Some Serological and Chemical Studies on Materials Extracted from an Amyolytic Streptococcus from the Rumen of the Sheep

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In recent years numerous types of amyolytic organisms have been shown to be present in the digestive tracts of animals (Van der Wath & Myburgh, 1948; Baker, Nasr, Morrice & Bruce, 1951; Masson, 1951; Huhtanen & Gall, 1953), and MacPherson (1953) has lately reported the isolation of a number of strains of amyolytic streptococci from the rumens of sheep fed on a variety of diets. The amylases of one of these strains of streptococci (BSO7) and of a rumen strain of *Clostridium butyricum*, and an intracellular, amylopectin-type polysaccharide from a pig caecum strain of *Cl. butyricum* have been described by Hobson & MacPherson (1952) and Hobson & Nasr (1951). Continuing the investigation of these streptococci we here report some findings on serologically active materials from the strain BSO7. Encapsulation appears to be common in rumen bacteria *in vivo* (Hobson & MacPherson, 1953a) so that besides their bacteriological interest, capsular polysaccharides may have some significance in ruminant nutrition as they represent one of the products of the breakdown of feeding-stuff polysaccharides by rumen micro-organisms.

The Lancefield (1933) and Fuller (1938) methods of extracting antigens from streptococci were found to give materials which were serologically not entirely comparable, although either method is commonly used for grouping pathogenic streptococci. Examination of the two extracts showed them to consist of protein and polysaccharide. In a number of cases a polysaccharide has been shown to confer the specificity for the organism (Kendall, Heidelberger & Dawson, 1937; Heidelberger, Kendall & Scherp, 1936; Mirick, Thomas, Curnen & Horsfall, 1944; Julianelle, 1926; Morgan, 1937), and since MacPherson & Oxford (1952) have shown that the capsules of these bacteria reacted with specific antisera, it appeared likely that a type-specific polysaccharide was part of the capsule, especially as other workers have shown that the majority of bacterial capsules, with the exception of those of some *Bacillus* spp. (Ivánovics & Bruckner, 1938) are composed mainly of polysaccharide. Attention was, therefore, directed to the preparation of polysaccharides from the medium in which the bacteria had grown (the 'metabolic liquid') and from the

bacterial capsules. A preliminary report of this work has already been given (Hobson & MacPherson, 1953b).

EXPERIMENTAL AND RESULTS

Methods and materials

Analytical methods. Before analysis the preparations were dried to constant weight *in vacuo* at 60° over P_2O_5 . In all determinations standard solutions were included along with the unknowns to calibrate the reagents.

Reducing sugars were determined using Somogyi's (1952) micro copper reagent in conjunction with a colorimetric reagent (Nelson, 1944) calibrated against glucose. A correction was made for the small loss of sugars during the hydrolysis (see Pirt & Whelan (1951) for glucose; similar results were found with galactose and rhamnose).

Rhamnose was measured by a microspectrophotometric method (Dische & Shettles, 1948) after standardizing against rhamnose monohydrate.

Glucosamine was determined with the reagents of Elson & Morgan (1933) calibrated with glucosamine hydrochloride and using a photoelectric colorimeter. *Total nitrogen* was determined by a micro-Kjeldahl method. *Uronic acids* were determined by the modification according to Bray, Humphris, Thorpe & Wood (1952) of the method of Hanson, Mills & Williams (1944). The reagents were calibrated against a mixture of galacturonic acid, galactose and rhamnose in the approximate proportions of the polysaccharide to obviate any effects of the sugars on the reagents (Hanson *et al.* 1944). *The ash content* was determined by incinerating a little of the material, in a platinum boat, to constant weight in a micro-muffle. *Total phosphorus* was determined by the method of Allen (1940) after combustion of the material with perchloric acid. *Acid hydrolysis* was carried out by heating the material (concentration approx. 0.5 mg./ml.) with 1.5N- H_2SO_4 for the stated time in a boiling-water bath in a lightly stoppered flask. For reducing-sugar determinations the hydrolysate was neutralized with 5N-NaOH and diluted to a known volume.

Paper chromatography. The acid hydrolysates were neutralized with $BaCO_3$, filtered, shaken alternately with small portions of cation- and anion-exchange resins (Amberlite IR 100H and IR 4B; Rohm and Haas Co., Philadelphia, U.S.A.) and evaporated to dryness. The residue was dissolved in an appropriate small volume of water and a suitable amount applied to the starting line of a strip of Whatman no. 1 paper along with reference sugars. The chromatograms were developed with the top layer of a benzene:butanol:pyridine:water mixture (1:5:3:3, v/v) for 24 hr. at room temp. After drying, the sugars were

coloured by spraying with a benzidine reagent (Bacon & Edelman, 1951). Other reagents were used to try to demonstrate the presence of ketoses or to colour hexosamines.

