240. *The Constitution of a Specific Somatic Polysaccharide from* M. tuberculosis (*Human Strain*).

By Sir Norman Haworth, P. W. Kent, and M. Stacey.

Two stable serologically specific polysaccharide fractions (having $[\alpha]_D^{18°}$ + 85° and $[\alpha]_D^{18°}$ + 25° in water respectively) together with a deoxyribonucleic acid derivative and bacterial glycogen have been isolated from heat-killed cells of *M. tuberculosis* (human strain).

The specific polysaccharide having $[\alpha]_D^{18°}$ + 85° was shown to originate mainly from the somatic part of the cell, while the specific polysaccharide having $[\alpha]_D^{18°}$ + 25° was shown to occur in close association with the cell lipoids.

The specific somatic polysaccharide was smoothly converted into its methyl ether, hydrolysis of which yielded 3 : 5-dimethyl methyl-D-arabofuranoside (21·9%), 3 : 4 : 6-trimethyl methyl-D-mannopyranoside (26·6%), 3 : 4-dimethyl methyl-D-mannopyranoside (20·2%), 2 : 3 : 4-trimethyl methyl-L-rhamnopyranoside (24·8%), and a methylated amino-sugar derivative (5·8%). This polysaccharide thus has a highly branched structure composed of mannopyranose, arabofuranose, and amino-sugar units with rhamnopyranose units forming terminal residues. Osmotic pressure measurements indicated a molecular weight of approximately 12,000.

For the first time D-arabofuranose was conclusively identified as a component of a naturally-occurring substance.

Complex carbohydrates of *M. tuberculosis* have been isolated from three sources, namely, from the somatic portion of the cell, from the cell lipoids, and from the culture medium.

Laidlaw and Dudley (*Brit. J. Exp. Path.*, 1925, **6**, 197) separated a specific somatic polysaccharide, glycogen, and nucleic acid by the action of dilute acid upon heat-killed organisms. The specific polysaccharide ($[\alpha]_D$ + 67°) reacted with serum from tuberculous animals in high dilution, and the authors reported that it gave positive tests for pentose and methyl pentose.

A similar dextrorotatory ($[\alpha]_D$ + 72°) specific polysaccharide was obtained by Maxim (*Biochem. Z.*, 1930, **223**, 404).

Heidelberger and Menzel (*Proc. Soc. Exp. Biol. Med.*, 1932, **29**, 631) separated a number of fractions by acid extraction of autolysed cells. It was shown that the principal serologically active fraction ($[\alpha]_D$ + 85°) contained D-arabinose and D-mannose. These authors (*J. Biol. Chem.*, 1937, **118**, 79; 1939, **127**, 221) found also a second specific somatic polysaccharide having a low dextrorotation.

The presence of D-galactose in polysaccharides of *M. tuberculosis* was shown by Gough (*Biochem. J.*, 1932, **26**, 248). Although the polysaccharides exhibited weakly acidic reactions, no glycuronic acid constituent could be detected among the products of hydrolysis.

Two serologically active polysaccharides were isolated by Chargaff and Schaefer (*J. Biol. Chem.*, 1935, **112**, 393) who showed that both contained D-arabinose and D-mannose, and reported for the first time that one contained a small amount of glucosamine.

Pentose-containing polysaccharides in the filtered culture medium were investigated by Meuller (*J. exp. Med.*, 1926, **43**, 9) who showed that some fractions had high serological activity.

The separation of polysaccharide from protein components was achieved electrophoretically by Seibert (*ibid.*, 1938, **68**, 413; *J. Biol. Chem.*, 1941, **140**, 55) who demonstrated the presence of at least two polysaccharides in the culture medium. One of these was a serologically active, nitrogen-containing substance with a molecular weight of 9000 (cf. the value of 7300 found by Tennet and Watson, *J. Immunol.*, 1942, **45**, 179).

In 1930, Masucci, McAlpine, and Glenn (*Amer. Rev. Tuberc.*, 1930, **22**, 699, 678) isolated a polysaccharide, $[\alpha]_D$ + 67°, of high serological activity from the culture medium, and showed that it contained D-arabinose and D-mannose.

Polysaccharides have been derived from a third source in *M. tuberculosis*, namely from the lipoid constituents. A systematic study of this group has been made by Anderson and his co-workers (*Chem. Reviews*, 1941, **29**, 225). From the phosphatide fraction a trisaccharide containing mannose and inositol was isolated, and in the acetone-soluble fats there were constituents which appeared to be esterified with trehalose.

The investigation showed that the principal lipoid component of the cells was a wax-like substance which, on alkaline hydrolysis, yielded a mixture of fatty acids and serologically active polysaccharide in which D-mannose, D-galactose, D-arabinose, and a trace of glucosamine were identified. The polysaccharide ($[\alpha]_D$ + 30°) reacted with immune serum in a high dilution (cf. following paper).

In spite of the immense number of polysaccharide fractions of seemingly divergent properties,

there appear to be two main fractions throughout. Both contain traces of nitrogen and phosphorus, D-arabinose, and D-mannose, and one has a lower pentose content and higher dextrorotation than the other.

No structural studies of any of the polysaccharides described have so far been attempted. It was the purpose of this present work to prepare relatively stable specific polysaccharide fractions in an essentially homogeneous form and to elucidate as far as possible some details of their configuration in order to gain information on possible immunising complexes of the tubercle bacillus.

In an extended search for high molecular complexes which might possess wider immunological properties than those of the hapten polysaccharides, there was obtained from a urea extract of heat-killed cells of *M. tuberculosis* a compound which contained a polysaccharide, $[\alpha]_D^{18°} + 28°$, in close association with deoxyribonucleic acid and lipoids, including the " acid-fast " mycolic acid.

