315. *Some Physical Properties of the Specific Polysaccharides from the Types I, II, and III Pneumococcus.*

By B. R. Record and M. Stacey.

Type I, II, and III pneumococcus polysaccharides have been obtained by the methods devised by Heidelberger and his colleagues (for references see Boyd, "Fundamentals of Immunology", Interscience, 1942) in relatively undegraded forms, and some of their general properties are described.

Ultracentrifuge and diffusion measurements have been made on the polysaccharides from the three types in order to determine their molecular weights and to gain knowledge of their molecular homogeneity and shape. The specimens showed deviations of considerable magnitude from the van't Hoff ideal, necessitating the extrapolation of both sedimentation and diffusion constants to infinite dilution in order to obtain a valid estimate of molecular weight. All three polysaccharides are polydisperse and consist of molecules of greatly extended shape.

A good deal of knowledge is now available from American investigations on the general chemical properties of several of the type specific polysaccharides of the pneumococcus. In one case, that of Type III, the chain structure of the repeating unit has been determined in some detail (Reeves and Goebel, *J. Biol. Chem.*, 1941, **139**, 511). Some ten years ago, one of us (M. S.) had the

opportunity of preparing and purifying some of the pneumococcus type specific polysaccharides in Professor M. Heidelberger's laboratory at Columbia University Medical School, while the other (B. R. R.) was able to examine these preparations in Professor Svedberg's laboratory at Uppsala. Circumstances beyond our control have delayed publication of these results until now.

Each type of polysaccharide was obtained from a virulent 24-hour culture which was grown, as described in the experimental section, in liquid medium containing, as essentials, glucose, phosphate, and either peptone or meat infusion broth. The polysaccharide was thrown out of the sterilised, filtered, and concentrated solution by means of alcohol. It was separated from extraneous protein material by being shaken for short periods in aqueous solution with chloroform and butanol, and was purified by fractional precipitation methods. Finally it was obtained in the form of the sodium salt which was a white fibrous powder having typical properties as shown in Table I for the Types I, II, and III material.

TABLE I.

Specific pneumococcus polysaccharide.	$[\alpha]_D^{20°}$ (sodium salt).	Equiv. (free acid).	Ash, %.	N, %.	P, %.	$\eta_{sp.}^{18°}$ (in water).	$\eta_{sp.}^{18°}$ (in 1% sodium chloride).
Type I	+230°	460	5·8	5·6	0·12	13·8	2·9
Type II	+ 59	740	3·8	0·34	0·18	12·8	2·5
Type III	− 36	340	5·9	0·35	0·15	18·8	4·1

It will be noted that some of these properties differ from those recorded by previous authors (see Brown, *J. Immunol.*, 1939, **37**, 445). In the case of both Types I and II polysaccharides the optical rotation was definitely lower than had previously been recorded. Despite many attempts at purification, avoiding strong reagents, it was not possible to increase the numerical value of the specific rotation of either polysaccharide. In the case of the Types II and III polysaccharides it was observed that every fraction contained a low but significant nitrogen and phosphorus content. It is considered that these elements are combined in a prosthetic group forming a firmly bound integral part of the macromolecule. Treatment of the viscous solution of the polysaccharide with alkali rapidly caused a degradation of the molecule as evidenced by an irreversible fall in relative viscosity and liberation of an insoluble flocculent nitrogen-containing fragment. It appears that the integrity of the macromolecule in each polysaccharide depended upon the presence of the firmly bound prosthetic group.

Ultracentrifuge and Diffusion Measurements.—In an investigation of the osmotic pressure–concentration relationship of a wide variety of polysaccharide derivatives in organic solvents (Carter and Record, *J.*, 1939, 660), it was shown that deviation, often considerable, from the ideal solution laws frequently occurred. Anomalies of a similar kind are found in the sedimentation of long-chain polymers such as polystyrenes (Signer and Gross, *Helv. Chim. Acta.*, 1934, **17**, 59, 335, 726); cellulose derivatives (Signer and Tavel, *ibid.*, 1938, **21**, 535; Kraemer, *Ind. Eng. Chem.*, 1938, **30**, 1200; Gralen, Dissertation, Uppsala, 1944; Jullander, *Arkiv Kemi, Min. Geol.*, 1945, **21***A*, No. 8). These deviations are manifest in a decrease in the sedimentation rate with increasing concentration, necessitating extrapolation to zero concentration, where the molecules no longer interfere with each other, in order to obtain a true value for the sedimentation constant of the free molecules. In diffusion also, the variation in the rate of diffusion with concentration leads to skew distribution curves in all but the most dilute solutions, and special precautions are necessary in deducing the true diffusion constant under conditions which permit free diffusion of the molecules. (See, *e.g.*, diffusion experiments on methylated and acetylated cellulose by Polson, *Kolloid Z.*, 1938, **83**, 172.) Methylated glycogen exhibits such anomalous effects only to a minor extent (see Record, this vol., p. 1567) evidently possessing a shape not far removed from the spherical. The capsular polysaccharides prepared from the pneumococcus, on the other hand, exhibit considerable deviations from the ideal behaviour in both sedimentation and diffusion. This section of the paper is concerned with the extrapolation of these two properties to zero concentration and the calculation of molecular weights and frictional ratios from the data so obtained for each of the three polysaccharides.

