Composition of an Extracellular Polysaccharide Produced by *Sphaerotilus natans*[1]

ELIZABETH GAUDY AND R. S. WOLFE

Department of Microbiology, University of Illinois, Urbana, Illinois

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ABSTRACT

GAUDY, ELIZABETH (University of Illinois, Urbana), AND R. S. WOLFE. Composition of an extracellular polysaccharide produced by *Sphaerotilus natans*. Appl. Microbiol. **10**:200–205. 1962.—The capsular polysaccharide of *Sphaerotilus natans* has been isolated, purified, and analyzed. Chromatographic and chemical analyses performed on acid hydrolyzates of the purified material have shown that the major components are fucose, galactose, glucose, and glucuronic acid in approximately equimolar amounts. Glucose and glucuronic acid are believed to occur as an aldobiuronic acid unit.

Although slimy deposits of *Sphaerotilus natans* are common in streams, industrial plants, and sewage treatment works, the chemical composition of the slime layer of this organism has not been investigated. In the present study a polysaccharide has been purified from the extracellular slime of *S. natans* described by Gaudy and Wolfe (1962) and its component sugars identified.

The extracellular polysaccharides of bacteria have been the subject of several recent reviews (Salton, 1960*a*; Wilkinson, 1958; Tomcsik, 1956; Evans and Hibbert, 1946). The polysaccharide described below is qualitatively similar in composition to several of the polysaccharides produced by enteric bacteria, of which the *Aerobacter-Klebsiella* group has been studied in detail (Wilkinson, Dudman, and Aspinall, 1955; Aspinall, Jamieson, and Wilkinson, 1956; Dudman and Wilkinson, 1956). Five strains of *Klebsiella aerogenes* were found to produce type-specific polysaccharides containing either three or four of five sugars (glucose, galactose, fucose, mannose, and uronic acid) in different proportions.

Although the studies of Linde (1913), Wuhrman and Mechsner (1960), and Romano and Lugananni (1961), do not present a consistent picture of the chemical composition of the sheath of *S. natans*, the polysaccharide described in the present communication appears distinct and unrelated to the structures analyzed by these authors. A preliminary report of these findings has appeared (Gaudy and Wolfe, 1961).

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MATERIALS AND METHODS

Organism and conditions. The strain of *S. natans* used in these studies has been described previously (Gaudy and Wolfe, 1962). The medium used for polysaccharide production contained: glucose, 0.5%; peptone, 1.0%; $MgSo_4 \cdot 7H_2O$, 0.02%; $CaCl_2$, 0.005%; $FeCl_3 \cdot 6H_2O$, 0.001%; deionized water. Sterile potassium phosphate buffer, pH 7.1, was added before inoculation to a final concentration of 0.01 M. Each Fernbach flask, containing 500 ml of medium, was inoculated with 1 ml of an aqueous suspension of cells from a 24-hr slant culture on 0.2% yeast extract agar. For smaller batches, cells were grown in 500-ml Erlenmeyer flasks, each flask containing 100 ml of medium. All cultures were incubated at 30 C for 5 days without shaking.

Hydrolysis. The polysaccharide was hydrolyzed in 5 M HCl at 100 C in a glass-stoppered bottle. For reducing sugar determinations, the concentration of polysaccharide was 1 mg per ml of 5 M HCl, samples being removed and neutralized with an equal volume of 5 M NaOH; f chromatography, 5 mg per ml solution were used, wat and HCl being removed in vacuo in a desiccator over concentrated H_2SO_4 and NaOH pellets.