This polysaccharide, the " lipoid-bound " polysaccharide, described in the following paper, could be isolated from the complex by means of alkaline hydrolysis, and fractionation methods showed it was essentially homogeneous. This lipoid-bound polysaccharide reacted with tuberculosis anti-serum in high dilution (1 : 2,000,000) when submitted to the precipitin test. When the main lipoid constituents had been removed by means of urea from heat killed *M. tuberculosis* cells, there remained the somatic portion of the cells from which on strong alkaline hydrolysis a second specific polysaccharide the " somatic " polysaccharide, $[\alpha]_D^{18°} + 85°$, was isolated.

Isolation of the Polysaccharides.—As a first approach to the problem of procuring relatively large quantities of both polysaccharides, it was considered that stable fractions, which would not readily undergo further degradation, could be obtained on alkaline hydrolysis of the whole cells so that by subsequent fractionation methods, using chiefly precipitation from solvents, reasonably homogeneous material might be obtained.

In view of the high lipoid content of the cells, as much fatty material as possible was first removed with organic solvents (ether, acetone, etc.), and then the dry partly defatted residue was warmed with alkali which served to liberate the more firmly bound lipoids and enabled the polysaccharides to pass into solution. The solution containing the polysaccharide was acidified, and some insoluble matter separated off on the centrifuge. The liquid was then freed from inorganic and other low-molecular impurities by dialysis. To the resulting solution were added several volumes of alcohol, and the crude polysaccharide thus thrown down was again dialysed and reprecipitated from an acid solution by addition of alcohol or acetone. The initial polysaccharide mixture was dextrorotatory, contained small amounts of nitrogen and phosphorus, and had weak acidic properties. It gave also a positive Dische test (Dische, *Z. Mikrochem.*, 1910, **8**, 4) for deoxyribonucleic acid, the presence of which or a derivative thereof was confirmed by an absorption band at 2650 A.

It was clear at this stage that the crude product was a mixture of various substances.

Purification. In a typical separation the polysaccharide mixture was resolved into various constituents by fractional precipitation from an aqueous solution with alcohol. Ten initial fractions were separated the first of which was composed mainly of inorganic matter. The second and third fractions contained a substance which imparted a reddish-brown or purple coloration with iodine and which closely resembled the glycogen isolated by earlier workers (Chargaff and Schaefer, 1935, *loc. cit.*; Heidelberger and Menzel, 1937, *loc. cit.*). The three fractions which then separated had essentially similar properties, appeared to be fairly homogeneous, were free from glycogen, and contained only traces of nucleic acid. The somatic polysaccharide was present in almost pure state in these first precipitates ($[\alpha]_D + 80°$).

A mixed fraction was next separated, and finally another three fractions, having similar properties, were separated; these ($[\alpha]_D + 25°$) were mainly composed of a second polysaccharide, which was identical with the lipoid-bound polysaccharide. This again gave a strong test for deoxyribonucleic acid and also had a strong absorption band (at 2650 A.).

The somatic polysaccharide was refractionated by alcoholic precipitation from an aqueous solution. The sub-fractions obtained all had closely similar properties though a trace of deoxyribonucleic acid still remained throughout. This impurity was not precipitated by addition of lanthanum acetate or acetic acid, and it was probable therefore that a deoxyribonucleoside was a contaminant. It was finally removed as described later during the course of methylation and fractionation of the methylated derivatives.

In the case of the lipoid-bound polysaccharide, the nucleic acid derivative was removed by passing an aqueous solution of the fraction repeatedly through ion-exchange resins. The

purified product was recovered in good yield by alcoholic precipitation of the acidified final solution.

Examination of both polysaccharides revealed that the weak acidity was correlated with the nitrogen and phosphorus contents and was doubtless due to the presence of a trace of a nucleic acid derivative. Preliminary qualitative examination showed that both polysaccharides contained a pentose and a small amount of an amino-sugar, and that both products were readily hydrolysed by dilute acids to give a mixture of reducing sugars.

Nature of the Somatic and Lipoid-bound Polysaccharide Fractions.—The place of origin of the two polysaccharide fractions in the bacterial cell was further studied by exhaustive removal of the ether-soluble lipoids from the cells. These cells were extracted with warm dilute alkali, and the polysaccharide isolated from this solution consisted almost entirely of the somatic fraction (above). On the other hand saponification of the numerous samples of the lipoidal constituents of the cell, separated by various methods, afforded only a polysaccharide having properites closely resembling those of the polysaccharide originally isolated, and in no case was any material having a high dextrorotation isolated from the lipoidal " capsular " material. It was thus confirmed that the polysaccharide having $[\alpha]_D + 85°$ is mainly of somatic origin, and that the polysaccharide having $[\alpha]_D + 25°$ is associated with the lipoids which occur in what is termed the waxy layer of the cell.

On further prolonged treatment of either of the purified polysaccharides with alkali, no changes could be detected and the fractions were recovered intact from solution. Thus it is considered that these two substances represent alkali-stable components of the specific polysaccharides of the cell. Further, when both fractions were separately submitted to the precipitin test with human tuberculous antisera, they reacted in a dilution as high as 1 : 2,000,000. This result indicates the specific nature of the polysaccharide fractions, and although, owing to the vigorous mode of their isolation, some degradative changes were likely, the fact is established that part at least of those portions of the molecule responsible for the reaction with immune serum are relatively alkali-stable. In view of this and of the fact that the fractions do not undergo detectable changes on prolonged treatment with alkali, it is reasonable to suppose that this part of the structure of the polysaccharide molecule was not appreciably modified during the subsequent process of methylation described herein.

Methylation of the Somatic Polysaccharide.—The polysaccharide was smoothly methylated by methyl sulphate in the presence of sodium hydroxide. The product had a methoxyl content of *ca.* 40% after six treatments, and this value was not increased by further methylations. The methylated derivative, obtained in the form of a brown gum, was purified by repeated fractional precipitation from organic solvents. The principal fractions had essentially identical properties, *i.e.*, methoxyl content (39·2%), optical rotation $[\alpha]_D^{18°} + 125°$ in chloroform, a very low viscosity, nitrogen, 0·3%, phosphorus content, nil.

