4. The action patterns of P-enzyme and β-amylase are compared.

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The Occurrence of Methyl Ethers of Rhamnose and Fucose in Specific Glycolipids of Certain Mycobacteria

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Individual strains of mycobacteria may produce a large number of complex lipids and it is therefore of interest, from the point of view of strain identification, to inquire whether or not certain of these lipids are produced only by organisms of a particular type or species.

Randall & Smith (1953) isolated a previously unknown lipid from three strains of *Mycobacterium bovis* by adsorption chromatography. The substance was characterized by a distinctive infrared spectrum and was shown in this way to be absent from nine strains of *Mycobacterium tuberculosis* (see also Smith, Harrell & Randall, 1954). Moreover, Randall & Smith (1953) found that the lipids of two strains of *M. tuberculosis* could be distinguished one from the other by the combined techniques of

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adsorption chromatography and infrared spectroscopy (see also Kubica, Randall & Smith, 1956). Further application of these techniques led to the discovery of two additional specific lipids, one produced only by strains of *Mycobacterium avium*, the other produced only by photochromogenic acid-fast bacteria (Smith, Randall, Gastambide-Odier & Koevoet, 1957). The species-specific character of these lipids was confirmed (Smith, Randall, MacLennan, Putney & Rao, 1960*b*) by an examination of 70 strains of mycobacteria. These included *M. tuberculosis*, *M. bovis* and *M. avium*, photochromogens and non-photochromogens of the 'atypical' acid-fast group (Runyon, 1955, 1959) and a number of other species of mycobacteria.

It is now known that these specific substances are glycolipids that contain characteristic *O*-methylated 6-deoxyhexoses in glycosidic linkage (MacLennan, Smith & Randall, 1960; Smith *et al.* 1960*b*), and the general name of 'mycoside' has been proposed for them (Smith, Randall, MacLennan & Lederer, 1960*a*). The lipid moiety of mycoside A, the characteristic mycoside of photochromogenic strains, contains mycocerosic acid (see Noll, 1957) and an aromatic alcohol in ester linkage. Similarly, the lipid moiety of mycoside B, characteristic of *M. bovis*, contains a phenolic alcohol esterified with either mycocerosic acid or with a branched-chain acid of lower molecular weight (Smith *et al.* 1957, 1960*a*, *b*). Mycosides A and B do not contain nitrogen or phosphorus.

The present paper reports the occurrence and tentative identification of sugars in mycosides A and B. A preliminary report has already appeared (MacLennan *et al.* 1960).

MATERIALS AND METHODS

Bacteria. The strain of *M. bovis*, BCG-Phipps was obtained from Dr Max Lurie, Phipps Institute, Philadelphia, Pa., U.S.A. The photochromogenic strain, P-8, was provided by Dr E. Runyon, Veterans Administration Hospital, Salt Lake City, Utah, U.S.A.

Cultivation of bacteria. Organisms were grown at 37° in static culture in the medium of Wong & Weinzirl (1936). Cultures of P-8 were harvested at between 30 and 46 days, cultures of BCG-Phipps at 42 days.

Extraction of lipids. Lipids were extracted with ethanol–ether, washed, filtered and dried, as described in detail by Smith *et al.* (1960*b*).

Adsorption chromatography on columns. For the preparation of alumina an aq. 40% (w/v) NaOH soln. was added dropwise to an aq. 30% (w/v) $Al_2(SO_4)_3,18H_2O$ soln. until a faint permanent precipitate formed. A large excess of aq. NH_3 (sp.gr. 0·88) was then added. The precipitate was washed with tap water until free from NH_3 and then washed, successively, with four changes each of distilled water, methanol and ether. Finally, the preparation was heated at 100° for 1 hr.

Silicic acid was prepared by pouring commercial sodium silicate soln.–water (1:1, v/v) into an excess of 4 N-HCl. The precipitate was washed and heated as described for alumina.

Hyflo Super-Cel (Johns–Manville Co., New York, N.Y.) was washed successively with methanol and $CHCl_3$, then dried at 100°. It was mixed intimately with the adsorbents before these were packed into columns.

Adsorption chromatography on glass-fibre sheets. Sheets of glass-fibre paper (no. X-934-AH, H. Reeve Angel and Co. Inc., 52 Duane Street, New York, N.Y.; the Whatman glass-fibre filter paper GF/A has similar properties) measuring 18 cm. × 10 cm. were impregnated with silicic acid as described by Brown, Yeadon, Goldblatt & Dieckert (1957); or with alumina by the method used by Bush (1952) for the impregnation of paper, except that cold aq. 30% (w/v) $Al_2(SO_4)_3,18H_2O$ soln. was used and $CaCl_2$ was omitted from the washing procedure.

The impregnated papers, whether treated with alumina or silicic acid, were washed in running tap water and then, successively, in three changes each of distilled water, methanol and ether.

The adsorbent sheets, or 16 cm. × 2 cm. strips cut from them, were spotted with 50–200 μg. amounts of lipids (contained in about 10 μl. of $CHCl_3$ soln.) on a starting line drawn 2·5 cm. from the bottom of the paper. The papers were stood in a 1 cm. depth of solvent (benzene, $CHCl_3$, methanol, and mixtures of these solvents) until the solvent front reached the upper edge of the paper. The time taken was normally 20 min. for alumina and 1–2 hr. for silicic acid. The papers were then dried in air, sprayed with aq. 50% (v/v) H_2SO_4 and heated to dryness over a hot plate. Black deposits revealed the location of organic matter. Except in detail the method is that described by Dieckert & Reiser (1956) and Brown *et al.* (1957).