Chromatography. Five solvent systems (designated A through E) were used for separation and identification of the sugar residues in the hydrolyzate: A) ethyl acetate-pyridine-water, 8:2:1 (Whistler and Kirby, 1956); B) ethyl acetate-acetic acid-water, 3:1:3 (organic layer) (Jermyn and Isherwood, 1949); C) isopropanol-pyridine-acetic acid-water, 8:8:1:4 (Gordon, Thornburg, and Werum 1956); D) *n*-butanol-acetic acid-water, 2:1:1 (Whistler and Conrad, 1954); E) *n*-butanol-pyridine-water, 6:4:3 (Whistler and Conrad, 1954). For maximal separation, descending chromatograms were used and the solvent was allowed to drip off the end of the paper for several hours. $R_{glucose}$ values were calculated for the unknown spots and for known sugars chromatographed in the same manner. Identification of the sugars was then confirmed by simultaneous chromatography with standards in the same solvent systems. Chromatograms were sprayed with a solution composed of 2-aminobiphenyl, 1.69 g; oxalic acid, 0.9 g; glycerol, 5 ml; water, 10 ml; and acetone, 84 ml, and then were heated at 105 to 110 C for 3 to 5 min (Gordon et al., 1956).

For quantitative chromatography, a technique similar to

that recommended by Putnam (1957) was used. The dried HCl hydrolyzate was dissolved in water to give a solution corresponding to 10 mg per ml of original material, and 100 μl were applied to each spot at the origin of the chromatogram. Smaller spots of 30 μl each were applied at both edges of the chromatogram, and after development in solvent system E, the butanol-pyridine-water system, and air drying, these edge indicator strips were cut off and sprayed to locate the areas of the separated components. Each component was then cut out and eluted with water; the elution technique was checked by quantitative recovery of standards. The eluates were lyophilized, taken up in a known volume of water, and used for chemical analysis and for chromatography in an acid solvent system with standard sugars.

Chemical analysis. The reducing sugar content of hydrolyzed and unhydrolyzed samples was determined by the phenol-dinitrosalicylate method (Borel, Hostettler, and Deuel, 1952). The method of Lowry et al. (1951) was used for protein determinations on unhydrolyzed samples, using crystalline bovine serum albumin (Armour) as standard.

The following sugars were determined quantitatively by the specific modifications of the methods of Dische (1947), Dische and Shettles (1948), and Dische, Shettles, and Osnos (1949) as recommended by Ashwell (1957). Fucose was determined by the cysteine-H_2SO_4 reaction for methylpentoses. Glucose and galactose were determined quantitatively by the cysteine-H_2SO_4 method for hexoses. The cysteine-H_2SO_4 reaction for mannose, which is qualitative also for glucose and galactose, was used for the identification of glucose. The carbazole reaction for uronic acids was used for glucuronic acid determination. A Cary 14[2] recording spectrophotometer was used for comparing absorption spectra of products formed by knowns and unknowns in the various reactions. Quantitative readings were made on the Beckman[3] model DU spectrophotometer.

Results

Isolation and properties of crude slime. Since most of the slime adhered firmly to the cells, it was found most convenient to harvest the slime and cells together and wash them free of medium before attempting to separate the slime layer. Accordingly, the cells, with adherent slime, were harvested by centrifuging for 45 min at 10,000 rev/min in a Servall[4] SS-1 centrifuge (SS-34 rotor) and washed three times with deionized water. After the third washing, an extremely viscous material was obtained, which consisted of small pieces of free slime and very large masses of slime with single cells and short filaments embedded throughout. This slimy material was treated in a blender for 2 min, resulting in a suspension of low viscosity in which most of the slime had been reduced to very small fragments. Centrifugation for 40 min at 12,000 rev/min removed many of the cells, leaving an opalescent suspension of slime particles still containing some cells and cell debris.

The suspension was brought to pH 1 or 2 with 6 M HCl and three volumes of 95% ethanol were added (Hanby and Rydon, 1946). The mixture was allowed to stand for 15 min with frequent stirring and then centrifuged at 10,000 rev/min for 20 min. The precipitate, largely cells and cell debris, was discarded and the supernatant was neutralized with 5 M NaOH. A flocculent precipitate began to form within ½ to 1 hr. After standing at room temperature for 10 or 12 hr, the precipitate was removed by centrifugation at 10,000 rev/min for 20 min. The supernatant was discarded.