Methods and Results.—The methods employed follow precisely those described in the work on methylated glycogen (Record, *loc. cit.*). The polysaccharides prepared as described in the first part of this paper were stored in a vacuum over phosphic oxide. The dried specimens dissolve slowly in dilute salt solutions to form viscous solutions. Since they contain small quantities of sodium and are probably sodium salts, care must be taken to make up the solutions

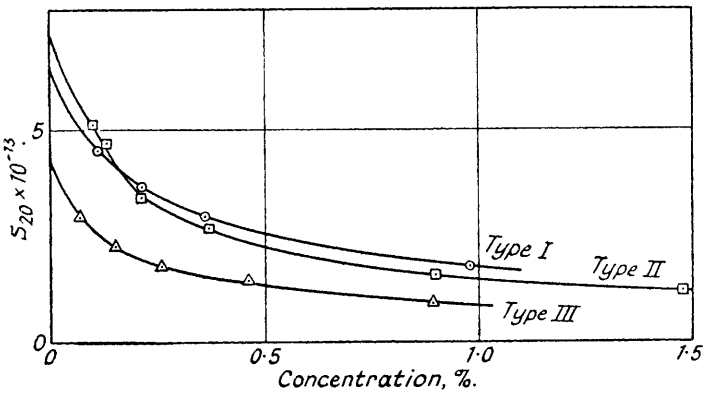
in sodium chloride of sufficient concentration to suppress any Donnan effects. With the exception of a few measurements in 0·5M-sodium chloride to check this point, 0·15M-sodium chloride was used throughout the present work and was found adequate in sedimentation and diffusion with the concentrations employed.

Sedimentation Velocity.—All runs were made in the Svedberg oil turbine centrifuge at the maximum routine speed of 70,000 r.p.m. (centrifugal field = 360,000 × gravity). A preliminary run on a 1% solution of the Type I polysaccharide showed a single and extremely sharp sedimentation boundary. The boundary showed no tendency to broaden as the run progressed, and indeed appeared as sharp at the end of the run (4 hours duration) as at the beginning. This behaviour suggested, at first sight, a substance possessing a high degree of molecular homogeneity and a low diffusion constant. On repeating the run on a 0·1% solution, however, not only did the boundary show a considerable broadening with time, but the sedimentation took place more than twice as rapidly. It is evident that this anomalous behaviour is the result of considerable departure from the ideal solution laws and that, if the sedimentation constant is to be used for the calculation of molecular weight, investigation of the sedimentation–concentration relationship and extrapolation to infinite dilution become essential.

The sedimentation constant determined at intervals during a run shows a tendency to increase as the run progresses, owing to a progressive dilution of the solution resulting from the combined effects of (*a*) the increase in the centrifugal field with increasing distance from the

FIG. 1.

Pneumococcus polysaccharides. Sedimentation–concentration curves.



axis of rotation, and (*b*) the sector shape of the cell. Owing to the difficulty of obtaining a well-defined drift in the calculated sedimentation constant during a run, a mean value has been taken over the duration of each run. This value refers strictly to a solution of slightly lower concentration than that placed in the cell at the commencement of the run. The error has been ignored in the present work since it has a negligible effect on the extrapolated value at infinite dilution. The sedimentation constants thus obtained, corrected to a medium of water at 20°, are given in Table II, together with the extrapolated values, $c \longrightarrow 0$. The general form of the sedimentation–concentration curves is shown in Fig. 1. The limiting values of s ($c \longrightarrow 0$) may be obtained from these curves, but can be extrapolated more conveniently from the 1/sedimentation–concentration curves which approximate more closely to straight lines.