A typical sample of the main bulk of the fractionated methylated polysaccharide was readily hydrolysed by means of methanolic hydrogen chloride, and the mixture of glycosides so obtained was separated by distillation in a high vacuum into a series of fractions. The still-residue was rehydrolysed with methanolic hydrogen chloride and the products fractionally distilled as before. A study of the resulting glycosidic fractions showed that five principal constituents were present and these were characterised as follows :

(*a*) 2 : 3 : 5-Trimethyl methyl-L-rhamnoside, identified by conversion into the free sugar and thence into the anilide, identical with that made under similar conditions from 2 : 3 : 4-trimethyl L-rhamnose.

(*b*) A dimethyl methylpentoside fraction which underwent rapid hydrolysis with N/20-sulphuric acid, a change which suggested the presence of furanose structures. The free sugar was oxidised by bromine to the 1-carboxylic acid which was converted *via* the lactone into 3 : 5-dimethyl D-arabonamide. On further methylation, the dimethyl methylpentoside gave a trimethyl methylpentoside which after hydrolysis was oxidised with bromine water. The product afforded a crystalline specimen of 2 : 3 : 5-trimethyl D-arabonamide.

(*c*) A fraction, having properties corresponding to those of a trimethyl methylhexoside, was converted on further methylation and subsequent hydrolysis into 2 : 3 : 4 : 6-tetramethyl D-mannose from which was formed the anilide. This fraction was hydrolysed by being heated with N-sulphuric acid, and crystalline 3 : 4 : 6-trimethyl D-mannopyranose was isolated.

(*d*) The fourth main constituent was methylated further and hydrolysed to a free sugar which was readily converted into 2 : 3 : 4 : 6-tetramethyl D-mannose anilide. Hydrolysis of this fraction with N-sulphuric acid afforded crystalline 3 : 4-dimethyl D-mannopyranose.

(*e*) A small quantity of an amino-sugar derivative was isolated in the following way :

specimens of the nitrogen-containing glycosidic fractions were mixed and warmed at 100° with 2N-sodium hydroxide for 8 hours in order to remove the *N*-acetyl group. The liquid was neutralised and exhaustively extracted with ether. The ethereal solution, after being dried, was saturated with dry hydrogen chloride, and a syrupy hydrochloride thereby precipitated. This was separated, washed with ether, dried, and *N*-acetylated with methanol and acetic anhydride. A small amount of crystalline product was isolated. The syrupy hydrochloride had a methoxyl content of 32·4% (suggesting dimethyl methylhexosamine hydrochloride). The substance has not yet been identified.

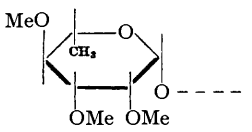
Careful search among the residual syrups gave further small amounts of the above substances but failed to reveal any constituents other than (*a*), (*b*), (*c*), or (*d*).

By reference to the refractive indices and methoxyl contents of the glycosidic fractions a quantitative assay of the amounts of the various constituents was made, the amount of the amino-sugar derivative being calculated also from the nitrogen contents of the various fractions. The following results were obtained: 3 : 5-dimethyl methyl-D-arabinoside (22·0%); 2 : 3 : 4-trimethyl methyl-L-rhamnoside (24·7%); 3 : 4 : 6-trimethyl methyl-D-mannoside (26·8%); 3 : 4-dimethyl methyl-D-mannoside (20·2%); a dimethyl methyl-2-acetamidohexoside (5·9%). This corresponded to a molecular proportion of 25%, 25%, 25%, 20%, and 5% respectively which clearly indicates a molecular ratio of 5 : 5 : 5 : 4 : 1.

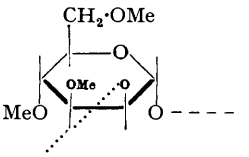
Discussion.

The isolation of a 2 : 3 : 4-trimethyl methyl-L-rhamnoside in 25% yield from the methylated polysaccharide indicates that this sugar can only be linked glycosidically through the position 1, and therefore the whole of the L-rhamnopyranose constituents form end groups linked as in (I).

By the identification of 3 : 4 : 6-trimethyl methyl-D-mannopyranoside it is clear that D-mannopyranose is a constituent unit of the polysaccharide chain and is linked through the 1 and 2 position as in (II). Other mannose units, also in the pyranose form, are linked as in (III) through the 1, 2, and 6 position as deduced from the isolation of 3 : 4-dimethyl methyl-D-



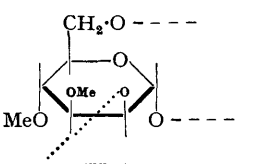
(I.)



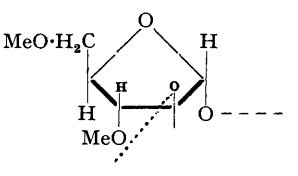
(II.)

mannoside. Since an appreciable amount (20%) of this dimethyl mannose is present the polysaccharide must have a highly branched structure, and this is in keeping with the high percentage (25%) of terminal residues isolated.

The occurrence of 3 : 5-dimethyl methyl-D-arabinose is of considerable importance, since it is the first time that the presence of this rare sugar in the furanose form has been conclusively demonstrated in a substance of biological origin. This pentose must be linked through the 1 and 2 positions as in (IV).



(III.)

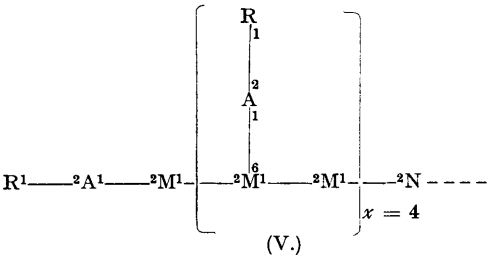


(IV.)

The mode of linkage of the amino-sugar has not yet been decided, but it is clearly not an end group.

