

6. 2:4-Dihydroxyquinoline does not yield an ethereal sulphate in the rabbit but is conjugated (20–40%) with glucuronic acid. The glucuronide was proved to be 2-quinolonyl-4-glucosiduronic acid.

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Studies in Immunochemistry

15. THE SPECIFIC POLYSACCHARIDE OF THE DOMINANT 'O' SOMATIC ANTIGEN OF *SHIGELLA DYSENTERIAE**

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The preparation and properties of a material possessing the immunological specificity and activities of the 'O' somatic antigen of *Shigella dysenteriae* have been described by Morgan (1937), Morgan & Partridge (1940, 1941*a*) and Davies, Morgan & Mosimann (1954), who showed it to be a protein-polysaccharide-phospholipid complex; this was the 'endotoxin' of earlier workers. The phospholipid component can be removed from the antigenic complex by the dissociating action of the highly polar solvent formamide and its elimination does not appear to affect any of the known immunological or toxic properties of the original antigen. Treatment of the antigenic material, after removal of the phospholipid component, with dilute acetic acid at 100° brings about the separation of the polysaccharide and protein components. The latter material is a lipoprotein (Davies & Morgan, 1953), and is identical with the 'conjugated protein' of Morgan & Partridge (1940, 1941*b*); the lipid component is insoluble in ether but soluble in chloroform whereas the phospholipid removed from the native antigenic complex by formamide is soluble in ether.

These materials will be described in a later communication.

The polysaccharide component remains in solution after hydrolysis of the antigen with acetic acid and can be readily separated from the water-insoluble conjugated protein. The polysaccharide can be extracted from the whole organism with weak acetic acid at 100° (Morgan, 1931, 1936) and has been shown to contain glucosamine, galactose and rhamnose (Morgan, 1936, 1938; Partridge, 1948).

The antigenic complex yields an undegraded polysaccharide by repeated precipitation from solution in formamide with ethanol (Morgan & Partridge, 1940) or by treatment with cold dilute alkali or with 90% (w/v) phenol (Morgan & Partridge, 1941*a*). The undegraded polysaccharide differs from the degraded form, which results from the use of mild acid, in certain of its properties; it is viscous and will recombine with the conjugated protein component to give an antigenic complex (Partridge & Morgan, 1940).

Lipopolysaccharides have been obtained from a number of species of bacteria; Goebel & Adams (1943) isolated an 'F' heterophile polysaccharide from *Pneumococcus* which only differed from the

* Part 14 of this series, Gibbons & Morgan, 1954, **57**, 283.

'C' somatic polysaccharide of the same organism (Tillet, Goebel & Avery, 1930), in that it contained a lipid component. Jesiatz & Goebel (1952) prepared a lipopolysaccharide from *Shigella sonnei*. By extraction with warm aqueous phenol, analogous materials have been isolated from several species of Gram-negative bacteria, including *Sh. dysenteriae*, by Westphal, Lüderitz & Bister (1952), Westphal, Lüderitz, Eichenberger & Keiderling (1952) and Lüderitz & Westphal (1952). Westphal and his collaborators found that the phospholipid components of their preparations were soluble in chloroform but not in ether.

In this paper three polysaccharide preparations are described which are all obtained from *Sh. dysenteriae* and all possess the dominant specific serological character of that organism. They differ from one another, however, in their chemical and physical nature and also in some of their immunological properties. One of these materials is the degraded polysaccharide haptan already referred to, another is an undegraded polysaccharide and the third a lipopolysaccharide. These substances can all be obtained directly from the organisms by different methods of extraction, but the degraded polysaccharide and lipopolysaccharides were, in the work described in the present paper, prepared from the purified and apparently homogeneous conjugated protein-polysaccharide complex which had been extracted from the bacteria with diethylene glycol.

MATERIALS AND METHODS

Source of material. Degraded polysaccharide and lipopolysaccharide were obtained from a preparation of the 'O' somatic antigenic complex which had been examined for homogeneity and in which no impurities could be detected by the methods of ultracentrifuging, electrophoresis and fractional solubility (Davies *et al.* 1954). The antigenic complex was extracted, together with simple undegraded polysaccharide, by diethylene glycol from acetone-dried cells of *Sh. dysenteriae*.

Materials for analysis. Samples of the extraction products were thoroughly dialysed against distilled water at 0–2° and dried from the frozen state. The freeze-dried materials absorbed approximately 15% of their weight of moisture if left exposed to the air. They were further dried to constant weight *in vacuo* at 78° before making into solutions of known concentration in water.

Total nitrogen. This was determined by the Kjeldahl method using the distillation apparatus of Markham (1942) and the mixed bromocresol green + methyl red indicator of Ma & Zuazaga (1942).

Phosphorus. This was determined on samples containing 5–30 µg. of P by a modification of the method of Fiske & Subbarow (1925). Labile P was taken to be the amount estimable after hydrolysis for 10 min. in N-HCl at 100°.

Sugars. Glucosamine was estimated by a modification of the colorimetric method of Elson & Morgan (1933) and *N*-acetylglucosamine by the method of Morgan & Elson (1934)

and Aminoff, Morgan & Watkins (1952). Rhamnose was determined by the method of Dische & Shettles (1948) and galactose by a method based on that of Dische, Shettles & Osnos (1949). The method of Somogyi (1937) was used to measure reduction.

Chromatography. Except where otherwise stated, chromatograms were run on Whatman no. 1 paper for approximately 17 hr. with ethyl acetate–pyridine–water (Jermyn & Isherwood, 1949) and sprayed with anisidine hydrochloride in butanol (Hough, Jones & Wadman, 1950). An 'Agl' micrometer syringe was used to deliver small known volumes of solutions on to papers for quantitative estimations on eluates of the individual sugars after their chromatographic separation.

Absorption measurements. Absorptions at specific wavelengths were measured in a 'Unicam' ultraviolet spectrophotometer; other absorption measurements were carried out in a Hilger Spekker absorptiometer.

Ultracentrifuging. Preparative ultracentrifuge runs were carried out in a Spinco Model 'L' Ultracentrifuge at 2–4°. Figures quoted for the force in *g* refer to the force exerted at the centres of the tubes and represent average values. Analytical ultracentrifuge runs were carried out in a Spinco Model 'E' machine except where otherwise stated.

Diffusion. Measurements were made with apparatus based on the Gosting–Kegeles–Longsworth version of the Gouy diffusimeter, using a cell and boundary sharpening methods similar to those described by Coulson, Cox, Ogston & Philpot (1947) and Creeth (1952).

Antigenicity. This was measured as the capacity of the materials to elicit the formation of specific *Sh. dysenteriae* agglutinins or precipitins, or of Forssman heterophile antibodies in the rabbit. Animals were injected intravenously with 0.5 ml. samples of fractions dissolved in sterile saline. Agglutinins were tested for using a saline suspension of heat-killed smooth homologous organisms adjusted, by measurement of opacity, to 0.2 mg./ml. The degree of agglutination was read after incubating the test serum and suspension for 2 hr. at 55° and leaving overnight at 0–2°. Normal rabbit sera rarely show agglutinins above a dilution of 1 in 2. Precipitin titres were read after incubating the test serum and solution at 37° for 2 hr. and leaving at 0–2° overnight. Heterophile antibodies were measured by observing the degree of haemolysis after incubating tubes containing dilutions of serum in a volume of 0.5 ml., guinea pig complement (0.5 ml. of a 1 in 20 dilution) and one drop of a 50% suspension of washed sheep erythrocytes at 37° for 45 min. and leaving overnight at 0–2°.

Toxicity. This was estimated by injecting groups of not less than ten white mice (body weight, 18–22 g.) intraperitoneally for each of six dilutions of the test material in sterile saline. Deaths were recorded over 4 days, most of the animals which died did so on the second day.