The material thus obtained was a clear, colorless gel which was soluble in water, forming a very viscous, opalescent solution. By dissolving the gel in a minimal amount of water and centrifuging at 10,000 rev/min for 30 min, the small amount of remaining cells and cell debris could be removed. The supernatant was then dialyzed against four changes of deionized water, 3 liters each, for 24 hr at 4 C to remove ethanol and inorganic ions. The resulting opalescent, viscous solution (fraction A) formed a tough, completely transparent film when dried at 70 C. On addition of water, the film swelled, became opalescent, and finally dissolved. This material was used for preliminary chemical analysis.

Purification of fraction A. The dissolved gel (fraction A) was reprecipitated with 95% ethanol. A fractional precipitation using ethanol at ½, 1, 2, 3, 4, and 5 volumes yielded only one fraction, that precipitating at 4 volumes of ethanol. It was necessary to add a few drops of dilute NaCl solution to initiate precipitation. The gelatinous precipitate was collected by centrifugation and was washed once with 95% ethanol, twice with acetone, once with ether, and air dried. The dried material (fraction B) was light brown in color and somewhat gummy.

TABLE 1. *Separation of protein and polysaccharide components of crude slime*

Fraction	Description	Sample I	Protein		Sample II	Reducing sugar	
		μg dry wt	μg	%	μg dry wt	μg	%
A	Precipitated, dialyzed slime	115	65	57*			
		330	84	25	330	210	64
B	Reprecipitated, dried	156	85	54	500	300	60
C	Water extract of B	192	50	26	480	330	69
D	Residue from extraction	43	43	100	500	0	0
E	Fraction C, reprecipitated, dried	1,000	39	3.9*	500	385	77
		1,000	18	1.8	500	385	77

* Protein analyses are given for two separate preparations of fraction A and fraction E.

[2] Applied Physics Corporation, Monrovia, Calif.
[3] Beckman Instruments, Inc., Fullerton, Calif.
[4] Ivan Sorvall, Inc., Norwalk, Conn.

Fraction B was dissolved in deionized water at a concentration of 1.5 mg per ml. An insoluble residue remained, which was removed by centrifugation at 13,000 rev/min for 45 min (fraction C). The supernatant (fraction D), a clear, bluish opalescent solution, was quite viscous and gave a strongly positive Molisch test. The insoluble fraction, C, was yellowish white, was not viscous when suspended in water, and gave a negative Molisch test. Both of these fractions were analyzed for protein and reducng sugar and fraction C was discarded.

Fraction D was made strongly alkaline by addition of $\frac{1}{5}$ volume of 5 M NaOH. One volume of 95% ethanol was then added and the mixture kept at 4 C for 30 min. The gelatinous precipitate was harvested by centrifugation, washed twice with 95% ethanol, once with acetone, once with ether, and then air dried.

The final product (fraction E) was a fine, dry, white powder, containing only 1.8 to 3.9% protein. This material was used for chromatographic and chemical studies.

Protein content of slime. Protein and reducing sugar analyses of various fractions obtained during purification of the polysaccharide are presented in Table 1. The protein content of the initial crude gel preparation varied between wide limits, as shown by the protein analyses of two different batches of fraction A.

The sample containing 57% protein was hydrolyzed in 6 M HCl in an evacuated sealed tube at 110 C for 20 hr. The hydrolyzate was analyzed for amino acid content,