Negative staining of capsules by the wet indian ink method. The films were prepared as described by Mackie & McCartney (1950) and examined by phase contrast illumination. Before making the films the pH of the sample was adjusted to 7 to prevent agglutination of the ink particles. High salt concentrations were also found to agglutinate the ink.

Preparation of sera. The sera were obtained from rabbits after a course of intravenous injections of formalized suspensions of the organisms over a period of some weeks as described by MacPherson (1953). Lancefield's group D serum was obtained from Burroughs, Wellcome and Co., London. The homologous immune sera were standardized before use by agglutination of a standard cell suspension.

Precipitin tests. Serial dilutions of the appropriate antigenic material in neutral solution were prepared and layered on top of the immune serum in Dreyer's agglutination tubes, and after incubation for 30 min. at 37° were examined for interfacial precipitate. The results obtained in this way were confirmed by complement-fixation tests.

Precipitin reactions in gels. The method used is described by Oudin (1952), a mixture of agar and BSO7 immune serum being used as internal reactant, the external reactants being solutions of the purified polysaccharides.

Culture of the organism BSO7

(a) *For Fuller extraction.* An 18 hr. nutrient broth culture (1 ml.) was inoculated into 4 l. of medium containing 1% Lab Lemco (Oxo Ltd., London), 1% peptone (Evans Medical Supplies Ltd., London), 0.5% NaCl (A.R.), 0.5% glucose (A.R.) which was incubated at 40° for 48 hr. The medium, with the exception of the glucose, was sterilized by autoclaving for 15 min. at 121°, and the glucose, sterilized by filtration, added as a 50% solution after cooling. The initial pH of the medium was 6.8.

(b) *For Lancefield extraction.* An 18 hr. culture (1 ml.) was inoculated into 4 l. of medium containing 1% peptone, 0.5% yeast extract (Difco, dehydrated. Difco Co., Detroit, Michigan, U.S.A.), 0.5% Lab Lemco, 0.5% Bacto-tryptone (Difco), and 0.5% glucose and incubated for 48 hr. at 40°. The medium was sterilized as before, the initial pH being 7.6.

(c) *For preparation of polysaccharides from the metabolic liquid and capsules.* The medium used was as in (a).

Preparation and analysis of the extracts

Fuller extraction. The procedure used was similar to that described by Fuller (1938), but as larger quantities of cells were used it was modified as follows. The cells were removed from a 48 hr. culture on a Sharples 'Supercentrifuge' and after being washed twice with sterile water on an angle centrifuge the sediment was suspended in formamide (B.D.H. Laboratory Reagent; 40 ml.) and heated at 150° in an oil bath for 15 min. After cooling, acid-alcohol (100 ml. containing 95 ml. ethanol, 4 ml. water, 1 ml. conc. HCl) was added with stirring and the precipitate removed on the centrifuge. To the supernatant liquid, acetone (200 ml.) was added and the precipitate centrifuged off after 10 min., dried by trituration with acetone and finally in a vacuum desiccator over P₂O₅.

Lancefield extraction. The washed cells were prepared from 4 l. of a 48 hr. culture, as for the formamide extraction.

The organisms were suspended in 0.05N-HCl (80 ml.) containing a few drops of congo red (0.2% aqueous solution). 1N-HCl was then added until the indicator turned slate blue. The whole was then immersed in a boiling-water bath for 10 min., when a pink colour developed, and, after rapid cooling, was centrifuged. The sediment was discarded. After addition of a few drops of phenol red (B.D.H. indicator) the clear supernatant was neutralized with 1N-NaOH and centrifuged. The supernatant solution was usually used for precipitin tests without further treatment. However, in order to obtain a solid for analysis the polysaccharide was precipitated by the addition of acetone (2 vol.) and dried in acetone and *in vacuo* over P₂O₅. Precipitation of the material did not appear to alter the serological reactions. For precipitin tests a known amount of the material was dissolved as far as possible in physiological saline (0.85% NaCl) and any insoluble residue centrifuged off, dried, and weighed to give the amount of material in solution.

