The Chemical Characterization and Enzymatic Synthesis of Mannolipids in Micrococcus lysodeikticus*

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SUMMARY

The major glycolipid of Micrococcus lysodeikticus has been isolated in pure form and has been shown to have the structure of α-D-mannosyl-(1→3)-α-D-mannosyl-(1→3)-diglyceride. The presence of two enzymes involved in glycolipid biosynthesis in M. lysodeikticus has been established. One of the enzymes is associated with the particulate cell fraction and catalyzes the formation of α-D-mannosyl-(1→3)-diglyceride from guanosine diphosphate mannose and 1,2-diglyceride. The enzyme is specific for 1,2-diglyceride and manifests maximal activity on 1,2-diglycerides containing branched chain fatty acyl groups. The reaction is stimulated by Mg++ ion and shows an absolute requirement for an anionic surface active agent. Although long chain alkyl surface-active agents and straight chain fatty acid salts stimulate the reaction, the most effective surface-active agents are the branched chain fatty acids common to M. lysodeikticus. A second enzyme present in the soluble cell fraction catalyzes the conversion of enzymatically prepared α-D-mannosyl-(1→3)-diglyceride to α-D-mannosyl-(1→3)-α-D-mannosyl-(1→3)-diglyceride.

Although recent studies have provided considerable insight into the biochemistry of bacterial phospholipids, relatively little is known about bacterial glycoacylglycerides, which have been discovered only recently (1, 2). For this reason, we have undertaken a study of the chemical structure and biosynthesis of glycolipids in Micrococcus lysodeikticus. The structure of the major glycolipid of this organism has been shown to be α-D-mannosyl-(1→3)-α-D-mannosyl-(1→3)-diglyceride. Studies in vitro have shown that M. lysodeikticus contains an enzyme that catalyzes the formation of α-D-mannosyl-(1→3)-diglyceride; a second enzyme catalyzes the conversion of this intermediate to α-D-mannosyl-(1→3)-α-D-mannosyl-(1→3)-diglyceride (Fig. 1). Preliminary reports on some aspects of this study have been published (3, 4).

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EXPERIMENTAL PROCEDURE

Materials

Unless otherwise stated, all chemicals used were analytical reagent grade. D-Mannose and methyl-α-D-mannopyranoside were purchased from Pfannstiel Laboratories, Inc. We are indebted to Dr. J. H. Law, Department of Biochemistry, University of Chicago, for samples of the various cationic and anionic detergents used in the study; to Dr. I. Goldstein, Department of Biochemistry, University of Michigan, for a sample of methyl-β-D-mannoside; and to Dr. C. E. Ballou, Department of Biochemistry, University of California, for samples of partially methylated mannose derivatives. Dr. W. E. M. Lands, Department of Biochemistry, University of Michigan, generously provided samples of diglycerides derived from pig liver and egg yolk lecithin, and monoglycerides. DL-α,β-Dipalmitin was a gift of Dr. R. Pieringer, Department of Biochemistry, Temple University, and dioleylglyceryl ether was a gift from Mr. P. Thomas, Department of Chemistry, Harvard University. Diglyceride derived from rat liver lecithin was prepared by a minor modification of the method of Hanahan, Brockerhoff, and Barron (5), with the use of Clostridium welchii toxin (kindly provided by Mr. G. Hensley of this Department) as a source of the phospholipase C. Fatty acids and methyl esters were purchased from Applied Science Laboratories. Suspensions of the fatty acid sodium salts, at a concentration of 0.03 M or less, were prepared by warming an aqueous suspension of the fatty acids containing a quantity of NaOH sufficient to bring the pH to 8.3. Suspensions of the diglycerides in H2O, at a concentration of 10 μmoles per ml, were prepared by sonic dispersion (under nitrogen in the case of diglycerides containing polyunsaturated fatty acids) with a Bronwill Biosonik I instrument operated at 60% of maximum probe intensity. Unlabeled GDP-α-mannose and GDP-α-mannose-14C uniformly labeled in the mannose moiety and α-mannose-1-P were synthesized according to the procedure of Roseman et al. (6). Uniformly labeled α-mannose-14C was purchased from Schwarz BioResearch. Activated charcoal (12 X 50 mesh, Pittsburgh Chemical Company) was washed with ethanol and water prior to use.

Methods

Analytical Methods—Unless indicated otherwise, acid hydrolysis of lipids was carried out in 2 N H2SO4 at 100° for 2 hours,
while alkaline hydrolysis was performed with 0.1 to 0.5 N KOH in 25 to 50% CH3OH at 60-70° for 2 hours. Phosphorus was determined by the methods of King (7) or Chen, Toribara, and Warner (8), as modified by Ames and Dubin (9), and protein was determined by the biuret method (10). Hexose was measured by the anthrone (11) or reducing sugar (12) methods, or both. Glycerol was determined by the periodate oxidation-chromotropic acid technique of Korn (13). Under the conditions of the assay, the presence of mannose in an amount equivalent to glycerol caused the values for glycerol to be high by 10 to 15%. This interference was corrected by preliminary determination of the mannose content of the sample. Esterified fatty acids (14) and free fatty acids (15) were determined by published methods. Quantitative periodate oxidation studies on mannosylmannosylglycerol were performed by the iodometric method of Dyer (16). Semiquantitative estimation of glycolipid in column effluents was made by the anthrone modification of Radin (17). Optical densities were determined with a Beckman DU or a Gilford spectrophotometer, and optical rotations were measured on a Rudolph polarimeter. NMR (nuclear magnetic resonance) analysis, kindly performed by Dr. Donald Hollis of this Department, was carried out on a Varian A-60 NMR spectrometer. Samples, previously equilibrated with D2O, were run at a concentration of 10 to 15 mg/0.5 ml of D2O containing 1% sodium 2,2-dimethylsilapentanesulfonate as internal standard. Carbon and hydrogen analyses were performed by Galbraith Laboratories.

Radioactivity Measurements—Measurement of 14C and 3H was performed with a Packard Tri-Carb liquid scintillation counter. Lipid solutions were evaporated in a scintillation vial with a stream of nitrogen and then treated with a drop of bromine to destroy carotenes. The excess bromine was then removed under reduced pressure, and 15 ml of toluene containing 4 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis(5-phenyloxazolyl)benzene (POPOP) per liter were added. Aqueous samples were counted directly in 10 ml of a mixture containing 750 ml of dioxane, 125 ml of anisole, 125 ml of 1,2-dimethoxyethane, 4 g of 2,5-diphenyloxazole, and 100 mg of 1,4 bis(5 phenyloxazolyl)benzene. Efficiency of counting was determined by addition of radioactive standards to samples.

Radioactivity on paper chromatograms was measured with a Vanguard paper strip scanner.