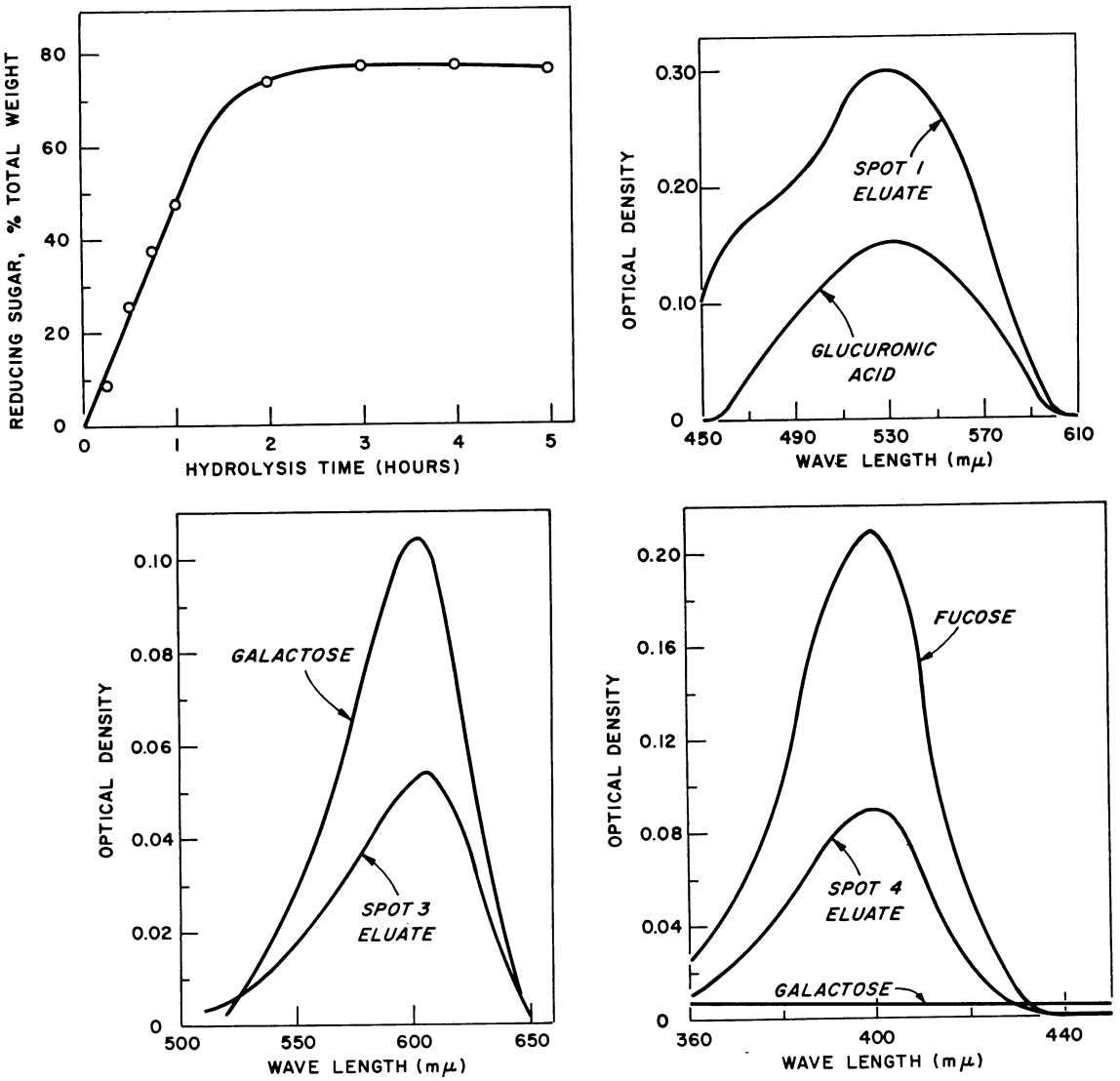

FIG. 1 (*top, left*). *Hydrolysis of the capsular polysaccharide in 5 M HCl at 100 C. Reducing sugar was measured as glucose.*

FIG. 2 (*top, right*). *Absorption spectra of reaction products of glucuronic acid and the material eluted from spot 1, in the carbazole reaction for uronic acids.*

FIG. 3 (*bottom, left*). *Absorption spectra of secondary reaction products of galactose and the material eluted from spot 3, in the cysteinesulfuric acid test for hexoses.*