EXPERIMENTAL

Preparation of the degraded polysaccharide

The preparation of this material was described in detail by Morgan (1936); the methods which were used in that investigation and have again been employed are, therefore, only given in outline. A sample of the purified protein-polysaccharide complex was hydrolysed in 1% (w/v) acetic acid at 100° for 4 hr. in an atmosphere of nitrogen and allowed to stand at 0–2° overnight. The insoluble conjugated

protein was separated by centrifugation, washed once with 1% (w/v) acetic acid, once with distilled water, and the washings added to the original supernatant solution. The moist sedimented protein was dried from the frozen state; this product amounted to approximately 25% of the weight of the antigen used. The supernatant solution remaining after separation of the protein was concentrated at 16° to about one-third of its original volume and the degraded polysaccharide precipitated by the addition of 10 vol. of ethanol. The precipitated material was recovered by centrifuging after standing at 0–2° overnight, redissolved in water and fractionated with ethanol which contained a small amount of potassium acetate to facilitate flocculation. In a typical experiment 4.5 g. of the protein-polysaccharide complex yielded polysaccharide which, after fractionation with ethanol, gave the materials whose analyses are detailed in Table 1.

Table 1. Fractionation of 'Shiga' degraded polysaccharide with ethanol

No. of vol. ethanol added	Wt. recovered	Total N (%)	Total P (%)
2	18.3 mg.	5.7	1.2
8	3.0 g.	2.2	0.14
Over 8	14.5 mg.	0.9	0.12

The fraction precipitated between the levels of 2 and 8 vol. of ethanol was redissolved in a small volume of 50% (v/v) aqueous acetic acid, and glacial acetic acid was added to bring the concentration up to 90%. At this level a small amount of material containing 2.5% N and 1.7% P was removed by centrifuging and the polysaccharide recovered by the further addition of acetic acid to a concentration of 96% (v/v); this fraction contained 1.9% N and 0.04% P. Some material recovered from the final supernatant solution by evaporation of the solvents, dialysis and freeze-drying did not differ significantly in its composition from the main fraction and was therefore combined with it. The product appeared from examination in fractional solubility tests, carried out as described later, to be of constant composition. Preliminary quantitative estimations of the three component sugars, acetylglucosamine, galactose and rhamnose showed, however, that only about 75% of the weight of the material could be accounted for. The material was therefore treated further to find whether any change occurred in the analytical figures; as no significant change was found the following procedure is only described in outline.

The polysaccharide was dissolved in cold distilled water at 1% (w/v) concentration and centrifuged at 105 000 g for 4 hr.; a very small amount of material was deposited. The supernatant solution was buffered at pH 8.5 by the addition of phosphate and incubated at 37° for 24 hr. with crystalline trypsin (10 mg.) in the presence of toluene. The material was recovered by the addition of 10 vol. of cold ethanol, redissolved and deproteinized by shaking several times with a butanol-chloroform mixture according to the method of Sevag (1934). The aqueous phase was then dialysed against distilled water and dried from the frozen state. The recovered polysaccharide was redissolved in freshly distilled pyridine, and acetylated by the addition of acetic anhydride; the acetylated material was recovered by pouring the solution

into ether, as described by Morgan (1936). The fully acetylated polysaccharide was dissolved in acetone and precipitated by the addition of water, redissolved in acetone and reprecipitated by adding a mixture of ethanol and ether. The material was deacetylated by warming at 37° for several hours in acetone to which a slight excess of N aqueous KOH had been added, and recovered, after dialysis against distilled water, by precipitation with ethanol. The precipitate was redissolved, the solution clarified by centrifuging at 105 000 g for 3 hr., dialysed and dried from the frozen state. The final product contained 1.9% N and no detectable P.

Examination of the degraded polysaccharide for homogeneity

Fractional solubility. Tests were carried out in the following way. A quantity of dried material (100–200 mg.) was shaken in a small volume of solvent (about 10 ml.). When a part had dissolved it was centrifuged and the material recovered from the supernatant solution. To the sediment a fresh volume of solvent was added and the process repeated until all the material had dissolved. Such a test was carried out using diethylene glycol as solvent, each fraction was recovered by pouring into several volumes of acetone, washing the precipitated polysaccharide with acetone to remove glycol, removal of residual acetone *in vacuo*, redissolving in water and drying from the frozen state. No significant differences were found in the analytical figures of the five fractions obtained (Table 2). A further test was carried out using 50% (v/v) aqueous acetone as solvent, each of the five fractions obtained was recovered by removing the acetone by evaporation and freeze-drying the resulting aqueous solutions. Again no significant differences were found in the analytical figures of the fractions and the material therefore showed no evidence of inhomogeneity. Dialysis was avoided in these tests because the degraded polysaccharide is of relatively small molecular weight and a little loss is normally found on dialysis.

Table 2. A fractional solubility test on the degraded polysaccharide using diethylene glycol

Fraction	Wt. (mg.)	N (%)	Rhamnose (%)	Rhamnose/N
1	35.4	1.9	33.4	17.6
2	37.3	1.9	32.7	17.3
3	21.8	1.9	32.7	17.3
4	15.3	1.9	32.3	17.0
5	22.0	1.9	33.5	17.7

Electrophoresis. Samples of the polysaccharide were examined in the Tiselius electrophoresis apparatus at 0.5% (w/v) concentration in phosphate buffer pH 8.0 and acetate buffer pH 4.1. Photographs obtained in these runs are shown in Fig. 1 (c and d). No evidence of inhomogeneity was found. The material travels slowly, towards the anode, but somewhat faster at pH 8 than at pH 4.1. Spreading of the boundaries was fairly rapid owing to diffusion because the molecule is relatively small. Exact mobility measurements have not been made.

Ultracentrifuging. The pictures shown in Fig. 1 (a and b) were obtained with degraded polysaccharide at 0.5 and 1% (w/v) concentrations respectively at pH 8 (phosphate buffer) and pH 4.5 (acetate buffer). Only one component is revealed at both pH values. Spreading of the boundaries is rather rapid; this does not appear to be due entirely to diffusion and the material is probably somewhat polydisperse.

Chemical composition of the degraded polysaccharide

The material dissolved very readily in water to give a clear non-viscous solution. Analytical figures

for the purified polysaccharide were C, 43.1%; H, 7.34%; N, 1.9%; no detectable P; ash 0.11%; $[\alpha]_D^{20} + 98^\circ$ (2%, w/v concentration in water).

The component sugars were identified by Morgan (1936, 1938); glucosamine was shown to be present, derivatives of galactose and rhamnose isolated, and the presence of acetyl groups demonstrated by the isolation from the material of silver acetate. Partridge (1948) considered, as a result of paper chromatographic analysis, that no other reducing sugars were present.

Estimation of the component sugars. Approximate values for each of the three sugars were obtained by the methods already referred to and the results were used to make more accurate estimations which were carried out as follows.

Polysaccharide (1 mg.) was hydrolysed for 8 hr. at 100° with 0.5 N-HCl, an hydrolysis curve having previously shown that the amount of glucosamine and the total reduction had reached maximum values after this period and the specific rotation a minimum value ($[\alpha]_D^{20} + 53^\circ$). Under these conditions of hydrolysis deacetylation of the amino groups takes place. Amounts of the hydrolysate which contained 100 μ g. of the material were used as samples for estimation after neutralization with NaOH. Standards were set up to contain the three sugars in approximately their correct amounts, i.e. rhamnose 33 μ g., galactose 28 μ g. and glucosamine base in different amounts covering the range of 17.5–27.5 μ g. An internal glucosamine standard which was included gave the expected recovery. The amount of glucosamine base found was 22%, the equivalent weight of acetylglucosamine being 27.5%. This would correspond to a N content of 1.7%, but a value of 1.9% has consistently been found by the Kjeldahl method. The rhamnose content of the material was estimated without previous hydrolysis. Samples of polysaccharide (100 μ g.) were heated with H_2SO_4 under the conditions described by Dische & Shettles (1948) and the difference in the absorption intensity at 400 and 430 m μ . of the solutions after the addition of cysteine hydrochloride taken as a measure of the amount of rhamnose present. At these two wavelengths the absorption due to galactose and glucosamine is equal and they do not interfere with the estimation. Standards were set up to contain 28 μ g. of galactose, 25 μ g. of acetylglucosamine and recrystallized rhamnose hydrate equivalent to amounts of anhydrous rhamnose in the range of 27.5–37.5 μ g. An internal rhamnose standard gave the expected recovery. The polysaccharide was estimated to contain 33% of rhamnose. As it was intended to estimate galactose on samples eluted from paper chromatograms, this method was first tried with rhamnose in order to determine whether the same figure could be obtained. A sample of polysaccharide was hydrolysed for 8 hr. at 100° with N- H_2SO_4 and neutralized with $Ba(OH)_2$. Two paper chromatograms were set up, one having five spots of rhamnose solution containing 27 μ g. in a volume of 15 μ l., the second having five spots of a neutral, salt-free, degraded polysaccharide hydrolysate containing 118.5 μ g. in a volume of 15 μ l. The papers were run overnight (17 hr.) with ethyl acetate–pyridine–water and the outer two strips of each paper cut away and sprayed to reveal the position of the sugar containing areas. Using these strips as guides, areas of paper (8 sq.cm.) containing