Determination of the amino acid content of the Fuller extract. To determine whether the nitrogen content of the Fuller extract was due to protein or hexosamine, a determination of the α -amino-nitrogen content of a hydrolysate of the material was made. A portion of the material was hydrolysed in a sealed evacuated tube in 6N-HCl for 24 hr. at 105°. The HCl was removed by distillation *in vacuo* and the residue diluted to a known volume. The α -amino nitrogen was determined by the ninhydrin/CO₂ method (Van Slyke, Dillon, MacFadyen & Hamilton, 1941), the pH of the buffer used being 4.7 and the volume of solution 3 ml. Two-dimensional chromatograms of the oxidized hydrolysate were developed in aqueous phenol and collidine solvents, the phenol in an atmosphere of coal gas. The amino acids were coloured by spraying with ninhydrin.

The results of analysis and the precipitin reactions of both extracts are shown in Tables 1 and 2. The sugars found in each hydrolysate were the same. The nitrogen content of the formamide extract was due to both protein and hexosamine-containing polysaccharide. The amino acids found on chromatograms of this material were aspartic and glutamic acids, glycine, threonine, alanine, histidine, proline, valine, leucine, isoleucine, methionine, phenylalanine, tyrosine, arginine and lysine. Serine was probably present but the resolution of that and the glutamic acid was not complete. Hexosamine was also shown on these chromatograms. α : ϵ -Diaminopimelic acid, which was found in a number of micro-organisms (Work & Dewey, 1953), was not present in this extract. Synge (1953) has examined four strains of amylolytic rumen streptococci and found this acid to be absent from all. While the formamide extract gave a weak reaction with group D antiserum, no reaction could be detected with the acid extract. Both showed positive reactions with the homologous immune serum, but while the acid extract did not react with homologous serum after the solution had been stored at room temperature for 48 hr., the reaction of the formamide extract was unaltered after a similar period.

Table 1. *Analysis of serologically active materials from an amylolytic rumen streptococcus* BSO 7

Preparation	Yield (mg.)*	Sugars (as glucose) after hydrolysis† (%)	Ash (%)	N (%)	Uronic acid (%)	Rhamnose (%)	Sugars in hydrolysate‡
Fuller (formamide) extract	101.1	34.9	—	8.6§		—	Glucose, galactose, rhamnose, ribose, hexosamine
Lancefield (acid) extract	309.5	41.0	—	4.3		13.9	Glucose, galactose, rhamnose, ribose, hexosamine
Metabolic liquid polysaccharide (18 hr. culture)	284.0	37.4	—	3.8		4.4	Glucose, galactose, rhamnose, ribose
Metabolic liquid polysaccharide (48 hr. culture)	1400.3	11.2	74.7	0.6	6.3	1.5	Glucose, galactose, rhamnose, xylose
Capsular polysaccharide (48 hr. culture)¶	113.4	58.2	7.2	0.7	22.0	17.6	Galactose, rhamnose, glucose (trace)

* In 5 l. of culture.

† For 6 hr. in 1.5N-H₂SO₄ at 100°; 3 hr. hydrolysis gave slightly lower results.

‡ Detected by paper chromatography.

§ 69.2% of this was α -amino nitrogen.

|| Qualitative test showed positive result.

¶ Phosphorus content, 0.2%.

Table 2. *Precipitin titres of serologically active materials from an amylolytic rumen streptococcus* BSO 7

Preparation	Precipitin titre (mg./ml. $\times 10^5$)*	
	BSO 7 immune serum	Lancefield group D serum
Fuller (formamide) extract	1200	12 000 (or rather less)
Lancefield (acid) extract	120.6	No reaction
Metabolic liquid polysaccharide (18 hr. culture)	71.0	55 800
Metabolic liquid polysaccharide (48 hr. culture)	1404 (800†)	10 800
Capsular polysaccharide (48 hr. culture)	35.7 (13.0†)	453 000

* Least concentration of preparation to give a positive precipitin reaction.

† Complement-fixation titration.

Schmidt (1952) in his studies of group A streptococcal polysaccharide digested the protein in the cells with pepsin. An attempt was made to remove the protein from an acid extract prepared from *Streptococcus faecalis* (N.C.T.C. no. 775) by digestion with crystalline trypsin (Armour Laboratories, Lindsay Street, London, E.C. 1) but analysis of the resulting material showed no improvement in the nitrogen, reducing-sugar content (after hydrolysis), or precipitin titre, over the untreated extract, so the method was not further investigated.

Preparation of a polysaccharide from the metabolic liquid. Since it was possible to demonstrate the presence of a serologically active material in the cell-free supernatant from liquid cultures (Mac-

Pherson, 1953) of the streptococci, it was thought that a polysaccharide uncontaminated by intracellular material might easily be obtained from this. The method used was a modification of that of Kendall *et al.* (1937), whose method has also been used by Loewenthal (1938) for the isolation of a polysaccharide from haemolytic streptococci. A young culture was used, as this would be less likely to contain material from autolysed cells.