FIG. 4 (*bottom, right*). *Absorption spectra of reaction products of fucose, galactose, and material eluted from spot 4, in the cysteine-sulfuric acid method for methylpentose.*

using the Beckman/Spinco amino acid analyzer,[3] and found to contain all the amino acids of a typical protein. Total amino acids recovered from the column amounted to 51 % of the dry weight of gel applied. The relative molar quantities of amino acids found were very similar to those reported by Roberts et al. (1957) for the cellular protein of *Escherichia coli*. Protein and nucleic acid contents of the gel, calculated on the basis of absorption at 280 and 260 mμ, were 68 % and 5 %, respectively.

As shown by the decreasing protein content of successive fractions obtained during the purification procedure, it was possible to separate the protein and carbohydrate components of the crude slime without the use of methods capable of covalent bond breakage. All centrifugations were carried out at 4 C or below. Preparations were never heated above room temperature and were allowed to stand at room temperature only at a neutral pH. When prolonged storage was necessary, samples were frozen.

Hydrolysis of polysaccharide. The time course of hydrolysis of the purified polysaccharide in 5 M HCl is shown in Fig. 1. Hydrolysis time beyond 3 hr resulted in no further release of reducing sugar. The discrepancy between total reducing sugar (calculated as glucose) and total dry weight is at least partially explained by the failure of uronic acids to develop full color with reducing sugar reagents, the color developed by glucuronic acid being approximately 41 % of that developed by an equal amount of glucose.

TABLE 2. *Chromatographic identification of sugar residues*

Spot no.	Solvent system	Distance traveled from origin			
		Unknown	Glucuronic acid	Galactose	Fucose
		cm	*cm*	*cm*	*cm*
1b	A	0.3	0.4		
	B	7.6	7.8		
	C	17.5	17.4		
	E	4.0	4.0		
3	A	9.0		8.9	
	C	29.1		29.0	
	D	17.0		17.0	
	E	18.6		18.6	
4	A	18.6			18.6
	C	33.9			33.9
	D	24.7			24.7
	E	25.9			25.9
5	B	7.1 (16.8)*	7.0		

Solvent systems: A, ethyl acetate-pyridine-water, 8:2:1. B, ethyl acetate-acetic acid-water, 3:1:3 (organic layer). C, isopropanol-pyridine-acetic acid:water, 8:8:1:4. D, *n*-butanol-acetic acid-water, 2:1:1. E, *n*-butanol-pyridine-water, 6:4:3.

* Figures given are for two components obtained from spot 5 eluted from a chromatograph developed in solvent E and rechromatographed in solvent B. Faster moving component is lactone form.

Chromatographic identification of sugars. In Table 2 are given distances traveled from the origin for unknown components and for known sugars chromatographed simultaneously. Spots are numbered in order of distance traveled, beginning at the origin, in the butanol-pyridine-water solvent (system E). Spots 1 (two components), 3, and 4 were the major residues of the hydrolyzate. Spots 2 and 5 were only faintly discernible when large amounts of material were applied to the paper, disappearing entirely with application of smaller volumes which were sufficient to produce highly colored spots for the other three components.

The very distinctive color reaction for uronic acids with the 2-aminobiphenyl oxalate spray reagent was obtained for spot 1. A bright orange color developed almost immediately after heating was begun, changing on further heating to pink, then to purple. Spot 1 dissociated into two components (designated 1a and 1b) when eluted from solvent E and chromatographed in an acid solvent, B. The colors produced by the two components differed slightly, that of 1a, the major but slower-moving one, being more brownish. The faster-moving minor component, designated as spot 1b in Table 2, had the same rate of movement as glucuronic acid. Evidence (described below) obtained from analysis of eluted spot 1a supports the conclusion, based on its chromatographic behavior, that this component was an aldobiuronic acid. Attempts to hydrolyze the eluted material in 10 M HCl at 100 C were unsuccessful. No spots were detected on chromatograms of the hydrolyzate.