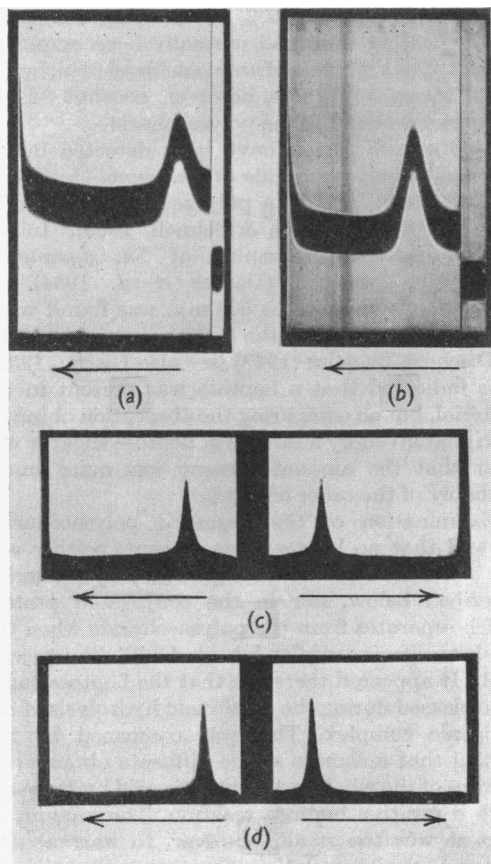


Fig. 1. Degraded polysaccharide, sedimentation and electrophoresis. (a) Ultracentrifuge pattern, concentration 0.5% (w/v) at pH 8.0 (phosphate-NaCl, $I=0.2$), force 260 000 g, exposure at 104 min.; (b) ultracentrifuge pattern, concentration 1.0% (w/v) at pH 4.5 (acetate-NaCl, $I=0.2$), force 260 000 g, exposure at 183 min.; (c) electrophoresis pattern, concentration 0.5% (w/v) at pH 8.0 (phosphate, $I=0.2$), exposure at 4 hr.; (d) electrophoresis pattern, concentration 0.5% (w/v) at pH 4.1 (acetate, $I=0.1$), exposure at 4 hr. Direction of movement shown by arrows, anodic on electrophoresis.

rhamnose were cut out and each eluted into a volume of 1 ml. of water. Estimations of rhamnose were carried out in triplicate with the solutions containing known amounts of the sugar, with those containing unknown amounts in the polysaccharide hydrolysate and on eluates from blank pieces of paper of equal size taken from parts of the same sheets in the area over which the solvent had travelled. The recovery from known rhamnose samples was $100 \pm 3\%$ and 32.2% of rhamnose was found in the polysaccharide. This is in good agreement with the figure obtained directly using unhydrolysed samples of polysaccharide.

Galactose was estimated on polysaccharide samples ($100 \mu\text{g.}$) which had been treated in the same way as for the rhamnose estimation. In this case, however, the mixtures were left for 48 hr. when a blue colour developed which was measured at $603 \text{ m}\mu$. The presence of methylpentose gives rise to low values and the absorption at $603 \text{ m}\mu$. was not always directly proportional to the amount of galactose present; it could sometimes be made so by the addition of mannose ($100 \mu\text{g.}$) to all standards and unknowns as suggested by Dische *et al.* (1949). Galactose standards were set up to contain amounts of sugar in the range of $15\text{--}30 \mu\text{g.}$; rhamnose ($33 \mu\text{g.}$) and acetylglucosamine ($25 \mu\text{g.}$) were included in each standard. Internal galactose standards failed to give the expected results but the amount of galactose present in the polysaccharide appeared to be in the range of $25\text{--}29\%$.

Neither this method nor the method 'PCyR1' described by Dische *et al.* (1949) appeared to be altogether satisfactory when applied to the polysaccharide material, and on this account an estimation was carried out on galactose samples eluted from paper chromatograms. This was carried out exactly as previously described for rhamnose, $30 \mu\text{g.}$ samples of galactose gave a 97% recovery, the paper blanks an insignificant reading, and the polysaccharide was estimated to contain 27% of galactose.

The total sugars estimated amount to 87.5% of the weight of the polysaccharide, but adjusted for the addition of water on hydrolysis (approximately 9.5%) the figure becomes 78% .

Total reduction. Standard curves were constructed for the three sugars present over the range of $100\text{--}500 \mu\text{g.}$ and these were compared with that obtained for glucose. Taking glucose as 100% the other sugars gave the following comparative figures under the conditions used: galactose 72.5% , rhamnose 64.5% , glucosamine 95.5% (and acetylglucosamine 36.5%). A sample of polysaccharide was found to give 70.5% reduction by this method calculated as glucose or 93.6% when adjusted for the three sugars actually present in their estimated amounts. Allowing for the weight of water added on hydrolysis this figure becomes approximately 85% .

The nature of a possible additional component. It appeared from the above results that about $15\text{--}20\%$ of the polysaccharide was unaccounted for and that some other component remained undetected. No reducing sugars other than those already found could be demonstrated by paper chromatography using the following solvent systems; collidine-lutidine-water, ethyl acetate-pyridine-water, butanol-pyridine-water and phenol.

The possibility that a sugar alcohol might be present could not be substantiated; a range of sugar

alcohols and inositol were found to be readily detected on paper chromatograms by spraying with AgNO_3 .

By potentiometric titration a curve was obtained using synthetic sugar mixtures made up in the light of results of the quantitative estimations already described which did not deviate significantly from a curve obtained using a polysaccharide hydrolysate and no evidence for the presence of an aldonic acid lactone could be found.

Synthetic mixtures of sugars were made up to simulate a polysaccharide hydrolysate and heated with acid, after which estimations were carried out to find whether any significant losses occurred during hydrolysis. Small losses were actually found and losses of up to 10% occurred on somewhat longer heating than had normally been employed when 3% of a mixture of amino acids were included. These losses would not, however, account for the discrepancy found in the polysaccharide.

Aldoheptose sugars have been detected in the specific lipopolysaccharide of *Sh. sonnei* (Jesiatis & Goebel, 1952), and in a polysaccharide extracted from *Sh. flexneri* (Slein & Schnell, 1953). In the somatic antigenic complex of *Sh. dysenteriae* previously described (Davies *et al.* 1954), an absorption maximum at $505 \text{ m}\mu$. was found when a sample was tested by the H_2SO_4 -cysteine reaction of Dische & Shettles (1948) (see also Dische, 1953). This indicated that a heptose was present in the material, but on comparing the absorption obtained with that given by a sample of heptose sugar it was clear that the amount present was quite small, probably of the order of $2\text{--}3\%$.

Examination of the degraded polysaccharide showed that no heptose was present, neither was any contained in the undegraded polysaccharide described below, nor in the conjugated protein which separates from the polysaccharide when the whole antigenic complex is heated with dilute acetic acid. It appeared therefore that the heptose sugar was released during the acetic acid hydrolysis of the antigenic complex. This was confirmed by the finding that a sample of the diffusate obtained on dialysis of the whole antigen acetic acid hydrolysate gave a positive heptose reaction. The amount of heptose was too small, however, to warrant any attempt at its isolation from the quantity of somatic antigen available.