The cells were removed from 1 l. of an 18 hr. culture (see above) by centrifuging at +1° (all subsequent operations were carried out at +1°). The sediment was washed with two lots of 0.1M phosphate buffer (pH 7.0; total 130 ml.) and the washings added to the original supernatant. To the clear liquid (approx. 1 l.) ethanol (2 vol.) was added and the whole stored at +1° for 48 hr. The precipitate was removed on the centrifuge and dissolved in water (200 ml.) containing hydrated sodium acetate (10 g.) and glacial acetic acid (5 ml.). The solution was divided, for convenience, into two portions and each deproteinized by shaking with successive lots of a mixture of CHCl₃ (25 ml.) and butanol (2.5 ml.) (Sevag, 1934). The final aqueous solutions were combined and recentrifuged and to the clear liquid 2 vol. of ethanol were added. After storing for 18 hr. the fine precipitate was removed on the centrifuge, dissolved in water, diluted to 50 ml. and centrifuged. A portion (1 ml.) was removed for precipitin tests, and the polysaccharide quantitatively precipitated from the remainder of the supernatant by addition of ethanol (2 vol.) and dried in acetone as before. The results of analysis and the precipitin titrations are given in Tables 1 and 2.

Preparation of the capsule polysaccharide. Morgan (1937) described several organic solvents which would dissolve polysaccharide from a number of organisms. Of these, ethylene and diethylene glycols appeared to be the best. Seastone (1939) removed the capsules from group C haemolytic

streptococci with phosphate buffer, pH 7.0, and with 1% aqueous chloroform. All these reagents were tried on small portions of washed cells of *streptococcus* BSO7. Wet indian ink preparations showed no reduction in capsule size after treatment, and no precipitate was formed on addition of excess ethanol to the supernatant liquids. The streptococcus cells were then heated in sodium acetate-acetic acid solutions of different pH and it was found that a few minutes heating at 100° in a solution of about pH 2 removed the capsules, without apparently damaging the cells. (This method is similar to that of Porges (1905) who used hot 0.25N-HCl for removing the capsules of *Klebsiella pneumoniae*.) The method was adopted for the large-scale preparation of the capsule polysaccharide.

The cells from 5 l. of a 48 hr. culture were removed on the Sharples centrifuge and washed three times with distilled water on an angle centrifuge. To the wet suspension of cells (100 ml.; concentration of organisms approx. 10^{10} /ml.) 0.2M sodium acetate (100 ml.; hydrated salt made up in 85% (v/v) aqueous acetic acid) and Tween 80 (Honeywell and Stein Ltd., London; 0.2 ml.) were added and the whole was heated in a boiling-water bath with frequent shaking. At intervals small samples were removed, neutralized with 5N-NaOH and examined in wet indian ink preparations. The decrease in size of the capsules on heating is shown in Table 3. In the medium the average diameter of the cells and capsules was $2.4\ \mu$; after washing and centrifuging the diameter was $2.1\ \mu$.

After 25 min. the majority of the cells were decapsulated, their appearance before and after acid treatment being shown in Fig. 1a, b. The suspension was rapidly cooled, centrifuged and the sediment washed with water. The supernatant and washings (300 ml.) were divided into two portions and deproteinized by shaking with CHCl_3 and butanol as before. To the final aqueous layer ethanol (2 vol.) was added and after storing at +1° for 48 hr. the precipitate was removed on the centrifuge and dissolved in water (50 ml.) to give a clear, viscous solution. This was centrifuged, a portion removed for precipitin tests and the polysaccharide quantitatively precipitated from the remainder by addition of a little NaCl and ethanol (150 ml.). The polysaccharide was dried by trituration with acetone and in a vacuum desiccator over P_2O_5 to give a white powder entirely soluble in water to a clear, viscous solution.

A polysaccharide was prepared by the method previously described after filtering and concentrating the metabolic liquid (5 l.) from the above culture to approx. 650 ml. *in vacuo*. The qualitative analysis of this material was similar to that previously prepared from the 18 hr. culture, but it contained a very large amount of ash (Table 1). This was probably mainly caused by evaporation of the liquid to a small volume before the first precipitation.

