THE SPECIFIC POLYSACCHARIDES OF TYPES I, II, AND III PNEUMOCOCCUS*

A Revision of Methods and Data

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For some years it has become increasingly evident that the methods originally proposed (1) for the isolation of the soluble specific substance (S) of pneumococcus were in need of revision. Avery and Goebel (2) and Enders and Pappenheimer (3) showed that more cautious manipulation of Type I pneumococcus or its culture filtrate led to the isolation of a product chemically and immunologically different from S I as originally described. Avery and Goebel presented evidence that these differences were due to a labile acetyl group which was removed by the treatment with alkali called for in the original method (1). We therefore undertook the preparation of the specific polysaccharides of Types II and III pneumococcus (S II and S III) without the use of alkali, and found no chemical differences (4) from the preparations made as originally described.

Avery and Goebel (2) had also shown qualitatively that the acetyl S I precipitated antibody from Type I antipneumococcus horse serum which the alkali-treated, deacetylated S I failed to throw down. Our quantitative determinations on a Type I antibody solution showed that the new product precipitated 2.0 mg. of antibody nitrogen as compared with 1.3 mg. thrown down by the deacetylated product. However, the S II and S III preparations which had not been alkali-treated failed to precipitate more antibody from homologous antipneumococcus horse serum than did the older preparations.

At this point we extended our quantitative study of the precipitin

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reaction between S III and homologous antibody (5) to rabbit antisera, and at once found that S III preparations which appeared identical in chemical properties, including reactivity with horse antisera, precipitated widely differing amounts of antibody from a given homologous rabbit serum (4). Similar results were obtained with S II. This was in accord with Enders and Pappenheimer's observation (3), that Type I antipneumococcus rabbit sera showed greater differences with S I prepared in various ways than did homologous horse sera.

In a search for the reason for these differences methods have been worked out for the isolation of the specific polysaccharides from culture filtrates without the use of heat, strong acid, or alkali. In general the procedure consists of the concentration of the culture filtrate *in vacuo* to a convenient volume; separation of the polysaccharide from salts and protein degradation products by repeated precipitation with alcohol in the presence of sodium acetate and acetic acid; removal of proteins by denaturation with chloroform and butyl alcohol (a modification of the method used by Sevag (6)); and elimination of any glycogen or starch present by methods depending upon the properties of the individual polysaccharides. The products were isolated as the neutral sodium salts. These were obtained entirely devoid of color and yielded solutions characterized by extraordinarily high viscosity.

EXPERIMENTAL

Since it was found necessary to vary the procedure slightly with different preparations, the following directions are given as a general guide rather than as an exact formula to be followed. The quantity of alcohol needed to precipitate the polysaccharides completely and the number of precipitations necessary to free the polysaccharides from impurities also depended to some extent upon the amount of polysaccharide present and the character of the impurities.

1. Preparation of the Specific Polysaccharide of Type I Pneumococcus, Preparation S 120.—10 liters of phosphate meat infusion broth containing 0.3 per cent of added glucose were seeded with a highly virulent Type I pneumococcus, strain 230.[1] After 72 hours at 37°C., 1 per cent of phenol was added and the culture

[1] Recent mouse passage is necessary for good yields of the polysaccharide. After a period of 3 weeks between the last mouse passage and the preparation of the polysaccharide the yield was reduced 80 per cent.