The lipopolysaccharide recovered from the antigenic complex by treatment with alkali or phenol retains the heptose sugar.

Molecular size of the degraded polysaccharide

End-group assay. While following the further degradation of the polysaccharide on continued heating at 100° in acetate buffer (pH 4), under conditions comparable to those used in the separa-

tion of the material from the antigenic complex, it was found that both reducing power and acetylglucosamine could be measured on the polysaccharide itself without further degradation.

Acetylglucosamine was measured on samples of intact material. Polysaccharide samples (3.75 and 7.5 mg.) gave amounts of colour equivalent to 30 and 57 μg . respectively of acetylglucosamine or 0.8% of the weight of the material. From these figures a minimum mean molecular weight of the order of 28 000 can be deduced.

Samples of the polysaccharide (15 mg.) gave an amount of reduction equivalent to 50 μg . calculated as glucose by the method of Somogyi. Acetylglucosamine was found to give only 36.5% of the amount of reduction given by glucose under the conditions used, the figure of 50 μg . could therefore be adjusted to 138 μg . on the assumption that acetylglucosamine was responsible for all this reduction. This is equivalent to 0.9% of the weight of the polysaccharide, and as this is so close to the acetylglucosamine figure obtained by direct estimation, the adjustment would seem to be a legitimate one. From this figure a minimum mean molecular weight of the order of 25 000 can be deduced.

Polysaccharides may have one, but usually have more than one, non-reducing end group, but in order to have more than one reducing end group a link of other than the usual glycosidic type would have to be present in the molecule. If no such unusual structure is present in this instance the minimum molecular weight would be equal to the true molecular weight.

Similar estimations carried out on samples of polysaccharide after heating at 100° for 92 hr. at pH 4 showed that further degradation had taken place and that the material was then composed of oligosaccharides having a mean molecular weight of about 2500; these would contain about fifteen sugar residues. The total reduction (8% calculated as acetylhexosamine) agreed well with the acetylglucosamine figure obtained by direct estimation (8.25%), from which it is deduced that all the reducing end groups are acetylglucosamine residues.

Sedimentation. Samples of polysaccharide were examined at concentrations of 1.0, 0.5 and 0.25% (w/v) in acetate buffer pH 4.5, $I = 0.1$ and containing 0.1M-NaCl, and the sedimentation coefficients determined. The partial specific volume was calculated from density measurements made pycnometrically using a 2% solution of polysaccharide in the same acetate buffer as that employed in the ultracentrifuge runs. A value of 0.648 was found. The corrected S_{20} values showed considerable change with concentration and the curve could not be extrapolated. The $1/S_{20}$ values, however, could be

extrapolated with more confidence (Fessler & Ogston, 1951), as can be seen from Fig. 2 (a). $1/S_{20} \times 10^{-13}$ at zero dilution was 0.582 giving $S_{20} = 1.72 \times 10^{-13}$.

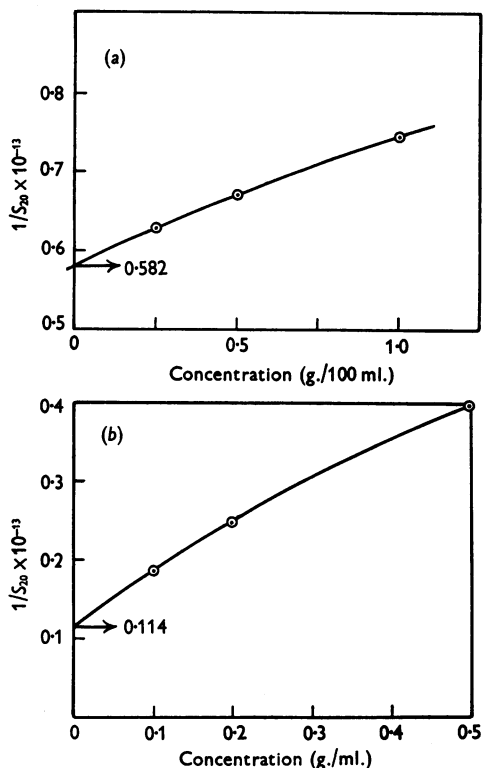


Fig. 2. Sedimentation coefficients. (a) Degraded polysaccharide, measurements made at pH 4.5 in acetate buffer, $I = 0.1$ containing 0.1M-NaCl; (b) undegraded polysaccharide, measurements made at pH 8.0 in phosphate buffer, $I = 0.1$ containing 0.1M-NaCl.

Diffusion. The diffusion coefficients were measured in a Gouy diffusion apparatus with an optical path length from centre of cell to photographic plate of 283.9 cm. The cell used had a path length of approx. 1.8 cm. All measurements were carried out at 20°. The results were calculated from the rate of movement of the outermost fringe away from the undeviated slit image. The diffusion coefficients so obtained correspond to those resulting from the height:area method in conventional refractive index gradient procedures. The polysaccharide was dissolved in and dialysed against pH 4.5 acetate buffer, $I = 0.1$, containing 0.1M-NaCl, for 3 days at 2° and thereafter stored at 2°. Dilutions were made from a stock 0.5% solution by weight. Points 1, 3 and 4 (Fig. 3a) were determined within 1 week from the start of the dialysis; point 2 was obtained

after 4 weeks storage and is in good accord with the other points showing that the material is quite stable in solution in this buffer at 2°. The variation in diffusion with concentration is small. A straight line through the three points at the higher concentrations gives an extrapolated value for D_{20} ($c=0$) = 4.35×10^{-7} . After correction for the viscosity of the buffer this gives a value of 4.61×10^{-7} for diffusion in water.

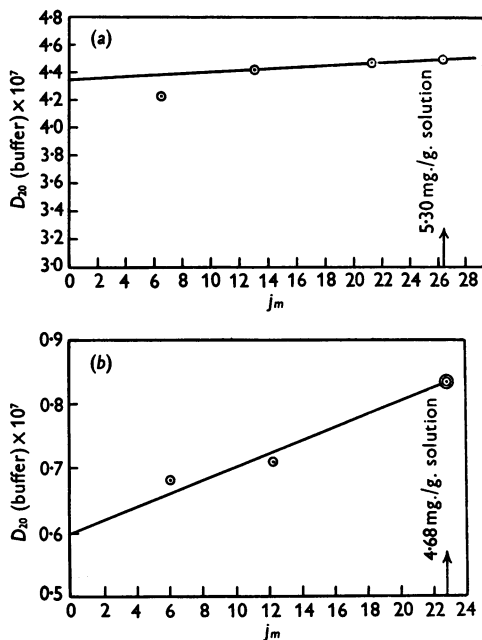


Fig. 3. Diffusion coefficients. (a) Degraded polysaccharide, measurements made at pH 4.5 in acetate buffer, $I=0.1$ containing 0.1 M-NaCl; (b) undegraded polysaccharide, measurements made at pH 8.0 in phosphate buffer, $I=0.1$ containing 0.1 M-NaCl. j_m is proportional to the concentration and has the significance given, e.g. by Gosting, Hanson, Kegeles & Morris (1949).

This result when combined with the sedimentation coefficient and partial specific volume in the Svedberg formula $M = RTs/D(1 - V)$ gives a value of 26 000 for the molecular weight, in excellent agreement with that deduced by the end-group assay, showing that the physical units consist simply of the chemical molecules and are not aggregates of these.

The simple undegraded polysaccharide

Preparation. In a previous communication (Davies *et al.* 1954), the extraction of acetone-dried cells of *Sh. dysenteriae* with diethylene glycol was described. After the extracts had been fractionated

with acetone and with $(\text{NH}_4)_2\text{SO}_4$ to remove un-specific contaminating materials, and the phospholipid component also removed, examination in the analytical ultracentrifuge revealed that two components were present. One substance sedimented rapidly and was somewhat polydisperse; this was shown to be the conjugated protein-polysaccharide complex; the second substance travelled much more slowly and gave a very sharp peak. The considerable difference in sedimentation rate suggested the possibility that these two materials might be separable by differential high-speed centrifuging.

