Identification of the Major Glycolipid from *Mycoplasma* sp., Strain J as 3,4,6-Triacyl-β-D-glucopyranose*

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ABSTRACT: *Mycoplasma* sp., strain J, contains two glycolipids, cholesteryl β-D-glucoside and 3,4,6-triacyl-β-D-glucopyranose. The structure of the latter was proven by standard chemical procedures. Glycolipids comprise about 20% of the total lipids of the organism. The 3,4,6-triacylglucose constitutes almost 80% of the total glycolipid. Its content remains constant during growth, in contrast to the cholesteryl glucoside. Unsaturated fatty acids, mainly oleic, predominate in the acylated glucose. A structural function is postulated for this glycolipid.

Glycolipids have been found to comprise a major portion of the total lipids of the mycoplasmas which utilize hexoses as their carbon and energy source (Shaw and Smith, 1967). The glycolipids of *Mycoplasma laidlawii* have been identified as 1-[*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl] 2,3-diglyceride, 1-[*O*-α-D-glucopyranosyl] 2,3-diglyceride (Shaw *et al.*, 1968), and a carotenyl β-D-glucoside (Smith, 1963a,b). *Mycoplasma mycoides* contains a galactofuranosyl diglyceride (Plackett, 1967). *Mycoplasma* sp., strain J, contains two glycolipids (Smith and Koostra, 1967), one of which is cholesteryl β-D-glucoside (Rothblat and Smith, 1961). The second glycolipid now has been identified as 3,4,6-triacyl-β-D-glucopyranose.

Materials and Methods

The organism was cultured in a lipid-free medium supplemented with cholesterol and sodium oleate as previously described (Smith and Koostra, 1967). The 24-hr cultures were harvested by centrifugation and the sedimented organisms lyophilized. Total lipids were extracted by three treatments with 40 volumes of chloroform–methanol (2:1, v/v). After drying *in vacuo* at 40°, the residue was freed of nonlipid material by passage through Sephadex G-25 (Wells and Dittmer, 1963). The purified total lipids were separated into neutral lipid, glycolipid, and phospholipid fractions on a silicic acid (Unisil, Clarkson Chemical Co., Williamsport, Pa.) column (2 × 8 cm) eluting with 150 ml of chloroform, 200 ml of acetone, and 250 ml of chloroform–methanol (1:10, v/v), respectively. The acetone eluate which contains both glycolipids (Smith and Koostra, 1967) was dried *in vacuo* and further separations were carried out by preparative thin-layer chromatography.

Thin-layer chromatographic plates (1 mm thick) were prepared using silica gel H (E. Merck AG, Darmstadt, Germany). Glycolipid separation was accomplished by development of the plates in chloroform–methanol (9:1, v/v). Two spots were seen, both being detectable with iodine vapor and both exhibiting a positive sodium periodate–Schiff reaction. The cholesteryl glucoside possessed an R_F of 0.1–0.15 and the unknown glycolipid an R_F of 0.4–0.5 after solvent flow of 13 cm (Smith and Koostra, 1967). The area containing the unknown glycolipid was scraped from the plates and eluted with chloroform–methanol (1:1, v/v). Usually two to three separations on thin-layer plates resulted in purification as indicated by the appearance of only one spot on thin-layer plates (250 μ thickness) in a variety of solvent mixtures, *e.g.*, chloroform–methanol (8:2 and 6:4, v/v) and chloroform–methanol–water (65:25:4, v/v). This purified lipid served as the material for analytical examinations.

Infrared absorption spectra were obtained with a Beckman IR-5A spectrophotometer. Samples were examined as liquid films on crystals of sodium chloride or in pellets of potassium bromide. Optical rotation of the free sugar was determined in water solution using a Rudolph Model 63 polarimeter. Gas–liquid partition chromatography of methyl esters of fatty acids was conducted in a Beckman GC-2A instrument equipped with a ThermoTrac temperature programmer as previously reported (Smith and Koostra, 1967). Gas–liquid partition chromatography also served as one method for the identification of the anomeric configuration and the ring size of the sugar component. Trimethylsilyl ethers of the methyl glucosides were separated by employing matched 6-ft columns of diethylene glycol succinate (20%) on Chromosorb W 42–60 at a temperature of 180°. Helium was used as carrier gas at a flow rate of 95 cc/min. Other conditions were: current, 250 mA; sensitivity, 1; thermal conductivity detector. The fully methylated sugars were separated on the columns of diethylene glycol succinate with a carrier gas flow rate of 95 cc/min at 165 and 180°; on matched 6-ft columns of silicone SE-30 (15%) on Chromosorb W 80–100 with a gas flow rate of 95 cc/min at 265°. Identifications were based upon retention times of standards prepared as described later (Aspinall, 1963; Bishop, 1964).