The reproducibility of R_F values from batch to batch of impregnated papers was poor. The practice was therefore introduced of using only those batches of paper which had the same degree of impregnation, as determined by direct weighing of equal areas, and the same adsorptive behaviour, as measured by the order and degree of separation of five Brockmann alumina standardization dyes.

Paper partition chromatography. Descending chromatograms were run on Whatman no. 1 paper and sprayed when the solvent front had travelled about 50 cm. from the starting line.

Paper electrophoresis. The method used was that described by Foster (1952) but without a special cooling mechanism.

Determination of 6-deoxyhexoses. The H_2SO_4–cysteine method of Dische & Shettles (1948) was used. In examining the specificity of this reaction (see below) both 3 and 10 min. heating periods were used and the tubes were read at 2 and 24 hr. (see Dische & Shettles, 1948).

By means of the H_2SO_4–cysteine reaction the amounts of 6-deoxyhexose in the mycosides were determined. Solutions of the mycosides in $CHCl_3$ were evaporated to dryness in tubes on a water bath. Water (1 ml.) was added and the test performed in the usual manner. The mycosides are immiscible with water but despite the heterogeneous hydrolysis system 10 min., or even 3 min., was an adequate heating period.

Infrared spectroscopy. Dry lipid samples were pressed to a suitable thickness on salt plates. The spectra were re-

corded over the 2–15 μ region on a Perkin–Elmer double-beam spectrophotometer, model 21. Full details have been presented elsewhere (Smith *et al.* 1960*b*).

RESULTS

Isolation of the mycosides

Mycosides A and B can be isolated from crude extracts by chromatography on columns of Magnesol (Smith *et al.* 1960*b*) or of silicic acid, preferably after a preliminary purification by precipitation at low temperature from solution in a chloroform–methanol mixture or by passage through an alumina column.

The progress of purification can be followed conveniently by infrared spectroscopy. The aromatic moieties of the mycosides contribute a distinctive infrared-absorption band at 6·6 μ to their spectra, so that the intensity of this band roughly indicates the proportion of mycoside present in a sample. As purification proceeds so the other characteristic features of the mycoside spectra appear.

Mycoside A. The method described by Smith *et al.* (1954) for the purification of mycoside A was followed, in general; a 10% (w/v) soln. of P-8 'total lipid' (10·3 g.) in chloroform–methanol (1:1, v/v) was left at 5° overnight. The precipitate that formed was discarded and the supernatant fluid cooled to −30° and left overnight. The precipitate (4·1 g.), enriched in mycoside A, was dissolved in chloroform (300 ml.) and silicic acid powder (100 g.) was added. The mixture was gently stirred for 24 hr. and filtered under suction. The residue was washed with chloroform (100 ml.) at the pump and then gently stirred in chloroform–methanol (300 ml.; 9:1, v/v) for 24 hr. After filtering and washing with the same solvent (100 ml.), the combined filtrates were evaporated to dryness. The dry lipid (1·5 g.) was dissolved in the minimum volume of chloroform and chromatographed with this solvent as developer on a column of silicic acid–Hyflo Super-Cel (1:1, w/w; 40 g.). After the emergence from the column of about 700 mg. of impurity, a fraction (700 mg.) rich in mycoside A was obtained. This was rechromatographed as before to yield 330 mg. of 'good' mycoside A. The material was dissolved in 2–3 ml. of chloroform and 50 ml. of methanol was added. The mixture was refluxed and then left at −30° overnight. The heavy flocculent precipitate that formed was filtered off, dissolved in chloroform and evaporated to dryness. The material was a hard, sticky, almost transparent solid that dried from solution in chloroform with a characteristic puckered surface. Mycoside A had m.p. 109°; $[\alpha]_D^{20} - 37 \pm 1°$ in chloroform (*c*, 1) (Found: C, 72·6; H, 10·8; OMe, 8·5%). Nitrogen and phosphorus were not detected. The mol.wt. (Rast) was roughly 1000. Fig. 1 (*a*) shows the infrared spectrum. The supernatant fluid from the second −30° precipitation yielded a yellow material, otherwise resembling mycoside A, on evaporation. The infrared spectrum was indistinguishable from that of mycoside A.

Mycoside B. An ethanol–ether extract (1 g.) of strain BCG-Phipps was dissolved in the minimum volume of chloroform–methanol (99:1, v/v) and chromatographed in this solvent on a column of alumina–Hyflo Super-Cel (1:2, w/w; 50 g.). A fraction (150 mg.) rich in mycoside B was collected and applied to a column of silicic acid–Hyflo Super-Cel (1:1, w/w; 40 g.). The column was developed first with chloroform and then with chloroform–methanol (99:1, v/v). Mycoside B (70 mg.) emerged in the second solvent; it dried as a wax with a pearly translucence. It had m.p. 23°; $[\alpha]_D^{20} - 26 \pm 1°$ in chloroform (*c*, 1) (Found: C, 77·1; H, 12·3; OMe, 4·0%). Nitrogen and phosphorus were not detected. The mol.wt. (Rast) was roughly 1000. Fig. 1 (*b*) shows the infrared spectrum of mycoside B.