Chromatographic Methods—Silica acid used in gradient elution chromatography consisted of a mixture of 2 parts of Mallinckrodt silicic acid, prepared according to Bloomfield and Bloch (18), and 1 part of Unisil, 200 to 325 mesh silicic acid (Clarkson Chemical Company). The latter was used for all batchwise elution chromatography. Routine gradient elution chromatographic analysis of lipid samples (5 to 100 mg) was performed on a column 1 X 25 cm containing 6 g of the above silica acid mixture in CHCl3. Samples were applied in a small volume of chloroform, after which the column was eluted with 20 ml of CHCl3. Fractions of 5.5 ml were collected. The column was then connected to a gradient apparatus designed after that of Wren (19), consisting of a 250-ml cylindrical bottle mixing chamber containing 200 ml of CHCl3 and a 250-ml Erlenmeyer flask reservoir containing 200 ml of CHCl3-CH3OH (1:1). In the case of CHCl3-acetone gradients, the solvent volumes were 200 ml of CHCl3 and 150 ml of acetone, respectively.

Paper chromatography was performed at room temperature on Whatman No. 1 paper. The solvent systems were as follows: A, butan-1-ol-pyridine-0.1 N HCl (5:3:1); B, propan-2-ol-H2O (7:3); C, ethyl acetate-pyridine-H2O (12:5:4); D, propan-2-ol-pyridine-H2O (12:4:1); and E, propan-2-ol-acetic acid-H2O (8:2:1). For chromatography of methylated hexoses, paper impregnated with 0.02 M sodium borate was utilized with the following solvents: F, butan-1-ol-0.1 M sodium borate (20:5) (upper phase); and G, butan-1-ol-ethanol-H2O (5:1:4) (upper phase). Carbohydrates were detected with periodate-benzidine (20), and methylated derivatives of hexoses were detected with ninilie trichloracetate (21). All systems were allowed to run for 12 to 18 hours except A and B, which were run for 40 and 30 hours, respectively.

Thin layer chromatography was performed with Silica Gel G (Brinkmann) as a layer 0.5 mm thick. For preparative purposes, samples were applied as a streak at the origin. Lipids were recovered from zones scraped from the thin layer plate (previously moistened by spraying with water) by extraction with several 2-ml portions of CHCl3-CH3OH (1:1). The resulting extract was concentrated to dryness and redissolved in
that the extract was collected directly in a scintillation vial. Recovery of radioactive components from the plates for counting was accomplished in the same manner, except that the extract was collected directly in a scintillation vial.

Lipids were visualized by spraying the plates with rhodamine 6G (0.001% in acetone, followed by 0.001% in water). Glycolipid was detected by aniline-diphenylamine spray (22).

Gas chromatography was performed in an E.I.R. Company gas chromatograph equipped with an ionization detector. For analysis of fatty acids, the acids were converted to the methyl esters with diazomethane and analyzed on a 6-foot glass column packed with 19% diethylene glycol succinate on 60 to 80 mesh Gas Chrom P (Applied Science Laboratories). The column was operated at 165° with an inlet pressure of 60 ml per min. Comparison was made with authentic standards, including the methyl esters of 12- and 13-methyltetradecanoic acids. The methylglycosides of the partially methylated mannose derivatives, kindly performed by Dr. C. E. Ballou, was carried out on a neopentyl glycol succinate column operated at 170°.

Growth of Cells and Preparation of Crude Extract—One-liter cultures of *M. lysodeikticus* ATCC 4698 were grown overnight at 30° in 2-liter flasks on a rotary shaker. The growth medium consisted of 1% Bacto-peptone (Difco), 0.5% NaCl, and 0.1% yeast extract. Cells were harvested by centrifugation when the optical density reached 400 Klett units (660-rnM filter), and then passed on ice for 3 to 5 min. After centrifugation for 5 to 10 min, the upper 0.9% NaCl layer was removed and discarded. For routine assays for mannosylglyceride synthesis, the lower layer was transferred directly to a scintillation vial for counting. When complete analysis for mannosylglyceride, mannosylmannosylglyceride, and mannotolipid III was desired, as in the case of crude extracts, the sample was evaporated to dryness under reduced pressure, dissolved in a small volume of chloroform, and analyzed for 14C-mannolipid by chloroform-methanol gradient elution chromatography. Occasionally, mannosylglyceride synthetase preparations catalyzed the formation of relatively low amounts of mannotolipid III (10% of the total 14C-mannose-containing lipid). With these preparations, mannotolipid III was separated from the mannosylglyceride in the following manner. The lipid extract was evaporated to dryness, dissolved in 1 ml of CHCl3, and applied to a column (1 X 7 cm) of Unisil silicic acid in CHCl3. Following elution with 18 ml of CHCl3, the column was eluted with 10 ml of acetone. This effluent contained the mannosylglyceride. When desired, mannotolipid III could be recovered from the column by subsequent elution with 8 ml of chloroform-methanol (1:1).

Preparation of Mannosylmannosylglyceride Synthetase—An acetone powder of the crude extract was prepared as described above for the 100,000 X g particulate fraction, with the use of 6 volumes of acetone per volume of crude extract. The acetone powder was stable for several months. To 250 mg of the acetone powder suspended in 7.5 ml of 0.02 M Tris-HCl, pH 7.6, 7.5 ml of saturated (NH4)2SO4 were added with stirring. The mixture was stirred for 15 min, and then centrifuged at 20,000 X g for 10 min. The pellet was redissolved in 1 ml of CHCl3, and applied to a column (1 X 7 cm) of Unisil silicic acid in CHCl3. Following elution with 18 ml of CHCl3, the column was eluted with 10 ml of acetone. This effluent contained the mannosylglyceride. When desired, mannotolipid III could be recovered from the column by subsequent elution with 8 ml of chloroform-methanol (1:1).
mannosylmannosyldiglyceride synthetase (3-fold purified) had a specific activity of 6.5 mmol of mannosylmannosyldiglyceride formed per mg of protein per hour.

**Assay for Mannosylmannosyldiglyceride Synthesis**—Incubation mixtures containing mannosylmannosyldiglyceride synthetase were extracted in the same manner as indicated for mannosyldiglyceride assay, except that at termination of the incubation 2 mg of total lipids of *M. lysodeikticus* were added as carrier. In cases in which mannosylmannosyldiglyceride synthetase was measured by the use of unlabeled mannosyldiglyceride and GDP-\(^{14}\)C-mannose, the lipid extract, which contained \(^{14}\)C-mannosylmannosyldiglyceride as the only labeled lipid, was counted directly. When \(^{14}\)C-labeled mannosyldiglyceride and GDP-mannose were the precursors, separation of the labeled precursor lipid and the labeled mannosylmannosyldiglyceride was accomplished either by gradient elution chromatography or by thin layer chromatography. In the latter instance, the lipid sample was applied to the thin layer plate as a streak 2 cm long. After elution in Solvent I, the zones corresponding to mannosyldiglyceride and mannosylmannosyldiglyceride were removed, and the lipids were counted.