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Mild deacylation of the intact lipid was carried out according to the procedure of Benson and Maruo (1958). More vigorous saponification was accomplished by refluxing for 30 min in 1 N methanolic KOH. Acid hydrolysis of the intact lipid was performed by refluxing in 2 N HCl for 48 hr.

The rate of utilization of sodium periodate by the intact lipid was measured at room temperature by the arsenite method described by Cheronis and Ma (1964) except for the use of dioxane rather than water as the solvent for the samples. Attempts to use the spectrophotometric method failed because of high-background absorbance. For qualitative determination of the end product of sodium periodate oxidation, the reaction was terminated after 18 hr by the addition of ethylene glycol and the acylated end product was extracted with chloroform and dried *in vacuo*. The residue was deacylated at 37°, neutralized with Dowex 50W (H^+) resin, filtered, and dried *in vacuo*. The water-soluble extract was used for paper chromatography. Since the possible effect of the sodium periodate oxidation procedure on the ester linkages of the lipid was unknown, commercial glucose pentaacetate and glucose tetra- and pentapalmitate, synthesized by the method described by Whistler (1945), were used as standards. No utilization of sodium periodate occurred with these compounds, nor were these acylated glucoses degraded by the conditions employed. Thus the ester linkages appeared stable during periodate oxidation.

Formation of the methyl glucosides of the intact lipid, of the free sugar derived from the intact lipid, and of 2,3,4,6-tetramethylglucose (Pierce Chemical Co., Rockford, Ill.) was accomplished either by refluxing in anhydrous methanol and Dowex 50W (H^+) resin (Bollenback, 1963) or in anhydrous 0.5% methanolic HCl (Horton and Hutson, 1963). Trimethylsilyl derivatives of the unknown methyl glucoside and standard α- and β-methyl glucosides were prepared by the method of Sweeley *et al.* (1963). Complete methylation of methyl glucosides was performed by the procedure of Carter *et al.* (1961) using fresh silver oxide and methyl iodide in dioxane. Standard 2,3,5,6-tetramethylglucosides were prepared by methylation of the methylglucofuranoses prepared according to the procedure of Phillips (1954).

Fatty acid esters were determined by hydroxamate formation (Rapport and Alonzo, 1955); reducing sugar by the Park–Johnson (1949) method; hexose by the anthrone reaction; glucose and galactose by their respective oxidases (Worthington Biochemicals Corp., Freehold, N. J.); glycerol by reduction of nicotinamide adenine dinucleotide by α-glycerophosphate dehydrogenase (EC 1.1.1.8) after glycerol phosphorylation by glycerol kinase (EC 2.7.1.31) and adenosine triphosphate (Hohorst, 1963).

Identification of sugars and their methyl glycosides and fully methylated derivatives was made or confirmed by ascending paper chromatography on Whatman No. 1 filter paper strips using the following solvent mixtures: 1-butanol–acetic acid–water (4:1:5, v/v), 1-butanol–pyridine–water (6:4:3, v/v), and 1-butanol–ethanol–water (5:1:4, v/v). Paper chromatography of glycerol also was carried out using isopropyl alcohol–5% boric acid (7:1, v/v) as solvent (Ikawa *et al.*, 1966). These compounds were detected with alkaline silver nitrate, sodium periodate–potassium permanganate, and sodium periodate–Schiff reagents.

Results

Cellular Composition. The yield and lipid composition of a typical 100-l. culture of *Mycoplasma* sp., strain J, is shown in Table I. The acylated glucose and cholesteryl glucoside constitute 79 and 21% of the total glycolipid respectively.

TABLE I: Lipid Composition of *Mycoplasma* sp., Strain J.