Chromatographic test for homogeneity

Samples (50 μg.) of the mycosides were spotted on silicic acid-impregnated strips of glass-fibre paper and these were developed as chromatograms in stoppered tubes containing a range of benzene–chloroform and chloroform–methanol mixtures. Fig. 2 illustrates the relationship of R_F to solvent composition. In chloroform containing 0·1–1·0% (v/v) of methanol the R_F of mycoside B was always greater than that of mycoside A. When mixtures of the mycosides were chromatographed in chloroform–methanol (99:1, v/v) two distinct spots were obtained; the $R_{\text{mycoside B}}$ of mycoside A was 0·5–0·7 in this solvent.

Mycoside B gave no indication of inhomogeneity in any of the solvents tested but mycoside A gave two spots, one strong and one weak, estimated as roughly 5% of the total, in chloroform containing 0·5–2·0% (v/v) of methanol. Samples of the two components were isolated by elution with chloroform–methanol (9:1, v/v) from silicic acid chromatograms. The infrared spectrum of the slower, weak component differed in certain respects from that of the main component (mycoside A), although there was an overall similarity. When the major component was rechromatographed it gave a single spot indicating that the slower material did not arise from breakdown of mycoside A on the adsorbent.

The relative mobility of mycosides A and B on silicic acid was reversed on alumina-impregnated glass-fibre paper, although the substances migrated in the same range of solvent composition.

On chromatograms impregnated with either adsorbent the R_F values of the mycosides fell sharply and continuously in chloroform–methanol mixtures containing more than 50% (v/v) of methanol. This effect could be attributed only in part to the low solubility of the mycosides in methanol as it did not occur on unimpregnated glass-fibre paper until the methanol concentration reached 90% (v/v).

Sugar components of the mycosides

Samples of the mycosides were hydrolysed with N-sulphuric acid in sealed ampoules at 100° for 16 hr. The water-soluble fraction was then removed and the residual lipid hydrolysed again in the same manner. The pooled water-soluble fractions from the two hydrolyses were extracted twice with chloroform and then neutralized with barium hydroxide, centrifuged to remove barium sulphate, and evaporated to dryness *in vacuo* at room temperature. The syrup thus obtained was examined by the conventional methods of paper chromatography. The *p*-anisidine hydrochloride reagent (Hough, Jones & Wadman, 1950) revealed three sugars in mycoside A, but only one of these in mycoside B. It will be convenient to refer to these sugars as the *X*, *Y* (in

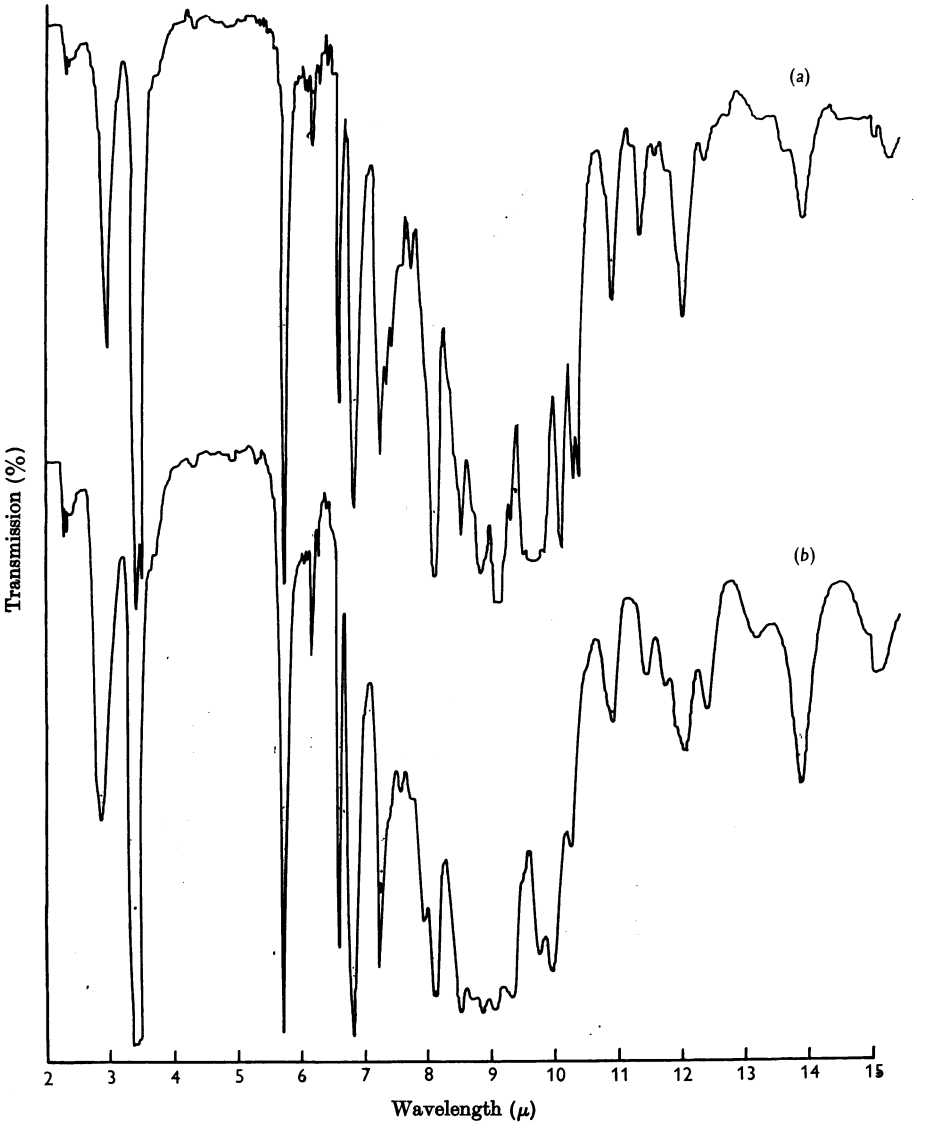

Fig. 1. Infrared-absorption spectra of mycobacterial glycolipids. (*a*), Mycoside A; (*b*) mycoside B.