allowed to stand overnight. It was then centrifuged in a Sharples centrifuge and the effluent concentrated to 1 liter under reduced pressure, keeping the temperature below 35°. 100 gm. of crystalline sodium acetate were dissolved in the concentrated broth and 1250 ml. of 95 per cent alcohol were added with constant stirring. The polysaccharide separated as a white curdy mass which settled rapidly. After standing overnight the supernatant was poured off and the precipitate centrifuged. Usually a three layer separation was obtained as described (1), but in cases in which only a single volume of alcohol was used the phosphates and other salts were not thrown out and no syrupy layer was found. The three layer separation was also not obtained in the absence of phosphates. If the broth used is phosphate-free, purification of the polysaccharides may often be greatly facilitated by addition of sufficient phosphate to bring about the three layer separation. A solution of 10 gm. of sodium acetate in 250 ml. of water was made acid to litmus with acetic acid and the alcohol precipitate (or middle layer) was dissolved in this and reprecipitated with 300 ml. of 95 per cent alcohol. The centrifuged precipitate was dissolved in 250 ml. of water containing 10 gm. of sodium acetate and 5 ml. of glacial acetic acid, and the turbid solution was shaken with 50 ml. of chloroform and 10 ml. of *n*-butyl alcohol for 30 minutes (*cf.* 6). On centrifugation a semisolid emulsion separated between the aqueous layer and the chloroform. The solution and the chloroform were poured off and the layer of emulsion was washed with two 50 ml. portions of water which were saved for washing later emulsion layers. 50 ml. of chloroform and 10 ml. of butyl alcohol were added to the aqueous layer and the shaking was repeated. On centrifugation a smaller semisolid emulsion layer was formed and this was treated as before. Shaking with chloroform was repeated as long as an emulsion layer formed. Seven shakings were usually required. The washings of the emulsion layers were combined and shaken with fresh additions of chloroform as long as an emulsion layer formed, and were then combined with the main aqueous solution, the total volume now being 350 ml. The polysaccharide was precipitated with 500 ml. of 95 per cent alcohol, redissolved in 250 ml. of water, and the solution tested for phosphate and glycogen. Phosphate may be removed either by repeated precipitations with alcohol in the presence of sodium acetate and acetic acid or with glacial acetic acid in the presence of sodium acetate. Glycogen may be left behind by precipitating the S I from a salt-free aqueous solution with copper acetate. In the present instance the solution was free from phosphate but gave a strong iodine test for glycogen. 20 ml. of a saturated solution of copper acetate slightly acidified with acetic acid were added and the resulting bluish precipitate was centrifuged off. The supernatant remained clear when more copper acetate was added. The precipitate was dissolved in 50 ml. of 20 per cent sodium acetate solution and 5 ml. of glacial acetic acid, and reprecipitated with an equal volume of alcohol. This was repeated until the precipitate was free from copper, after which it was dissolved in 100 ml. of water and the solution again tested for glycogen. If this is present the copper precipitation is repeated. The S I was finally precipitated with redistilled alcohol in the presence of a small

amount of sodium acetate, washed with redistilled alcohol, filtered, and dried. Yield: 0.9 gm. of the neutral sodium salt of S I.

2. Preparation of the Specific Polysaccharide of Type II Pneumococcus, Preparation S 84.—10 liters of a 4 day culture of Type II pneumococcus, strain B 39, were treated in the same way as for Type I. No differences were observed up to the point at which the copper acetate precipitation was made. However, only one-third of the S II was thrown down as the copper salt. This fraction, S 84 A, which was free from glycogen, was reprecipitated as the copper salt and isolated separately.

The copper-soluble fraction, S 84 B, was freed from copper salts by several precipitations with alcohol in the presence of acetic acid and sodium acetate and was redissolved in a small volume of water. Even this concentrated solution failed to react with copper acetate. As glycogen was present in this fraction it was removed by adjusting the pH to 6.5 and adding a small amount of saliva. After a few minutes the iodine test was negative. To remove protein impurities added in the saliva 5 gm. of sodium acetate and 2.5 ml. of acetic acid were added, and the solution (volume 100 ml.) was repeatedly shaken with chloroform and a little butyl alcohol until an emulsion layer was no longer formed. Since the polysaccharide solution still contained nitrogen it was adjusted to contain 5 gm. of sodium acetate per 100 ml. and was chilled and precipitated with 5 volumes of glacial acetic acid. The precipitate was centrifuged in the cold, taken up in 50 ml. of 5 per cent sodium acetate solution, and again precipitated with 5 volumes of acetic acid. This was followed by two precipitations with alcohol from 5 per cent sodium acetate solution and one precipitation with redistilled alcohol, after which the polysaccharide was washed with redistilled alcohol, filtered, and dried.