**Lipid Extraction and Isolation of Mannosylmannosyldiglyceride**—Total lipid was isolated from packed cells by extraction in 20 volumes of CHCl\(_3\)-\(\text{CH}_2\)OH (2:1). The mixture was stirred at room temperature for 4 to 12 hours, after which time insoluble material was removed by filtration through a Buchner funnel. The filtrate was washed with 0.20 volume of 0.9% NaCl solutions; after both phases had cleared, the CHCl\(_3\) layer was concentrated under reduced pressure. From 100 g of cells, wet weight, approximately 1.0 g of crude lipid was obtained. Mannosylmannosyldiglyceride was purified by gradient elution chromatography on silicic acid, first with a CHCl\(_3\)-CH\(_2\)OH gradient and then with a CHCl\(_3\)-acetone gradient. In the case of the CHCl\(_3\)-CH\(_2\)OH gradient, the manno lipid was eluted in tubes 23 to 26, while with CHCl\(_3\)-acetone it was found in tubes 32 to 37 (see "Chromatographic Methods"). An alternative method more applicable to large scale isolation was the following. To a 100-g column of Unisol silicic acid packed in CHCl\(_3\), 1 g of total lipid was added. The column was eluted with 3 to 5 column volumes of CHCl\(_3\). In either case, final purification of the diglyceride, contaminated with carotenes, was eluted with 2 to 4 column volumes of CHCl\(_3\). The diglyceride, contaminated with carotenes, was chromatographed on silicic acid by the use of a CHCl\(_3\)-acetone gradient. Frequently, rechromatography in the same system was necessary to free the mannosylmannosyldiglyceride of all carotene. An alternative method for final purification was preparative thin layer chromatography in Solvent I. The yield of pure mannosylmannosyldiglyceride from 1.0 g of crude lipid was 75 to 150 mg.

**Preparation of Mannosylmannosyldiglycerol**—To a solution of 150 mg of mannosylmannosyldiglyceride in 1.5 ml of CH\(_2\)OH, 1.0 ml of 1 N KOH and 7.5 ml of H\(_2\)O were added. The mixture was heated at 70° for 1.5 hours and then allowed to stand at room temperature for 2 hours. The solution was chilled, adjusted to pH 2.5 with concentrated HCl, and extracted with four 10-ml portions of ether. After removal of traces of ether from the aqueous phase with a stream of nitrogen, the solution was deionized with mixed bed resin (prepared by mixing equal volumes of Dowex 50-H\(^+\) and Dowex 1-CO\(_2\)\(^-\)). The resulting solution of mannosylmannosyldiglycerol was concentrated to a syrup under reduced pressure at 45°. Treatment of the syrup with acetone resulted in crystallization of the mannosylmannosyldiglycerol (see "Results").

**Exhaustive Methylation of Mannosylmannosyldiglycerol**—Mannosylmannosyldiglycerol was methylated by a slight modification of the method of Hakomori (24). To 88 mg (1800 \(\mu\)molues) of NaH, contained in a 3-necked flask equipped with a thermometer and a gas inlet through which dry nitrogen was passed, 4.5 ml of redistilled dimethylsulfoxide were added. The mixture was stirred by means of a magnetic stirrer for 45 min at 60°. The solution was cooled and then 1.5 ml of it were added to a solution of 21.7 mg (50 \(\mu\)molues) of mannosylmannosyldiglycerol in 3.5 ml of dimethylsulfoxide. An additional 2.0 ml of dimethylsulfoxide were added, and the mixture was stirred at room temperature for 5 min. After this period, 0.5 ml of methyl iodide was added and the mixture was stirred for 20 min. Addition of a second portion of NaH-treated dimethylsulfoxide (1.0 ml) and methyl iodide (0.3 ml) was repeated in the same manner. After 20 min, the mixture was poured into 40 ml of cold H\(_2\)O. The resulting mixture was extracted thoroughly with three 20-ml portions of CHCl\(_3\). The combined CHCl\(_3\) extract was washed with four 30-ml portions of H\(_2\)O, filtered through Na\(_2\)SO\(_4\), and evaporated to dryness under reduced pressure. The yield of permethylated mannosylmannosyldiglycerol, probably contaminated with some dimethylsulfoxide, was 32.6 mg (120% yield).

Hydrolysis of the permethyl derivative was accomplished by treatment with 2 n H\(_2\)SO\(_4\) for 7 hours in a boiling water bath. After removal of H\(_2\)SO\(_4\), from the mixture with Dowex 1-CO\(_2\)\(^-\), the solution was concentrated to dryness under reduced pressure. Methanolysis was accomplished by refluxing a solution of the permethyl derivative in anhydrous methanol containing 6% HCl for 16 hours. The reaction mixture was then evaporated under reduced pressure and placed under a high vacuum in the presence of NaOH pellets for 15 min.

**Purification of 1,3-Diglyceride**—Crude diglyceride was isolated by CHCl\(_3\)-CH\(_2\)OH gradient elution of the total lipid of *M. lysodeikticus* as described for the isolation of mannosylmannosyldiglyceride. The diglyceride, contaminated with carotenes, was eluted in tubes 9 to 12. Alternatively, larger quantities were prepared by silicic acid chromatography of the total lipids. The diglyceride, contaminated with fatty acids, carotenes, and other nonpolar material, was eluted with 2 to 4 column volumes of CHCl\(_3\). In either case, final purification of the diglyceride was accomplished by preparative thin layer chromatography in Solvent H. The zone containing the diglyceride was scraped free of the plate, and the diglyceride was eluted with ether.

Diglyceride which contained fatty acyl groups labeled with \(^3\)H in the methyl-terminal portion of the branched chain was prepared by growing *M. lysodeikticus* (250-ml culture) in the presence of uniformly labeled l-isoleucine-\(^3\)H (250 \(\mu\)C, 200 \(\mu\)C per \(\mu\)molue) (25). The \(^3\)H-labeled lipids were isolated and the diglyceride was purified as described above. Alkaline hydrolysis of the diglyceride followed by determination of the radioactivity in the resulting glycerol and fatty acids indicated that all of the label was in the fatty acyl portion of the diglyceride.

**Analysis of 1,3-Diglyceride**—To approximately 1 \(\mu\)molue of purified diglyceride dissolved in 0.5 ml of CH\(_2\)OH, 0.5 ml of 0.2 n NaOH was added. The mixture heated at 60-70° with occasional shaking. After 1 hour, the solution was adjusted to pH 2 with H\(_2\)SO\(_4\), and the volume was brought to 1.0 ml
with H$_2$O. Fatty acid was extracted from the aqueous phase with ether and determined colorimetrically. The aqueous phase was analyzed for glycerol as previously indicated.

The determination of the position of the acyl chains of the diglyceride was carried out by a modification of the procedure of Matson and Volpenheim (26). 1-14C-Palmitoyl chloride, prepared from 1-14C-palmitic acid (27), was used to acylate the diglyceride. The resulting triglyceride was purified by thin layer chromatography and then subjected to lipase hydrolysis. The incubation mixture contained 6.6 mg of 1-14C-palmitoyl-labeled triglyceride, 0.216 ml of 1 M Tris-HCl (pH 8.0), 0.012 ml of 45% CaCl$_2$, 0.005 ml of 1% aqueous bile salts (Difco No. 3), and 0.024 ml (80 mg per ml) of pancreatic lipase (steapsin). After incubation for 45 min at 45°C with shaking, the reaction was stopped by the addition of 5.0 ml of CHCl$_3$-CH$_2$OH (2:1) and 0.01 ml of concentrated HCl. A duplicate mixture was treated in the same manner, except that incubation was omitted. An aliquot of both reaction mixtures was analyzed for free fatty acid content. A second aliquot of each was subjected to thin layer chromatography in Solvent H, along with standard samples of mono-, di-, and triglycerides and fatty acid. After visualization of the various lipids with rhodamine 6G, the zone corresponding to each class of compounds was scraped from the plate and counted.

