A Mannose- and Erythritol-Containing Glycolipid from *Ustilago maydis**

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ABSTRACT: Erythritol- and mannose-containing glycolipids have been found in the intracellular lipids of a strain of the corn smut fungus, *Ustilago maydis*. The lipids have been fractionated by Florisil column chromatography and the major component has been obtained in relatively pure form. This compound contains equimolar amounts of mannose and erythritol.

The chromatographic behavior and infrared spectrum indicated that the material contained unsubstituted hydroxyl groups. Fatty acid analysis showed only four acyl residues per molecule of erythritol mannoside. The glycolipid was oxidizable with lead tetraacetate which resulted in the destruction of the erythritol subunit, but not the mannose. The nature of the lead tetraacetate oxidation products indicates that all of the mannose hydroxyl groups are substituted and that the three free hydroxyl groups are on erythritol. The fatty acid constituents were found to range from C_{12} to C_{18} with the C_{16} acids predominating. It is proposed that the major glycolipid component has the structure 4-*O*-(2,3,4,6-tetra-*O*-acyl-*β*-D-mannopyranosyl)-D-erythritol.

The only complex biological material known to contain erythritol as a constituent is an extracellular oil produced by a strain of the corn smut fungus, *Ustilago maydis* (Haskins *et al.*, 1955; Boothroyd *et al.*, 1956). This oil has been partially characterized as an acetylated disaccharide, containing both erythritol and mannose. This organism appears to be unique in its ability to produce large quantities of erythritol containing complex molecules and therefore should be an excellent choice for studying erythritol metabolism. This report is concerned with the chromatographic purification and identification of the erythritol-containing glycolipids of this organism and the determination of the structure of the major intracellular erythritol glycolipid. This compound has been characterized as 4-*O*-(2,3,4,6-tetra-*O*-acyl-*β*-D-mannopyranosyl)-D-erythritol.

Experimental Section

Culture Conditions. The organism employed in this study was derived from a culture of *U. maydis* PRL-627 kindly supplied by Dr. R. H. Haskins of the Prairie Regional Laboratory, Saskatoon, Saskatchewan. The culture medium was identical with that described by Haskins *et al.* (1955) except that anhydrous D-glucose replaced cerelose and calcium carbonate buffering was not employed. Stocks of the organism were maintained by allowing streak cultures to grow for several days at 30° on slants of potato dextrose agar. Mature slant cultures were stored at 4° and remained viable for several months. Liquid cultures were grown in 100 ml of culture medium in 500-ml erlenmeyer flasks at room temperature with constant shaking. Maximum cell mass was achieved after 3–4 days, and maximum lipid content after about 7 days. Subcultures were obtained by inoculating fresh medium after 3–7 days of growth. The cells were harvested by centrifugation at 10,000*g* after 7 days. The cells were washed once with distilled water and either frozen or used immediately for the lipid extraction.

Lipid Extraction. Total lipids were extracted by homogenizing the packed cells in ten volumes of chloroform–methanol (2:1, v/v) in a Waring blender. The nonextractable residue was removed by filtration through glass wool and was extracted again in the same manner with a second ten volumes of chloroform–methanol. The combined chloroform–methanol filtrates were taken to dryness *in vacuo* at 40°. After weighing, the crude lipid was extracted with two to three volumes of chloroform–methanol (9:1, v/v) leaving an insoluble residue which was discarded. The lipid solution was then taken to dryness, dissolved in chloroform, and aliquots of this solution were used for column chromatography. Recently we have found that dialysis of the chloroform solution against water for 24 hr eliminates contaminating carbohydrates and greatly extends the useful lifetime of the Florisil columns.

Florisil Column Chromatography. A 100–200-mg aliquot of the total lipid extract in 5 ml of chloroform was applied to a column of chloroform-washed and equilibrated Florisil (19 × 2.5 cm) prepared as described for the isolation of cerebroside sulfate (O'Brien *et al.*, 1964a). The charged column was eluted with 250-ml portions of the following eluents: chloroform, chloroform–methanol (9:1, v/v), and chloroform–methanol (2:1, v/v). The total eluate from each elution mixture was taken to dryness *in vacuo*, the weight of lipid was determined, and its purity was ascertained by thin-layer chromatography. A flow diagram outlining the lipid extraction and fraction procedure is shown in Figure 1.