Attempted identification of the uronic acid. Various spectrophotometric methods were tried without success to identify the uronic acid present in the whole polysaccharides and hydrolysates. Finally, portions of the metabolic liquid polysaccharide (48 hr. culture, above) were hydrolysed for 6 and 11 hr. in 1N sulphuric acid. Glucuronolactone and

galacturonic acid controls were heated for the same times in acid solution. After cooling, the solutions were diluted and barium chloride was added in sufficient amount to precipitate most of the sulphate ions. The precipitate was removed on the centrifuge,

Table 3. Removal of capsules from an amylolytic rumen streptococcus BSO7 with sodium acetate-acetic acid

Time of heating in 100° water bath (min.)	Overall diameter of cell + capsule (μ .)*
0	2.1
10	2.1
20	1.4
25	1.0†

* Measured with calibrated micrometer eyepiece across shortest diameter.

† Cells not visibly damaged.

Fig. 1. Removal of capsules in sodium acetate-acetic acid. Wet indian ink preparations. Phase-contrast illumination. Magnifications, $\times 1600$. (a) Pure culture of rumen streptococcus (BSO7) with capsules outlined by indian ink particles. (b) The same culture after removal of the capsules with sodium acetate-acetic acid.

washed, and the washings and supernatant liquid neutralized with 1N sodium hydroxide and evaporated to dryness *in vacuo*. 5N sulphuric acid (3-4 drops) was then added to each residue to acidify and moisten it and each was then extracted with two 10 ml. portions of pyridine at 100° for 10 min. (Malpress & Morrison, 1949). The extracts were evaporated to syrups at 40° or below *in vacuo* and the syrups were further dried as far as possible in a vacuum desiccator. The polysaccharide residue in the initial hydrolysate was very brown and gave a long streak on the chromatograms before spraying with a saturated solution of *o*-phenetidine and trichloroacetic acid in ethanol. Further pyridine extracts were, therefore, treated in aqueous solution in a boiling-water bath with active charcoal, filtered and evaporated to dryness to give a clear 'glassy' residue. Chromatograms of the hydrolysates together with the galacturonic acid and glucuronolactone controls were developed in pentanol:pyridine:water (7:7:6, v/v) and the top layer of a butanol:acetic acid:water mixture (4:1:5, v/v). (On some chromatograms in the latter solvent mannuronolactone was included.) On all the chromatograms the polysaccharide showed an initial spot about the position of the galacturonic acid controls with streaking below due to the presence of degradation products. No spots corresponding to the faster-moving glucuronic acid or lactone, or mannuronic acid could be detected. It seems, therefore, that the uronic acid present is galacturonic, although the presence of degradation products makes the chromatograms difficult to interpret.

The results of analysis and precipitin tests are shown in Tables 1 and 2. Precipitin tests were also carried out in agar-serum gels, the external reactant being the capsular polysaccharide (4.5 mg./ml. in water) and the polysaccharide from the metabolic liquid (13.6 mg./ml.). The capsular polysaccharide diffused farthest into the gel forming a single band of precipitate with a sharp leading edge and a rather diffuse upper part. The polysaccharide from the metabolic liquid formed a diffuse band which travelled about half the distance of the capsular polysaccharide and the front was not so well defined. Using a mixture of the two polysaccharides as external reactant two precipitate bands were formed in the gel, the distances travelled corresponding with those of the separate solutions.

DISCUSSION

Two methods are in general use for serological classification of streptococci. These are based on group and type specificity. In Lancefield's classification (Lancefield, 1928, 1933) streptococci having a common antigen are placed in the same group

designated by one of the letters A to N. Intestinal streptococci have, in general, been found to belong to group D. Groups A and D streptococci have been subdivided into types by Griffith (1934) and by Sharpe & Shattock (1952), respectively. These types possess, besides the common group antigen, a type-specific antigen which has been associated with polysaccharide in the capsule (Kendall *et al.* 1937). The group-specific substance has been shown to be intracellular and to be either protein or polysaccharide in nature (Heidelberger & Kendall, 1931, 1936).

In the experiments described, the Lancefield and Fuller methods, although intended to extract similar antigens, gave products with different serological reactions (Table 2), but of similar chemical composition. The great difference between the reagents, and the somewhat drastic procedures involved in the extractions, could be possible reasons for the discrepancies in the reactions of the products.

The polysaccharide isolated from the metabolic liquid of an 18 hr. culture had a lower nitrogen content than the acid and formamide extracts and a higher precipitin titre with the homologous-type serum, but in higher concentration it gave a reaction with the group serum. This was to some extent non-specific, as solutions of higher concentration gave a reaction with normal rabbit serum. The capsular polysaccharide reacted in extremely low concentration (35.7×10^{-5} mg./ml.) with the homologous-type serum and only in high concentration (4.5 mg./ml.) gave a faint reaction with the group D specific serum. No reaction was given by the polysaccharide in this concentration with normal serum. No conclusions can be drawn about the group-specific material in the organisms except to suggest that it is probably intracellular as it is found in the formamide extract of whole cells and in a greater amount in the metabolic liquid polysaccharide of the old culture (48 hr.) than of the 18 hr. culture, and the former would be expected to contain more autolysed cells. The injection of capsular polysaccharide into rabbits to see if it acts as a complete antigen has not been tried.