Synthesis of Mannosylglycerol—Mannosylglycerol was synthesized by a modification of the method reported by Fischer and Beensch for the synthesis of glucosylglycerol (28). To 25.0 ml of freshly prepared anhydrous glycerol, 1.8 g of d-mannose (previously dried under reduced pressure overnight) and 100 mg of Na$_2$SO$_4$ were added. Anhydrous HCl was bubbled into the solution until the HCl concentration was approximately 0.1 N. The flask was stopped and the solution was stirred at 30°C for 20 hours. The reaction mixture was neutralized with 2 N Na$_2$CO$_3$ (approximately 3 ml) and diluted to 60 ml with H$_2$O. After deionization with mixed bed resin, the solution was concentrated to 30 ml.

Isolation of mannosylglycerol was accomplished by chromatography on charcoal. A column (2.5 × 50 cm) in H$_2$O, 3.6 ml of the above solution of crude product were added. The column was connected to a linear gradient elution system containing 50 ml of H$_2$O in the mixing chamber and 500 ml of 33% ethanol in the reservoir. Fractions of 15 ml were collected; the flow rate was approximately 0.75 ml per min. A broad anthrone-positive peak was found between tubes 40 and 56. The tubes comprising the central portion of this peak (tubes 44 to 55) were pooled and concentrated under reduced pressure. The resulting syrup was dissolved in methanol, and a small amount of insoluble residue was by centrifugation. Evaporation of the methanol under reduced pressure afforded 142 mg (56% yield) of colorless syrup. Attempts to crystallize this material were unsuccessful. Paper chromatography revealed the presence of only one periodate-positive substance ($R_f$ = 0.82 in Solvent A, 0.58 in Solvent B, 0.92 in Solvent C, and 0.60 in Solvent D). The mannose to dry weight ratio of the substance was that expected for mannosylglycerol: 3.95% mannose per mg. The compound was nonreducing before acid hydrolysis and reducing after hydrolysis, and it contained mannose and glycerol in the ratio 1.00:1.05. Treatment with periodate released 1 eq of formaldehyde. The latter observation indicates that the mannosyl group is linked to position 1 or 3 of glycerol. The results of optical rotation measurements and NMR studies on mannosylglycerol are summarized in Tables II and III and Fig. 2. It is evident from these results, and from studies on the inhibition of concanavalin A-glycogen complex formation, that the synthetic mannosylglycerol is of the α-anomeric configuration (cf. parallel studies on mannosylmannosylglycerol). On one occasion, the synthesis of mannosylglycerol afforded a mixture consisting of approximately 85% of the α anomer and 15% of a substance chemically identical with the α anomer (including liberation of one equivalent of formaldehyde on periodate treatment), but having a lower chromatographic mobility ($R_f$ of mannose = 0.83 in Solvent C). Presumably, this compound is the β anomer of mannosylglycerol; however, the limited amount of it available has precluded confirmation of this possibility. On the basis of these results, the structure proposed for the synthetic mannosylglycerol is α-d-mannosyl-(1 → 3)-DL-glycerol.

RESULTS

Characterization of Mannosylmannosylglyceride

Chromatographically purified mannalipid was homogeneous, as judged by thin layer chromatography in three systems ($R_f$ = 0.56 in Solvent I, 0.29 in Solvent J, and 0.87 in Solvent K). Analysis indicated the absence of phosphorus and the presence of fatty acids (as esters), mannose, and glycerol in the ratio 2:2:1 (Table I, Rows I and IV). The mannose and glycerol were shown to be chromatographically identical with authentic samples upon chromatography in Solvents A, C, D, and E. The hexose was further characterized as mannose by gas-liquid chromatography of its pertrimethylsilyl derivative. Gas-liquid chromatography analysis of the fatty acids indicated that at least 75 to 80% of the fatty acids were a mixture of 12- and 13-methyltetradecanoic acids, the former being the predominant (approximately 70%) isomer (29, 25).

<table>
<thead>
<tr>
<th>Substance analyzed</th>
<th>Determination</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Intact lipid</td>
<td>Total phosphate</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>Ester groups</td>
<td>2.04</td>
</tr>
<tr>
<td></td>
<td>Fatty acids</td>
<td>2.00</td>
</tr>
<tr>
<td>II. Alkaline hydrolysate of lipid</td>
<td>Mannose (anthrone)</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>Mannose (reducing sugar)</td>
<td>&lt;0.05, &lt;0.02</td>
</tr>
<tr>
<td></td>
<td>Formaldehyde (periodate oxidation)</td>
<td>1.08</td>
</tr>
<tr>
<td>III. Acid hydrolysate of II</td>
<td>Mannose (reducing sugar)</td>
<td>2.15</td>
</tr>
<tr>
<td></td>
<td>Mannose (anthrone)</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>0.98</td>
</tr>
<tr>
<td>IV. Acid hydrolysate of lipid</td>
<td>Mannose (reducing sugar)</td>
<td>2.16, 2.02</td>
</tr>
<tr>
<td></td>
<td>Mannose (anthrone)</td>
<td>(2.00) a</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>0.84, 0.97</td>
</tr>
</tbody>
</table>

* The mannose content of the glycolipid was determined by the anthrone method after acid hydrolysis (IV). The value obtained in this determination was adjusted to 2.00 for purposes of comparison of the molar ratios of constituents; i.e. the results of all analytical determinations were normalized to the value obtained for mannose in IV.
TABLE II
Optical rotation of mannosides

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\left[\alpha\right]_D^\text{20}^\circ$</th>
<th>$M\left[\alpha\right]_D^\text{20}^\circ$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl-$\alpha$-d-mannopyranoside</td>
<td>+79.2</td>
<td>+15,400</td>
</tr>
<tr>
<td>Methyl-$\beta$-d-mannopyranoside</td>
<td>-49.0</td>
<td>-9,500</td>
</tr>
<tr>
<td>Mannosylmannosylglycerol</td>
<td>+91.4 ± 0.9</td>
<td>+39,600</td>
</tr>
<tr>
<td>Mannosylglycerol</td>
<td>+43.9 ± 0.2</td>
<td>+15,300</td>
</tr>
</tbody>
</table>

The nonreducing carbohydrate obtained upon alkaline hydrolysis of the mannolipid contained mannose and glycerol in the ratio 2:1 (Table I, Rows II and III). The fact that periodate oxidation of the nonreducing sugar yielded 1 eq of formaldehyde (Table I, Row II) suggests that the mannosyl groups are present in a disaccharide which is glycosidically linked to position 3 (or 1) of the glycerol moiety. Thus, the partial structure of the nonreducing alkaline hydrolysis product is mannosyl-mannosyl-(1 → 3)-glycerol. The most likely structure for the intact lipid is mannosylmannosyl-(1 → 3)-diglyceride, although the above analyses do not exclude a structure containing one fatty acyl residue esterified to the glycerol moiety and the second esterified to a hydroxy group of one of the mannosyl units. However, this possibility may be excluded on the basis of the biosynthetic studies (see below). Studies on the anomeric configuration and position of the glycosidic linkages of the mannolipid were facilitated by the availability of pure, crystalline mannosylmannosyl-(1 → 3)-glycerol. Treatment of an aqueous solution (100 mg per ml) of the alkaline hydrolysis product of the mannolipid with acetone resulted in the formation of white, needle-like crystals in nearly quantitative yield. The crystals were collected, washed with acetone, and dried under reduced pressure at 60°C; m.p. 134–136°C (corrected), before and after recrystallization. Paper chromatography revealed one periodate-positive spot in Solvents A (Rf = 0.37), C (0.55), and D (0.77).