In the light of these results it will be seen that several structures for the somatic polysaccharide are possible, one type being shown in (V). In this representation all those linkages engaging the glycosidic groups are temporarily depicted as being in the α-form since the direction of the change of rotation on acid hydrolysis of both the original polysaccharide ($[\alpha]_D$ + 85° ⟶ 22°) and its methylated derivative ($[\alpha]_D$ + 125° ⟶ + 47°) is in a negative direction.

Based on the nitrogen content of the methylated polysaccharide it will be seen that if a single amino hexose unit only is present in one molecule of the polysaccharide, then x will be 4.



(V.)

R represents L-rhamnopyranose M represents D-mannopyranose.
A ,, D-arabofuranose. N ,, an amino-hexose.

(The numerals represent the points of attachment.)

The structure of the somatic polysaccharide in (V), elucidated by the methylation technique, represents only the type of the minimum repeating pattern of the entire polysaccharide. The somatic polysaccharide molecule may be any multiple of the suggested structure. In the case of this somatic polysaccharide, that part of the macromolecule shown in (V) may be linked glycosidically through its amino-sugar residue to one constituent of a second similar repeating unit. Extension of this arrangement would indicate more completely the macromolecular structure, information on which might be obtained from osmotic pressure and other physical determinations.

Osmotic Pressure Investigation.—A method for the determination of the molecular weight of substances soluble in organic solvents has been developed by Mr. C. Graff-Baker in our department, based on the earlier findings of Carter and Record (*Chem. and Ind.*, 1936, **55**, 218). A full description of the apparatus and the experimental procedure is to be published later.

Investigation of the osmotic behaviour of chloroform solutions of the fractionated methylated somatic polysaccharide (OMe, 39·2%; $[\alpha]_D$ + 125° in chloroform) indicated that the minimum molecular weight of the methylated derivative was 12,000. The molecular weight of the methylated fraction as derived from (I) is 3898. Thus it is clear that at least 3 such residues are joined together in the intact polysaccharide molecule.

Periodate Oxidation.—Some results kindly obtained for us by Dr. J. K. N. Jones and Mr. T. G. Halsall in Professor Hirst's laboratory are of particular interest. These workers have developed reliable methods for the determination of the number of end-groups in polysaccharide molecules (Hirst *et al.*, *Nature*, 1945, **156**, 785). In the method, the polysaccharide is submitted to oxidation by periodate ions. For each pyranose end group of the polysaccharide, one mole of formic acid is liberated. From the amount of formic acid liberated and the amount of periodate taken up in the oxidation, the number of end-groups can be calculated.

This method has been applied to the somatic polysaccharide. The experimental value, shown below, for the somatic polysaccharide indicates the presence of 4 rhamnose end-groups in every 20 sugar units, as compared with 5 rhamnose end-groups found by the methylation technique and shown in (V).

Thus, accepting the osmotic pressure measurements by which three such structures are shown to be mutually linked (*e.g.*, through the amino sugar and through a rhamnose unit), it is clear that the somatic polysaccharide will have at least 13 end-groups in every 60 sugar units. This is in close agreement with periodic oxidation results.

Experimental.

The cells used consisted of a human strain of *M. tuberculosis* grown on a synthetic medium, and were obtained from the Ministry of Agriculture and Fisheries laboratories (Weybridge), through the courtesy of Dr. Green and his colleagues.

Isolation of a Crude Polysaccharide Mixture.—Moist steam-killed cells (856 g.) were extracted with acetone (1 l.) for 72 hours in a Soxhlet apparatus. The residue was then extracted with ether until no further lipoidal material was removed. Evaporation of acetone and ethereal extracts afforded 31 g. of lipoids.

The dry mainly-defatted cells were warmed with sodium hydroxide (1 l. of N) for 110 hours. The solid matter was separated and the supernatant liquor was acidified and centrifuged. To the liquid so obtained was added alcohol (3 vols.) and the crude polysaccharides removed. This material ($[\alpha]_D^{14°}$ + 36·6°; ash content 31·8%) gave strong Molisch and Bial's (pentose) tests.

The crude product was dissolved in water (250 c.c.), acidified (Congo-red) with dilute hydrochloric

acid, and dialysed in a cellophane membrane. The contents of the membrane were centrifuged and the supernatant liquid treated with alcohol (5 vols.). The product was separated and dried ($[\alpha]_D$ + 85°; 4 g.; ash content 0·6%).

By repetition of this typical experiment, there were obtained 40 g. of starting material which gave positive Molisch, Dische, and phosphate tests and had $[\alpha]_D^{19°}$ + 52·5° in water. This material had an acid equivalent of 2,064, and ash content of 3·9%, and gave a red coloration with iodine.

The ash content could be reduced still further (0·6%) by repeated precipitation from an aqueous acid solution or by prolonged dialysis of an aqueous solution.

Fractionation of the Crude Polysaccharide.—The material was fractionated in quantities of 10 g., each dissolved in 100 c.c. of water. Alcohol was added dropwise to the aqueous solution until a precipitate appeared. This was separated, and more alcohol added to the supernatant liquor until a second precipitate was obtained. Repetition of this procedure afforded 9 fractions, a tenth fraction being isolated by the evaporation of the final alcoholic mother liquors (see Table I).

TABLE I.

No.	Wt. (g.).	$[\alpha]_D$.*	Ash, %.	Bial.	Molisch.	Fehling.	Iodine.	Dische.	Remarks.
1	0·05	+17°	14·9	±	+++	±	Nil	±	—
2	0·13	+61	7·5	+	+++	±	Brown	±	Bacterial glycogen.
3	0·29	+82	3·4	+	+++	—	Reddish-brown	—	—
4	3·00	+85	1·6	++	+++	—	Nil	±	Mainly specific somatic polysaccharide plus traces of nucleic acid derivative.
5	0·89	+79	1·1	++	+++	—	Nil	±	
6	0·28	+96	2·0	++	+++	—	Nil	±	Mixed fractions.
7	0·40	+44	0·5	++	+++	—	Nil	+	—
8	3·75	+28	2·5	++	+++	—	Nil	++	Mainly lipoid-bound specific polysaccharide plus nucleic acid derivative.
9	0·10	+27	1·7	++	+++	—	Nil	+	
10	0·12	+29	1·3	+	+++	—	Nil	+	

* In N/2-sodium hydroxide.