	gm.
Yield of sodium salt of copper-precipitable S II	0.163
" " " " " copper-nonprecipitable S II	0.302
Total	0.465

The S II content in the original broth was determined according to Reference 7. 1 ml. of the broth contained 0.59 mg. or a total of 0.590 gm. of S II in the 10 liters used. Recovery, 78 per cent.

Preparation S 85.—9 liters of an 18 hour culture of Type II pneumococcus were worked up as above except as follows: Glycogen was removed with saliva after the first shaking with chloroform. The proportion of copper-precipitable S II, S 85 A, was much greater in this lot. Of 406 mg. of S II present in the broth 300 mg. were isolated from the copper-precipitable fraction and 60 mg. from the nonprecipitable fraction. The latter portion was not rigorously purified.

3. Preparation of the Specific Polysaccharide of Type III Pneumococcus.—A number of procedures for the isolation of S III were tried before the simplified method given in the following paragraph was found. Thus in preparation S

105 B, the culture filtrate was concentrated *in vacuo* to 1/10 its original volume. After three precipitations with 1.5 volumes of alcohol, part of the protein contained in the material was removed by 40 per cent saturation with sodium sulfate at 37°. The S III was then precipitated by completely saturating the solution with sodium sulfate. After removing the sodium sulfate by repeated precipitations of the S III with alcohol and acetic acid in the presence of sodium acetate the product was found to contain 2.4 per cent of nitrogen. This was removed by twice precipitating the S III as the barium salt with barium chloride. The barium was removed by repeated precipitations from 20 per cent sodium acetate solution with acetic acid and alcohol. The S III was isolated as the neutral sodium salt.

Similar methods were used in preparation S 107 with the omission of the sodium sulfate precipitations.

Preparation S 108.—9 liters of a 4 day culture of Type III pneumococcus, strain A 66, were worked up in the same way as the Type I culture. Only minor differences in behavior were noted. Thus, in the presence of sodium acetate less alcohol was needed to precipitate this polysaccharide, 1 volume of alcohol instead of 1.25 volumes being sufficient. Instead of giving a test for glycogen this preparation showed a distinct blue color when tested with iodine. This starch-like substance remained in the supernatant when the S III was precipitated with copper. Yield of the neutral sodium salt of S III: 1.08 gm.

4. Chemical and Physical Properties of Specific Polysaccharides of Types I, II, and III Pneumococcus.—The chemical and physical properties of different preparations are summarized in Table I. In this table the preparation designated S I old was prepared by the short method as described in an earlier paper (8); S 91 A by concentrating the culture filtrate on the steam bath and isolating the polysaccharide without the use of alkali or strong acid; S 120 as described above. Of the Type II preparations, S II old was prepared by the original method (1) but was fractionated by precipitation with copper; S 80 A was obtained from heated broth without the use of strong acid or alkali, and S 83 E, S 84 A, S 84 B, and S 85 A from broth concentrated *in vacuo* and isolated without the use of strong acid or alkali. Of the Type III preparations, S III old and A 66 were prepared as in Reference 1, S 102 from broth concentrated on the steam bath and isolated without the use of strong acid or alkali; S 105, S 107, and S 108 by methods described in this paper.