mycoside B also) and *Z* sugars, in order of increasing *R* values. The three spots obtained with *p*-anisidine were of roughly the same intensity but those of *X* and *Y* had a reddish-brown colour, whereas *Z* gave a green–brown spot. The *R* values of all three sugars (Table 1) were sufficiently high to exclude from consideration all of the aldopentoses, aldohexoses and aldoheptoses and the common ketoses and deoxy-sugars.

The transient red colour reaction given by the mycoside sugars with a vanillin–perchloric acid spray reagent (Godin, 1954; Lambou, 1957; MacLennan, Randall & Smith, 1959) suggested that they were derivatives of one or more 6-deoxyhexoses. Spray reagents of broad specificity such as ammoniacal silver nitrate (Partridge, 1946) did not reveal substances other than the mycoside sugars in the water-soluble fraction of acid hydrolysed mycosides A and B and, since this fraction also contained the greater part of the methoxyl (Zeisel) originally present in the mycosides themselves, it appeared very probable that the *X*, *Y* and *Z* sugars were alkyl ethers of 6-deoxyhexoses. This interpretation was supported by the apparent stability of the sugar substituent to treatment with alkali. A sample of mycoside A (68 mg.) was heated with propan-2-ol (6·5 ml.), water (0·1 ml.) and potassium hydroxide (130 mg.) for 1 hr. under reflux in an atmosphere of nitrogen (Noll & Bloch, 1955). Much of the mycoside remained unsaponified after this treatment and subsequent acid hydrolysis liberated the three sugars as before.

Variation of the period of acid hydrolysis did not reveal additional sugars. Samples of the mycosides were hydrolysed with N-H_2SO_4 for ½ hr. at 100°. Water-soluble products were removed and fresh acid was added to the lipid residue. Hydrolysis was renewed for 4 hr., and again for periods of 8 hr. and 24 hr., removing the water-soluble products at each stage. Paper chromatography of the products from mycoside A revealed that at ½ hr. a preponderance of the *X* sugar was present, *Y* and *Z* being low or absent, whereas in the final hydrolysate the *X* sugar could not be detected and *Z* was in excess of *Y*. Since it had been shown (above) that the three sugars were present in mycoside A in roughly equal amounts these observations suggest that the sugars were released at different rates by acid hydrolysis. For mycoside B the *Y* sugar was detected at all stages of hydrolysis but *X* and *Z* were absent.

Isolation of the mycoside sugars

An additional sample of mycoside A was prepared from 12 g. of pooled ethanol–ether extracts from a number of photochromogenic strains. This sample, prepared by chromatography successively on alumina and on silicic acid columns as described above for mycoside B, gave an infrared spectrum indistinguishable from Fig. 1 (*a*) and yielded on hydrolysis three sugars chromatographically identical with those previously found.

The water-soluble fractions from four successive 16 hr. hydrolyses of mycoside A (380 mg.) were pooled, neutralized, concentrated and applied as a band along sheets of Whatman no. 1 paper that had previously been washed by running as chromatograms with distilled water as developing solvent for 48 hr. The chromatograms were developed with butan-1-ol–pyridine–water (6:4:3, by vol.) and the

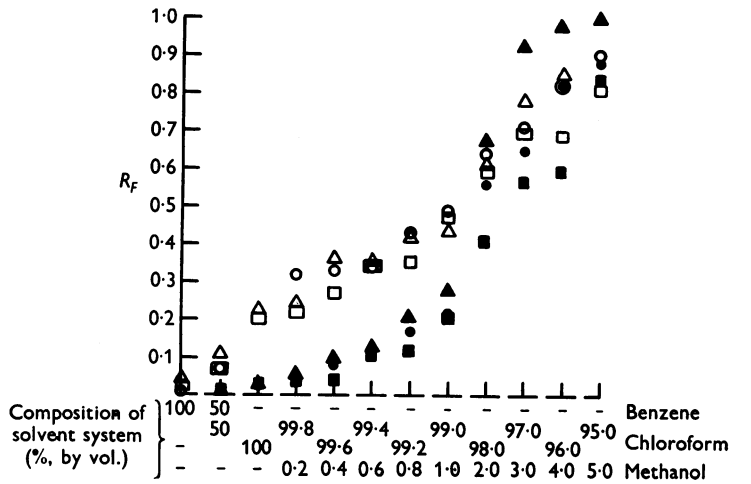

Fig. 2. Diagram illustrating variation of R_F values of mycosides A and B when chromatographed in various solvent systems on glass-fibre sheets impregnated with silicic acid. ●, ▲, ■, Replicate determinations of mycoside A; ○, △, □, corresponding replicates of mycoside B.

three sugars eluted separately with water after determining their location on the chromatograms by spraying marker strips with *p*-anisidine hydrochloride reagent. Subsequent chromatographic examination revealed that each of the three sugars was virtually free from contamination by the others.