Paper and Thin-Layer Chromatography. Lipids were chromatographed on activated plates of silicic acid G using chloro-

* From the Department of Biological Sciences and The Graduate Program in Biochemistry and the Department of Pathology of the School of Medicine, University of Southern California, Los Angeles, California. *Received December 23, 1968.* Supported in part by National Institutes of Health Grant AM 06667 and Research Career Development Award K-3-HD-12,662 to J. S. O.

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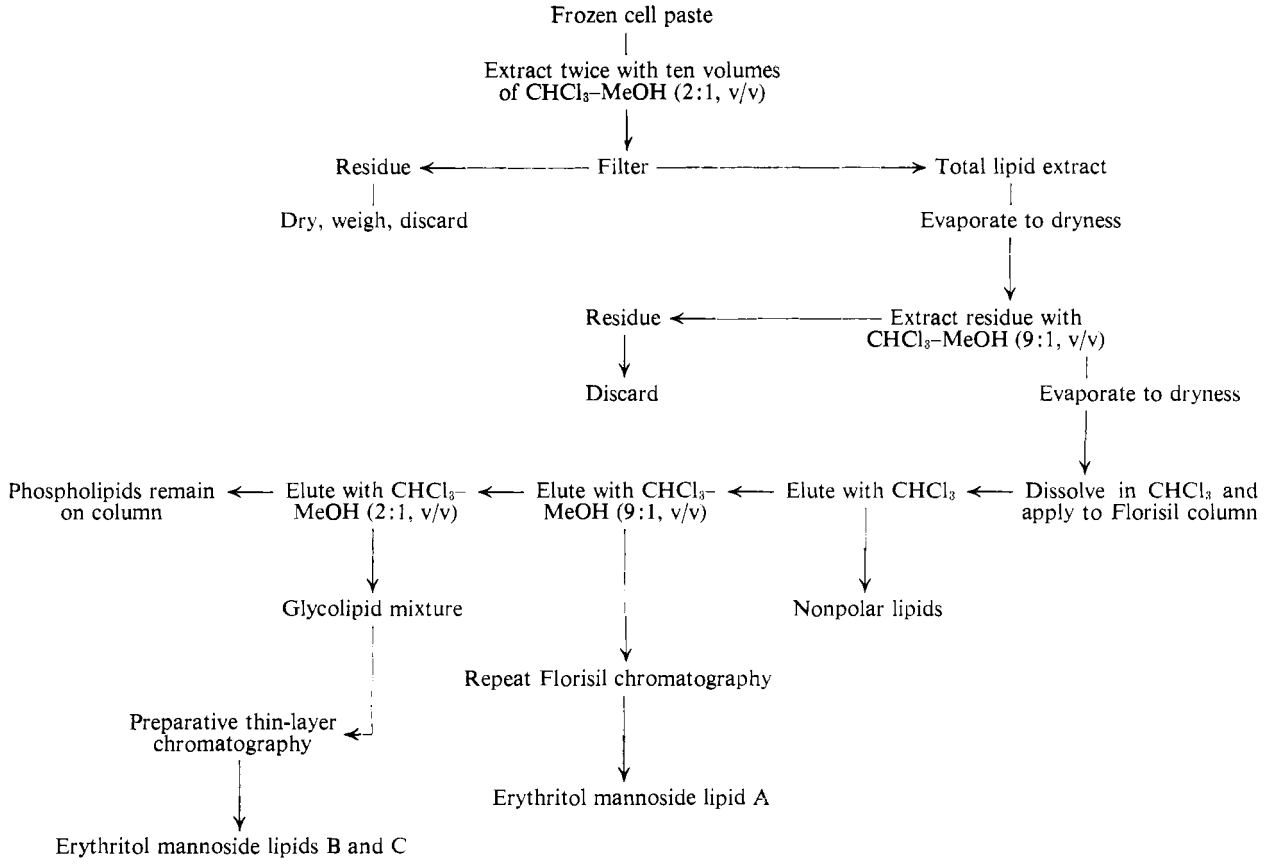