	Yield (g)	% Dry Wt Organism	% Total Lipid
Dry cell weight	3.00	100	
Total lipid	0.48	16.0	100
Neutral lipid	0.14	4.7	29.4
Glycolipid	0.10	3.4	21.4
Phospholipid	0.23	7.8	48.8

Structural Analysis. Acid and alkaline hydrolysis and mild deacylation of the unknown glycolipid resulted in the production of only fatty acids and glucose. The ratio of fatty acid ester to glucose based on 12 determinations from three different lots of lipid was 2.93 ± 0.25. No glycerol, inositol, phosphate, or ninhydrin-positive material could be detected by previously described methods (Smith and Koostra, 1967). The amount of glucose determined as reducing activity of the deacylated product was equivalent to the amount of hexose present as determined by the anthrone reaction. This ratio of 1.02 indicated that a simple hexose and not an oligosaccharide existed in the intact lipid. That this hexose was glucose was proven by its R_F (0.62 in butanol–pyridine–water; 0.22 in butanol–acetic acid–water) on paper chromatograms and its reactivity with glucose oxidase but not with galactose oxidase. Furthermore, the ratio of the hexose determined by anthrone to glucose determined by glucose oxidase approximated 1.0.

The previously reported positive reaction of this lipid with sodium periodate–Schiff reagent indicated the presence of unsubstituted vicinal hydroxyl groups. The slow reactivity of the intact lipid and its deacylation product in contrast to the rapid reaction obtained with open-chained vicinal hydroxyls suggested the presence of a pyranose or furanose ring. Infrared absorption at 925 cm^{-1} by the intact lipid also was suggestive of a ring structure (Davidson, 1967; Barker *et al.*, 1956). The existence of the hexose as a ring and the presence of three fatty acid ester groups, by elimination, allow for the existence of only one pair of unsubstituted vicinal hydroxyl groups.

The positive reaction for reducing sugar by the intact lipid indicated the hydroxyl group on C-1 of glucose is free. Thus the unsubstituted vicinal hydroxyls should be at C-1 and C-2 while the acyl groups should be at C-3, C-4, and C-6 or C-3, C-5, and C-6 depending upon whether the ring is a pyranose or a furanose. In either case oxidation with sodium periodate followed by deacylation should yield arabinose. The product of these sequential reactions exhibited R_F values of 0.33 and 0.26 in butanol–pyridine–water and butanol–acetic acid–water, respectively (standard arabinose: R_F 0.35 and 0.28). Reduction of this product with sodium borohydride in pH 9 sodium borate buffer yielded a compound with R_F values of 0.41 and 0.32 in the above solvent mixtures (standard arabinitol: R_F 0.43 and 0.32).

The rate of periodate utilization proceeded by apparent first-order kinetics as shown in Table II. The reaction was complete in about 36 hr. This slow utilization was suggestive of a *trans* configuration of the vicinal hydroxyl groups, a result borne out by the identification of the anomeric configuration at C-1 as β described later.

TABLE II: Rate of Utilization of Sodium Periodate by the Acylated Glucose from *Mycoplasma* sp., Strain J.[a]

Time (hr)	Glucose (anthrone) (μmoles)	Sodium Periodate Consumed (μmoles)	Sodium Periodate Consumed (μmoles): Glucose (μmoles)
4	4.72	1.05	0.21
20	5.74	3.19	0.56
24	11.84	7.70	0.65
72	7.56	7.26	0.96
120	7.56	6.72	0.89

[a] Each time period represents a separate oxidation.

The small amount of material available made impossible an accurate determination of the optical rotation. However, the rotation was unmistakenly positive, $[\alpha]_D^{23}$ +71.3 ± 20.4°. Thus the glucose appeared to be of the D configuration.

The infrared spectrum exhibited a weak absorption peak at 897 cm^{-1} identical with that exhibited by β-D-glucose pentaacetate. No absorption was observed in the 840–850-cm^{-1} region as found with α-D-glucose and α-D-glucose pentaacetate. Such a spectrum together with the slow utilization of sodium periodate suggested that the anomeric configuration was β. Formation of the methyl glucoside of the intact lipid by refluxing in anhydrous methanolic HCl followed by mild deacylation yielded a product with an R_F value of 0.53 in butanol–acetic acid–water, identical with the R_F of standard methyl β-D-glucopyranose. Gas chromatography of the trimethylsilyl derivative of the unknown glucoside (prepared by refluxing in methanol and resin) on the diethylene glycol succinate column resulted in two peaks with retention times of 5.8 and 6.4 min (standard α-methyl glucoside, 5.8 min; β-methyl glucoside, 6.4 min). It would appear that isomerization occurred during glycoside formation in the presence of the resin. Most of the experimental evidence favors the existence of the β configuration in the natural lipid which probably reflects the greater relative stability of the β anomer.