Analyses for nitrogen were made by a modified micro Kjeldahl method. The acetyl content was determined by hydrolyzing with 25 per cent *p*-toluenesulfonic acid solution, to which a small amount of barium hydroxide had been added, in an all glass reflux apparatus heated in a bath of boiling saturated sodium chloride solution for 2 to 4 hours. The acetic acid formed was distilled in a current of steam in a micro Kjeldahl apparatus. Successive 100 ml. samples of the distillate were collected, heated to boiling under reflux, and then cooled in an ice bath in a current of carbon dioxide-free air. They were then titrated with N/70 sodium hydroxide using phenolphthalein as indicator. In general all of the

TABLE I

Properties of Specific Polysaccharides of Pneumococcus

Preparation No.	Ash as Na	Nitrogen*	$[\alpha]_D$*	Neutral equivalent*	Acetyl*	Uronic anhydride*	Reducing sugars after hydrolysis*	Relative viscosity of 0.10% solution in 0.9% NaCl	$\eta_{rel.}$ 0.2% solution in H_2O	Amino nitrogen*	P*
	per cent	*per cent*	*degrees*		*per cent*	*per cent*	*per cent*			*per cent*	*per cent*
Type I											
SI old	0.3	5.12	+305	417	3.4	65				2.5	0.00
S 91 A	0.0	5.22	+294	590	10.0†			1.10			0.25
S 120	3.42	4.62	+278	650‡	7.1	56	30	1.69§	9.0‖	2.0	0.00
Type II											
SII old	0.0	0.16	+52	1000	1.9†			1.04			
S 80 A, Cu	0.1	0.20	+58	870	3.8†			1.04			
S 83 E	2.2	0.46	+53	1020‡	1.0		100	1.31			
S 84 A	2.3	0.40	+54	980‡	1.3	19.1	95	1.38			
S 84 B	2.35	0.73	+58	960‡		17.6	86	1.54			
S 85 A	3.70	0.14	+55	600‡	0.4	19.8		1.64**	10.6		0.00
Type III											
S III old	0.7	0.10	−38.0	351				1.04			
A 66	0.2	0.06	−37.3	350				1.09			
S 102	3.4	0.43	−30.7	351				1.18			
S 105	6.3	0.22	−32.5	340‡	0.6	48.2	84	2.64††	27.6‡‡		
S 107	6.1	0.27	−32.8	360‡	0.9			2.06	24.7§§		
S 108	6.5	0.08	−36.0	330‡	0.5	51		3.14	32.9		0.00
S 108 acid	6.2	—	−36.0	350‡				2.23	31.6		

* Calculated to the ash free basis. † Determined as in Reference 9. ‡ Calculated from the ash content.

§ After 6 hours' heating at 100° pH 6.4 in a sealed tube this value fell to 1.04. Treatment with N/2 NaOH at 37° for 48 hours gave $\eta_{rel.}$ = 1.20.

‖ " " " " " " " " " " " " " " " " 1.22.

** " " " " " " " 6.6 " " " " " " " " " 1.34. Treatment with N/2 NaOH at 37° for 24 hours gave $\eta_{rel.}$ = 1.51.

†† " " " " " " in a current of air " " " " 1.21.
in a sealed tube " " " " 1.34.

‡‡ 1.39 mg. per ml. §§ 2.14 mg. per ml.

acetic acid was contained in the first two fractions. The uronic anhydride was determined by the method of Burkhart, Baur, and Link (10) and reducing sugars, after acid hydrolysis, by the Hagedorn-Jensen method (11). Viscosities were measured in 0.9 per cent salt solution with an Ostwald viscometer at 20°C. As the values for the viscosity in water are greatly affected by the presence of small amounts of salt they are less useful for comparing different preparations than are the values in salt solutions.

In Table II are summarized data on the maximum amount of antibody nitrogen specifically precipitable by the different polysaccharide preparations from homologous antisera produced in the rabbit and in the horse. In order to remove antibodies that might react with material from the culture other than the polysaccharide (12) all of the antisera were first completely absorbed with somatic carbohydrate "C" (13) derived from heterologous strains of pneumococcus, and with pneumococcus protein obtained from an R strain. As described in previous papers (5), a slight excess of the polysaccharide was mixed with 1.0 ml. of the serum or antibody solution at 0° in a total volume of 4 ml., and the quantity of antibody nitrogen in the washed precipitate was determined by the micro Kjeldahl method. Rabbit sera were allowed to stand for 48 hours in the refrigerator after mixing with the polysaccharide, and the horse sera for 24 hours to insure completion of the reaction.