FIGURE 1: Flow diagram outlining the extraction and fractionation of the intracellular lipids of *U. maydis*.

form–methanol–ammonium hydroxide (43:6:1, v/v) as the developing solvent. Chloroform–methanol (99:1, v/v) was used as developing solvent for analyzing the lead tetraacetate cleavage products. Components on thin-layer chromatograms were detected by spraying with 70% sulfuric acid saturated with potassium dichromate followed by charring at 110° for 5–10 min (Mangold, 1961). Glycolipids were specifically detected by spraying with anthrone in sulfuric acid followed by heating at 110° (Mangold, 1961). A periodate–benzidine spray system (Viscontini *et al.*, 1955) was used to detect components with adjacent free hydroxyl groups. For preparative thin-layer chromatography the total lipid extract was applied as a band at the origin and the bands of various components were visualized by spraying lightly with water until the bands became visible. After air drying, the bands containing the material of interest were scraped from the plates and eluted from the silica gel with chloroform–methanol (2:1, v/v).

Samples for chromatographic analysis of the carbohydrate components were prepared by hydrolyzing the lipid fractions in 2 N HCl at 110° for 1.5 hr in sealed ampules. Carbohydrate constituents were separated by either ascending or descending chromatography on Whatman No. 1 paper in 1-butanol–acetic acid–water (4:1:5, v/v). Components were detected with the periodate–benzidine spray reagents (Viscontini *et al.*, 1955) or with a silver nitrate–sodium hydroxide dipping procedure (Trevelyan *et al.*, 1950). Estimations of relative proportions of mannose and erythritol were obtained using the silver nitrate procedure followed by clearing the background with a 10% ammonium hydroxide solution. The relative density of the spots was determined with a Photovolt scanning densitometer, comparing the results with those obtained from a standard mixture of the two carbohydrates run simultaneously.

Analytical Procedures. Infrared spectra in carbon tetrachloride were obtained with a Perkin-Elmer Model 421 spectrophotometer.

Mannose was analyzed by the phenol–sulfuric acid procedure (Giudici and Fluharty, 1965). Initially the lipids were hydrolyzed in 2 N HCl before sugar analysis. Later, excellent results were obtained by dissolving the dried lipid in the 80% phenol before water was added. This resulted in an excellent dispersion of the lipid in the water and the hexose content agreed closely with that obtained after acid hydrolysis. Methyl α-D-mannopyranoside, which was subjected to all steps of the assay, was used as a standard.

Ester groups were estimated by the hydroxamate procedure of Rapport and Alonzo (1955). β-D-Glucose pentaacetate was used as standard, and the extinction coefficient at 530 mμ for the fatty acid hydroxamate–ferric ion complex was assumed to be equal to that of the acetyl hydroxamate complex.

Gas–Liquid Partition Chromatography. Methyl esters of the fatty acid constituents were prepared by transmethylation of lipid samples in 5% potassium hydroxide in methanol for 17 hr at room temperature or by heating the lipid at 60° for 1 hr in 5% hydrogen chloride in methanol. Methyl esters were extracted into hexane which was then removed by evaporation on a rotary evaporator. The fatty acid esters were then analyzed by gas–liquid partition chromatography on columns

TABLE I: Composition of *U. maydis* Lipids.

		% Total Lipid by Weight
Neutral lipids		19.2
Glycolipids		29.4
A	19.4	
B	1.5	
C	8.8	
Phospholipids		51.4