Spot 2 has not been identified. Its $R_{glucose}$ value corresponds approximately to that of the amino sugars, but no color was obtained with ninhydrin spray. Since this component was present only in trace amounts, sufficient material for chemical analysis was not obtained.

The chromatographic identity of spot 3 with galactose and of spot 4 with fucose was well established. Rates of movement in four solvent systems and color reactions with the spray were identical with those of the known sugars. Since galactose and glucose have very similar values of R_F in most solvent systems, spot 3 was sprayed with glucose oxidase (Glucostat)[5] (Salton, 1960*b*). The reaction was negative; controls with glucose were positive.

Spot 5 also gave the distinctive uronic acid color with the 2-aminobiphenyl oxalate reagent. When eluted from the butanol-pyridine-water chromatogram and chromatographed in the ethyl acetate-acetic acid-water solvent (system B), spot 5 also dissociated into two components, one of which moved at the same rate as glucuronic acid. On the basis of this evidence and the colorimetric analysis discussed below, spot 5 was identified as glucurone, the lactone form of glucuronic acid.

Three other components, present in even smaller amounts than spots 2 and 5, gave very faint reactions with ninhydrin spray. It is probable that these represent traces

[5] Worthington Biochemicals Company, Freehold, N. J.

of amino acids released from the small amount of protein remaining after the final purification.

Chemical analyses. The modifications of the cysteine-H_2SO_4 reaction with sugars described previously were used both as supporting evidence of the identity of the sugar residues and as a quantitative measure of the amount of each sugar present in the hydrolyzate. The carbazole reaction was employed in a similar manner for uronic acid. Results of quantitative analyses of the eluates of each spot, from a chromatogram to which a known total amount of reducing sugar had been applied, are given in Table 3. Absorption spectra of the reaction products formed by the sample and the corresponding known sugar are presented in Fig. 2, 3, and 4. Figure 2 shows the absorption spectra obtained with spot 1 and with glucuronic acid in the carbazole reaction. The identical absorption maxima indicate the presence of uronic acid in the sample. However, there is a shoulder at approximately 470 mμ in the spectrum of the eluate, indicating the presence of a second compound, not a uronic acid. It is believed that this represents the hexose moiety of the aldobiuronic acid postulated as a component of spot 1. Although aldobiuronic acids are extremely resistant to hydrolysis (Pigman and Goepp, 1948), the conditions of the reaction (boiling for 20 min in concentrated sulfuric acid) are probably sufficiently severe to effect at least partial hydrolysis. The hexose moiety of the aldobiuronic acid was identified by the modification of the cysteine-sulfuric acid reaction designed as a qualitative test for galactose, glucose, and mannose (Ashwell, 1957). Standards were prepared containing the three hexoses and each hexose plus glucuronic acid, in amounts approximately equal to those measured in the eluate. Data upon which the identification of the hexose as glucose was based are presented in Table 4. The value $(D_{375-400}) - (D_{350-375})$ should be negative for glucose, zero for galactose, and positive for mannose. The standards conform to these criteria (the galactose standard contained traces of glucose), and the value obtained for the eluate is not only negative but also very close numerically to that obtained with the combined glucose-glucuronic acid standard. The composition of this standard was based on quantitative determinations of hexose (by the cysteine-sulfuric acid modification for hexoses in general) and of uronic acid by the carbazole reaction for the eluate of spot 1.

Figure 3 shows absorption spectra for secondary reaction products of galactose and the eluate from spot 3, obtained with the cysteine-sulfuric acid test for hexose. Figure 4 shows absorption spectra obtained with fucose and the eluate from spot 4 in the cysteine-sulfuric acid modification for measurement of methylpentose. A galactose standard was included to check the specificity of the reaction.