In an attempt to purify the specific polysaccharide, fractions 4 and 5 (above) were dissolved in water (40 c.c.) and reprecipitated by the gradual addition of alcohol. Three sub-fractions were thus obtained (Table II), but these were still contaminated by the deoxynucleic acid.

TABLE II.

No.	Alcohol added (c.c.).	Wt. (g.).	$[\alpha]_D$.	Dische.	Bial.	Iodine.
4*a*	30	0·3	+74°	±	+	Nil
4*b*	80	0·2	+87°	—	±	Brown
4*c*	190	2·35	+85°	±	+	Nil

Investigation of the Nucleic Acid Derivative in the Polysaccharide Fractions, $[\alpha]_D$ + 85° *and* $[\alpha]_D$ + 25°.—(i) The fractions were examined by the Dische test (Dische, *loc. cit.*); fraction 4 gave only a weakly positive reaction whereas the test was strongly positive with fraction 8.

(ii) Spectrophotometric examination of fractions 4, 8 and 4*a* revealed that each exhibited an absorption band at 2600—2650 A. Attempts to precipitate the derivative from an aqueous solution of the polysaccharide material indicated that intact nucleic acid was not present but that the contaminant was probably a nucleoside. From the spectrographic determinations, it was calculated that the fractions 4, 8, and 4*a* would contain 3·9, 13·7, and 2·5% respectively of such a substance.

(iii) The nucleic acid derivative was not precipitated from an aqueous solution by either lanthanum acetate or dilute or glacial acetic acid.

Separation of the Nucleic Acid Impurities by Adsorption on an Ion-exchange Resin.—The lipoid-polysaccharide (fraction 8; $[\alpha]_D^{17°}$ + 28°; 50 c.c.; 2% solution) was twice passed slowly through a "Zeocarb" resin and then 10 times through a "Deacidite" resin. The removal of nucleic acid was followed by the Dische test which initially was strongly positive and finally was only weak.

The final traces of the nucleic acid were removed when the polysaccharide was precipitated with alcohol from the acidified (pH 2) final liquor. A small precipitate (8*a*; 0·05 g.) having a strong Dische test was first obtained. The acid alcoholic mother liquor was neutralised, and immediately a flocculent precipitate (8*b*; 0·7 g.) of polysaccharide, $[\alpha]_D^{16°}$ + 25°, was obtained.

Alkaline Hydrolysis of Polysaccharides, $[\alpha]_D^{17°}$ + 85° *and* $[\alpha]_D^{17°}$ + 25°.—A 0·5% solution of the somatic polysaccharide, in 2N-sodium hydroxide, was heated on a water-bath for 20 hours. No change in optical rotation ($[\alpha]_D^{17°}$ + 85°) could be detected, and the substance was recovered unchanged in good yield.

Similarly, no changes were detected when a 0·5% solution of lipoid polysaccharide in 2N-sodium hydroxide was treated in the same way.

Location of Polysaccharides, $[\alpha]_D^{17°}$ + 85° *and* $[\alpha]_D^{17°}$ + 25°.—(i) Dry defatted cells (12 g.) were shaken at room temperature for 12 hours with sand and water (40 c.c.). The cell debris was separated (centrifuge) and the supernatant liquor treated with N-sodium hydroxide (40 c.c.) at 100° for 20 hours. The solution was acidified (Congo-red) with acetic acid, filtered, and treated with alcohol (4 vols.). The resulting precipitate was taken up in water, dialysed for 24 hours, and reprecipitated (yield, 0·035 g.).

The product, which was evidently a somatic polysaccharide, had the following properties: iodine coloration, −ve; Dische test, −ve; Molisch test, +++ve; $[\alpha]_D^{17°}$ + 78° in N/2-sodium hydroxide.

(ii) 10 G. of ether-soluble lipoids separated from the organisms were saponified by being heated at

100° for 20 hours with sodium hydroxide (N; 50 c.c.). The liquor was filtered, acidified (Congo-red), and the resulting precipitate removed (centrifuge). To the supernatant liquid was added alcohol (5 vols.). The resulting precipitate was purified by dialysis in a cellophane membrane; it then had the following properties: iodine coloration, −ve; Dische test, ±ve; Molisch test, +++ve; $[\alpha]_D^{17°}$ + 27° in N/2-sodium hydroxide.

Serological Activity.—The polysaccharides, $[\alpha]_D^{17°}$ + 85° and $[\alpha]_D^{17°}$ + 25° (fractions 4*c* and 8), were submitted to the precipitin test with tuberculosis anti-sera. Both reacted in a dilution of 1 : 2,000,000.

TABLE III.

Properties of Polysaccharides, $[\alpha]_D^{18°}$ + 85° *and* $[\alpha]_D^{18°}$ + 25°.

	Somatic polysaccharide.	Lipoid-bound polysaccharide.
$[\alpha]_D^{18°}$	+85°	+25°
Ash, %	1·4	2·5
Inorganic phosphorus, %	0·6	0·5
Organic phosphorus, %	0·3	1·4
Nitrogen, %	0·73	1·7
Acid equivalent	2,300	1,470
Nucleic acid, %	2·5	13·7
Serological activity	1 : 2,000,000	1 : 2,000,000
Ehrlich test	±	+

These approximate very closely to the specific polysaccharides isolated by Heidelberger and Menzel (*J. Biol. Chem.*, 1937, **118**, 79).