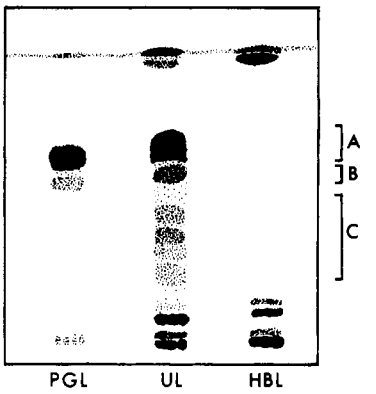

FIGURE 2: Thin-layer chromatogram showing the erythritol mannoside lipids of *U. maydis*. The first band on the left (PGL) is the purified sample of the major erythritol mannoside lipid. It consists primarily of a single component referred to in the text as component A. There is also a small contamination by a slower moving material designated as component B. The second band is the unfractionated lipid extract (UL). It contains rapidly migrating nonpolar lipids, components A and B, a slower broad band designated component C, and two or three components which migrate slowly or not at all. It can be seen that component C probably contains several subfractions. The third band is a sample of unfractionated human brain lipids (HBL) in which the two slow-moving bands above the origin are cerebrosides. The chromatographic medium was activated silicic acid G and development was with chloroform–methanol–ammonium hydroxide (43:6:1, v/v). The lipids were visualized by charring with dichromate–sulfuric acid.

of 10% diethylene glycol succinate as described previously (O'Brien *et al.*, 1964b). Fatty acids were identified by comparing their retention times with standards. They were quantified by triangulative peak areas.

Lead Tetraacetate Oxidation. A 5-mg sample of lipid was taken to dryness in a glass-stoppered centrifuge tube. To this was added 2 ml of a 5-mg/ml solution of lead tetraacetate in glacial acetic acid. The reaction was gently shaken for 4 hr at room temperature. After this time 1.5 ml of a 3.2-mg/ml solution of oxalic acid in glacial acetic acid was added; an amount slightly in excess of that required to reduce and complex all of the tetravalent lead. After mixing, the suspension was allowed to stand for a few minutes and then the lead oxalate suspension was centrifuged. The supernatant was decanted through glass wool and the precipitate was washed by suspending it in 1.5 ml of glacial acetic acid, centrifuging, and decanting. The supernatants were combined, taken to dryness *in vacuo*, and the resulting residue was dissolved in 1 ml of chloroform. There was a small amount of a chloroform insoluble residue which was discarded.

Chemicals. Common solvents, salts, and sugars of analytical grades were obtained from local chemical suppliers. Florisil (60–180 mesh) was purchased from the Fisher Scientific Co. β-D-Glucose pentaacetate, methyl α-D-mannopyranoside, and glyceryl monooleate were obtained from Calbiochem. Lead tetraacetate was purchased from K & K Laboratories. Standard mixtures of fatty acid methyl esters were obtained from Applied Science Laboratories, Inc. The sugar beet molasses, required in small amounts for the culture medium, was kindly supplied by the Holly Sugar Co. All solvent ratios given in the text are on a volume basis and solvents were redistilled prior to use.

Results

Between 0.5 and 1.0 g (dry weight) of cells was obtained per 100-ml culture, of which 20–40% was extractable into the total lipid fraction. There was no evidence of extracellular oil production by the cultures employed for the studies reported here. The distribution of the crude *U. maydis* lipid after thin-layer chromatography in the chloroform–methanol–ammonia (43:6:1, v/v) solvent is shown in Figure 2. Since this solvent is somewhat less polar than that usually employed for glycolipids from animal tissues the distribution of human brain lipids are also shown for comparison. The major glycolipid components of the fungus are less polar than any of the human brain glycolipids. When the lipids of *Ustilago* were fractionated by Florisil chromatography the chloroform fraction contained nonpolar lipids, and no glycolipids. The chloroform–methanol (9:1, v/v) eluate contained glycolipid components A and B plus a small amount of component C. The chloroform–methanol (2:1, v/v) fraction contained all three glycolipid fractions. Phospholipids remained on the column and were estimated by difference in weight between the amount of lipid applied and that recovered.