Identification of mycoside sugars as derivatives of 6-deoxyhexoses

The *X*, *Y* and *Z* sugars each gave a rapidly fading orange–red colour when chromatograms were sprayed with a vanillin–perchloric acid reagent. An examination of the specificity of this reaction showed that six of the eight structurally isomeric 6-deoxyhexoses and several methyl ethers of rhamnose reacted, whereas the aldohexoses did not react (MacLennan *et al.* 1959). Different colour reactions were given by 6-deoxyhexitols, 2-deoxypentoses and 2-deoxyhexoses, 3-deoxyhexoses, 2:6- and 3:6-dideoxyhexoses. However, it is not certain that the test is entirely specific since 6-deoxyketohexoses were not tested and it appeared possible that some 7-deoxyaldoheptoses might also react like rhamnose (see below).

In another test for 6-deoxyhexoses (Dische & Shettles, 1948) the chromophore formed by the reaction of each of the *X*, *Y* and *Z* sugars with a sulphuric acid–cysteine reagent resembled that formed by rhamnose and by fucose in having an absorption peak in the range 370–430 mμ with a maximum at about 400 mμ. Hexoses do not give this reaction and pentoses can be distinguished from rhamnose (Dische & Shettles, 1948). The ketoses L-rhamnulose and L-fuculose are said to resemble rhamnose in the test (Wilson & Ajl, 1957).

The specificity of the sulphuric acid–cysteine reaction was examined further. All eight 6-deoxyhexoses were examined but since 6-deoxy-D-altrose was available only as 2:3:4-tri-*O*-benzoyl-6-deoxy α-D-altroside it was tested in this form, by using a solution in chloroform as described (above) for the intact mycosides. Although the eight sugars gave similar absorption curves with maxima at about 400 mμ there were striking quantitative differences. When the 396 – 426 mμ value of each sugar (30 μg.) was read against an L-rhamnose calibration curve (20–75 μg.), it appeared that L-rhamnose and L-fucose were quantitatively alike, whereas 6-deoxy-L-glucose gave 150–180 % of the L-rhamnose value and 6-deoxy-D-gulose about 120 %, in agreement with the earlier observations of Dische & Shettles (1948) and Gibbons (1955). Repeated tests on 6-deoxy-L-talose gave a figure of 150 % and, with much less certainty because of the very small number of tests performed, 6-deoxy-D-allose and 6-deoxy-L-idose gave 150–180 %, and 6-deoxy-D-altrose about 200 %. Although 6-deoxy-L-galactitol, 2-deoxy-D-galactose and 2:6-dideoxy-D-*ribo*-hexose did not resemble L-rhamnose in reaction (see also Gibbons, 1955) a sample of 7-deoxy-L-*glycero*-L-*galacto*-heptose was found to resemble L-rhamnose qualitatively but gave only about 1/15th of the 396 – 426 mμ value. It is possible that other 7-deoxyaldoheptose sugars might react more strongly, in view of the known variability within the 6-deoxyaldohexose series.

Mycosides A and B contained 26 and 6 % respectively of 6-deoxyhexose, estimated as L-rhamnose. The addition of a tenfold excess of phthiocerol dimycocerosate (Noll, 1957), which has at least one component in common with the lipid moiety of mycoside A, did not interfere with the test, beyond producing black specks which it was necessary to remove before measuring light-absorptions. In another test duplicate samples of mycoside A were hydrolysed with N-sulphuric acid for 24 hr. at 100°. The products were partitioned between chloroform and water and the 6-deoxyhexose content of both fractions was determined. Two-thirds of the 6-deoxyhexose found was present in the water-soluble fraction but the total 6-deoxyhexose value was similar to that found in direct determinations on mycoside A, again suggesting that the lipid moiety did not interfere.

Demethylation of the mycoside sugars

Seven methyl ethers having the pyranose ring can be formed from each of the eight structurally isomeric 6-deoxyhexoses. Many of these sugars have not been synthesized and very few have been found in Nature. Identification of an unknown methyl ether cannot therefore be entirely based on direct comparisons with authentic methyl ethers but must depend on an identification of the parent unsubstituted sugar, followed by a determination of the number and position of the methyl groups.

The mycoside sugars were demethylated with boron trichloride following generally the method of Allen, Bonner, Bourne & Saville (1958). An aqueous solution of the sugar, equivalent to 1–4 mg. of dry sugar syrup, was evaporated to dryness in a 5 ml. round-bottomed Quickfit flask. Dry dichloromethane (0·1–0·2 ml.) was added and the flask was cooled to −78° in powdered solid carbon dioxide. Boron trichloride was distilled into the flask through a Quickfit microdistillation apparatus, the condenser being cooled with air which had passed over solid carbon dioxide. The delivery tube from the condenser was drawn to a blunt tip within the collecting flask so that the drops of condensate delivered (0·1–0·2 ml.) could be counted. The final product was dissolved in water, run

slowly through a small column (0·5 g. in 5 mm. internal diam. tubing) of Amberlite MB-3 resin and concentrated to small volume before application to chromatograms.