Methylation of the Somatic Polysaccharide, $[\alpha]_D^{18°}$ + 85°.—The polysaccharide (25 g.) was methylated in 5 g. portions by sodium hydroxide and methyl sulphate, in the following manner: the material (5 g.) was dissolved in water (25 c.c.) and warmed to 35°. To this solution, 300 c.c. of 30% sodium hydroxide and 100 c.c. of methyl sulphate were added in aliquot tenth portions every 10 minutes; 30 c.c. of the sodium hydroxide being added first in order to maintain alkaline conditions. The reaction mixture, which was vigorously agitated during the addition, was stirred for a further 2 hours at 35° after all the reagents had been added.

The reaction liquid was then raised to 100° for 30 minutes and allowed to cool while still being stirred. The liquid was cooled in ice and acidified (Congo-red) with dilute sulphuric acid. The material which separated at this stage was carefully removed. The acidified liquor was dialysed (parchment) for 48 hours and concentrated to a small volume (30 c.c.). This solution containing some product, and the partially methylated material obtained on acidification, were mixed and remethylated.

After this second methylation almost all the product separated on acidification. A small amount of remaining material was extracted with chloroform from the solution.

After 5 methylations, the product was acetylated by treatment with methyl alcohol (40 c.c.) and acetic anhydride (10 c.c.) for 48 hours at room temperature. In this way any free amino-residues in the amino-sugar, the presence of which was indicated by a positive Ehrlich test in the original polysaccharide, would be acetylated at the nitrogen atom and would thus be in a suitable form for the hydrolysis of the methylated polysaccharide.

The methoxyl content was constant (39·9%) after 6 methylations. Yield 4·2 g. By repetition of this procedure, 20 g. of methylated polysaccharide were obtained from 24 g. of starting material. An attempt was made to increase the methoxyl content of the derivative by methylation with methyl iodide and silver oxide. After several such treatments, the derivative was recovered in good yield, with the methoxyl content unchanged. It was concluded, therefore, that a fully methylated product was obtained by the 6 initial methylations.

Fractionation of the Methylated Somatic Polysaccharide.—Since traces of deoxyribonucleic acid were present in the starting material it was necessary to ascertain whether or not these had been removed during methylation. The methylated derivative (5·4 g.), which was a brown viscid gum, was dissolved in dry chloroform (42 c.c.), and to the solution was added light petroleum (b. p. 60—80°) until a precipitate formed. This was removed and more petroleum added to the mother liquor until a second precipitate formed. In this way 4 fractions were separted, the fifth being obtained on evaporation of the final mother liquors. A typical fractionation is shown in Table IV.

TABLE IV.

No.	Petroleum added (c.c.).	Wt. (g.).	Ash, %.	OMe, %.	$[\alpha]_D$.*	Remarks.
M1	70	0·15	32·2	—	—	Inorganic impurity.
M2	100	2·35	Trace	39·5	+129°	Methylated polysaccharide.
M3	150	2·1	—	39·9	+125	—
M4	170	0·9	—	39·2	+122	—
M5	evaporation	0·5	—	43·1	+ 83	Not examined.

* In chloroform.

The fractions M2 and M3, consisting of essentially homogeneous methylated polysaccharide, were pale-yellow, glass-like solids, containing 0·3% of nitrogen and having no characteristic absorption band in the ultra-violet region. The material was phosphorus free.

Methanolysis.—A typical sample (0·44 g.) of the methylated polysaccharide ($[\alpha]_D^{20°}$ + 125° in chloroform) was boiled in methanolic hydrogen chloride (1%; 45 c.c.). The hydrolysis was followed polarimetrically and the optical rotation became constant ($[\alpha]_D^{20°}$ + 47°) after 5 hours; this value did not change when the concentration of the acid was increased to 5% and the heating resumed.

The methanolysis proceeded more rapidly when another sample of the methylated polysaccharide ($[\alpha]_D^{20°}$ + 125° in chloroform) was heated with 5% methanolic hydrogen chloride, and the rotation became constant ($[\alpha]_D^{20°}$ + 45°) after 3 hours.

In this way, 9 g. of the fractionated methylated polysaccharide were hydrolysed. The acid was neutralised (litmus) with silver carbonate and the silver residue carefully washed with methanol. More silver residues were removed from the methanolic solution of hydrolysis products by addition of a little light petroleum. The solution was then evaporated to dryness by aeration at 50°, giving a mixture of glycosides (8·8 g.).

Fractionation of the Mixed Glycosides.—The mixed glycosides (8 g.) were fractionally distilled in a Widmer flask in a high vacuum. The fractionation was controlled by noting frequently the change in the refractive index of the distillate. The rate of distillation was so adjusted that the process occupied about 24 hours. Nine fractions (I to IX) were thus collected (Table V). The still residue was re-hydrolysed with methanolic hydrogen chloride, and the further yield of mixed glycosides obtained was fractionally distilled in the manner described giving 5 fractions (X to XIV) (Table VI). The final still residue amounted to 0·9 g.

TABLE V.

No.	Vap. temp./ 0·02 mm.	Bath temp.	Wt. (g.).	$n_D^{17°}$.	OMe, %.	Remarks.
I	71—76°	106—116°	0·81	1·4471	51·5	Mobile colourless liquid.
II	76—78	117—122	0·87	1·4520	53·0	,, ,, ,,
III	78—81	122—125	1·02	1·4532	52·1	,, ,, ,,
IV	79—83	125—130	0·42	1·4550	50·1	Immobile colourless syrup.
V	83—84	133—138	0·32	1·4549	47·5	,, ,, ,,
VI	90	138—140	0·04	1·4569	48·3	,, ,, ,,
VII	94—97	150	0·72	1·4590	47·2	Viscid yellow syrup.
VIII	100	155—160	0·67	1·4630	47·8	,, ,, ,,
IX	>100	170—190	0·07	1·4640	44·4	,, ,, ,,

TABLE VI.