Table I gives the lipid composition of *U. maydis*. The glycolipids constitute between one-quarter and one-third of the total lipids. Component A comprises two-thirds of the glycolipids. By repeated column fractionation of the (9:1) fraction we were able to obtain a purified sample of component A. Fractions containing one or more of the more polar glycolipids B and C free from other materials were obtained by refractionation of the (2:1) components once again on Florisil or by preparative thin-layer chromatography. The only carbohydrate components detected in these glycolipids were erythritol and mannose. The molar ratios of mannose and erythritol in several glycolipid fractions are given in Table II. In each case erythritol and mannose were present in nearly equivalent proportions. Upon mild alkaline hydrolysis, each glycolipid gave rise to a single carbohydrate component which migrated slower than either erythritol or mannose. We believe this component to be erythritol mannoside since it gave rise to nearly equivalent proportions of erythritol and mannose on subsequent acid hydrolysis. The most highly purified sample of component A was contaminated by 5% or less of component B, but was employed for structural studies since it was felt that the small degree of contamination present would not seriously affect the conclusions. The chromatographic behavior of the glycolipid A (and the other glycolipids) is suffi-

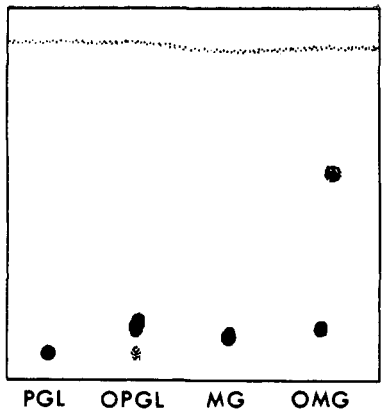

FIGURE 3: Thin-layer chromatogram of lead tetraacetate oxidation products. The chromatography was carried out on activated silicic acid G with chloroform–methanol (97:3, v/v) as developing solvent, and the materials were detected by charring with dichromate–sulfuric acid. The first spot on the left is unoxidized *Ustilago* glycolipid (PGL). The second spot is the same material after oxidation (OPGL). The third spot is unoxidized glycerol monooleate (MG), and the fourth is the monoglyceride after cleavage (OMG).

ciently polar to suggest that all the erythritol mannoside hydroxyl groups are not acetylated. The infrared spectrum of the purified component shows a strong absorption band near 3500 cm^{-1} characteristic of free hydroxyl groups. The strength of this band suggests that more than one unesterified hydroxyl function might be present.

Table III shows the results of duplicate determinations of mannose and ester groups on two different samples of the purified lipid. These analyses indicate that four hydroxyl groups out of the seven present in the erythritol mannoside are esterified leaving three free hydroxyl groups.

When a thin-layer chromatogram of the purified lipid was sprayed with the diol-specific periodate–benzidine spray reagent a positive test was observed, indicating that adjacent free hydroxyl groups were present. A sample of glycolipid A was then oxidized with lead tetraacetate in glacial acetic acid. Thin-layer chromatography of the chloroform-soluble material present after the reaction revealed that the original lipid had been completely converted into a much less polar derivative, while a control sample of glycolipid A, which was subjected to the identical procedure in the absence of oxidant, remained unaltered. The mannose content of the lipid before oxidation was 0.81 μmole/mg, while that of the oxidation product was 0.83 μmole/mg indicating that the mannose moiety was unaltered by oxidation with lead tetraacetate. After acid hydrolysis of the tetraacetate-treated lipid, mannose was readily detected in the hydrolysate but erythritol was absent. A sample of the tetraacetate-treated lipid was reduced with diborane in anhydrous tetrahydrofuran (Brown and Subbarao, 1960). After acid hydrolysis of this oxidized and reduced material no evidence was found for any product of erythritol oxidation. If glycerol had been present, it would have been detected on the paper chromatogram. The diol-selective sprays are much less sensitive for ethylene glycol and the presence of this compound would have been difficult to demonstrate with the amounts of material available for this assay.

The possibility exists that one of the fatty acyl residues could be esterified to the primary hydroxyl group of erythritol, distal to the point of mannose attachment. This would give rise to an esterified glycolaldehyde upon lead tetraacetate oxidation. To test this possibility the cleavage products of the purified *Ustilago* lipid were compared with those from a sample of mixed α- and β-glyceryl monooleate. The results are seen in Figure 3. The purified *Ustilago* lipid was converted into a single faster migrating component. The monoglyceride mixture, which migrated as one spot before oxidation, contained a new fast-moving component which corresponds to an esterified glycolaldehyde. No similar cleavage product could be detected from the *Ustilago* lipid indicating that the primary hydroxyl group of erythritol is unesterified.