The results of glycerol and mannose analysis, as well as of carbon and hydrogen analysis, were those expected for the monohydrate of mannosylmannosylglycerol.

\[
\text{C}_{41}H_{72}O_{14} \cdot \text{H}_{2} \text{O} (434.38)
\]

Calculated: C 41.47, H 6.98

Found: C 41.35, H 6.84

The results of glycerol and mannose analysis, as well as of carbon and hydrogen analysis, were those expected for the monohydrate of mannosylmannosylglycerol.

Three approaches were taken in order to establish the anomeric configuration of the glycosidic linkages. First, optical rotation studies on mannosylmannosylglycerol indicated a strong positive rotation, of the order of magnitude expected only if both glycosidic linkages were of the $\alpha$-anomeric configuration (Table II). Second, the finding of Dr. I. Goldstein\(^1\), that mannosylmannosylglycerol inhibited complex formation between concanavalin A and glycogen indicated the presence of at least one $\alpha$-anomeric linkage, that linking the two mannosyl units. As shown by Goldstein, Hollerman, and Smith (30), inhibition of complex formation by the nonreducing terminal unit of glycosides is highly specific for $\alpha$-mannoside and $\alpha$-glucosides. Finally, NMR studies served to confirm the assignment of the $\alpha$-anomeric configuration to both glycosidic linkages. Two resonance lines in the range expected for $\alpha$-glycosides (31, 32) were evident for mannosylmannosylglycerol (Table III and Fig. 2). Authentic $\alpha$- and $\beta$-mannosides fell within the range previously reported for these anomers.

In order to establish the position of linkage between the mannosyl groups, mannosylmannosylglycerol was exhaustively methylated. One aliquot of the resulting permethyl derivative was hydrolyzed, and the products were subjected to analysis by paper chromatography; another aliquot was methanolyzed

\(^1\) I. Goldstein, unpublished studies.
and analyzed by gas-liquid chromatography. The results of these analyses are shown in Table IV.

Paper chromatography revealed the presence of two methylated mannose derivatives with mobilities identical with those of 2,3,4,6-tetra-O-methyl- and 2,3,4,6-tetra-O-methylmannose. However, treatment of the mixture of the two methylmannose derivatives with periodate followed by chromatography revealed that both hexose derivatives were stable to periodate. The only tri-O-methylmannose derivative stable to periodate is 2,4,6-tri-O-methylmannose, the expected trimethyl derivative if the linkage between hexose units is 1 → 3.

Confirmation of the fact that one of the methylated derivatives was 2,3,4,6-tetra-O-methylmannose and that the other was not 2,3,4,6-tetra-O-methylmannose was obtained by gas-liquid chromatography of the methylmannosides of these compounds (Table IV, Column 3). Further gas chromatographic analysis, with the use of all the possible tri-O-methylmannose derivatives as standards (kindly performed by Dr. C. E. Ballou), established that the second methylated mannose derivative was 2,4,6-tri-O-methylmannose (Table IV, Column 4). Thus, the linkage between the two mannose units must be 1 → 3. Final confirmation of this assignment was obtained by quantitative periodate oxidation studies on mannolipid III will be reported at a later date.

**Enzymatic Synthesis of Mannosyldiglyceride**

As was previously reported (3), three 14C-mannose-containing lipids are formed from GDP-14C-mannose in crude extracts. As shown in Fig. 3 (top), these three compounds were separated by gradient elution chromatography. Evidence will be presented to support the contention that the first peak represents mannosyl-β-diglyceride, while the second is mannolipid III. The structure of the third 14C-labeled lipid, mannolipid III, is not yet known. In contrast to crude extracts, the particulate mannosyldiglyceride synthetase preparation catalyzes the formation of only mannosyldiglyceride (Fig. 3, middle), while a preparation obtained from the soluble cell fraction catalyzes the formation of mannolipid III (Fig. 3, bottom). Studies on the formation of mannolipid III will be reported at a later date.

**Properties of Mannosyldiglyceride Synthetase**—The dependence of mannosyldiglyceride synthesis on enzyme concentration and the time course of the reaction are illustrated in Fig. 4. The requirements of GDP-mannose and 1,2-diglyceride for mannosyldiglyceride synthesis are illustrated in Fig. 5. Mannose and mannose-1-P cannot substitute for GDP-mannose. Maximal rates of mannosyldiglyceride synthesis require Mg2+ ion, a relatively high ionic strength, and a salt of a long chain fatty acid. The requirement for Mg2+ ion is illustrated in Table VI. Optimal activity requires a final ammonium sulfate concentration of 0.06 to 0.25 M. Above 0.31 M salt, the reaction is inhibited, whereas in the absence of added salt the activity is reduced to 70% of the maximal value. (NH4)2SO4 was routinely added to provide a medium of high ionic strength; however, sodium citrate, Na2SO4, Tris-maleate, or ammonium acetate can be used instead of (NH4)2SO4. In order to investigate the effect of high ionic strength on the reaction, preliminary studies on the heat inactivation of the enzyme in the absence and presence of the various cofactors and substrates were undertaken. Preincubation for 3 min at 40° of a mixture of enzyme, buffer, and MgCl2 resulted in complete loss of activity. Addition of (NH4)2SO4 to the preincubation mixture reduced loss of activity to approximately 50%, whereas addition of the other components, either singly or in combination, did not prevent inactivation. Thus it appears that the major function of (NH4)2SO4 is to stabilize the enzyme.

A study of mannosyldiglyceride synthesis as a function of the structure of fatty acid salt revealed that the fatty acids of M.

---

**TABLE IV**

<table>
<thead>
<tr>
<th>1. Compound</th>
<th>Paper chromatography</th>
<th>Gas chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-Tetramethyl-D-mannose</td>
<td>0.81</td>
<td>0.82</td>
</tr>
<tr>
<td>3,4,6-Tetramethyl-N-mannose</td>
<td>0.55</td>
<td>0.52</td>
</tr>
<tr>
<td>2,3,4-Trimethyl-D-mannose</td>
<td>0.68</td>
<td>0.72</td>
</tr>
<tr>
<td>2,3,6-Trimethyl-D-mannose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,4,6-Trimethyl-D-mannose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>0.81</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>0.69</td>
<td>0.73</td>
</tr>
</tbody>
</table>

* Gas chromatography was performed on the methylmannoside derivatives of these compounds.

Stable to periodate treatment.

