphyrin apoprotein c with acid-acetone failed to liberate porphyrin. (f) Treatment of synthetic porphyrin apoprotein c by mercuric sulfate in 50% acetic acid gave a single porphyrin which was identical with hematoporphyrin as regards spectral properties, HCl number, and paper chromatography.

Properties of Synthetic Cytochrome c-like Compound—Absorption spectra of the synthetic cytochrome c-like compound purified by paper electrophoresis are shown in Fig. 1. Spectra of both ferrocytochrome c-like and ferrocytochrome c-like compound were found to be slightly lower than those of natural cytochrome c. The synthetic cytochrome c-like compound was not autooxidizable and did not combine with oxygen or carbon monoxide. The catalytic activity in the sucinic dehydrogenase system was found to be about 55% of natural cytochrome c. Ultracentrifugation studies on synthetic cytochrome c-like compound showed a single peak, and both sedimentation pattern and coefficient were identical with those of natural cytochrome c. The possibility suggested by Sano and Granick (5) that cytochrome c may be formed by the interaction of the —SH groups of apocytochrome c peptide with protoporphyrinogen during its autoxidation has been definitely demonstrated by this experiment.

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The Chemical Characterization and Enzymic Synthesis of a Bacterial Glycolipid*

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(Received for publication, June 15, 1964)

In 1961, M. G. Macfarlane isolated a new glycolipid, mannosyl diglycercide, from Micrococcus lysodeikticus, NCTC 2063 (1). Recently, a glucosyl diglyceride and a glycolysyl diglyceride containing both glucose and galactose were found in Streptococcus faecalis (2). These reports, as well as others in which the presence of uncharacterized glycolipids in eubacteria was noted (3), suggest that these compounds may be more common components of bacterial lipids than previously suspected. In this communication, the isolation and chemical characterization of a novel mannosylmannosyl (1 — α)-diglyceride is reported, and evidence bearing on the enzymic synthesis of this compound as well as two other mannose-containing lipids is presented.

M. lysodeikticus, ATCC 4698, was grown at 30° to the late logarithmic phase in a medium containing 1% peptone, 0.5% NaCl, and 0.1% yeast extract. Lipids were extracted by the

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† Clayton Scholar.
method of Folch, Lees, and Sloane Stanley (4). Preliminary separation of the mannosyl lipid was accomplished by elution from silicic acid with acetone. The mannosyl lipid, obtained in the acetone eluate, was further purified by gradient elution from a silicic acid column with acetone. The mannolipid, obtained in the acetone eluate, was further purified by gradient elution from a silicic acid column with acetone.

The purified mannosyl lipid (5 mg per 10 g of packed cells) was at least 95% pure as judged by thin layer chromatography in four solvent systems, contained no detectable phosphorus, and ex-  mansose = 1.00, were 0.37, 0.55, and 0.77, in Solvents 1, 3, and 4, respectively.

The results of quantitative analyses on the intact lipid and on the products of alkaline and of acid hydrolysis are summarized in Table 1. Mannose was determined by the reducing sugar (9) and anthrone (10) methods. In one experiment (Group C), the nonreducing carbohydrate obtained upon alkaline hydrolysis was isolated by preparative paper chromatography, hydrolyzed in acid, and then subjected to analysis.

It is evident from the experiments in Groups A, B, and C that the same amount of fatty acid is estimated, whether determined by ester groups (11) or by fatty acid liberation (12) upon alkaline hydrolysis, and also that the ratio of fatty acid to mannosyl is approximately 1:1. Analysis of the nonreducing carbohydrate product of alkaline hydrolysis (Group B) reveals that the production of 1 mole of formaldehyde by periodate oxidation (13) per 2 moles of glycosidically bound mannosyl. Subsequent acid hydrolysis releases 1 mole of glycerol (13) per 2 moles of mannosyl (Group C). This stoichiometric relationship between glycerol and mannosyl is confirmed when analysis is carried out after acid hydrolysis of the intact lipid (Group D). That the product of alkaline hydrolysis is nonreducing but produces 1 mole of formaldehyde upon periodate oxidation suggests that 2 moles of mannosyl are linked glycosidically and that the resultant disaccharide is glycosidically bound to an α position of glycerol. On the basis of these data, the suggested structure of the glycosyl lipid, mannosylmannosyl (1→α)-diglyceride, is that shown in Fig. 1. The anomeric configuration of the glycosidic bonds, as well as the linkage between the two mannosyl groups, remains to be established.

Neufeld and Hall (14), with the use of spinach chloroplasts, have demonstrated the enzymic synthesis of a series of galacto- lipids; these products were similar to, but not identical with, the galactolipids found generally in plant material.

In view of the known role of GDP-β-mannose as a mannosyl donor, it seemed possible that this compound might be involved in the formation of mannosyl-containing lipids. Cells were disrupted by means of a French pressure cell, and a cell-free supernatant was obtained by centrifugation at 3,000 × g. Incubation of uniformly labeled GDP-β-mannose-14C with this supernatant resulted in the formation of labeled mannosyl-containing lipids (Table II, Experiment I). The enzymic activity was localized primarily in the pellet obtained upon centrifugation of the 3,000 × g supernatant at 100,000 × g for 1 hour (Experiment II). Small, but significant, stimulation of the activity was obtained by the addition of lipids obtained by chloroform-methanol extraction of cell extracts (Experiment III). Presumably these lipids supplement the system with more of the endogenous lipid that apparently serves as acceptor for the mannosyl group. The nature of the lipid acceptor or acceptors has not yet been studied.

