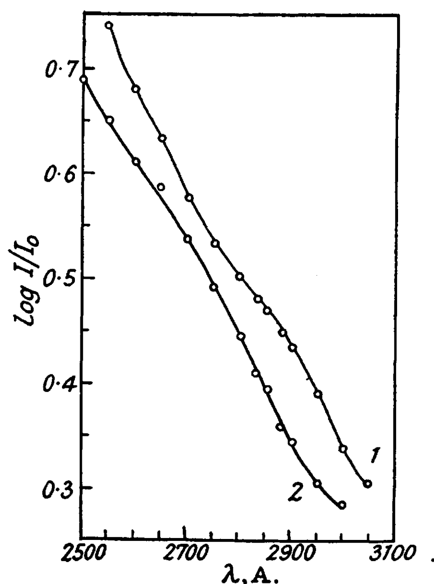
79. *Structure of an Antigenic Polysaccharide Isolated from Tuberculin.*

By P. W. Kent.

A substance which had been isolated from the human-type *M. tuberculosis* (strain H37.A33) and which, in a previous communication, was reported to be electrophoretically homogeneous and capable of eleciting antibodies, has been found to consist of a polysaccharide component (92%) and a lipoid component (8%). The polysaccharide component contained nitrogen and gave positive tests for a 2-amino-sugar. Chromatographic analysis of the hydrolyzed material showed that the principal monosaccharide constituent was glucose and that glucosamine was present. The polysaccharide component was investigated by the methylation technique. The products of hydrolysis of the methylated derivative consisted almost entirely of 3 : 4 : 6-trimethyl glucose. The polysaccharide therefore consists mainly of glucopyranose units linked in the 1 : 2-positions. The molecular weight of the native substance measured by a light-scattering technique was of the order $4{\cdot}9 \times 10^5$ (cf. 1×10^5 obtained previously from ultra-centrifuge investigations).

It has been shown (Seibert, Stacey, and Kent, *Biochem. Biophys. Acta*, 1949, **3**, 632) that an antigenic polysaccharide can be isolated from culture filtrates of certain types of *M. tuberculosis*. This polysaccharide (designated polysaccharide II in order to differentiate it from the non-antigenic polysaccharide I also present in tuberculin-bearing filtrates) contained nitrogen—a finding consistent with detection of an amino-sugar component in the material. The preliminary examination, whilst it revealed the presence of glucose as the major monosaccharide component, also indicated the presence of some non-reducing constituent, and this was further substantiated by the slight amount of specific absorption in the ultra-violet region (2800—2880 A.). The absorption band disappeared (Fig. 1) when a solution of the native substance was treated with benzene at pH 11 at 80°. The polysaccharide was recovered from the aqueous solution by precipitation with alcohol after acidification (it was then purified by dissolution in water, dialysis, and reprecipitation). The benzene layer was dried and the solvent distilled off, yielding a small amount of lipoid material which appeared to be a mixture of fatty acids and corresponded to 8% of the original material. The quantity obtained was too small for further study.

Fig. 1.
Ultra-violet absorption spectra.



(1) Native, (2) lipoid-free polysaccharide.

The specific rotation of the original polysaccharide II was +160° (*c*, 0·25 in water) and after defatting was +183·5° (*c*, 0·22 in water). The amount of reducing sugar, measured by the Shaffer–Hartmann technique (*J. Biol. Chem.*, 1921, **47**, 477), also increased from 84% to 94·1%. The serological activity of the native and the defatted material was studied by Dr. F. B. Seibert of the Henry Phipps Institute by means of the precipitin reactions, antitubercle bacilli horse serum (A5807) being used, and both were found to react in dilutions of 1 : 200,000 (see Table I). The properties of the substances are summarised in Table I.

It seems therefore that the substance is a lipo-polysaccharide containing 1 part of lipoid to 11 parts of polysaccharide, moving as an entity on electrophoresis but dissociated in aqueous alkali at slightly elevated temperatures, and that the lipoid constituent is not apparently involved in the reaction with homologous serum in the precipitin test. The native material

was readily hydrolyzed by acid and in this respect differed from Anderson's fatty acid–polysaccharide waxes (*Chem. Reviews*, 1941, **29**, 225). The equilibrium value, $[\alpha]_D$ +52°, was reached after 2 hours' heating with N-acid. In the sugars so produced only glucose could be

TABLE I.

Comparison of native and lipoid-free polysaccharide.