Purified diethylene glycol extracts were, therefore, centrifuged in a Spinco Model 'L' machine at 1% (w/v) concentration in water. In the supernatant solutions remaining after centrifugation for 3 hr. at 105 000 g a viscous polysaccharide was found which differed from the sedimented material in not being opalescent and in having a substantially lower N content. The sedimented antigenic complex was separated from the traces of free polysaccharide it retained by repeated sedimentation. The free undegraded polysaccharide was likewise freed from traces of the antigenic complex by repeated centrifuging at 105 000 g; each time the supernatant solution was removed and respun. Some loss of polysaccharide into the sediment was inevitable in the course of removing the last traces of antigenic material. The solution was finally freeze-dried when the N and P figures showed no further reduction.

Origin of the undegraded polysaccharide. When freed from the protein-polysaccharide complex the polysaccharide could be precipitated from aqueous solution with acetone between the levels of 50 and 60% (v/v) acetone concentration and also with $(\text{NH}_4)_2\text{SO}_4$ between the levels of 35 and 40% saturation with salt. The antigenic extracts had originally been freed from un-specific contaminants by precipitation between these levels.

A sample of conjugated protein-polysaccharide complex in which no evidence of inhomogeneity could be found, was stable on storage in solution at pH 7.0 (phosphate buffer) for several months and did not tend to dissociate (a process known to take place in alkaline solution, the products being a simple protein and a lipopolysaccharide). At no stage in the treatment of the organisms or of the extracts obtained from them, were alkaline conditions used. In one experiment in which organisms were extracted with diethylene glycol the whole procedure was carried out at 0°; in the crude diethylene glycol product, however, the undegraded polysaccharide component could be detected by its characteristic sharp peak in the analytical ultracentrifuge even at this earliest stage of extraction.

In order to know whether the amount of free undegraded polysaccharide differed in successive

extracts, a batch of acetone-dried organisms weighing 80 g. was extracted in the usual way with diethylene glycol 3 times and the extracted material purified by acetone and ammonium sulphate fractionations, each extract being kept separate throughout the process. Details of the products are shown in Table 3. Portions of each extract were dissolved in water, centrifuged at 105 000 g for 4 hr. and the sediments and materials remaining in the supernatant fluids recovered. Details of the products are shown in Table 4. The amounts of material in the supernatant fluids, which, from their N contents are seen to be very largely undegraded polysaccharide, decrease by about half in successive extracts.

Table 3. *The products of a diethylene glycol extraction of 'Smooth' Sh. dysenteriae*

Extract	Yield (g.)	N (%)	P (%)
1	1.49	4.2	0.50
2	0.60	4.5	0.63
3	0.22	6.7	0.60

Table 4. *The products of high-speed centrifuging of Sh. dysenteriae diethylene glycol extracts*

Extract	Wt. of starting material (mg.)	Wt. recovered (mg.)	Wt. (%)	N (%)	P (%)
1	364	Ppt. 117	—	6.9	0.8
		Supt. 221	65	3.0	0.3
2	251	Ppt. 161	—	5.0	0.8
		Supt. 78	33	2.8	0.2
3	177	Ppt. 138	—	7.4	0.66
		Supt. 28	17	3.0	0.3

Table 5. *A fractional solubility test on undegraded polysaccharide using trimethylene glycol*

Fraction	Wt. (mg.)	N (%)	P (%)	N/P
1	15.4	2.3	0.07	32.8
2	37.3	2.2	0.07	31.4
3	18.3	2.25	0.08	28.1
4	31.2	2.4	0.08	30.0
5	22.2	2.25	0.08	28.1

These results strongly suggest that the undegraded polysaccharide is extracted directly from the organisms and does not arise from the antigenic complex by dissociation in the course of the further treatment of the extracts.

Homogeneity. A specimen of undegraded polysaccharide was examined for homogeneity in a fractional solubility test carried out as previously described and using trimethylene glycol as solvent. Five fractions were obtained whose analysis showed only small differences which were probably due to experimental error and are not thought to indicate

heterogeneity. The results of this test are shown in Table 5.

Electrophoresis runs were carried out at pH 8.0 in phosphate buffer and pH 4.1 in acetate buffer, both at 0.25% (w/v) concentration. Photographs obtained in these examinations are shown in Fig. 4c, d. The material travelled very slowly anodically at both pH values and there was little spreading due to diffusion even after 21 hr. running. Exact mobility measurements were not made.

Photographs obtained in analytical ultracentrifuge runs at pH 8.0 in phosphate buffer and pH 4.5 in acetate buffer showed a sharp peak which moved much more slowly than that given by the degraded polysaccharide. Again no evidence of inhomogeneity was obtained (Fig. 4a, b).

Chemical properties. The material dissolved slowly in water to give a clear solution having a viscosity of 7.8 at 1% (w/v) concentration at 20° relative to water at that temperature. Analytical figures found were C, 43; H, 7.2; N, 2.20; P, 0.075; ash 0.55%. Specific rotation $[\alpha]_D^{20} + 94^\circ$ in water (c, 1).

Examination by paper chromatography after acid hydrolysis of the material revealed the three sugars previously found in the degraded polysaccharide. On hydrolysis with 1% acetic acid at 100° for 4 hr. or with 15% acetic acid for a shorter period, in an atmosphere of nitrogen, about 5% of the weight of the material could be recovered as an alkali-soluble, acid-, ether- and chloroform-insoluble component which contained 7.5% N and 1.4% P. This material would appear to be similar to the conjugated protein but with a lower N content. Insufficient of this fraction was available for further study. The acid-soluble product was identical with the degraded polysaccharide already described.

Quantitative estimations of the component sugars were carried out as described for the degraded polysaccharide and the following figures obtained: rhamnose 30%, galactose 25–26% and acetylglucosamine 22% (estimated as glucosamine base). Approximately 70% of the material is thus accounted for as sugars after allowing for the weight of water added on hydrolysis.

Molecular size. It was not possible to measure the minimum molecular weight by end-group assay, the methods of reduction (Somogyi, 1937) and acetylhexosamine estimation (Morgan & Elson, 1934) were insufficiently sensitive. From this fact it was concluded that the molecule is substantially larger than that of the degraded polysaccharide.

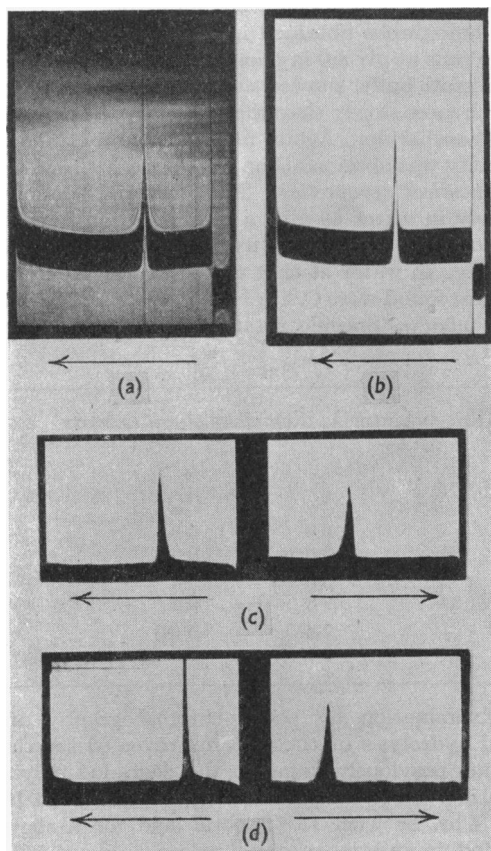


Fig. 4. Undegraded polysaccharide, sedimentation and electrophoresis. (a) Ultracentrifuge pattern, concentration 0.25% (w/v) at pH 8.0 (phosphate-NaCl, $I=0.2$), force 260000 g, exposure at 96 min.; (b) ultracentrifuge pattern, concentration 0.25% (w/v) at pH 4.5 (acetate-NaCl, $I=0.2$), force 260000 g, exposure at 118 min.; (c) electrophoresis pattern, concentration 0.25% (w/v) at pH 8.0 (phosphate, $I=0.2$), exposure at 21 hr.; (d) electrophoresis pattern, concentration 0.25% (w/v) at pH 4.1 (acetate, $I=0.1$), exposure at 5 hr. Direction of movement shown by arrows, anodic on electrophoresis.