The results of the precipitin reactions in gels suggest that the metabolic liquid polysaccharide is of somewhat higher molecular weight than the capsular polysaccharide, or is linked to protein not present in the capsular polysaccharide, as it diffused a smaller distance into the gel. As each solution formed only one precipitate band it may be assumed that there is only one constituent in each preparation capable of giving a precipitin reaction with the BSO7 immune serum. This is supported by the results of ultracentrifuge examination, reported in the Addendum, which show that the capsular polysaccharide is essentially homogeneous in molecular size.

Galactose, rhamnose and uronic acid were common to the sugar constituents of the hydrolysates of the preparations. Glucose was also present in a similar concentration in the hydrolysates of the acid and formamide extracts and the two polysaccharides isolated from the metabolic liquid. This glucose may be present in an intracellular polysaccharide (it has been found in hydrolysates of whole group A streptococci by Consden & Stanier (1952) and the metabolic liquid might be expected to contain material from some autolysed cells), or it may be due to some extent to adsorption from the medium on to the precipitates. The latter is possible, as traces of arabinose were found in hydrolysates of a metabolic liquid polysaccharide and control experiments showed that small amounts of this sugar were present in the medium together with the glucose. The small amount of xylose in the metabolic liquid (48 hr. culture) polysaccharide is at present unexplained, although it has been found in some bacteria (Belozerskiĭ, 1952; Hofman, 1953). The presence of ribose in the acid and formamide extracts and traces in the metabolic liquid (18 hr. culture) polysaccharide suggest that nuclear material is contained in these preparations. No ketose sugars were found in hydrolysates, but some hexosamines could be detected in chromatograms of the hydrolysates of the acid- and formamide-extracted polysaccharides. The purified capsular polysaccharide is composed of galactose, rhamnose, and a uronic acid, probably galacturonic acid, with a trace of glucose. Like some other polysaccharides containing uronic acids (see, for example, Hough, Jones & Wadman, 1952) the initial rate of acid hydrolysis was rapid, but after 3 hr. in 1.5N sulphuric acid only a slow increase in reducing sugars with time of heating was found (after correcting for the slight destruction of the sugars by acid). This was not increased by the use of 3N acid, which greatly increases the rate of destruction of the sugars. Galactose, rhamnose and galacturonic acid each have a lower reducing power than glucose with the reagents used, and this and the slow rate of hydrolysis would account for the apparently low reducing-sugar content after hydrolysis.

A purification of the type-specific material from this streptococcus has thus been achieved and it has been shown to be a capsular polysaccharide in conformity with the results obtained with other bacteria. Schmidt (1952) obtained a polysaccharide from group A streptococci containing 26.1% glucosamine, 60.8% rhamnose and 2.6% nitrogen which reacted in high dilution with group A serum. Consden & Stanier (1952) reported glucose, rhamnose, uronic acid and hexosamine in unpurified hydrolysates of group A streptococci. Hofman (1953) found galactose, ribose, rhamnose and xylose in hydrolysates of *Nitrosomonas*. Salton (1952)

found glucose, galactose, rhamnose, and hexosamine in cell wall preparations of *Streptococcus faecalis*. Polysaccharides containing uronic acids have been isolated from the pneumococci and the polysaccharide from this amylolytic streptococcus resembles that of type II pneumococcus (Kent, 1952) which contains glucose, rhamnose and glucuronic acid.

SUMMARY

1. Materials extracted from an amylolytic rumen streptococcus by the Lancefield (1933) and Fuller (1938) methods have been compared for serological activity and chemical composition. Similar tests have been made on polysaccharides prepared from the metabolic liquid.

2. A method has been developed for removing the capsules from these streptococci.

3. The capsular polysaccharide has been shown to possess a high type-specific serological activity.

4. An analysis of the capsular polysaccharide has been carried out and its main constituents have been shown chromatographically to be galactose, rhamnose and a uronic acid, probably galacturonic, the analysis being; reducing sugars, as glucose, after acid hydrolysis, 58.2%; N, 0.7%; P, 0.2%; ash, 7.2%; uronic acid, 22.0%; rhamnose, 17.6%.

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ADDENDUM

A Physico-Chemical Examination of the Capsular Polysaccharide from an Amylolytic Sheep Rumen Streptococcus

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The capsular polysaccharide from the amylolytic rumen streptococcus BSO7 described in the preceding paper has been examined in the ultracentrifuge, and in addition its partial specific volume and limiting viscosity number* have been determined.