The fatty acid composition of the purified *U. maydis* erythritol mannoside lipid is presented in Table IV. The C_{16} fatty acids comprise almost half of the total. The C_{14} acids are the next most predominant with the C_{12} and C_{18} acids making up the remainder. The majority of the fatty acids are saturated. The monoenoic acids represent about 25% of the total and the dienoic acids only 5%. No acetate or short-chain fatty acids (6–10 carbons) were detected. Branched-chain fatty acids were not observed.

TABLE II: Relative Amounts of Erythritol and Mannose in Glycolipid Fractions.[a]

Glycolipid Fraction	Density Ratio Mannose: Erythritol	Molar Ratio Mannose: Erythritol
A	1.20	0.98
B	1.26	1.03
C	0.94	0.77
Standard mixture	1.67	1.36

[a] The standard mixture contained a 2:1 weight ratio of mannose to erythritol. The density was that of the silver oxide stain on a chromatogram of the water-soluble material. Chromatography and determination of the density of the spots are described in the text.

TABLE III: Mannose and Fatty Acid Analyses of Purified Glycolipid.

Sample	Mannose (μmole/mg)	Sample	Fatty Acid (μmole/mg)
1a	0.65	A_1	2.38
1b	0.80	A_2	2.88
11a	0.65	B_1	3.00
11b	0.69	B_2	2.96
	Av 0.70		Av 2.78

Fatty acid groups per mannose = 4.0.

Discussion

The impetus to look for erythritol in the intracellular lipids of *U. maydis* resulted from our initial inability to achieve

TABLE IV: Fatty Acid Composition of Purified *U. maydis* Glycolipid.

Peak No.	Identity	% Total	Peak No.	Identity	% Total
1	12:0	10.8	7	16:1	11.3
2	13:0	0.2	8	16:0	34.5
3	14:1	9.0	9	18:2	4.5
4	14:0	22.1	10	18:1	4.9
5	15:1	0.3	11	18:0	1.8
6	15:0	0.6			

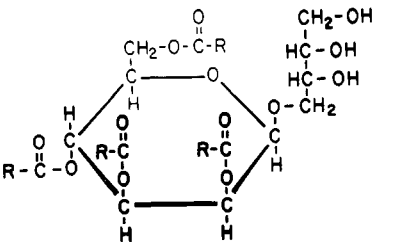

FIGURE 4: Proposed structure for the major erythritol mannoside glycolipid of *U. maydis*.

extracellular oil production by the fungus. Although the organisms employed in this study were derived from the strain which Haskins *et al.* (1955) have reported to be the most active in oil production, it is possible that extended storage and repeated transfers resulted in the selection of a strain somewhat different from that originally studied. The gross characteristics of our cultures have remained constant for several years. The cells are rich in lipids, and numerous intracellular globules, presumably lipid droplets, can be observed microscopically. Recently we have observed copious extracellular oil production in some cultures, and these cells seem identical with those from other cultures studied earlier. We believe that the variability in extracellular oil production only reflects minor differences in growth and culture conditions.

The proposed structure for the major glycolipid component as a 4-*O*-(2,3,4,6-tetra-*O*-acyl-β-D-mannopyranosyl)-D-erythritol (Figure 4) is consistent with all of the experimental information. Both chromatographic behavior and infrared spectra suggest that unesterified hydroxyl groups are present. Analysis of fatty ester content showed that only four of the seven hydroxyl groups present on the presumed erythritol mannoside backbone are substituted. Since erythritol and not mannose is destroyed by lead tetraacetate oxidation, at least two of the three free hydroxyl groups must occupy adjacent positions on the erythritol. The fact that no glycoaldehyde fatty acid ester is produced by the diol-cleaving reagent coupled with the failure to detect glycerol after oxidation, reduction, and hydrolysis establishes that all three unesterified hydroxyl groups are on erythritol. Therefore, each of the four mannopyranosyl hydroxyls must carry a fatty acyl substituent. The failure to detect glycerol as the eventual product of erythritol breakdown also establishes that mannose is linked to the polyol through one of the primary hydroxyl groups. This structure is in marked contrast to other glycolipids where the polyol is acylated and the glycone relatively unsubstituted. Thus the erythritol mannoside lipids constitute a unique new class of glycolipid.