Sedimentation constants were obtained at concentrations of 0.5, 0.2 and 0.1% (w/v) concentration at pH 8.0 (phosphate buffer, $I=0.1$, containing 0.1 M-NaCl) in the Svedberg oil-turbine ultracentrifuge. When the values of $1/S_{20} \times 10^{-13}$ were plotted

against concentration a curve was obtained (Fig. 2b), which extrapolated to 0.114 at zero dilution, giving $S_{20} = 8.76 \times 10^{-13}$.

The figure (0.639), obtained for the partial specific volume and used for correcting the S_{20} values, was measured pycnometrically on a 1.5% (w/v) solution of polysaccharide in the same pH 8 buffer as that used in the ultracentrifuge runs; solutions more concentrated than 1.5% were difficult to handle on account of their viscosity.

The diffusion constant was measured in the manner already described for the degraded polysaccharide. The material was dissolved in and dialysed against a pH 8 phosphate buffer, $I=0.1$, containing 0.1 M-NaCl. Dilutions were made by weight from a 0.5% stock solution. Owing to the high viscosity and low diffusion coefficient of the material a high degree of accuracy of measurement of the diffusion constant could not be attained in this case. The dependence of diffusion on concentration is considerable and this results in asymmetrical concentration gradients. Extrapolation to zero concentration (Fig. 3b) leads to an approximate value for $D_{20} (c=0) = 0.60 \times 10^{-7}$ which after correction for the viscosity of the buffer gives 0.62×10^{-7} for the diffusion coefficient in water.

Combined with the sedimentation coefficient and the anhydrous partial specific volume in the Svedberg formula this gives a figure of 950000 for the molecular weight.

Lipopolysaccharide

Preparation. Lipopolysaccharide was prepared from a sample of the protein-polysaccharide antigenic complex by the method of Westphal, Lüderitz & Bister (1952) after removal of the ether-soluble phospholipid. The antigen was dissolved in water at 1% (w/v) concentration and the solution warmed to 65°. An equal volume of 90% (w/v) phenol at 65° was added and mixed to give one phase. The solution was kept at 65° for 45 min. and then cooled at 0°. This brought about a separation of aqueous and phenol phases, which was hastened by centrifuging. The phases were recovered independently, dialysed against distilled water at 0° until free from phenol, and freeze-dried. The phenol-insoluble water-soluble material contained lipopolysaccharide, the phenol phase yielded an alkali-soluble protein containing 13–14% N.

A convenient source of the lipopolysaccharide proved to be the side fraction precipitated between the levels of 20 and 35% saturation with $(\text{NH}_4)_2\text{SO}_4$ which accumulated during the large-scale extraction of protein-polysaccharide antigen with diethylene glycol (Davies *et al.* 1954). This fraction contained a considerable amount of the antigenic material but was contaminated with small quantities of nucleoprotein. The material was extracted with warm

45% phenol as already described and the lipopolysaccharide obtained could be precipitated from the aqueous phase between the levels of 35 and 40% saturation with $(\text{NH}_4)_2\text{SO}_4$ and by this means freed from material showing light absorption at 260 $m\mu$. The product was dissolved in water at 1% (w/v) concentration and freed from ether-soluble phospholipid by pouring into 10 vol. of an ether-ethanol mixture (1:1, v/v) containing 0.5% of conc. HCl at -10° . This process was repeated twice, the precipitated material was then dialysed and freeze-dried.

Homogeneity. Examination in the analytical ultracentrifuge revealed one component at pH 8.0 and pH 4.5 (phosphate and acetate buffers re-

spectively, $I=0.1$, containing 0.1 M-NaCl). Photographs taken of these runs are shown in Fig. 5*a, b*. The material sedimented very rapidly and sedimentation coefficients were not determined. From the rate of movement of the boundary, however, and from the observation that sedimentation was more rapid at pH 4.5 than at pH 8.0, it is apparent that the material is more highly aggregated in acid solution but it would appear to have a particle size of several million even at pH 8.0.

Electrophoresis at pH's 8.0 and 4.1 (phosphate and acetate buffers, $I=0.2$ and 0.1 respectively) showed only one component; the substance travelled very slowly. Exact mobility measurements were not made (Fig. 5*c, d*).

Chemical composition. The purified material contained 2.04% N, 0.8% P of which 30% was labile P (P released in 10 min. on heating at 100° in N-HCl), $[\alpha]_D^{20} + 95^\circ$ in water ($c, 1.5$). The polysaccharide dissolved readily in water to give an opalescent solution. On hydrolysis with dilute acetic acid as previously described, some material separated from solution; this was recovered by centrifuging, washed with dilute acetic acid and freeze-dried. This acid-insoluble fraction failed to dissolve on shaking with ether but dissolved in chloroform. The CHCl_3 -soluble material amounted to about 7% of the weight of the lipopolysaccharide. The acid soluble component was identical with the degraded polysaccharide described above.

Unlike the degraded and undegraded polysaccharide, the lipopolysaccharide gave a definite absorption at 505 $m\mu$. when tested by the H_2SO_4 -cysteine reaction, owing to the presence of a small amount of heptose sugar. Different heptose sugars gave different amounts of absorption in the reaction and as the sugar present in this instance has not been identified its exact amount could not be estimated. It would appear, however, to compose less than 5% of the weight of the polysaccharide.

Biological properties of the polysaccharides

The capacity of the materials to induce the formation of specific agglutinins in the rabbit was tested by injecting samples intravenously at 3- to 4-day intervals. Some results are shown in Table 6. The degraded polysaccharide appears to be non-antigenic, the undegraded polysaccharide induces agglutinins in an amount which could be accounted for by contamination to the extent of only 1% with the highly active conjugated protein-polysaccharide complex. From the method of preparation such a degree of contamination cannot be excluded and the tests for homogeneity are not sufficiently sensitive to detect so small an amount of a second component. The lipopolysaccharide is also relatively poorly active and its activity is also most probably due to the same cause.

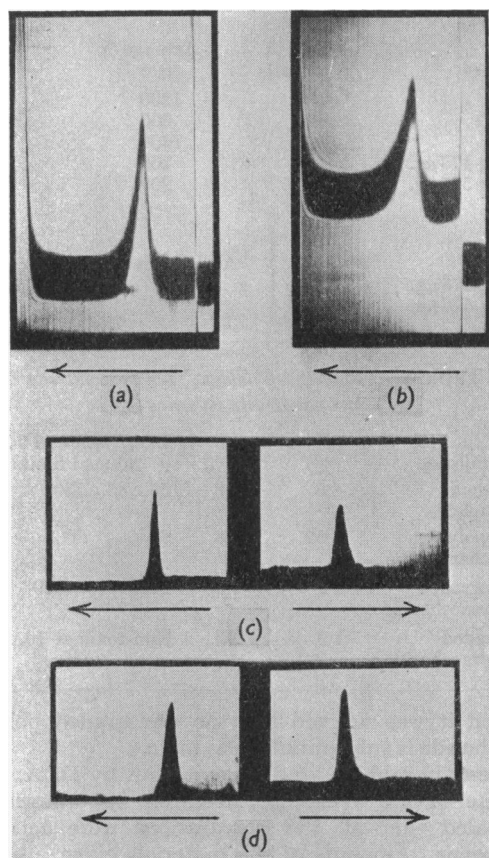


Fig. 5. Lipopolysaccharide, sedimentation and electrophoresis. (a) Ultracentrifuge pattern, concentration 1% (w/v) at pH 8.0 (phosphate-NaCl, $I=0.2$), force 260 000 g, exposure at 32 min.; (b) ultracentrifuge pattern, concentration 1% (w/v) at pH 4.5 (acetate-NaCl, $I=0.2$), force 260 000 g, exposure at 12 min.; (c) electrophoresis pattern, concentration 0.4% (w/v) at pH 8.0 (phosphate, $I=0.2$), exposure at 20 hr.; (d) electrophoresis pattern, concentration 0.4% (w/v) at pH 4.1 (acetate, $I=0.1$), exposure at 20 hr. Direction of movement shown by arrows, anodic on electrophoresis.