METHODS

Solution. The dried polysaccharide was dissolved in a buffer containing 0.2M-NaCl, 0.0633M- Na_2HPO_4 and 0.0033M- KH_2PO_4 (pH 7.8). A 1% (w/v) solution was diluted by weight as required and used for all measurements. (Weights were corrected for 7.2% of ash present in the material.)

Sedimentation. Rates of sedimentation were determined using an electrically driven 'Spinco' ultracentrifuge (Specialized Instruments Corporation, Belmont, California,

U.S.A.) at rotor speeds of 1000 rev./sec. The pressure in the rotor chamber was less than 1 μ . Hg, and the temperature rise in the rotor itself was about 0.6°/hr. (the refrigerating system was not utilized). Movement of the boundary was followed using a modified Philpot-Svensson optical system. The magnification of this lens system was 2.22, constant over the whole field, and the average distance of the reference line from the axis of rotation was taken as 5.730 cm. (at 1000 rev./sec.). Measurement of the rates of sedimentation were made at several concentrations, the distance through which the boundary had moved being measured, to 0.02 mm., directly from the photographic plate with a travelling microscope. Sedimentation constants were evaluated from the slope of $d \log_{10} x/dt = S/2.303$, x being measured in cm. from the centre of rotation and t in seconds from an arbitrary origin, and converted to water at 20°.

Viscosity. An Ostwald type viscometer was used, the essential dimensions being as follows: diameter of capillary, 0.5 mm.; length of capillary, 12.5 cm.; working volume, 2 ml.; mean pressure head, 13.0 cm.; efflux time for solvent, 83.8 sec.; temp. 22.5°. The viscosity number (η_{sp}/c) was determined at several concentrations (where $\eta_{sp} = \eta_{rel} - 1$, and η_{rel} = solution/solvent viscosity ratio), and the limiting

* The nomenclature used in this paper is in accordance with the 'Report on Nomenclature in the Field of Macromolecules of the International Union of Pure and Applied Chemistry', reported in *J. Polym. Sci.* (1952), **8**, 257.

viscosity number $[\eta]$ obtained from the graph of η_{sp}/c against c , as

$$[\eta] = \lim_{c \rightarrow 0} (\eta_{sp}/c).$$

Partial specific volume. The densities of solutions of several concentrations were determined using a pycnometer, and the partial specific volume ($\bar{v}$) calculated from the equation (Svedberg & Pedersen, 1940)

$$(1 - \bar{v}\rho) = (1 - w/m) dm/dw,$$

where ρ is the density of a solution for which the weight fraction of solute is w and the mass of a given volume m ; dm/dw was determined from the linear graph of m against w .

RESULTS

In 0.2M sodium chloride buffered with phosphate at pH 7.8 the presence of only one component in the polysaccharide was apparent from the sedimentation determinations. The boundaries appeared symmetrical and, for higher concentrations, sharp. Typical sedimentation diagrams are shown in Fig. 1. The values of the sedimentation constants (S_{20}) for three concentrations are recorded in Table 1. Owing to lack of material it was not possible to examine a wider range of concentrations, but a marked dependence of sedimentation constant on concentration was observed. The limiting value of S_{20} was determined using a method suggested by Jullander (1945). In this method S_{20} and $S_{20} \times \eta_{rel}$ are both plotted against c on the same graph and since

$$\lim_{c \rightarrow 0} S_{20} = \lim_{c \rightarrow 0} S_{20} \times \eta_{rel}$$

the two functions approach the same limiting value and hence define it more exactly. Fig. 2 shows these results and the limiting value of S_{20} is seen to be $3.25 \pm 0.2 \times 10^{-13}$.

Experimentally determined values of η_{sp}/c are also given in Table 1, together with the extrapolated value of the limiting viscosity number, 3.75. The value for the partial specific volume of the material was found to be 0.60.