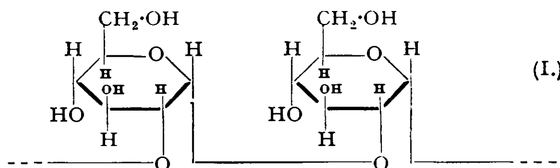
	Native.	Defatted.
$[\alpha]_D$ in water	+160°	+183·5°
Log I/I_0 at 2830 A. (c = 0·25% in water)	0·480	0·410
Reducing sugar, % (max. value)	84·2	94·1
Precipitin titre	1 : 200,000	1 : 200,000

detected, but there was a positive test for amino-sugar. Glucose had been identified earlier both as the anilide and as the diethyl mercaptal penta-acetate.

Paper-chromatographic examination of the hydrolyzed polysaccharide (Partridge, *Nature*, 1946, **158**, 270; *Biochem. J.*, 1948, **42**, 238) showed that one principal reducing sugar was present, namely glucose. A second weakly-reducing band was detected, corresponding to glucosamine both in its reduction of silver nitrate and in its colour with ninhydrin. The presence of glucosamine was confirmed by the formation of the *N*-(2 : 4-dinitrophenyl) derivative by treatment of the hydrolyzed polysaccharide with 1-fluoro-2 : 4-dinitrobenzene and sodium hydrogen carbonate. Chromatographic examination of the product showed that only dinitrophenylglucosamine and glucose were present (Kent, *Research*, 1950, **3**, 427). There was no evidence of the presence of amino-acids (Kent, Lawson, and Senior, *Science*, 1950, in the press).

The mixture of glucosides formed by methanolysis of the methylated polysaccharide had OMe 53·2% (a trimethyl methylhexoside requires 52·54%) and this was taken as indicating that the fraction consisted mainly of a trimethyl methylglucoside. Paper-chromatographic examination of the hydrolyzed mixed glycosides (Brown, Hirst, Hough, Jones, and Wadman, *Nature*, 1948, **161**, 720) showed the presence of only one principal band and this corresponded to 3 : 4 : 6-trimethyl glucose. The absence of any significant quantity of di- or tetra-methyl sugar suggests either a long-chain structure with little branching (which is in agreement with the sedimentation and diffusion investigation of the material) or a closed-chain structure. The trimethyl methylglucoside (*A*) (n_D^{18} 1·4640) was almost entirely soluble in light petroleum but there remained a small amount of insoluble material (*B*) (n_D^{18} 1·4660), the quantity of which did not permit further investigation. The glucoside *A* was hydrolysed with dilute mineral acid, to yield a syrupy free-sugar derivative. This free sugar did not yield a crystalline anilide but with phenylhydrazine in acid solution yielded 3 : 4 : 6-trimethyl glucosazone; it was oxidized by bromine water to the corresponding acid. Distillation of the acid in a high vacuum gave a non-crystalline lactone (OMe 42·6%) which afforded a good yield of crystalline 3 : 4 : 6-trimethyl D-gluconophenylhydrazide. The corresponding non-crystalline amide gave a positive Weerman test indicative of an α-hydroxy-group.

This evidence indicates that in the polysaccharide moiety a large part of the glucose residues are linked through the 1 and the 2 position and are in the pyranose form [as (I)]. The failure to detect any dimethyl methylhexoside in the hydrolyzate of the methylated polysaccharide suggests that the polysaccharide may have little branched-chain structure. The slow rate of oxidation of the polysaccharide by potassium periodate, under controlled conditions, precludes the possibility of a wholly dextran-like structure (Kent, *Science*, 1949, **110**, 689).

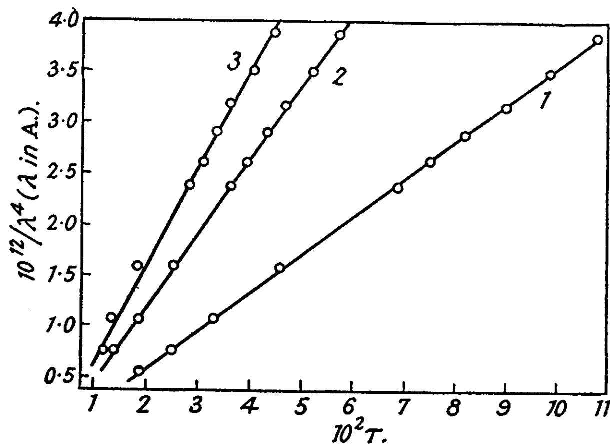


The molecular weight of the native material calculated from diffusion constant–sedimentation data was of the order of 1×10^5. In dilute aqueous solution, the material exhibited the common Tyndall blue colour. In a preliminary examination of such solution by the light-scattering technique (Debye, *J. Appl. Physics*, 1944, **15**, 338; *J. Physical Chem.*, 1947, **51**, 18; Ostler, *Chem. Reviews*, 1948, **43**, 319; Mark, " Chemical Architecture," Interscience, 1948, p. 121) it was found that the turbidity (from λ 4000 to 6000 A., measured on a Beckman absorption spectro-