Identification of the 6-deoxyhexoses

Authentic samples of the eight 6-deoxyhexoses were examined by paper chromatography in two solvent systems (MacLennan *et al.* 1959) and by paper electrophoresis in borate buffer at pH 9·5 as described by Foster (1952). With this combination of methods each of the sugars could be distinguished with certainty from the others, with the exception of the 6-deoxy-D-allose/6-deoxy-D-gulose pair which could be distinguished readily from other 6-deoxyhexoses but not from each other (these two sugars can be separated on chromatograms with a phenol–water solvent). Fig. 3 illustrates the order and degree of separation of the sugars achieved by the two methods. $R_{rhamnose}$ values on paper chromatograms in two solvent systems have been published elsewhere (MacLennan *et al.* 1959). Numerical values have not been assigned to the relative mobilities of the sugars on paper electrophoresis because variation in these values was greater than on paper chromatograms. However, the separations illustrated in Fig. 3 are based on comparisons of pairs of sugar examined as mixtures and also in adjacent positions on electrophoretograms. The degrees of separation of the sugars were rarely less than those illustrated (Fig. 3).

The demethylation product of the *Y* sugar gave two spots with the *p*-anisidine spray reagent on paper chromatography and paper electrophoresis. One spot was due to unchanged *Y* sugar, the other had *R* values corresponding to rhamnose. When rhamnose was added to demethylated *Y* before testing, the putative rhamnose spot was intensified but no new spot was formed. It was concluded that the *Y* sugar is a methyl ether of rhamnose.

The demethylation product of the *Z* sugar contained unchanged *Z* and rhamnose but a third spot was detected, which did not correspond to any of the 6-deoxyhexoses. It was subsequently found that *Z* was a dimethyl ether of rhamnose so that the unidentified spot was clearly due to a monomethyl ether arising from partial demethylation.

In a similar manner the demethylation product of *X* was identified as fucose. Unchanged *X* was also present.

Final identification of the mycoside sugars

Table 1 records some *R* values of the mycoside sugars and of a number of authentic *O*-methylated sugars, measured in two solvent systems. The solvent, containing ethanol, and the reference sugar tetramethylglucopyranose were chosen so that *R* values could be compared with the published results obtained when this solvent and reference compound were used in a study of 10 methyl ethers of fucose (Gardiner & Percival, 1958) and of the mono- and di-methyl rhamnopyranoses (Brown, Hough & Jones, 1950; Butler, Lloyd & Stacey, 1955).

The paper-electrophoretic mobilities of the mycoside sugars were compared with those of authentic sugar ethers, the criterion for dissimilarity being that a mixture of unknown and authentic sugar should give rise to two spots. The order of separation of the mycoside sugars on paper electrophoresis was the reverse of that on paper chromatography. All three sugars were slower than rhamnose, itself the slowest of the 6-deoxyhexoses.

Further distinctions among sugars with similar *R* values were made on the basis of colour reactions with spray reagents, for example *p*-anisidine (Hough *et al.* 1950; Brown *et al.* 1950; Butler *et al.* 1955; Andrews, Hough & Jones, 1955). The triphenyltetrazolium chloride–sodium hydroxide

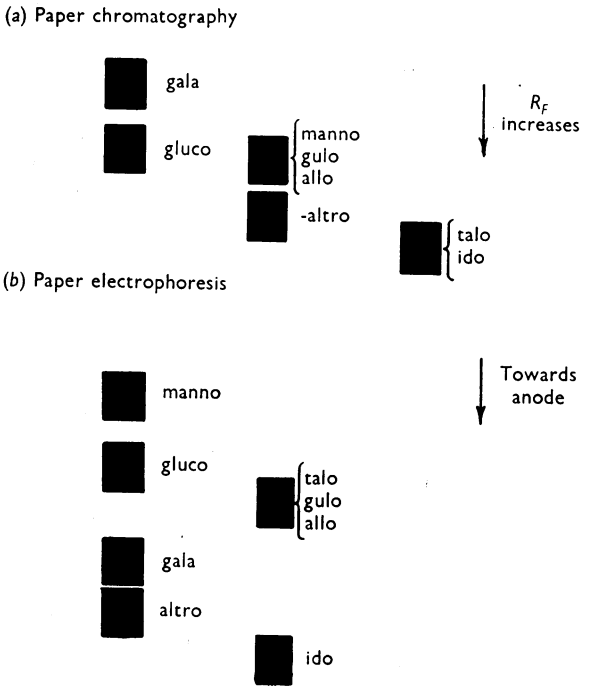

Fig. 3. Order and degree of separation of the 6-deoxyhexoses by paper chromatography (butan-1-ol–pyridine–water; 6:4:3, by vol.) and paper electrophoresis (borate buffer, pH 9·5). Each of the eight 6-deoxyhexoses is denoted by the configurational prefix of the corresponding aldohexose, e.g. manno-, 6-deoxymannose; allo-, 6-deoxyallose.

Table 1. *$R_{rhamnose}$ (R_{Rh}) and $R_{tetramethylglucopyranose}$ (R_{TMG}) values of methyl ethers of 6-deoxyhexoses*

Solvent 1, butan-1-ol–ethanol–water (4:1:5, by vol., upper phase); solvent 2, butan-1-ol–pyridine–water (6:4:3, by vol.). Chromatograms were sprayed with *p*-anisidine hydrochloride and heated at 110° for 3–5 min.