Table 6. *Agglutination titres of sera obtained by intravenous injection of 'Shiga' polysaccharide preparations into rabbits*

Material injected	N (%)	P (%)	Total dose ($\mu\text{g.}$)	Titre (average from four rabbits)
Antigenic complex	4.5	0.75	1	20
	4.5	0.75	3	320
	4.5	0.75	30	1560
Degraded polysaccharide	1.9	Nil	3000	Nil
Undegraded polysaccharide	2.2	0.08	30	10
	2.2	0.08	575	200
Lipopolysaccharide	2.05	0.8	30	75
	2.05	0.8	575	280

Table 7. *Forssman heterophile antibody induction by 'Shiga' polysaccharide preparations: haemolysis titres*

Material injected	Doses	No. of animals	Average titre
Lipopolysaccharide	$4 \times 10 \mu\text{g.}$	2	1200
	$5 \times 10 \mu\text{g.}$	2	600
	$2 \times 25 \mu\text{g.}$	5	1400
	3 totalling $175 \mu\text{g.}$	3	700
	5 totalling $575 \mu\text{g.}$	3	200
Undegraded polysaccharide	$3 \times 10 \mu\text{g.}$	2	All less than 50
	$5 \times 10 \mu\text{g.}$	2	
	$2 \times 25 \mu\text{g.}$	4	
	3 totalling $175 \mu\text{g.}$	2	
	5 totalling $575 \mu\text{g.}$	2	

The capacity of the materials to induce the formation of Forssman heterophile antibody was examined and some of the results obtained are shown in Table 7. The undegraded polysaccharide is completely inactive but the lipopolysaccharide is a strong heterophile antigen; the degraded polysaccharide was not examined in this instance but had previously been found to be totally inactive. The haemolysis titres were found to increase up to the second or third injection but further doses caused the titre to decrease; this fall was not observed for the relatively weak agglutinins found in some of these sera. It appeared that a series of small doses of lipopolysaccharide induced haemolysins as readily as larger doses, but rabbits are very variable in their response to the Forssman antigen and the number of animals used was not sufficient to warrant a firm conclusion on this point.

Precipitation titres were measured using a serum prepared by the injection of eight intravenous doses totalling $625 \mu\text{g.}$ of conjugated protein-polysaccharide complex. The degraded polysaccharide precipitated at a dilution of 1 in 12 million, the undegraded material at 1 in 3 million and the lipopolysaccharide at 1 in 2 million.

The results of the measurement of toxicity of the materials are given in Table 8. The lipopolysaccharide retains all the toxicity of the antigen from

Table 8. *Toxicity of 'Shiga' polysaccharide preparations in mice*

Material injected	N (%)	P (%)	LD ₅₀ ($\mu\text{g.}$) and 95% fiducial limits
Antigenic complex	4.5	0.8	55...85...130
Lipopolysaccharide	2.05	0.8	80
Undegraded polysaccharide	2.2	0.08	220...370...620
Degraded polysaccharide	1.9	Nil	Non-toxic at 10 mg.

which it was derived but the undegraded polysaccharide is substantially less toxic.

Tests in rabbits, kindly carried out by Dr A. C. White of the Wellcome Research Laboratories, revealed that all the preparations were active pyrogens. Amounts of the materials given intravenously to groups of three rabbits which induced the minimum significant rise of temperature ($0.6^\circ/\text{kg.}$ rabbit), were: degraded polysaccharide $5 \mu\text{g.}$, undegraded polysaccharide $0.05 \mu\text{g.}$, lipopolysaccharide $0.002 \mu\text{g.}$ and the full antigen (conjugated protein-polysaccharide complex) $0.002 \mu\text{g.}$ Whereas the degraded polysaccharide has less than one-thousandth of the activity of the lipopolysaccharide, the undegraded material has considerable activity.

DISCUSSION

Polysaccharides in what is probably their native condition, in various states of degradation and combined with proteins or lipids, have been prepared by different workers from many bacterial species. A polysaccharide has now been obtained in three different states from cultures of *Sh. dysenteriae* and in each instance no impurities have been demonstrable by the usual methods employed in examining large molecules for homogeneity. The physical, chemical and immunological properties of these materials have been compared.

Undegraded polysaccharide extracted together with the antigenic protein-polysaccharide complex by diethylene glycol accounted for more than half of the weight of the first extract of the bacteria but decreased by about half in successive extracts; this suggests the possibility that it composes the outermost layer of the cell and that the protein-polysaccharide complex lies beneath. The undegraded polysaccharide retains a small amount of conjugated protein-like material which accounts for the nitrogen content being significantly higher than that of the degraded material.

In the course of extracting the somatic antigenic complexes from several types of *Sh. flexneri* by means of diethylene glycol or pyridine, Goebel, Binkley & Perlman (1945) found that specific polysaccharide haptens were present in the supernatant solutions when the protein-polysaccharide complexes had been precipitated at 66% (v/v) level of acetone. The *Sh. dysenteriae* undegraded polysaccharide differs from the *Sh. flexneri* haptens in being precipitated together with the antigenic complex by 60% (v/v) of acetone.

The degraded polysaccharide is produced from the antigenic complex or from whole organisms by treatment with hot dilute acetic acid. From a study of the results of further hydrolysis of the polysaccharide under the same conditions something of the nature of the degradation can be deduced by extrapolation to the starting time; there appears to be a splitting of glycosidic links between acetylglucosamine and another sugar, since the total reduction due to end groups corresponds to the acetylglucosamine estimated, at least until the polysaccharide chains have broken down to lengths of about 15 sugar residues.

By quantitative estimation of the component sugars it was hoped that the polysaccharide could be totally accounted for in terms of its sugar constituents. A substantial discrepancy was found, however, and this problem remains for further investigation.

The lipopolysaccharide contained about the same amount of phosphorus as the antigen from which it

was derived. It has been found with some other Gram-negative organisms that lipopolysaccharide extracted directly from the bacteria by the phenol method has a much higher P content (i.e. a higher phospholipid content) than material extracted by the same method from the somatic antigen, (diethylene glycol or trichloroacetic acid product), where the P content is limited by the amount of ether-insoluble phospholipid present in the isolated somatic antigen. Samples of lipopolysaccharide with a high phospholipid content are very poorly soluble or insoluble in water.

The rate of sedimentation of the lipopolysaccharide indicated that the material, like the protein-polysaccharide complex, was aggregated to a very large particle size and on that account sedimentation coefficients were not measured.

The degraded polysaccharide is non-antigenic and non-toxic. The undegraded material is relatively weakly toxic and very poorly antigenic; the toxicity and antigenicity of preparations decreased progressively as their content of protein-polysaccharide complex decreased. It seems possible that since the antigenic complex is polydisperse some of the smallest molecules of the material do not differ in size sufficiently from those of the undegraded polysaccharide to be separable in the preparative ultracentrifuge and that the undegraded polysaccharide completely freed from amino acid containing material might be wholly without toxicity and antigenicity in the rabbit.