meter) was accurately proportional to λ^{-4}. (Fig. 2). The molecular weight obtained from the measurements was $4·9 \times 10^5$ (Fig. 3). This value is only approximate as backward and

FIG. 2.

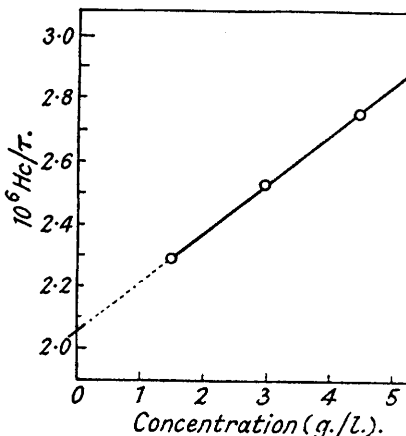
Relation between turbidity (τ) *and* $1/\lambda^4$ *at various concentrations.*



Concentrations : 1, 4·430 *mg./c.c.* ; 2, 2·960 *mg./c.c.* ; 3, 1·48 *mg./c.c.*

FIG. 3.

Extrapolation of the turbidity relation against concentration.



forward scattering factors were not taken into account, but it is significant as it is of the same order as the value deduced from ultra-centrifuge determinations.

EXPERIMENTAL.

The native substance gave negative results in the Bial test for pentoses and in Tollens's reaction. The Molisch test was positive (+ + +), and the test (after preliminary acidic hydrolysis) for amino-sugars was positive (+) (Elson and Morgan, *Biochem. J.*, 1933, **27**, 1824). The substance had $[\alpha]_D^{19}$ +160° (*c*, 0·25 in water).

Action of Dilute Alkali and Benzene.—The native substance (0·394 g.) was dissolved in 0·001N-sodium hydroxide (20 c.c.) to which was added benzene (10 c.c.), and the mixture was heated at 80° under reflux for 20 hours. The solution was made acid (pH 5) with dilute acetic acid. The aqueous layer was dialysed against distilled water for 3 days and the defatted polysaccharide precipitated by addition of alcohol (5 vols.). The moist product was dissolved in water (10 c.c.) and dried by lyophylization, and finally over phosphoric oxide (yield, 0·352 g.).

The benzene layer was dried (K_2CO_3) and evaporated in a weighed flask. The resulting lipoidal non-crystalline solid (0·031 g.) was not soluble in water. A specimen of the native substance was hydrolyzed in the absence of benzene; after 1 hour a white flocculent precipitate separated.

The defatted polysaccharide had $[\alpha]_D^{19}$ +183·5° (*c*, 0·22 in water). An aqueous solution (0·25%), examined in the Beckman photospectrometer, showed only general absorption between 2500 and 3000 A. (*E* at 2800 A. = 0·480).

Absorption Spectra of the Native Polysaccharide.—An aqueous solution (0·25%) showed a small but distinct absorption between 2800 and 2870 A., *E* at 2830 A. being 0·480.

Acid Hydrolysis.—(i) The native polysaccharide (*c*, 0·2 g. per 100 c.c.) was hydrolyzed at 100° with 0·1N-sulphuric acid; the following observations were made: $[\alpha]_D^{19}$, initial, +165°; after 30 minutes, +183°; after 9 hours, +140°. (ii) On hydrolysis of the native substance with N-sulphuric acid at 100°, $[\alpha]_D^{20}$ fell to +52° (equilibrium in 2 hours).

Chromatographic Examination of the Monosaccharide Constituents.—(i) The acid-hydrolyzate (above) was neutralized with barium carbonate, and the solution filtered and evaporated to dryness. The residue was taken up in water (2 drops) and submitted to paper chromatography, with butanol–water–ethanol (40 : 50 : 10) as eluant. One main band (R_F, 0·18) was observed, corresponding to that given by glucose (0·18) when examined simultaneously. The band was detected by means of aniline hydrogen phthalate.