	Solvent 1		Solvent 2		
	R_{Rh}	R_{TMG}	R_{Rh}	R_{TMG}	Colour
X and 2-*O*-methylfucose	1·32	0·57	1·18	0·79	Red–brown
3-*O*-Methylfucose	1·18	0·53	—	—	Green–brown
3:4-Di-*O*-methylfucose	1·60	0·70	—	—	Green–brown
Y and 2-*O*-methylrhamnose	1·48	0·65	1·28	0·85	Red–brown
3-*O*-Methylrhamnose	1·36	0·60	—	—	Green–brown
Z and 2:4-di-*O*-methylrhamnose	1·96	0·86	1·46	0·96	Green–brown
2:3-Di-*O*-methylrhamnose	1·92	0·83	—	—	Red–brown
3:4-Di-*O*-methylrhamnose	1·98	0·88	—	—	Green–brown
2:3:4-Tri-*O*-methylrhamnose	2·32	0·99	—	—	Green–brown

spray (Wallenfels, 1950; Trevelyan, Procter & Harrison, 1950), which reacts only with those methylated aldoses in which position 2 is unsubstituted (for references see Gardiner & Percival, 1958) was of special value. The modified silver nitrate spray of Trevelyan *et al.* (1950) was found to react more slowly with 2-*O*-methyl sugars than with the 3-*O*-methyl compounds. In all tests where unknown sugars were compared with authentic compounds in their reactions with spray reagents, amounts were used which gave an intensity of reaction similar to that of the authentic sugar with *p*-anisidine.

The X *sugar.* This methyl ether of fucose behaved like a sample of 2-*O*-methylfucose in all tests applied. Paper chromatography and electrophoresis clearly distinguished the *X* sugar from 3-*O*-methyl-D-fucose (D-digitalose) on the basis both of *R* values and colour reaction (Table 1). Since the *R* values for 2- and 3-*O*-methylfucose and for 3:4-di-*O*-methylfucose (Table 1) are similar to those found by Gardiner & Percival (1958) under the same test conditions, it can be inferred from their data for other methyl ethers of fucose that the *X* sugar is neither a dimethylfucose nor 5-*O*-methylfucose. The *X* sugar, unlike 3-*O*-methylfucose, did not react with the triphenyltetrazolium chloride–sodium hydroxide spray. Since 3:4-di-*O*-methylfucose reacts (Gardiner & Percival, 1958), 4-*O*-methylfucose would also be expected to react. Therefore it was concluded that the *X* sugar is very probably 2-*O*-methylfucose. The *X* sugar reacted slowly with silver nitrate (Trevelyan *et al.* 1950) as did 2-*O*-methylglucose and 2-*O*-methylrhamnose but in contrast with the rapidly reacting 3-*O*-methyl ethers of glucose, fucose and rhamnose.

The Y *sugar.* This methyl ether of rhamnose could not be distinguished from 2-*O*-methylrhamnose in any of the tests applied. On paper chromatography and electrophoresis the *Y* sugar was readily distinguished in *R* from 3-*O*-methylrhamnose and from the three dimethylrhamnopyranoses (Table 1). It was therefore believed to be either 2- or 4-*O*-methylrhamnose because 5-*O*-methylrhamnose would be expected to have a relatively high *R* in the light of behaviour in the fucose ether series (Gardiner & Percival, 1958). The *Y* sugar did not react with the triphenyltetrazolium chloride–sodium hydroxide spray and reacted slowly with silver nitrate (Trevelyan *et al.* 1950); it was therefore considered to be 2-*O*-methylrhamnose.

The Z *sugar.* The high *R* values and low electrophoretic mobility of this methyl ether of rhamnose suggested that it might be a di-*O*-methylrhamnose. In agreement with this, demethylation yielded not only rhamnose but a second product which behaved as a monomethylrhamnose.

Butler *et al.* (1955) showed that 2:4- and 3:4-di-*O*-methylrhamnose had similar *R* values on paper chromatograms, whereas 2:3-di-*O*-methylrhamnose was distinctly slower and gave a characteristic reddish colour with aniline trichloroacetate. On paper electrophoresis, on the other hand, both the 2:3 and 2:4 diethers had low, similar mobilities, whereas the 3:4 diether had a high mobility. By this combination of methods each of the three sugars could be distinguished from the other two. The *Z* sugar was compared in these tests with authentic samples of the three sugars and was indistinguishable from 2:4-di-*O*-methylrhamnose. Moreover, demethylation of authentic 2:4-di-*O*-methylrhamnose yielded products whose behaviour on paper chromatography and electrophoresis was identical with those formed from the *Z* sugar.

The *Z* sugar and 2:4-di-*O*-methylrhamnose did not react with the triphenyltetrazolium chloride–sodium hydroxide spray or with a periodate–benzidine spray (Cifonelli & Smith, 1954); 3:4-di-*O*-methylrhamnose reacted with both reagents. The reaction of the *Z* sugar with silver nitrate (Trevelyan *et al.* 1950) was very slow. It was concluded that the *Z* sugar is 2:4-di-*O*-methylrhamnose.

DISCUSSION

Mycosides A and B differ from the majority of known mycobacterial lipids in that they are type specific (Smith *et al.* 1957, 1960*b*). A distinctive chemical feature is the presence of characteristic *O*-methylated 6-deoxyhexoses. Several sugars of this class occur also in a group of glycolipids, generically named mycoside C, produced only by strains of *M. avium* (Smith *et al.* 1957, 1960*a*, *b*); none of these sugars are the same as those found in mycosides A and B (A. P. MacLennan, unpublished observations). Outside the order Actinomycetales, strains of *Pseudomonas aeruginosa* produce a glycolipid composed of two molecules each of L-rhamnose and β-hydroxydecanoic acid (Jarvis & Johnson, 1949; Hauser & Karnovsky, 1957).