The high limiting-viscosity number suggested that the molecules were very asymmetrical, and this figure was used to obtain the approximate axial ratio ($1/d$) and hence the frictional ratio (f/f_0), as a preliminary to the molecular-weight calculation. (This procedure assumes that a value of $f/f_0 > 1$ indicates a deviation from spherical shape when f , the actual molar frictional coefficient $= M(1 - \bar{V}\rho)/S$, and f_0 , the molar frictional coefficient of a spherical, non-solvated particle $= 6\pi\eta N(3M\bar{V}/4\pi N)^{1/3}$.) f/f_0 can then be related to the axial dimensions $1/d$ of the equivalent ellipsoid, where $1/d = \text{length} (1) \text{ of major axis/diameter } (d) \text{ of equatorial axis.}$

The axial ratio ($1/d$) was calculated by the use of Simha's equation (Simha, 1945).

$$\eta_{sp}/\phi = 0.233 (1/d)^{1.696},$$

Fig. 1. Sedimentation diagrams for the capsular polysaccharide. Speed, 1000 rev./sec. Solvent, 0.2M-NaCl buffered with phosphate to pH 7.8. Concentration, 0.37 g./100 ml. Times of exposures (a) 37 min., (b) 129 min., (c) 142 min., (d) 177 min., from starting the centrifuge.

Fig. 2. Determination of limiting value of S_{20} by the method of Jullander (1945). For explanation see the text.

Table 1. S_{20} and η_{sp}/c values at different values of c for the capsular polysaccharide

Concentration, c (g./100 ml. solution)	Sedimentation constant (S_{20}) $\times 10^{13}$	Viscosity number (η_{sp}/c)
1.00	1.34	—
0.64	1.87	4.0
0.37	2.34	—
0.34	—	3.9
0.17	—	3.8
0.00	[3.25]*	[3.75]†

* See text and Fig. 2.

† By extrapolation.

when $20 \leq 1/d \leq 100$ and ϕ is the volume fraction of solute. For the concentration units used (g./100 ml. solution) this expression reduces approximately to

$$[\eta] 100 = 0.233 (1/d)^{1.698},$$

and from this the axial ratio ($1/d$) was found to be 77. This value represents a frictional ratio (f/f_0) of 3.5, assuming the particles to be prolate spheroids, and using the data of Herzog, Illig & Kudar (1933). (Any hydration effect has been ignored.) The molecular weight M was then calculated from the equation

$$M = 2.45 \times 10^{22} \bar{V}^{\frac{1}{3}} (f/f_0)^{\frac{1}{3}} [S/(1 - \bar{V}_p)]^{\frac{1}{3}}.$$

The frictional ratio (f/f_0) of 3.5 gave a value of about 90 000. If the molecule was assumed to be spherical and unsolvated (i.e. $f/f_0 = 1$), the value of 14 000 was obtained.

DISCUSSION

The results show that the polysaccharide is not obviously polydisperse and the high limiting-viscosity number, and the marked dependence of the sedimentation constant on concentration suggest that the molecules are very asymmetrical. (Factors causing a dependence of sedimentation constant on concentration have been discussed by Kinell & Rånby (1950), and Ogston & Woods (1953).) The value of 90 000 for the molecular weight calculated using the frictional ratio obtained from the viscosity measurements is regarded as being of the correct order of magnitude, whilst the figure of 14 000 obtained assuming the molecule is spherical represents the minimum value. The effect of hydration could, however, reduce the higher figure. Few measurements have been made on similar poly-

saccharides from other bacteria, but Record & Stacey (1948) have reported molecular weights of 171×10^3 , 540×10^3 , and 141×10^3 for pneumococcus types I, II and III polysaccharides. Results obtained with other polysaccharides have been reviewed by Greenwood (1952).

SUMMARY

1. The capsular polysaccharide from an amylo-lytic rumen streptococcus has been found to be homogeneous in the ultracentrifuge.

2. The material possesses a high limiting-viscosity number and hence a very extended shape in solution (the frictional ratio (f/f_0) = 3.5).

3. The molecular weight of 90 000 calculated using the frictional ratio obtained from the viscosity measurements is regarded as being more correct than that calculated assuming a spherical molecule.

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The Distribution and Metabolism of Phosphorus Compounds in Normal and Demyelinating Nervous Tissue of the Chicken

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Several studies have been reported on the composition and metabolism of the phospholipids and other phosphorus-containing compounds of the mammalian nervous system, both in health and during the classical Wallerian degeneration which nerve fibres undergo following transection (see Brante, 1949; and Johnson, McNabb & Rossiter, 1950).

Demyelinating changes also occur in man after poisoning with tri-ortho-cresyl phosphate (TOCP), and numerous extensive outbreaks of poisoning by

this compound have been described (Smith & Elvove, 1930*a, b*; Ter Braak, 1931; Germon, 1932; Sampson, 1938; Hunter, Perry & Evans, 1944). More recently, the biochemical effects (Earl & Thompson, 1952*a, b*; Earl, Thompson & Webster, 1953) accompanying the histological and clinical changes (Barnes & Denz, 1953) in experimental intoxication with this compound have been studied, and it has been found to be a potent inhibitor of the pseudo-cholinesterase present in nervous tissue.