TABLE 3. *Quantitative determination of sugar residues in eluates**

Spot no.	Sugar	Micrograms per spot	Per cent of total reducing sugar
1	Glucuronic acid	144 148	20.5
1	Glucose	135	19
3	Galactose	130	18
4	Fucose	160 147	22
5	Glucuronic acid (lactone)	27	4
Total recovery			83.5

* Total reducing sugar applied to chromatogram = 710 μg.

TABLE 4. *Identification of glucose in eluate of spot 1 by cysteine-sulfuric acid method qualitative for hexoses*

Sample*	Optical density			$D_{375-400} - D_{350-375}$
	375 mμ	400 mμ	350 mμ	
Eluate, spot 1	0.109	0.082	0.154	−0.018
Glucose	0.035	0.027	0.071	−0.028
Glucose + glucuronic acid	0.042	0.030	0.079	−0.015
Galactose	0.070	0.034	0.110	−0.004
Galactose + glucuronic acid	0.076	0.038	0.116	−0.004
Mannose	0.103	0.035	0.134	0.037
Mannose + glucuronic acid	0.110	0.038	0.144	0.041

* Glucose, glucuronic acid, galactose, and mannose all at 65 μg per ml.

Discussion

The conclusion that the extracellular slime produced by *S. natans* is polysaccharide in nature, and that the protein associated with the polysaccharide in the product initially isolated is primarily contaminating material from broken cells, is based on the following facts. (i) The protein content of the crude gel was quite variable, (ii) Microscopic examination showed many empty cell walls embedded in the masses of slime. (iii) Nucleic acid was also present in samples containing a high proportion of protein. (iv) The protein component had the amino acid composition of typical cellular protein, conforming fairly closely to the amino acid content of the cellular protein of *E. coli*. Although it has been reported by Scheuring and Hohnl (1956) that the protein of *S. natans* contained only traces of phenylalanine, methionine, cystine, or cysteine, their conclusions were based only on relative amounts of color obtained in chromatography of a protein hydrolyzate. (v) Methods capable of breaking covalent bonds were not employed in the separation of protein and polysaccharide. On the other hand, the small amount of protein which could not be removed from the purified preparation, may be covalently bound to the polysaccharide. Stacey (1946) has stated the opinion that all bacterial polysaccharides occur naturally in combination with bound protein or peptide residues, which he suggested may be remnants of the synthesizing enzyme.

The presence of galactose, fucose, and glucuronic acid in the hydrolyzate of the polysaccharide has been established by both chromatographic and chemical methods. The pres-

ence of glucose, probably bound in glycosidic linkage to glucuronic acid as an aldobiuronic acid, is suggested by chemical analyses. The aldobiuronic acid unit was first discovered and characterized in a bacterial capsular polysaccharide (Heidelberger and Goebel, 1926, 1927).

The calculation of percentage recovery of sugar residues based on the total dry weight of polysaccharide hydrolyzed is complicated by the difficulty of assaying the aldobiuronic acid as well as the degree of hydrolysis of this unit. If it is assumed that the amount of uronic acid determined is correct, the amount of reducing sugar found can be corrected for the low color production of uronic acid and 91% of the original material can be accounted for as reducing sugar and protein. Assuming that all the glucose found is present in nonreducing form, the total recovery is 104% for the hydrolyzate before concentration. Reducing sugar determinations contain a slight inherent error since they are calculated on the basis of a glucose standard.

The molar ratios of the four major components, based on moles of fucose, may be calculated as: fucose, 1.0; galactose, 0.77; glucose, 0.77; glucuronic acid, 0.80. The equivalence of glucose and glucuronic acid on a molar basis in the eluate lends further support to the suggestion that the major component of spot 1 is an aldobiuronic acid composed of glucose and glucuronic acid.

Skerman (1959) has reported the conversion of the capsular material of *S. natans* into the tubular sheath. In view of the recently reported analyses of the material of the sheath (Wuhrmann and Mechsner, 1960; Romano and Lugananni, 1961) and the composition of the capsule as determined in the present study, such a conversion would seem unlikely.

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