**TABLE V**

**Kinetics of periodate oxidation of α-d-mannosyl-(1→8)-d-mannosyl-(1→3)-diglycerol**

Periodate oxidation of 11.25 μmoles of mannosylmannosylglycerol was performed at 0° according to the iodometric method of Dyer (16). At the indicated intervals, 10% aliquots of the sample (1.12 μmoles) and the blank were removed, and periodate consumption was measured.

<table>
<thead>
<tr>
<th>Time</th>
<th>μmoles consumed per μ mole of mannosylmannosylglycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>(0)</td>
</tr>
<tr>
<td>5.5 min</td>
<td>1.06</td>
</tr>
<tr>
<td>30.0 min</td>
<td>1.29</td>
</tr>
<tr>
<td>3.0 hrs</td>
<td>2.22</td>
</tr>
<tr>
<td>22.0 hrs</td>
<td>3.00</td>
</tr>
<tr>
<td>30.0 hrs</td>
<td>2.98</td>
</tr>
<tr>
<td>72.0 hrs</td>
<td>3.03</td>
</tr>
</tbody>
</table>

* Blank value at zero time.
Mannolipids in M. lysodeikticus

Vol. 241, No. 11

Formation of mannolipids by crude cell-free extract.

Formation of mannosyl diacylglyceride by acetone powder of 100,000Xg pellet.

Formation of mannosyl mannosyl diglyceride by soluble preparation.

FIG. 3. Gradient elution chromatographic analyses of the 14C-mannose-labeled lipids produced by crude extracts of M. lysodeikticus (top), an acetone powder of the 100,000 × g pellet fraction (middle), and an acetone powder of the 100,000 × g supernatant fraction (bottom). ○--○ (bottom chromatogram) represents unreacted substrate, 14C-mannosyl diglyceride. Incubation and chromatographic conditions are indicated in the text.

Fig. 4. Dependence of mannosyl diglyceride synthesis on enzyme concentration and on time (inset). Standard incubation conditions were as follows (in micromoles in final volume of 0.39 ml): MgCl₂, 10; Tris-maleate, pH 8.3, 30; (NH₄)₂SO₄, 40; acetone powder, 4.0 mg (50% protein) dissolved in 0.08 ml of 0.02 M Tris-HCl, pH 7.5; GDP-[U-14C]-mannose (13,350 dpm), 0.187; 1,2-diglyceride, 0.50; and sodium salt of M. lysodeikticus fatty acids, 1.5. Incubation was performed with shaking at 30°C for 60 min.

lysodeikticus serve as the best "cofactor" for the reaction (Fig. 6). As shown earlier, 70 to 80% of the fatty acids of the lipids of this organism are the branched chain 12- and 13-methyltetradecanoic acids (29, 29). The superior activity of the branched chain acids is also seen in Fig. 7, where the mannosyl diglyceride synthesis at optimal fatty acid concentration is plotted as a function of straight chain fatty acid chain length. Tridecanoic acid shows the best activity, but it is still inferior to the mixed branched chain acids of M. lysodeikticus, pure 12-methyltetradecanoic acid, or a mixture of 12- and 13-methyltetradecanoic acids. In order to investigate whether the ratio of fatty acid

Table VI

Requirement of Mg²⁺ for mannosyl diglyceride synthesis

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Mannosyl diglyceride</th>
<th>µmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td></td>
<td>40.0</td>
</tr>
<tr>
<td>Omit MgCl₂</td>
<td></td>
<td>7.5</td>
</tr>
<tr>
<td>Omit MgCl₂; add MnCl₂</td>
<td></td>
<td>8.6</td>
</tr>
<tr>
<td>Omit MgCl₂; add Na₂SO₄</td>
<td></td>
<td>8.6</td>
</tr>
</tbody>
</table>
to diglyceride was an important factor in the stimulation caused by fatty acid, a study of the fatty acid concentration optimum at varying levels of diglyceride was undertaken. As is evident from Fig. 8, the optimum concentration of fatty acid is altered very little whether diglyceride is in excess (0.5 μmole) or is limiting (0.2 μmole). Addition of the diglyceride and fatty acid separately or together as a sonically treated mixture has no effect on the reaction. However, fatty acid alone apparently inhibits the enzyme since activity is reduced when it is added before, rather than after, the diglyceride.

An investigation of the activity of other anionic surface-active agents revealed that sodium hexadecyl sulfate was almost as active as the branched chain fatty acids, while other anionic substances were less active (Fig. 9). Two cationic surface-active agents, cetyltrimethylammonium bromide and benzylidinemethyltetradecylammonium chloride, were inactive, as were the neutral surface-active agents, Tween 80 (polyoxyethylene sorbitan monoleate) and BRIJ 35 (polyoxyethylene lauryl ether). Phosphatidylycerol could not replace the fatty acid salt. Moreover, phosphatidyglycerol, mannosylmannosyldiglyceride, or total lipid extracts caused 50 to 90% inhibition of mannosyldiglyceride synthesis by the complete system.

Characterization of Diglyceride Substrate—Preliminary chromatographic characterization of the neutral lipid required for mannosyldiglyceride synthesis indicated that it had the mobility of a diglyceride. Thin layer chromatography in Solvent II revealed the presence of one major component with an RF equal to that of a 1,2-diglyceride and a second minor component.
with an $R_F$ equal to that of a 1,3-diglyceride. The amount of the latter varied widely from preparation to preparation, but never constituted more than approximately 10 to 15% of the total diglyceride fraction. Final purification by thin layer chromatography removed this component. Analysis of the major diglyceride from M. lysodeikticus indicated a fatty acid to glycerol ratio of 1.87:1.00. The fatty acid composition, as determined by gas-liquid chromatography, was similar to that of mannosylmannosyldiglyceride. In order to establish with certainty that the compound was a 1,2-diglyceride, it was converted to the triglyceride by acylation with L-14C-palmitoyl chloride and then subjected to the action of pancreatic lipase. It is well known (cf. Reference 26) that pancreatic lipase preferentially hydrolyzes positions 1 and 3 of a triglyceride, resulting in the production of a 2-monoglyceride. Thus, assuming 100% hydrolysis of positions 1 and 3 of the labeled triglyceride formed from M. lysodeikticus diglyceride, one would expect to obtain 14C-labeled 2-monopalmitin if the original diglyceride had been 1,3, and 14C-palmitic acid and unlabeled monoglyceride if the diglyceride had been 1,2. The results of thin layer chromatographic analysis of the reaction products after lipase hydrolysis are shown in Table VII. It is evident that only trace amounts of 14C-labeled monopalmitin were formed, and that there is good agreement between the calculated and observed amount of 14C fatty acid released. Moreover, since the reaction did not go to completion, some 14C-labeled diglyceride is present, in addition to unhydrolyzed triglyceride. If the value for this diglyceride (8.2%) is added to that of the remaining triglyceride (28%), the result is in fair agreement (36%) with the calculated value of 43%. Thus, these results show that the diglyceride from M. lysodeikticus serving as a substrate for mannosyldiglyceride synthetase is 1,2-diglyceride.