5. Effect of Various Procedures on Reactivity of Polysaccharides with Rabbit Antisera.—In order to determine which of the preparative manipulations used in the original isolation of the polysaccharides were responsible for the lowered reactivity of the older preparations with rabbit antisera, the following experiments were performed.

Type I. Effect of Heat.—Solutions of S 120 containing 1.0 mg. per ml. in 0.9 per cent NaCl and 2.0 mg. per ml. in water at pH 6.4 were sealed in glass tubes and heated for 6 hours in a boiling water bath. The relative viscosity of the solution in salt fell from 1.69 to 1.05 while the viscosity of the solution in water fell from 9.0 to 1.22. 12.4 ml. of the heated aqueous solution containing 24.8 mg. of S 120 were precipitated with 90 ml. of alcohol with the addition of a few drops of sodium sulfate solution to assist flocculation. The precipitated polysaccharide was centrifuged off and the supernatant concentrated to dryness on the steam bath after the addition of 5 ml. of 10 per cent sodium hydroxide solution to prevent loss of acetic acid. The residue was taken up in water, acidified with sulfuric acid, and the volatile acids were determined. Blank analyses were made on the reagents used. Volatile acids equivalent to 0.44 mg. of acetyl group were found, or 25 per cent of the acetyl content of the original polysaccharide. Part of the heated polysaccharide was precipitated when the pH of the solution was brought to 3.6 with acetic acid (*cf.* 1).

Effect of Alkali.—To 12.5 ml. of solution containing 50 mg. of S 120 were added 3.13 ml. of 10 per cent sodium hydroxide solution to make the concentration of alkali 0.5 N. The solution was allowed to stand in an incubator at 37°C. for 48 hours and the alkali was neutralized with hydrochloric acid to pH 7.0

and the volume made up to 50 ml. The final concentration of sodium chloride was 0.91 per cent and the relative viscosity of the solution was 1.20. 10 ml. of

TABLE II

Maximum Antibody Nitrogen Precipitated at 0°C. by Pneumococcus Polysaccharides from Homologous Antisera

Type I

Preparation / Serum	S I old	S 91 A	S 120	S 120 heated	S 120 treated with alkali	S 120 heated and treated with alkali
	mg.	mg.	mg.	mg.	mg.	mg.
Rabbit 3.70	0.08	0.21	0.48	0.26	0.14	0.07
Rabbit 3.41	0.15	0.26	0.51			
Horse antibody solution B 77	0.62	0.71	0.75	0.72	0.65	

Type II

Preparation / Serum	S II old	S 80 A	S 83 A	S 84 A	S 84 B	S 85 A
	mg.	mg.	mg.	mg.	mg.	mg.
Rabbit RS 1	0.70	0.70	0.97	0.98	0.99	1.01
Horse antibody solution B 83	0.72	0.73		0.71	0.71	

Type III

Preparation / Serum	S III old	A 66	S 102	S 105	S 107	S 108	S 108 acid	S 108 acid, heated in air	S 108 acid + broth	S 108 acid + broth, heated in air
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Rabbit antibody solution B 53	0.33	0.62	0.56	0.78						
Rabbit serum 3.50	0.86	1.12	1.10	1.25*	1.18†	1.27	1.23			
Rabbit serum 3.51	0.94	1.24	1.05			1.45	1.44	1.36	1.43	1.24
Horse serum 607	1.34	1.34	1.32	1.37	1.37					

* S 105 after treatment with N/2 NaOH at room temperature for 24 hours precipitated 1.22 mg. antibody N from this serum.