As previously stated, type 1 absorption at 925 cm^{-1} suggested a pyranose ring structure. Proof was obtained by gas–liquid partition chromatography of the fully methylated glucose prepared by conversion of the acylglucose into its methyl glucoside followed by deacylation and methylation. The retention times found were as follows: diethylene glycol succinate column: unknown, 4.46 min at 180°, 7.39 min at 165°; 2,3,4,6-tetramethyl-β-D-glucoside, 4.45 min at 180°, 7.42 min at 165°; 2,3,5,6-tetramethyl-β-D-glucoside, 6.98 min at 180°; silicone column: unknown, 2.99 min; 2,3,4,6-tetramethyl-β-D-glucoside, 2.99 min; 2,3,5,6-tetramethyl β-D-glucoside, 3.65 min.

The fatty acid composition of the triacylglucose is shown in Table III. Unsaturated fatty acids comprised 83% of the total fatty acids. The $C_{18:1}$ acid no doubt represents the oleic acid provided as a growth requirement in the culture medium.

TABLE III: Fatty Acid Composition of Acylated Glucose from *Mycoplasma* sp., Strain J.

Fatty Acid	% Total Moles of Fatty Acid
12:0	0.2
14:0	0.5
14:1	0.5
160:0	5.2
16:1	4.8
Unknown	0.5
Unknown	0.6
18:0	7.9
18:1	62.5
18:2	15.2
Unknown	2.2

Effect of Age of Organisms on Glycolipid Content. The data are presented in Table IV. Increase in dry weight of the organisms followed a typical logarithmic growth pattern. The total lipid content varied little during the course of growth. The acylated glucose remained relatively constant. Greater fluctuation was noted in the content of cholesteryl glucoside. Lower levels were observed during the period of rapid multiplication with some accumulation occurring during the stationary phase of growth.

TABLE IV: Glycolipid Composition of *Mycoplasma* sp., Strain J, at Various Ages.

Time (hr)	Dry Wt Organisms (μg/ml of culture)	Total Lipid (% dry wt organisms)	μmoles/mg Dry Wt Organisms	
			Acylglucose	Cholesteryl Glucoside
4	2.81	16.2	0.033 ± 0.006	0.020 ± 0.001
8	3.63	13.7	0.033 ± 0.004	0.012 ± 0.002
12	4.64	14.5	0.030 ± 0.003	0.014 ± 0.001
16	7.32	12.1	0.032 ± 0.006	0.016 ± 0.002
24	12.18	11.9	0.031 ± 0.007	0.018 ± 0.003
33	11.91	12.3	0.031 ± 0.006	0.020 ± 0.003
48	12.25	13.0	0.027 ± 0.002	0.029 ± 0.004

Discussion

The glycolipids of *Mycoplasma* sp., strain J, are 3,4,6-triacyl-β-D-glucopyranose and cholesteryl β-D-glucopyranose. Acylated sugars represent a new class of lipids found in microorganisms. A tetraacylglucose with the probable structure of 3,4,6-tri-*O*-acetyl-2-*O*-laurylglucopyranose has been found in *Streptococcus faecalis* (Welsh *et al.*, 1968). Acylated sugar derivatives also were isolated from some gram-negative bacteria (*Aerobacter, Escherichia, and Pseudomonas*) but the harsh extraction procedures required suggest that these lipids are components of the lipopolysaccharide. Alkaline hydrolysis of these lipids yielded mono-, di-, tri-, and oligosaccharides composed of glucose, galactose, and arabinose. Vilkas *et al.* (1968) isolated from *Mycobacterium fortuitum* a glycolipid which has the structure of an asymmetric trehalose diester in which two fatty acids esterify the same pyranose ring.