A second band (R_F 0·1) was observed by treatment of the paper with silver nitrate. The same band gave a blue colour with ninhydrin and was therefore thought to be an amino-sugar (R_F for glucosamine, 0·08). (ii) The initial polysaccharide (15 mg.) was hydrolyzed in a sealed tube with N-sulphuric acid (1 c.c.) at 110° for 3 hours. The hydrolysate was neutralised with sodium hydrogen carbonate (0·05 g. excess), and the resulting solution was shaken with 1-fluoro-2 : 4-dinitrobenzene (3 drops) for 2 hours. Three drops of the reaction mixture were applied to the paper chromatogram which was eluted with the

above-mentioned mixture. A yellow band (R_F 0·74) was observed which corresponded to authentic *N*-2 : 4-dinitrophenylglucosamine (m. p. 167—169°; R_F 0·75) when investigated simultaneously on the same paper chromatogram. When the paper was sprayed with a solution of aniline hydrogen phthalate and warmed, the yellow band became brown (a characteristic of dinitrophenylamino-sugars) and another band corresponding to glucose appeared.

Action of Amylase.—The native polysaccharide (17 mg.) was incubated at 25° and at 35° with β-amylase obtained from soya bean by Dr. E. J. Bourne. There was no liberation of reducing sugar as measured by the Shaffer–Hartmann method.

Reducing-sugar Content.—(*a*) Native polysaccharide (2·50 mg.) was hydrolyzed with N-sulphuric acid (1 c.c.) for 4 hours at 100°, then neutralized with sodium carbonate, and the reducing sugar in the resulting solution was determined (Shaffer–Hartmann) : 2·34 mg. of glucose were found. The reducing sugar content calculated as glucose was 84·2% (constant value).

(*b*) Defatted polysaccharide (1·08 mg.) was hydrolyzed, neutralized, and titrated with Shaffer–Hartmann reagent, 1·13 mg. of glucose were found, equivalent to 94·1% calculated as glucose.

Methylation.—(*a*) The native polysaccharide (0·1 g.) was methylated by means of liquid ammonia (5 c.c.), sodium (0·2 g.), and methyl iodide (1 c.c.) since it could be demonstrated that under the action of liquid ammonia alone, the viscosity of the polysaccharide in water was not appreciably changed. The methylated product, however, was not wholly insoluble in the liquid ammonia and this method was abandoned in favour of the Haworth method.

(*b*) A typical methylation was carried out on 0·3 g. of the native polysaccharide by the use of 5N-sodium hydroxide (3 c.c.) and methyl sulphate (1 c.c.), added dropwise during 1 hour, the mixture being kept slightly alkaline throughout. The mixture was stirred for a further hour, and excess of methyl sulphate destroyed by heating the mixture rapidly to 90°. The liquid was neutralized by sulphuric acid, and the partly methylated derivative which separated was collected. After two such methylations the derivative was soluble in chloroform, and after four methylations the product was treated with methyl iodide and dry silver oxide, until the methoxyl content became constant (44·4%) ; 0·2 g. of product was obtained, which had been freed from gross impurities by precipitation with light petroleum.

Hydrolysis of the Methylated Polysaccharide.—The methylated polysaccharide (0·38 g.) was hydrolyzed by boiling methanolic hydrogen chloride (5 c.c.; 0·1 N.) ; $[\alpha]_D$ (originally +144°) became +107° in 10·5 hours (not constant). N-Methanolic hydrogen chloride caused $[\alpha]_D$ to fall to +66° (constant) in 2 hours. The solution was neutralized by silver carbonate and filtered and the solution treated with a small volume of light petroleum to precipitate suspended silver salts. After filtration, the solution was gently dried under a slightly reduced pressure, yielding a syrup (0·30 g.), $[\alpha]_D$ +68° (in methanol), n_D^{18} 1·4645 (Found : OMe 53·2%).

Examination of the Methylated Fraction.—(*a*) The methylated sugar (0·03 g.) was hydrolyzed by hot 0·5N-hydrochloric acid (2 c.c.) for 4 hours, neutralized (silver carbonate), filtered, and concentrated almost to dryness. Paper chromatography as above gave only one band (R_F 0·76) on a Whatman No. 1 filter paper (R_F for 3 : 4 : 6 trimethyl glucose, 0·74 under the same conditions).

(*b*) The partly methylated sugar (0·2 g.) was extracted several times with dry light petroleum. The solution yielded, on evaporation, 0·17 g. of material (*A*), n_D^{18} 1·4440, and 0·01 g. of a residue (*B*), n_D^{18} 1·4660.