† S 107 after heating 6 hours at 100° in a sealed tube precipitated 1.17 mg. antibody N from this serum.

this solution were sealed in a glass tube and heated in a boiling water bath for 6 hours.

Data on the reaction with antisera of the polysaccharide treated as described above are included in Table II.

Type II.—A solution of preparation S 83 E in phosphate buffer at pH 5.8 was heated in a small evaporating dish in a boiling water bath for 8 hours, keeping the volume constant by addition of water. A similar solution was sealed in a glass tube *in vacuo* and heated in a boiling water bath. Samples of the culture filtrate were treated in the same way. After cooling, the solutions were neutralized to pH 7.0 with sodium hydroxide and set up in excess with 1 ml. of rabbit serum in a total volume of 11 ml. at 0°C. and the antibody nitrogen precipitated was determined after 72 hours. The large volume taken was considered desirable to avoid errors due to the high salt concentration of the neutralized broth.

Antibody Nitrogen Precipitated from Rabbit Serum RS 1

	Unheated	Heated *in vacuo*	Heated in air
	mg.	*mg.*	*mg.*
Broth	1.07	1.02	0.86
S 83 E	0.94	0.94	0.84

The difference in the amount of nitrogen precipitated by the broth and the isolated polysaccharide is believed to be due to antibodies to somatic carbohydrate and protein remaining in the serum in spite of the preliminary absorption. Quantitative agglutinin determinations (12) showed that there was still 0.17 mg. of non-type specific antibody nitrogen in the serum removable with pneumococcus I R suspension. The viscosities of solutions of S 85 A containing 1 mg. per. ml. in 0.9 per cent sodium chloride solution were compared after heating at 100°C. in sealed tubes at pH 6.6 for 6 hours, after heating in a current of air for 6 hours, and after treating with N/2 sodium hydroxide solution at 37° for 24 hours. The maximum amount of nitrogen precipitated from rabbit serum RS 1 was also determined.

	S 85 A	S 85 A heated in sealed tube	S 85 A heated in current of air	S 85 A alkali-treated
Relative viscosity 1 mg. per ml. in 0.9 per cent NaCl	1.64	1.34	1.21	1.51
Mg. antibody N pptd. from 1 ml. RS 1	1.01	1.00	0.99	1.00

Type III.—Preparation S 105 was treated with N/2 sodium hydroxide solution for 24 hours at room temperature without greatly changing its power to precipitate rabbit antibody (Table II). Heating preparation S 107 for 6 hours in a sealed tube at 100° reduced its viscosity from 2.64 to 1.34 without changing its reactivity. Purification of part of preparation S 108 by precipitation with

strong hydrochloric acid at 0° as described in the earlier papers (1) reduced its viscosity (S 108 acid) from 3.11 to 2.23 without affecting its ability to react with rabbit antiserum. Heating this preparation at pH 5.5 in a sealed tube at 100° also failed to change its reactivity, but heating at 100° in a current of air reduced it slightly. However, when this preparation was heated in the presence of sterile broth in a stream of air the amount of antibody precipitated from a rabbit antiserum was reduced to a still greater extent (Table II).

6. Dialysis Experiments.—In an earlier paper (14) it was reported that while S III would not diffuse through a collodion membrane from an aqueous solution into water, it would diffuse if the solutions both inside and out of the dialysis bag contained 10 per cent sodium chloride. The experiment has been repeated on the S III preparations described in this paper.

Collodion bags were made in test tubes with 6 per cent parlodion-ether-alcohol-acetic acid mixture (15). Aqueous solutions of preparations S III old, S 102, S 108, and S 108 heated with broth, were placed in the bags, and they were immersed in beakers of distilled water for 18 hours. At that time the solutions in the beakers were tested for the presence of polysaccharide with homologous horse and rabbit antiserum. The solutions in the bags were then replaced by solutions of S III containing 10 per cent sodium chloride and dialysis continued against 10 per cent sodium chloride solution in the beakers. S 108 was also tested in the presence of 15 and 20 per cent sodium chloride.