The similarity of the glycolipids in streptococci and mycoplasmas extends also to the glycosyl diglycerides. The diglucosyl diglyceride of *S. faecalis*, *S. pyogenes*, and *M. laidlawii* is 1-[*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl] 2,3-diglyceride (Shaw and Baddiley, 1968). Taxonomic significance appears to be associated with the nature of the glycolipids in microorganisms. Although *S. faecalis* contains both types of glycolipid, the mycoplasmas examined thus far contain either acylated glucose or glycosyl diglycerides. Too few mycoplasmas have been examined to derive a positive conclusion.

Mycoplasmas utilizing hexoses for a carbon and energy source also contain steryl or carotenyl glucosides, while those incapable of hexose utilization have no glycolipids. β-D-Glucosides of carotenols also have been found in *Mycobacterium phlei* (Hertzberg and Liaaen-Jensen, 1967). Glucosides of plant sterols occur commonly, but mycoplasmas are the only bacterial source known at present. Esterified forms of steryl glucosides occur in some plants (LePage, 1964; Hou *et al.*, 1967), the acyl group being esterified to C-6 of the glucose moiety.

The function of the acylglucose is unknown. Its constancy during different stages of the growth cycle and the similarity of quantity per unit dry weight to the glucosyl diglycerides of *M. laidlawii* (Shaw *et al.*, 1968) suggest a structural role. These latter compounds do not turn over during metabolism of glucose in contrast to other lipids. The carotenyl glucoside, carotenyl esters, and phosphatidylglucose of *M. laidlawii* turn over rapidly during glucose metabolism (Smith, 1968) and have been postulated to serve in glucose transport (Smith, 1967). The same role has been ascribed to cholesteryl glucoside in strain J (Smith, 1963b). It is probable that the acylglucose in strain J possesses a structural function similar to the glucosyl diglycerides of *M. laidlawii*. An acylglucose possesses a structure analogous to these diglycerides and could effect an orientation postulated by Brundish *et al.* (1967) whereby such glycolipids could form clusters in which the hydrophilic regions would come together to form a pore extending through the membrane.

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Hydrogen–Deuterium Exchange of Cytochrome *c*. I. Effect of Oxidation State*

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ABSTRACT: The hydrogen–deuterium exchange of oxidized and reduced horse heart cytochrome *c* has been examined by infrared spectrophotometry. At pD 7.4, about 59% of the peptide hydrogens of ferrocytochrome exchange rapidly, *i.e.*, within 5 min, 20% slowly, and 21% are unexchanged after 24 hr. Comparable values for ferricytochrome are 68, 21, and 11%. The constant difference of 9–10% is in accord with previous observations that the oxidized and reduced cytochromes differ in conformation and that the reduced protein is the more compact (Margoliash, E., and Schejter, A. (1966), *Advan. Protein Chem. 21*, 113). Reversal of the redox condition of either cytochrome at the *initiation* of exchange instantly and completely alters the kinetic exchange curve to that typical of the new oxidation state. In contrast, oxidation of ferrocytochrome (or reduction of ferricytochrome) at intervals *after* initiation of exchange accelerates (or retards) the exchange reaction so as to *gradually* approach that characteristic of the new oxidation state. In this manner, it becomes apparent that the number of peptide hydrogens affected by the structural alteration is considerably larger than indicated from the 10% displacement of individual kinetic curves. This suggests that the oxidoreduction-induced change in conformation involves an appreciable portion of the cytochrome molecule.

Oxidation–reduction of the heme iron atom induces remarkable changes in the physicochemical properties of cytochrome *c*. Ferrocytochrome has greater thermal stability (Butt and Keilin, 1962) and resistance to proteolytic digestion (Nozaki *et al.*, 1957, 1958) than does ferricytochrome, and the two differ in antigenic characteristics (Margoliash *et al.*, 1967), in chemical reactivity of amino acid side chains such as methionine (Matsubara *et al.*, 1965) and tyrosine (Ulmer, 1966), and in the interaction of the heme group with ligands such as cyanide and azide (Theorell and Åkesson, 1941; Horecker and Kornberg, 1946; Horecker and Stannard, 1948). These, and related observations, have generally been interpreted to indicate that oxidized and reduced cytochrome *c* differ in conformation.

The nature of this conformational variation has been investigated recently by means of spectropolarimetry

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