Re-hydrolysis of the still residue from first fractionation.

No.	Vap. temp./ 0·02 mm.	Bath temp.	Wt. (g.).	$n_D^{17°}$.	OMe, %.	Remarks.
X	68—72°	110—125°	0·40	1·4481	51·0	Colourless mobile liquid.
XI	79—82	125—145	0·30	1·4516	52·1	,, ,, ,,
XII	100—110	150—160	0·62	1·4620	45·2	Viscid colourless syrup.
XIII	120—125	160—200	0·38	1·4712	41·4	,, ,, ,,
XIV	120—	220—	0·11	1·4749	33·3	Viscid yellow syrup.
Still residue		—	0·9	—	—	—

Identification of 2 : 3 : 4-*trimethyl methyl*-L-*rhamnoside and* 3 : 5-*dimethyl methyl*-D-*arabinoside.* A specimen of the mixed fractions I and X (0·4 g.) was methylated with silver oxide and methyl iodide. The fully methylated glycosides so obtained afforded two fractions on fractional distillation. The first of these fractions (IA) distilled at 110°(bath temp.)/11 mm. and had $n_D^{21°}$ 1·4370 and OMe, 58·9%. The second fraction (IB) distilled at 90—93°/0·2 mm. and had $n_D^{21°}$ 1·4445 and OMe, 57%.

The fraction IA was readily hydrolysed by N/20-sulphuric acid ($[\alpha]_D$ + 31·5° ⟶ + 18°, 6 hours) which suggested the presence of a furanose structure. The free sugar so obtained did not form a crystalline anilide when boiled with alcoholic aniline. This free sugar (0·2 g.) was therefore oxidised with bromine (0·5 g.) in water (10 c.c.) at room temperature for 5 days. Excess of bromine was removed by aeration, and the solution was neutralised with silver carbonate. After filtration, hydrogen sulphide was passed in and the filtered solution evaporated to dryness. The syrupy acid produced (0·1 g.) was extracted from the residue with chloroform, and lactonised by being heated at 100° in a vacuum for 3 hours. This syrupy lactone, on treatment with dry saturated methanolic ammonia (20 c.c.) for 24 hours, yielded an amide which was recrystallised from acetone; m. p. 135—136° alone or in admixture with an authentic specimen of 2 : 3 : 5-trimethyl D-arabonamide {Found : $[\alpha]_D^{17°}$ — 14° in water, OMe; 43·9%. Cf. $[\alpha]_D$ — 14° in water; OMe, 44·9% (Haworth, Peat, and Whetstone, *J.*, 1938, 1975)}.

Fraction IB resisted hydrolysis with N/20-sulphuric acid but was hydrolysed with N-sulphuric acid ($[\alpha]_D^{19°}$ + 14° ⟶ + 9°; 4 hours). The free sugar derivative ($n_D^{17°}$ 1·4561; OMe, 45%) was converted into the anilide by being refluxed for 5 hours with the theoretical amount of aniline in dry ethanol. The syrup obtained after removal of the solvent was crystallised from light petroleum; m. p. 112° alone or in admixture with 2 : 3 : 4-trimethyl L-rhamnose anilide (Found : $[\alpha]_D^{19°}$ + 127°; OMe, 32·6%).

A sample of fractions I and X was hydrolysed first with N/20 then by N-sulphuric acids for 6 hours ($[\alpha]_D$ + 29° ⟶ ± 0°). A specimen of the free sugar derivative was converted into the anilide, which had m. p. 111—112° alone or in admixture with 2 : 3 : 4-trimethyl rhamnose anilide. A second anilide (m. p. 118°; $[\alpha]_D^{18°}$ — 28·8° in water after 2 hours) crystallised from the mother liquors on being kept; it was probably a dimethyl arabinose anilide, but was clearly different from 2 : 3-dimethyl arabinose anilide, m. p. 139° (Smith, *J.*, 1939, 753), and 2 : 4-dimethyl arabinose anilide, m. p. 142—143° (*idem*, *ibid.*, p. 744).

A further specimen of the free sugar derivative of I (0·3 g.) was oxidised with bromine (1 g.) at room temperature for 2 days. Excess of bromine was removed, and the lactone formed by distillation of the acid at 138°/0·05 mm. Treatment of the lactone with methanolic ammonia furnished an amide which on recrystallisation from acetone had m. p. 144° ($[\alpha]_D$ — 11° in water; OMe, 32·7%). This substance (10 mg.) was treated with sodium hypochlorite (5 drops) for 12 hours, and to it was then added saturated

semicarbazide hydrochloride and sodium acetate; a precipitate of hydrazodicarbonamide (m. p. 254°) was obtained. It is considered that this amide is 3 : 5-dimethyl D-arabonamide (cf. 3 : 5-dimethyl L-arabonamide, m. p. 145°; $[\alpha]_D$ + 10°; OMe, 31·9%; Cunneen, Thesis 1940, University of Birmingham; White, *J. Amer. Chem. Soc.*, 1946, **68**, 272).

Further quantities of 2 : 3 : 4-trimethyl methyl-L-rhamnoside and 3 : 5-dimethyl methyl-D-arabinoside were identified in fractions II, III, IV, and XI.

Identification of 3 : 4 : 6-*trimethyl methyl*-D-*mannoside.* A specimen (0·3 g.) of fractions VII, VIII, and XII was methylated with silver oxide and methyl iodide. The methylated product (0·3 g.; $n_D^{19°}$ 1·4450; b. p. 90—95°/0·02 mm.; OMe, 60·6%) was hydrolysed by being heated with N-sulphuric acid ($[\alpha]_D^{20°}$ + 47° ⟶ + 41° after 8 hours), and a specimen of the free sugar (0·1 g.; n_D 1·4600; OMe, 52·2%) converted into the anilide which readily crystallised from acetone–light petroleum; m. p. 140° alone or in admixture with 2 : 3 : 4 : 6-tetramethyl D-mannose anilide.