Reaction of Dialysate with Immune Serum

Salt concetration	0		10 per cent		15 per cent		20 per cent	
Antiserum	Horse	Rabbit	Horse	Rabbit	Horse	Rabbit	Horse	Rabbit
S III old	−	−	++	++				
S 102	−	−	++	++				
S 108	−	−	−	−	−	−	−	−
S 108 (heated)	−	−	+					

7. Additional Data on Constitution of S I.—Work is in progress in this laboratory on the structure of the Type I pneumococcus specific polysaccharide. Analyses show that the alkali-treated partially deacetylated S I (Table I) contains 5.12 per cent of nitrogen and 65 per cent of uronic anhydride or one atom of nitrogen per molecular weight of 273 and one molecule of uronic anhydride for every 271 molecular weight.

Preparation S 120, not alkali-treated, with 4.62 per cent nitrogen, 56 per cent uronic anhydride, and 7.1 per cent acetyl contains one nitrogen for each 303, one uronic anhydride for each 314, and one acetyl group for each 605 molecular weight. In both preparations about half of the nitrogen is free amino nitrogen.

Acetylation of the deacetylated S I with acetic anhydride in the presence of sodium carbonate followed by treatment with N/2 sodium hydroxide at room

temperature gave a product which contained 4.43 per cent nitrogen, 54.7 per cent uronic anhydride, 13.2 per cent acetyl, and no free amino nitrogen. This corresponds to one nitrogen for each 316, one uronic anhydride for each 322, and one acetyl for each 326 molecular weight. In contrast to the original product and to the S 120, both of which are good buffers at the neutral point, this material titrates sharply with phenolphthalein as indicator and shows an acid equivalent of 319. This indicates that the carboxyl groups of the uronic anhydride are free and that none of the nitrogen is present as an acid amide.

Treatment of S I with nitrous acid destroys its serological activity (1) and gives a product which has one-third the reducing value of glucose as measured by the Hagedorn-Jensen (11) method.[2] Upon hydrolysis of S I with hydrochloric acid this same reducing value is obtained.

The data agree with the hypothesis that the basic unit of the molecule is a trisaccharide containing two molecules of uronic acid and an unidentified substance containing two atoms of nitrogen. As already indicated by the isolation of mucic acid (1) part of the uronic acid, at least, is galacturonic acid. After hydrolysis of S I with methyl alcoholic hydrochloric acid as described by Morell and Link (16) the crystalline methyl-*d*-galacturonide methyl ester monohydrate was isolated.

DISCUSSION

In the preceding portion of the paper revised methods have been given for the isolation of the specific polysaccharides of Types I, II, and III pneumococcus. The procedure is relatively simple, avoids the use of heat, alkali, or mineral acid, and results in the recovery of high yields of polysaccharide as entirely colorless sodium salts. For good yields recent animal passage of the pneumococcus strain appears essential. Since the manipulation is less drastic than any other hitherto proposed, except that of Sevag (6) which is followed in part, it is believed that the products represent the closest approach yet attained to the carbohydrates as they are given off by the pneumococcus cell to the surrounding culture medium.

The product isolated by Sevag (6) from Type I organisms contained 6.7 per cent nitrogen, 1.3 per cent amino nitrogen, and had an optical rotation $[\alpha]_D$ 217°.[3] The high nitrogen, low amino nitrogen, and optical rotation indicate the presence of a nitrogen-containing component not present in our products. The question of whether this component is

[2] Details of this work will be given in a later communication.

[3] Through a typographic error this value was given as 21.7° in Sevag's paper.

present as an impurity or is an integral part of the polysaccharide as it exists in the organisms cannot be settled at present.