Sufficient purified glycolipid has not been available to completely characterize the erythritol mannoside derived from it. However, we were able to establish that the disaccharide component contained equimolar proportions of erythritol and mannose, and that it behaved identically on chromatography and hydrolysis with the nonacylated erythritol mannoside isolated from the culture medium of this organism. The nonacylated disaccharide has been characterized s 4-*O*-β-D-mannopyranosyl-D-erythritol by Charlson and Perlin (1956) and Gorin *et al.* (1960) and the structure has been confirmed by synthesis (Gorin and Perlin, 1961). Since there is no indication of more than one erythritol mannoside in the organism, it seems reasonable to assume that the disaccharide moiety of the purified lipid is identical with that in the culture medium.

If one calculates the molecular weight based upon the proposed structure, assuming that the fatty acids have an average chain length of 16 carbons, a value of 1236 is obtained. If, on the other hand, the minimum molecular weight is calculated from the average mannose analysis of 0.827 μmole/mg ob-obtained with a dry sample of the purified lipid, a value of 1210 is obtained. The close correspondence of the theoretical and derived molecular weight calculations lends additional credibility to the proposed structure.

There is no evidence, at present, to suggest how the more polar minor erythritol mannoside lipids differ from the major component. Mannose and erythritol are present in equimolar amounts in all fractions, so these compounds do not represent a family of acylated mannose oligosaccharides varying in their mannose contents as has been found in the inositol mannosides from *Mycobacteria* (Lee and Ballou, 1965). Since the minor components have a somewhat more polar chromatographic behavior it is possible they are less fully acylated. The differences seem too great to be due simply to variations in chain length or degree of saturation of the fatty acids. It is also possible that the slower migration of the more polar components is due to the presence of hydroxy fatty acids which are known to occur in some strains of *U. maydis* (Lemieux, 1951, 1953). Preliminary studies suggest that fatty acids more polar than unsubstituted fatty acids are present in these fractions, but insufficient amounts of purified material have been available for structural analysis.

It is also unclear how the intracellular erythritol mannoside lipid is related to the extracellular oil studied by Boothroyd *et al.* (1956). Their analysis of long-chain fatty acid composition (based upon a fractional distillation of methyl esters) is not radically different from that expected on the basis of the composition reported here. Their yield of 0.67–0.90 g of methyl ester per g of oil (excluding methyl acetate) is compatible with an expected 0.87 g calculated for the structure we propose. Likewise their yield of 0.124–0.218 g of water-soluble material per g of oil after methanolysis approaches the value of 0.255 g expected for the proposed structure. However, the large amount of acetate which they report was not found in our purified fraction and their saponification number, which ranged between 245 and 312, is considerably higher than the value of 174 expected from our fatty acid analyses. One logical explanation of these differences is that the extracellular glycolipids have a composition similar to the intracellular

glycolipids except that the erythritol hydroxyls are esterified with acetate. Such a modification would not greatly lower the amount of methyl esters of long-chain fatty acids or water-soluble constituents which would derive per gram. Such a change would increase the saponification number and account for the reported yield of acetate. However, we have found that the extracellular glycolipids, produced recently by cultures in our laboratory, had chromatographic characteristics almost identical with the glycolipids extracted from the same cells. Therefore, it is also possible that the acetate and high saponification numbers reported previously reflect the presence of other low molecular weight constituents in the unpurified extracellular oil.

Acknowledgments

The authors wish to thank Dr. R. H. Haskins whose generous gift of a culture of *U. maydis* PRL 627 made this research possible and Dr. T. A. Giudici for carrying out the diborane reduction. They also acknowledge the technical assistance of Mr. R. Bishop and Mrs. S. L. Gaber on portions of the experiments presented.

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