In order to test the specificity of the enzyme, a survey of its activity toward a variety of natural and synthetic glycerides was made. The results, summarized in Table VIII, indicate that the enzyme shows maximal activity with a 1,2-diglyceride containing branched chain fatty acids, such as those found in M. lysodeikticus and Bacillus megaterium. Natural diglycerides from animal sources, containing unsaturated fatty acids, are less active, while synthetic 1,2-dipalmitin, 1,3-diglyceride, and the ether analogue of a 1,2-diglyceride, dioleoylglyceryl ether, are essentially inactive.

**Identification of Enzymatic Product**—The lipid extracts from 10 to 30 standard incubation mixtures were combined and purified by chromatography on silicic acid. Preliminary isolation was accomplished by batchwise chromatography, with the use of acetone to elute mannosyldiglyceride (see "Lipid Extraction and Separation of Mannosyldiglyceride"). The resulting crude mannosyldiglyceride was further purified by gradient elution chromatography with CHCl₃-CH₃OH. The purified mannosyldiglyceride was eluted in tubes 18 to 20. Phosphorus analysis revealed that the glycolipid contained less than 0.03 μmoles of phosphorus per μmole of glycolipid. An aliquot of the glycolipid was treated with 0.08 x NaOH in 20% CH₃OH for 2 hours at 70°, followed by acidification in the cold to pH 1 to 2 with H₂SO₄. The acidified hydrolysate was extracted with ether; analysis of the ether phase for fatty acids and the aqueous phase for mannose (based on 14C measurements) indicated that the mannosyldiglyceride contained fatty acids and mannose in the ratio 1.96:1.00. The remaining portion of hydrolysate was deionized with mixed bed resin, and aliquots of the resulting solution of the 14C-labeled neutral sugars were analyzed by paper chromatography in Solvents A, B, C, and D, along with authentic α-mannosyglycerol. A single 14C peak, coinciding with the periodate-positive spot due to α-mannosyglycerol, was evident in all systems. Another aliquot of the neutral sugar solution was mixed with α-mannosyglycerol, and the mixture was converted to the trimethylsilyl derivative. Upon gas-liquid chromatographic analysis, it was found that the 14C elution peak corresponded with the mass peak due to α-mannosyglycerol (retention time relative to the trimethylsilyl derivative of β-D-gluco-3,6-fucosyl = 6.44). Further proof that the alkaline hydrolysis product was mannosylyglycerol was obtained in a second experiment in which a quantity of the deacylated glycolipid sufficient for glycerol and mannose analysis was obtained in pure form by preparative paper chromatography. Acid hydrolysis of this purified material followed by paper chromatography in Solvent D revealed the presence of only glycerol and mannose; quantitative analysis indicated that these compounds were present in the ratio of 1.00: 1.06. On the basis of these experiments, it was concluded that

### Table VII

<table>
<thead>
<tr>
<th>Chromatogram zone</th>
<th>Compound</th>
<th>0 time</th>
<th>45 min</th>
<th>Calculated distribution of 14C</th>
<th>%</th>
<th>Observed distribution of 14C</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Monoglyceride</td>
<td>4 25 2 0</td>
<td>4 25 2 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Diglyceride</td>
<td>2 95 8.2 0</td>
<td>2 95 8.2 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Fatty acid</td>
<td>0 635 56.6 57</td>
<td>0 635 56.6 57</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Triglyceride</td>
<td>1133 324 28 43</td>
<td>1133 324 28 43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Total activity</td>
<td>1137 1158</td>
<td>1137 1158</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* If one assumes that the labeled triglyceride contained 14C-palmitate in position 3. Calculation was based on the fact that free fatty acid determination at 45 min indicated 87% conversion of the triglyceride to monoglyceride, with no accumulation of diglyceride assumed.

### Table VIII

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-Diglyceride</td>
<td>M. lysodeikticus</td>
<td>100</td>
</tr>
<tr>
<td>1,2-Diglyceride</td>
<td>B. megaterium</td>
<td>100</td>
</tr>
<tr>
<td>1,2-Diglyceride</td>
<td>Egg lecithin</td>
<td>50</td>
</tr>
<tr>
<td>1,2-Diglyceride</td>
<td>Rat liver lecithin</td>
<td>20</td>
</tr>
<tr>
<td>1,2-Diglyceride</td>
<td>Pig liver lecithin</td>
<td>10</td>
</tr>
<tr>
<td>dl-1,2-Dipalmitin</td>
<td>Synthetic</td>
<td>0</td>
</tr>
<tr>
<td>1,2-Diglyceride</td>
<td>M. lysodeikticus</td>
<td>0</td>
</tr>
<tr>
<td>1-Monoolein</td>
<td>Commercial</td>
<td>0</td>
</tr>
<tr>
<td>1-Monoolein</td>
<td>Commercial</td>
<td>0</td>
</tr>
<tr>
<td>dl-1,2-Dioleoylglyceryl ether</td>
<td>Synthetic</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>
the structure of the enzymatically formed glycolipid was \( \alpha-\alpha\)-mannosyl-(1 \rightarrow 3)-diglyceride.

In order to confirm the structure assignment of mannosyl-
diglyceride and, at the same time, to show directly the involve-
ment of 1,2-diglyceride in mannosylglyceride synthesis, experi-
ments utilizing \(^3H\)-labeled diglyceride and unlabeled
GDP-mannose were performed under the same conditions as
with unlabeled diglyceride and GDP-\(^{14}C\)-mannose (described
earlier). The results of gradient elution chromatographic anal-
ysis of the lipid components obtained after incubation are shown
in Fig. 10. When GDP-mannose is absent from the incuba-
tion mixture, the diglyceride remains as the only \(^3H\)-labeled
component. Addition of GDP-mannose results in the forma-
tion of a second component which is chromatographically identical
with pure \(^{14}C\)-mannosyldiglyceride added as a marker.

**Enzymatic Synthesis of Mannosylmannosyldiglyceride**

Thus far, only limited studies on the enzymatic synthesis
of mannosylmannosyldiglyceride have been performed. As
illustrated in Fig. 3 (bottom), an acetone-treated preparation
obtained from the 100,000 \( \times \) g supernatant of crude extracts
catalyzes the conversion of \(^{14}C\)-mannose-labeled mannosyl-
diglyceride to mannosylmannosyldiglyceride. That the product
of this enzymatic reaction is \( \alpha-\alpha\)-mannosyl-(1 \rightarrow 3)-\(\alpha\)-manno-
syl-(1 \rightarrow 3)-diglyceride was indicated by the following facts.

\(^{14}C\)-Mannosylmannosyldiglyceride was chromatographically identical
diglyceride obtained from whole cells when a mixture of the
two was analyzed by either gradient elution chromatography or
thin layer chromatography in Solvents I, J, and K. More-
over, \(^{14}C\)-containing deacylation product of enzymatically
formed mannosylmannosyldiglyceride chromatographed with
pure \( \alpha-\alpha\)-mannosyl-(1 \rightarrow 3)-\(\alpha\)-mannosyl-(1 \rightarrow 3)-glycerol.

Further information on the requirements for mannosyl-
diglyceride synthesis, with either mannosylglyceride (Experiment A) or GDP-mannose (Experiment B) as the labeled
precursor, is shown in Table IX. Although agreement in the
total yield of mannosylmannosyldiglyceride formed in Experi-
ments A and B may be fortuitous, inasmuch as different levels
of GDP-mannose and mannosylglyceride were used in each
experiment, it is clear that synthesis of mannosylmannosyldi-
glyceride requires mannosylglyceride, GDP-mannose, and Mg\(^{++}\).