The lipopolysaccharide is a powerful Forssman heterophile antigen and differs from the undegraded polysaccharide chemically in containing the chloroform-soluble phospholipid component and a heptose sugar. It seems likely that the possession of a lipid component is one of the essential features of a heterophile antigen. It should be noted, however, that there is another marked difference between the two materials which might affect their antigenic properties, viz. their particle sizes. The role of the conjugated protein now becomes a subject of much interest, the lipopolysaccharide Forssman antigen has a particle size comparable with that of the protein-polysaccharide complex and they both contain the same specific determinant groups, but the lipopolysaccharide is a very poor antigen as judged by its ability to induce the formation of agglutinins and precipitins in the rabbit. Yet the protein-polysaccharide complex is a very powerful antigen in this respect, although the conjugated protein component is itself a very weak antigen with a different specificity. The full antigenic complex is a potent Forssman heterophile antigen.

The significance of the presence of a heptose sugar in the whole antigen and lipopolysaccharide is not understood. Jesiatz & Goebel (1952) found a heptose sugar which composed about 20% of the

specific lipopolysaccharide of *Sh. sonnei*, Slein & Schnell (1953) isolated a phosphorylated aldoheptose from a polysaccharide they extracted from *Sh. flexneri*. It is not known whether the heptose in the *Sh. dysenteriae* products is phosphorylated, but P is present in these materials. Aldoheptose sugars have been found in other bacterial polysaccharides, amounting in one case to at least 60% of the weight of the material (Davies, 1954, unpublished), but the absence of heptose from the 'Shiga' degraded polysaccharide indicates that it is bound in a different way from the other sugar components of the antigen.

Pyrogenic activity has been considered to be due to the presence of a chloroform-soluble phospholipid component combined to polysaccharide (Westphal, Lüderitz, Eichenberger & Keiderling, 1952). This may not be the only factor involved, however, since the undegraded polysaccharide has considerable activity although very little phospholipid could be present as judged from its P content, no phospholipid could be isolated from the amount of undegraded polysaccharide available. Furthermore the degraded polysaccharide is not altogether without activity.

SUMMARY

1. Three polysaccharide preparations have been obtained from cultures of 'Smooth' *Shigella dysenteriae*. Each material has the specificity of the dominant 'O' somatic antigen of the homologous organism.

2. The materials showed no evidence of inhomogeneity on ultracentrifugal and electrophoretic analysis at acid and alkaline pH.

3. The degraded polysaccharide hapten, $[\alpha]_D^{20} + 98^\circ$; N, 1.9%, obtained from the isolated antigenic protein-polysaccharide complex contains acetylglucosamine (27.5%), rhamnose (33%) and galactose (27%). No P was present. Molecular weight 25 000–28 000 by end-group assay and 26 000 by sedimentation and diffusion measurements, showing the identity of the chemical and physical units. The degraded polysaccharide is neither toxic nor antigenic, but is pyrogenic in relatively large doses (2–5 $\mu\text{g./kg.}$).

4. The undegraded polysaccharide, $[\alpha]_D^{20} + 94^\circ$; N, 2.2%, extracted from the organisms with diethylene glycol differed from the degraded material in containing a few per cent of amino acids, and in having a molecular weight of the order of one million by sedimentation and diffusion measurements. The material is poorly toxic (mouse LD₅₀, 400 $\mu\text{g.}$), is weakly antigenic and is less active as pyrogen (0.05 $\mu\text{g./kg.}$) than the lipopolysaccharide or complete antigen.

5. The lipopolysaccharide, $[\alpha]_D^{20} + 95^\circ$; N, 2.04%;

P, 0.8%, extracted from the isolated antigenic protein-polysaccharide complex with phenol contains about 7% of chloroform-soluble, ether-insoluble phospholipid. The lipopolysaccharide is of very large particle size, is toxic (mouse LD₅₀, 80 $\mu\text{g.}$) and is a strong heterophile (Forssman) antigen, but weakly active in inducing the formation of specific agglutinins and precipitins in the rabbit. The material is also a very powerful pyrogen, active at 0.002 $\mu\text{g./kg.}$ in the rabbit.

We wish to thank Dr R. G. Wallis of the Microbiological Research Department, Porton, for carrying out diffusion measurements and Dr R. A. Kekwick of the Lister Institute of Preventive Medicine, London, for determining the sedimentation coefficient of the undegraded polysaccharide. Permission to publish that part of the work carried out at the Microbiological Research Department has been granted by the Chief Scientist, Ministry of Supply.

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6-Hydroxynicotinic Acid as an Intermediate in the Oxidation of Nicotinic Acid by *Pseudomonas fluorescens*

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As shown by Allinson (1943), many strains of *Pseudomonas fluorescens* can grow on a medium containing inorganic salts with nicotinic acid as a sole source of carbon and nitrogen. When thus grown, washed cell suspensions will oxidize nicotinic acid forming carbon dioxide and ammonia, amongst other end products. The formation of the oxidative system is adaptive (Koser & Baird, 1944; Nichol & Michaelis, 1947). More recent studies (Pinsky & Michaelis, 1952) suggest that the pyridine ring is split during the first stages of the oxidative attack and that either carbon 2' or carbon 6' is involved in the ring-opening reaction. In the present paper two lines of approach were used to study the oxidation. First, the oxidation of some halogen-substituted nicotinic acids was studied; the results indicated that carbon 6' rather than carbon 2' was involved in the ring-opening reaction. Secondly, the possibility that intermediates in the oxidation might accumulate during adaptation was examined and 6-hydroxynicotinic acid was isolated in the early stages.

EXPERIMENTAL

Organisms. Three strains of *Ps. fluorescens* were isolated from soil-enrichment cultures by Mrs M. Kogut, Microbiology Department, Sheffield (Kogut & Brodoski, 1953). The strain (KBI) used in most of the experiments was isolated from medium containing 0.1% sodium benzoate as a sole source of carbon; strain 221 was isolated from a citrate medium, and strain 224 from a succinate medium. The three strains grew readily in the inorganic medium of Koser & Baird (1944) containing 0.1% nicotinic acid as a sole source of carbon and nitrogen; cells thus grown are referred to as

'adapted cells'. Cells not grown with nicotinic acid are referred to as 'non-adapted cells' and were grown on the inorganic medium of Koser & Baird with 0.1% asparagine. The cultures were maintained by subculturing twice-weekly upon solid medium containing 1.0% yeast autolysate, 1.0% asparagine and 2.5% agar; cultures were incubated at 25°.

Preparation and handling of washed suspensions. Cells for manometric experiments were grown in 200 ml. batches contained in 1 l. penicillin culture flasks which were shaken at room temperature for 18–24 hr. Each flask was inoculated with a loop of the culture from the solid medium. The average yield of cells was 100–150 mg. dry wt./200 ml. medium. After growth, the cells were collected by centrifuging, washed twice and then suspended in 0.9% NaCl to give a suspension containing 10–12 mg. dry wt./ml. Dry wts. were estimated by drying a sample at 110° and subtracting the dry wt. of an equal vol. of 0.9% NaCl. In some experiments cells were grown on the surface of a medium solidified by the addition of 2.0% agar.

The oxidation of nicotinic acid was followed manometrically in a Warburg apparatus. Each flask contained cell suspension (1.0 ml.) and 0.1 M phosphate buffer (KH₂PO₄ adjusted with N-NaOH), pH 7.0 (0.5 ml.) in the main compartment. Nicotinic acid (0.5 ml.) was added from the side arm after equilibration for 20–30 min. at 30°. The centre well contained folded paper and 2N-KOH (0.2 ml.) to absorb CO₂. Rates of oxidation are expressed as μ moles O₂ absorbed/mg. dry wt. cells/hr. Both the rate and uptake were calculated by subtracting the O₂ uptake of a blank experiment with water instead of substrate. The value of the blank is also stated in most experiments.

Materials. The nicotinic acid used in preliminary studies was a specimen used as an analytical standard in the laboratory. It had been sublimed at 230° and later recrystallized from aqueous ethanol (Knight, 1937; Hughes & Williamson, 1952). This was assumed to be pure until it was found that immediately upon its addition to non-adapted cells there was an O₂ uptake of 0.25–0.5 μ mole O₂/ μ mole nicotinic acid added/20 min. but no corresponding

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