A specimen of the original mixed fractions VII, VIII, and XII (1 g.) was hydrolysed with N-sulphuric acid (25 c.c.) ($[\alpha]_{D_5}^{20°}$ + 53° ⟶ + 47° after 8 hours). The free sugar (0·85 g.; $n_D^{20°}$ 1·4778) was extracted with ether and crystallised from alcohol–ether. After recrystallisation, the substance, which strongly reduced Fehling's solution, had OMe, 40·6%, $[\alpha]_D^{20°}$ + 26° ⟶ + 6° in water (2 hours), and m. p. 100° alone or in admixture with 3 : 4 : 6-trimethyl D-mannopyranose (m. p. 102°; OMe, 41·8%; $[\alpha]_D^{18°}$ + 22° ⟶ + 8° in water; Haworth, Heath, and Peat, *J.*, 1941, 833).

The residue (0·2 g.) crystallised on standing at 0°, affording 3 : 4-dimethyl D-mannopyranose (see below).

Identification of 3 : 4-*dimethyl methyl*-D-*mannopyranoside.* A specimen (0·15 g.) of the mixture of fractions IX and XIII was methylated with silver oxide and methyl iodide. Hydrolysis of the product (0·12 g.; $n_D^{16°}$ 1·4556) with N-sulphuric acid (10 c.c.; $[\alpha]_D^{20°}$ + 27° ⟶ + 14°) afforded a free sugar derivative (0·08 g.; $n_D^{19°}$ 1·4670) which on treatment with ethanolic aniline gave a quantitative yield of 2 : 3 : 4 : 6-tetramethyl D-mannose anilide (m. p. 139° alone or in admixture with an authentic specimen).

A further specimen (0·3 g.) of fractions IX and XIII was hydrolysed with N-sulphuric acid (15 c.c.; $[\alpha]_D^{20°}$ + 46° ⟶ + 24° after 8 hours at 100°). The free sugar was extracted with ether. Evaporation of the ethereal extract gave a crystalline compound which, after recrystallisation from aqueous alcohol, had m. p. 113° alone or in admixture with 3 : 4-dimethyl D-mannopyranose monohydrate (Found : OMe, 27·5. Calc. : OMe, 27·48%; cf. Haworth, Hirst, and Isherwood, *J.*, 1937, 784).

Isolation of an amino-sugar derivative. On analysis it was found that the principal fractions obtained on distillation had the following nitrogen contents : mixed fractions I and X (0·27%); mixed fractions II, III, XI (0·33%); mixed fractions VII, VIII, XII (0·36%); mixed fractions IX, XIII (nil).

0·4 G. of each nitrogen-containing fraction was mixed and heated at 100° for 8 hours with sodium hydroxide (15 c.c.; 4N). The resulting solution was neutralised (phenolphthalein) with dilute sulphuric acid, extracted exhaustively with ether, and the extract then dried (Na_2SO_4) for 24 hours. After filtration, the dry ethereal solution was saturated with dry hydrogen chloride at 0°. A turbidity developed and a syrup separated. This was removed (0·03 g.; OMe, 32·4%) and washed with ether, then acetylated by being shaken for 12 hours with acetic anhydride (0·4 c.c.) and silver acetate (0·5 g.) in 10 c.c. of dry methanol. The filtered solution was evaporated to 2 c.c. and finally dried in a vacuum (at room temperature) over solid potassium hydroxide. A solid which was recrystallised from aqueous alcohol (m. p. 87°; 10 mg.) was obtained.

In view of the positive Ehrlich reaction of the original polysaccharide, the nitrogen contents, and the isolation of the above derivative (OMe, 32·4. Calc. for a dimethyl methylacetamidohexoside: OMe, 35·1%) it is suggested that this represents the amino-sugar constituent of the polysaccharide.

TABLE VII.

Assessment of the methylated sugar components of the methylated somatic polysaccharide.

Distillates, 6·35 g. Still residue, 0·88 g. (Ash, 13·4%).

Component.	Wt. (g).	% (by wt.).	*M*.	Mol. proportion.	% (mol.).	Molecular ratio.
3 : 5-Dimethyl methyl-D-arabinoside	1·40	22·0	192	7·29	29	5·05
2 : 3 : 4-Trimethyl methyl-L-rhamnoside	1·57	24·7	220	7·14	25·5	4·96
3 : 4 : 6-Trimethyl methyl-D-mannoside	1·70	26·8	236	7·20	25·6	4·99
3 : 4-Dimethyl methyl-D-mannoside	1·29	20·2	222	5·86	20·8	4·03
Dimethyl methylaminohexoside	0·38	5·9	263·5	1·44	5·1	1

Periodic Acid Oxidation (private communication from Dr. J. K. N. JONES).—51·54 Mg. of somatic polysaccharide were oxidised for 190 hours with sodium periodate (5 ml.; 0·274M), water (25 ml.), and potassium chloride (1 g.) with shaking. Excess of glycol was added at the completion of the oxidation, and the resulting solution titrated with barium hydroxide (6·77 ml.; 0·01N). (The initial substance was neutral.)

Thus 1 mol. of acid is given by 761 g. of polysaccharide. The unmethylated structure in (I) has *M*, 3051, *i.e.*, 3051 G. of polysaccharide give rise to 5 end-groups = 5 mols. of acid

610 g. of polysaccharide (theory) = 1 mol. of acid

But from experiment 761 g. of material = 1 mol. of acid

∴ No. of end groups (experimental) = 4

45·77 Mg. of the same polysaccharide were oxidised by the same method, and the uptake of IO_4^- was determined.

144 G. of polysaccharide took up 1 mol. of IO_4^-.

Structure (V) can take up 20 mols. of IO_4^- (assuming that the amino-sugar takes 1 mol.).

Thus, 153 g. of polysaccharide would take up 1 mol. of IO_4^- (theoretical value).

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