Isolation of 3 : 4 : 6-*Trimethyl* D-*Glucosazone.*—A portion of the fraction *A* (0·1 g.) was hydrolyzed with 0·1N-sulphuric acid (7 c.c.) {$[\alpha]_D$ +49° ⟶ +42° (8 hours)}. There was no further change when the concentration of acid was increased to N. The solution was neutralized (phenolphthalein) with aqueous sodium hydroxide, evaporated to dryness, and extracted with chloroform. Evaporation of the solvent yielded a syrup (0·08 g.) which on chromatography showed only one band (R_F 0·76) (R_F found for 3 : 4 : 6-trimethyl glucose, 0·74). Of this reducing syrup 30 mg. were refluxed with aniline (15 mg.) in dry ethanol (2 c.c.) for 3 hours, but no crystalline anilide was obtained. A further 40 mg., in acetic acid (1 c.c.; 1N.), were heated with phenylhydrazine hydrochloride (25 mg.) at 90° for 45 minutes and the orange solid which formed was filtered off and crystallized from a little alcohol; it had m. p. 78—80° alone or on admixture with authentic 3 : 4 : 6-trimethyl glucosazone (Haworth, Hirst, and Isherwood, *J.*, 1937, 782).

Identification of 3 : 4 : 6-*Trimethyl Gluconohydrazide.*—The partly methylated free sugar (0·35 g.) obtained by aqueous acidic hydrolysis of either the methylated polysaccharide or the products of methanolysis was dissolved in water (10 c.c.), and bromine (0·5 c.c.) added. The solution was kept at room temperature until it ceased to reduce Fehling's solution (3 days). The excess of bromine was removed by aeration, and the acid neutralized with silver carbonate. The filtrate was saturated with hydrogen sulphide, filtered, and evaporated to dryness. The product was extracted with chloroform and then distilled [b. p. 115—119° (bath-temp.)/0·05 mm.] and had n_D 1·4640 (0·26 g.) (Found : OMe, 42·6. Calc. for trimethyl gluconolactone : OMe, 41·5%). The lactone so obtained did not crystallize but with the calculated amount of phenylhydrazine at 100° for 20 minutes in water gave a crystalline product which, recrystallized from ethyl acetate, had m. p. 124—125° (Found : OMe, 28·2%) [3 : 4 : 6-trimethyl gluconophenylhydrazide (Haworth and Pannizon, *J.*, 1934, 154) (OMe, 28·5%) has m. p. 126°]. The lactone was treated with saturated methanolic ammonia at 0° for 4 hours. A syrup resulted with sodium hypochlorite at 0° and gave a product which with saturated semicarbazide hydrochloride yielded a hydrazodicarbonamide (Weerman, *Rec. Trav. chim.*, 1917, **36**, 16), m. p. 252—253°.

Molecular Weight by Light Scattering.—A solution of the dry dialyzed native material in water (4·43 mg./c.c.) was passed twice through a fine sintered-glass filter. From this two dilutions were made, giving solution containing 1·48 and 2·96 mg./c.c. respectively.

The linear relationship between turbidity (τ) and $1/\lambda^4$ was demonstrated for all three solutions examined in the range 6000—4000 A (Fig. 2). Results for 5000 A. and 21·1° are given in the following table. Fig. 3 is drawn from these results. If $H = 32\pi^3\mu^2(\text{solvent}).\Delta\mu^2/3N\lambda^4c^2$, where N = Avogadro's number, c = concentration, $\Delta\mu = \mu(\text{solution}) - \mu(\text{solvent})$, λ = wave-length in cm., and μ(solvent) was found to be 1·33327, then the following equation holds : $Hc/\tau = 1/M + 2Bc$ . . ., where τ = turbidity, M = molecular weight, and B is a coefficient.

$10^3 \times$ Concn. (c) (g./c.c.).	μ(solution).	$10^3\ \Delta\mu$.	τ.	$10^6\ Hc/\tau$.
4·43	1·33596	2·69	0·092	2·76
1·48	1·33417	0·900	0·037	2·27
2·96	1·33507	1·8	0·051	2·53

In Fig. 3, Hc/τ was plotted against c. Extrapolation to zero concentration gave an intercept value for $1/M = 2{\cdot}05 \times 10^{-6}$, whence the molecular weight $= 4{\cdot}9 \times 10^5$.

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FRICK CHEMICAL LABORATORIES, PRINCETON UNIVERSITY,
NEW JERSEY.
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