The sample of mycoside A used for analysis was not pure. It contained an estimated 5% of a substance whose infrared spectrum differed in certain respects from that of the bulk of the material. As mycoside A contains roughly equal quantities of its three sugar components, that is to say about 8% of each, it is plain that the impurity could not contribute to mycoside A the whole of any one of the sugars detected. It can also be said definitely that the 2-*O*-methylrhamnose found in mycoside A could not arise from the presence in this substance of mycoside B; the two mycosides were shown to have distinctly different *R* values on silicic acid-impregnated glass-fibre chromatograms.

Apart, however, from the presence of a minor impurity and the demonstrable absence of mycoside B, the question remains whether or not mycoside A is a single glycolipid containing three sugars or a mixture of glycolipids each containing one or more of the three sugars.

The minimum molecular weights of mycosides A and B, calculated from their measured contents of 6-deoxyhexose, and assuming that each is a single molecular species, are 1800 and 2700 respectively. These results are at variance with the measured molecular weights (Rast) of about 1000 for both compounds, but measurements of depression of freezing point can give erratic and erroneous results in the case of glycolipids (Asselineau, Choucroun & Lederer, 1950; E. Lederer, personal communication).

Until 1954 it could be said that only 3-*O*-methyl sugars had been found in Nature (Garrod & Jones, 1954) but since this time other methylated sugars have been discovered in bacteria and other organisms. The rhamnose ethers of the mycosides A and B have not been found elsewhere in Nature, so far as we are aware, but 2-*O*-methylfucose occurs widely in plant tissues (Andrews & Hough, 1958).

The scheme for the identification of *O*-methylated 6-deoxyhexoses presented here relies in the first place on the high specificity of the Dische & Shettles (1948) test and the vanillin–perchloric acid spray reagent (MacLennan *et al.* 1959) in revealing the class of substituted sugar. The technique of demethylation with boron trichloride (Allen *et al.* 1958) applied to samples (1–4 mg.) of sugar ether yields sufficient parent sugar to permit its identification by a combination of paper chromatography and paper electrophoresis, and may also provide information on the degree of methylation of the sugar. Other paper-chromatographic techniques for distinguishing among the 6-deoxyhexoses and among their 3-*O*-methyl ethers have been reported by Isherwood & Jermyn (1951) and by Krauss, Jäger, Schindler & Reichstein (1960). Finally, information on the number and position of substituent methyl groups may be provided by a number of specific spray reagents, notably triphenyltetrazolium chloride–sodium hydroxide (Trevelyan *et al.* 1950) and periodate–benzidine (Cifonelli & Smith, 1954), coupled with measurements of chromatographic and electrophoretic behaviour.

Mention should be made of the possible relationship between the mycosides and the previously described leprosols (Uyei & Anderson, 1931; Crowder, Stodola & Anderson, 1936; Butenandt & Stodola, 1939). The leprosols were isolated (Crowder *et al.* 1936) from ethanol–ether extracts of an organism originally believed (Uyei & Anderson, 1931) to be the leprosy bacillus. It would now be said (*Bergey's Manual*, 1957) that the 'highly chromogenic' organism which 'grows well on a synthetic medium' was not *Mycobacterium leprae*. Mycoside A and α-leprosol resemble each other in their resistance to saponification, their methoxyl content, melting points and ultraviolet-absorption spectra. Moreover, the leprosols are reported as giving a red colour with a vanillin–sulphuric acid reagent (Butenandt & Stodola, 1939). These resemblances have been shown to be very probably fortuitous: a sample of normethyl-β-leprosol kindly provided by Dr F. H. Stodola did not react in the Dische & Shettles (1948) test and its infrared spectrum was very different from that of mycoside A. Moreover, the leprosols give a positive reaction with a ferric chloride reagent, mycosides A and B do not (E. Lederer, personal communication).

SUMMARY

1. A glycolipid was isolated from ethanol–ether extracts of photochromogenic strains of 'atypical' acid-fast bacteria. It contained 2-*O*-methylfucose, 2-*O*-methylrhamnose and 2:4-di-*O*-methylrhamnose.

2. Another glycolipid, obtained from a strain of *Mycobacterium bovis*, contained 2-*O*-methylrhamnose alone.

3. A scheme for the tentative identification of small amounts of *O*-methylated 6-deoxyhexoses is described.

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Nicotinamide Mononucleotide Adenylyltransferase of Pig-Liver Nuclei

THE EFFECTS OF NICOTINAMIDE MONONUCLEOTIDE CONCENTRATION AND pH ON DINUCLEOTIDE SYNTHESIS

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Kornberg (1948, 1950) found that extracts of pig liver catalysed the reaction:

Nicotinamide mononucleotide + adenosine triphosphate ⇌ nicotinamide–adenine dinucleotide + pyrophosphate

owing to the presence of nicotinamide mononucleotide adenylyltransferase (nicotinamide–adenine dinucleotide pyrophosphorylase). Hogeboom & Schneider (1952) and Branster & Morton (1956) found that this activity was associated with the