While the preparations now reported may be artefacts just as were the older ones, they are certainly a step closer to the native substances themselves, and it is proposed to refer to these products as the specific polysaccharides of Types I, II, and III pneumococcus (S I, S II, S III). The distinction between acetyl S I and S I is not made, since Avery and Goebel (2) have made it obvious that S I, properly isolated, should contain the acetyl group.

An unexpected result is the finding that the specific polysaccharides of pneumococcus are not thermostable, as was thought when only qualitative methods were available for their study. As will be noted from the data in Table I the viscosity is the most sensitive indicator of the change on heating, dropping markedly without any accompanying change in reactivity with antisera (Table II) in the case of S II and S III, and with a decrease in precipitating power in the case of S I owing to the partial removal of acetyl. When air is admitted during the heating an even greater decrease in viscosity occurs, and this is accentuated in the presence of broth, resulting in a decrease in the precipitating power of all three polysaccharides. This is probably the reason for the inferior precipitating power toward rabbit antisera of the older preparations, including even the acetyl S I, since heating was a step in the isolation. From the marked diminution in viscosity it is probable that heating effects a partial depolymerization of the long, thread-like chains of native polysaccharide, for Staudinger (17) has shown a definite relation between the viscosity and chain length of polymer homologues. It would appear, however, that the depolymerization, if such it be, may proceed quite far before the ability of the specific polysaccharide to precipitate rabbit antisera is markedly affected. On depolymerization by more drastic treatment, such as partial hydrolysis by mineral acid, the precipitating power toward rabbit antisera may be entirely lost, while the amount of antibody thrown down from horse antisera merely diminishes (18). Acid cleavage is also accompanied by further decreases in viscosity (14).

In harmony with the above are the dialysis experiments with S III (page 568). It had been shown previously that S III prepared according to Reference 1 did not pass through a collodion membrane into

water, but dialyzed readily in the presence of 10 per cent salt solution (14). It has now been found that a new preparation of S III which had not been heated failed to dialyze even in the presence of strong salt, but that after heating with broth dialysis occurred.

It is believed that the simplest explanation of the above is that the unheated preparations have the largest particle size, or longest chain, and that heating results in a degradation of the molecule to smaller units.

The instability of the pneumococcus specific polysaccharides toward heat has thus been shown by three independent methods. In addition to the probable depolymerizing action of heat there is evidence that an oxidative process occurs in the presence of air, and that this is greatly enhanced in the presence of broth, under the conditions similar to those used in the older preparations (1, 2), by the catalysts present in this material (19).

For the above reasons, also, the S III reported by Hornus and Enders (20) would appear to be a degradation product and not a different substance. Possibly the added amount of antibody thrown down by the preparation is due to contamination with C substance.

Work on the constitution of the specific polysaccharide of Type I pneumococcus has been continued. Indirect evidence had previously been obtained of the presence of galacturonic acid in the molecule (1). This has now been confirmed by the actual isolation of the methyl glycoside of galacturonic methyl ester from the products of hydrolysis of S I by methyl alcoholic hydrochloric acid. The analytical data so far obtained are consistent with the assumption of a trisaccharide unit for the S I molecule, containing two molecules of uronic acid, possibly both galacturonic acid, and two atoms of nitrogen. Further work is in progress.

SUMMARY

1. The thermolability of the specific polysaccharides of Types I, II, and III pneumococcus has been shown by three independent methods: (*a*) diminution of the viscosity of solutions on heating; (*b*) decrease in the amount of antibody precipitated from homologous rabbit antisera; and (*c*) increased tendency (S III) to pass through a collodion membrane.

2. These effects may be explained most simply as a partial depolymerization under the influence of heat. In air, particularly in the presence of broth, oxidation also appears to be involved.

3. Improved and simpler methods of preparation based on these findings, are given for S I, S II, and S III. The resulting products precipitate more anti-S from homologous rabbit antisera than do the earlier preparations.

4. The methyl glycoside of methyl galacturonate has been isolated from the hydrolytic products of S I, and evidence of the ultimate structural unit obtained.

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