I am indebted to Dr. E. C. Heath for a generous sample of uniformly labeled GDP-β-mannose-14C.

### Table I

<table>
<thead>
<tr>
<th>Group and method of determination</th>
<th>Molar ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Intact lipid</strong></td>
<td></td>
</tr>
<tr>
<td>Ester groups</td>
<td>2.04</td>
</tr>
<tr>
<td><strong>B. Alkaline hydrolysate of lipid</strong></td>
<td></td>
</tr>
<tr>
<td>Fatty acids</td>
<td>2.00</td>
</tr>
<tr>
<td>Mannose (anthrone)</td>
<td>1.75</td>
</tr>
<tr>
<td>Mannose (reducing sugar)</td>
<td>&lt;0.05, &lt;0.02</td>
</tr>
<tr>
<td>Formaldehyde (periodate oxidation)</td>
<td>1.08</td>
</tr>
<tr>
<td><strong>C. Acid hydrolysate of B</strong></td>
<td></td>
</tr>
<tr>
<td>Mannose (reducing sugar)</td>
<td>2.15</td>
</tr>
<tr>
<td>Mannose (anthrone)</td>
<td>2.00</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>D. Acid hydrolysate of lipid</strong></td>
<td></td>
</tr>
<tr>
<td>Mannose (reducing sugar)</td>
<td>2.16, 2.02</td>
</tr>
<tr>
<td>Mannose (anthrone)</td>
<td>2.00</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.84, 0.97</td>
</tr>
</tbody>
</table>

* Based on a value of 2.00 for mannose after acid hydrolysis as determined by the anthrone method.
The complete incubation mixture contained Tris-maleate, pH 8.3, 25 μmoles; Tris-HCl, pH 7.6, 6 μmoles; GDP-mannose-¹⁴C (uniformly labeled), as indicated below; water; and enzyme (2 to 10 mg of protein) to a final volume of 0.35 ml. In Experiment 1, 67 μmoles (3,000 c.p.m.) of uniformly labeled GDP-mannose-¹⁴C, uniformly labeled GDP-mannose 1 P-¹⁴C, or uniformly labeled GDP mannose-¹⁴C were added. In Experiments 2 and 3, 33.5 μmoles of GDP-mannose were used. After incubation at 30° for 1 hour, 6.0 ml of chloroform-methanol, 2:1, were added. The mixture was warmed, mixed well, and filtered. The filtrate was washed with 1.8 ml of 0.9% NaCl and then evaporated to dryness. Carotenoids were destroyed by addition of several drops of bromine. Samples were counted in toluene solution containing the scintillators 2,5-diphenyloxazole and 1,4-bis-(5'-phenyloxazolyl) benzene at approximately 45% efficiency.

<table>
<thead>
<tr>
<th>Experiment and conditions</th>
<th>Mannose-¹⁴C incorporated</th>
<th>mpmoles/10 mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system, 100,000 X g supernatant</td>
<td>18.6</td>
<td></td>
</tr>
<tr>
<td>Complete system, 100,000 X g pellet</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Complete system, 100,000 X g supernatant, minus GDP-mannose, plus mannose-¹⁴C</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Complete system, 100,000 X g supernatant, minus GDP-mannose, plus mannose-¹⁴C</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Complete system, 100,000 X g supernatant</td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

In order to characterize the enzymic products, the ¹⁴C-mannosyl lipids obtained from a large scale incubation were purified by silicic acid chromatography. Three distinct ¹⁴C-mannosyl-containing lipids, designated I, II, and III in the order of their elution, were isolated. ¹⁴

Compound II was shown to be chromatographically identical with mannosylmannosyl (1 → α)-diglyceride on silicic acid chromatography and on thin layer chromatography in Solvents 1, 2, and 3. ² Acid hydrolysis of Compound II liberated ¹⁴C-mannose, nonradioactive fatty acids, and glycerol; alkaline hydrolysis released a radioactive neutral compound that was chromatographically identical with the product obtained upon alkaline hydrolysis of the mannosylmannosyl (1 → α)-diglyceride isolated from the cells.

The structures of Compounds I and III are not yet known. Upon acid hydrolysis, both lipids yielded mannose as the only radioactive product. The products of alkaline hydrolysis of

The structures of Compounds I and III are not yet known. Upon acid hydrolysis, both lipids yielded mannose as the only radioactive product. The products of alkaline hydrolysis of

Acknowledgments—I acknowledge with pleasure the assistance of Mr. Norman Argue and the valuable advice received from Drs. E. C. Heath and R. Edstrom.

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The Structures of Compounds I and III exhibited Rf values that are consistent with the possibility that Compounds I, II, and III are members of a homologous series containing 1, 2, and 3 mannose residues, respectively. Evidence supporting this idea in the case of Compound I was obtained upon analysis of the neutral product of alkaline hydrolysis of Compound I; the glycerol to mannose ratio was 1:1. Thus far we have been unable to isolate any detectable amounts of Compounds I and III from whole cells. ⁴

The Isolation of Cholesterol Sulfate from Bovine Adrenals*  

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Recently evidence was presented (1) that demonstrated that cholesterol-7α-²H sulfate-³⁴S, when injected into the artery

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† Recipient of a Fellowship from the Medical Research Council of Canada.
The Chemical Characterization and Enzymic Synthesis of a Bacterial Glycolipid
W. J. Lennarz


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