**DISCUSSION**

During the past year, it has become evident that the glycosyl-
diglycerides are common components of the lipids of many gram-
positive bacteria. From earlier studies, it was known that such
compounds were present in *M. lysodeikticus* (1) and *Staphylococcus
aureus* (2). While this study was in progress, reports ap-
peared concerning the occurrence of glycosylglycerides in
*Streptococcus faecalis* (33, 34); *Diophlococcus pneumoniae*, type I
(35, 36) and type XIV (37); *Lactobacillus plantarum* (34, 38);
*Lactobacillus casei* (34); *Bacillus subtilis* (34); *Staphylococcus
saprophyticus* (34) and *Streptococcus lactis* (34). Thus, glycosyl-
diglycerides containing galactose, glucose, or mannose appear
to be almost as ubiquitous in gram-positive bacteria as are
galactosylglycerides in photosynthetic microorganisms and
plants. In the present study with *M. lysodeikticus*, the structure
of the major glycolipid has been established to be \( \alpha-\alpha\)-mannosyl-
(1 \rightarrow 3)-\(\alpha\)-mannosyl-(1 \rightarrow 3)-diglyceride. The monomann-
osyldiglyceride is not detectable in whole cells; however, it is
formed as an intermediate in the biosynthesis of mannosyl-
mannosyldiglyceride. Originally, Macfarlane tentatively identi-
fied the major glycolipid in *M. lysodeikticus* as mannosylglyceri-
de (1). Our results do not agree with this conclusion, although
it is quite possible that differences in the strains of *M. lysodeikt-
icus* or variation in the growth conditions or both could markedly
affect the levels of the mono- and dimannosyldiglycerides found
in whole cells.

The complete structures of the glycolipids of *D. pneumoniae*
and of *S. aureus* have been elucidated recently. In both type I
(35, 36) and type XIV D. pneumoniae (37), the occurrence of α-β-glucopyranosyl(1 → 3)-diglyceride and α-β-galactopyranosyl(1 → 2)-α-β-glucopyranosyl(1 → 3)-diglyceride has been established. In S. aureus, the glycolipids consist of a mixture of α-glucosyl(1 → 6)-N-glucosylglyceride and α-glucosyl(1 → 3)-N-glucosylglyceride; the anomeric configuration of the glucosyl units remains to be established (39).

As was previously reported (3), crude, cell-free extracts of M. lysodeikticus catalyze the formation from GDP-[14C]-mannose of three mannose-containing lipids. The enzymatic formation of two of these lipids has now been studied in more detail. A particulate preparation, free of endogenous diglyceride, catalyzes the formation of a mannotrity the structure of which has been established to be α-N-mannosyl(1 → 3)-diglyceride. The reaction involves the transfer of the mannosyl moiety from GDP-mannose to the hydroxyl group of a 1,2-diglyceride, and requires Mg++ ion and an anionic surface-active agent. The enzyme manifests relatively high specificity for a diglyceride which contains branched chain fatty acyl groups. Of particular interest is the requirement for an anionic surface-active agent. This requirement, which is absolute, is best satisfied by the salts of the 12- and 13-methyltetradecanoic acids, which are the major fatty acids found in M. lysodeikticus, as well as in many other gram-positive bacteria (40). Straight chain fatty acids are less effective in this regard. In contrast, the synthetic surface-active agent, sodium hexadecyl sulfate, is almost as effective as the branched chain acids, although it becomes considerably more inhibitory at relatively low levels. At least two possible explanations for the function of the anionic surface-active agent in this reaction may be considered. One is that the surface-active agent alters the physical properties of the diglyceride aggregate in a way which facilitates interaction of this hydrophobic substrate with the enzyme. Another is that association of the surface-active agent with the enzyme or with the enzyme-containing particles provides a hydrophobic surface suitable for interaction of substrate with the enzyme. Two other instances in which anionic surface-active agents are required in enzymatic reactions involved in bacterial lipid metabolism have recently come to light (41). It is evident, however, that in the present instance any definitive studies on the function of the surface-active agent are precluded by the impurity of the enzyme preparation. So far, attempts to purify the enzyme have been thwarted by its lability.

The conversion of mannosyldiglyceride to mannosylmannosyldiglyceride has thus far been studied in less detail than mannosyldiglyceride synthesis. The enzyme catalyzing this reaction, unlike the mannosyldiglyceride synthetase, is present in crude extracts in a soluble form. The formation of mannosylmannosyldiglyceride requires GDP-mannose, mannosyldiglyceride, and Mg++. As yet, no indication of a lipid or surfactant "cofactor" requirement for this reaction has become evident. With partially purified preparations of this enzyme, as well as with crude extracts, the rate of synthesis of mannosylmannosyldiglyceride is considerably slower than the rate of mannosyldiglyceride synthesis. Obviously, this is not the case in vivo, since mannosylmannosyldiglyceride, rather than mannosyldiglyceride, is the major lipid extractable from whole cells of M. lysodeikticus. It is hoped that further studies on mannosylmannosyldiglyceride synthesis will serve to clarify this situation.

It appears that the formation of glycolipid in M. lysodeikticus via the transfer of a mannosyl unit from GDP mannose to a diglyceride, followed by addition of a second hexose unit to form the mannosylmannosyldiglyceride, may represent the route utilized for mono- and diglycosyldiglyceride synthesis in general. Neufeld and Hall (42) have demonstrated that crude spinach chloroplast extracts catalyze the formation of galactolipids from GDP-galactose. More recently Kaufman et al. (37) have shown that the biosynthesis of pneumococcal glycolipids involves the successive addition of glucose (from UDP-glucose) and galactose (from UDP-galactose) to form galactosylglycerolipid. In neither study, however, was the involvement of diglyceride directly demonstrated.

Finally, it is of interest to consider the possible function of glycolipids in the bacterial cell. As shown earlier by Macfarlane (29), virtually all the lipids in M. lysodeikticus are localized in the cell membrane. Moreover, it is known that the membrane of this organism contains a mannan (43), the structure of which is currently under investigation in this laboratory. Similarly, the pneumococci, which contain galactosylglycerolipid, also produce a polysaccharide which contains galactosylglucosyl units (44). Another example in which cellular polysaccharide and glycolipids contain identical hexose units is found in the mycobacteria (45, 46). It may be that the role of these glycolipids is strictly a structural one, and that the interaction of the relatively hydrophobic glycolipids with the polysaccharides somehow facilitates binding of these polysaccharides to the lipid-rich membrane. Another possibility is that the biosynthesis of these cell envelope-bound lipids and polysaccharides is in some way interrelated. This possibility is particularly intriguing in view of the recent reports which implicate lipophilic intermediates in bacterial cell wall (47) and lipopolysaccharide biosynthesis (48, 49).

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In a recent private communication, B. Kaufman and S. Roseman have indicated that diglycerides stimulate pneumococcal glycolipid synthesis in vitro.
The Chemical Characterization and Enzymatic Synthesis of Mannolipids in *Micrococcus lysodeikticus*

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