One result of the large variation in the sedimentation constant with concentration has just been discussed, *viz.*, a tendency of the calculated s values to increase during a run owing to progressive dilution. Another consequence of this variation is seen in the exceptionally sharp sedimentation boundaries in all but the most dilute solutions. The smaller molecules which tend to lag behind the main boundary find themselves in a region of low concentration where their sedimentation velocity is greatly increased, while the larger particles sedimenting in a region of high concentration will have a relatively small sedimentation velocity; the net effect is an artificial sharpening of the sedimentation boundary. This effect calls for caution in deducing information about the degree of polydispersity from the rate of boundary spreading.

In many of the runs the boundaries were so sharp as to involve the deviation of only one of the scale divisions, representing only about 1/100 part of the distance down the centrifuge cell. In cases of this kind the diagonal Schlieren method of Philpot (*Nature*, 1938, **141**, 283), which

TABLE II.

Sedimentation constants (corrected to water at 20°) of pneumococcus polysaccharides in 0·15M-sodium chloride.

Type I.		Type II.		Type III.	
Concn., g./100 g. of 0·15M-NaCl.	$s_{20} \times 10^{13}$.	Concn., g./100 g. of 0·15M-NaCl.	$s_{20} \times 10^{13}$.	Concn., g./100 g. of 0·15M-NaCl.	$s_{20} \times 10^{13}$.
0·114	4·43	1·475	1·17	0·917 *	1·20
0·209	3·59	0·904	1·56	0·890	1·056
0·355	2·98	0·366	2·80	0·447	1·53
0·98	1·88	0·210	3·47	0·270	1·88
Limit $(c \longrightarrow 0)s_{20} = 6·5 \times 10^{-13}$		0·126	4·48	0·158	2·40
		0·084	5·11	0·089	2·95
		1·390 *	1·30	Limit $(c \longrightarrow 0)s_{20} = 4·3 \times 10^{-13}$	
		0·504 †	2·26		
		Limit $(c \longrightarrow 0)s_{20} = 7·2 \times 10^{-13}$			

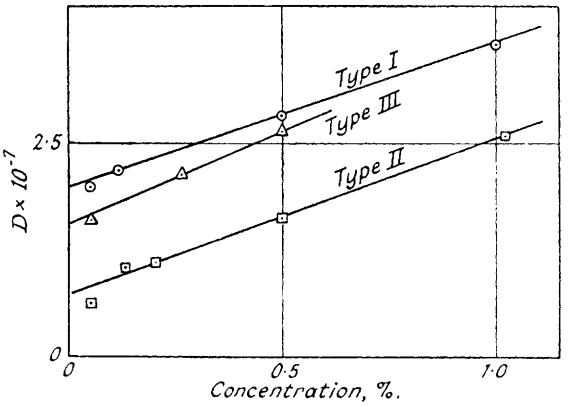
* These runs were made in 0·5M-NaCl.

† Run carried out at the Lister Institute using the Diagonal Schlieren Optical System (see Fig. 2).

was developed after most of the present work had been carried out, has advantages over the scale method, as it permits a more accurate determination of the boundary position and also of the degree of boundary spreading. The untouched photograph in Fig. 2 was obtained by means of this optical system installed by Dr. A. S. McFarlane on the oil-turbine centrifuge at the Lister Institute, London. It represents the sedimentation diagram for a 0·5% solution of the Type II polysaccharide in which the sharpening effect is most marked, but which is characteristic of all the three polysaccharides examined.

FIG. 3.

Pneumococcus polysaccharides. Diffusion–concentration relationship.



Diffusion.—The large increase in the sedimentation constant with diminishing concentration is in a direction corresponding to an apparent increase in the " effective " molecular weight of the particles on dilution. A similar effect in the same direction is found in diffusion. Thus when a 0·5% solution of the Type I polysaccharide in 0·2M-sodium chloride was allowed to diffuse against 0·2M-sodium chloride, an asymmetric concentration distribution was observed. Diffusion took place much more rapidly in the solution than in the solvent, so that the concentration gradient after a given time was steeper on the solvent side than on the solution side of the boundary. In order to follow the variation in the diffusion constant with concentration and at the same time avoid the difficulty of dealing with skew concentration distributions, one solution was allowed to diffuse against another of slightly different concentration, the concentration difference being taken as small as possible compatible with accurate definition of the concentration distribution. With differences of not more than 0·2%, practically symmetrical curves were obtained. The curves were analysed by the moment method by calculating the standard deviation for the whole curve from the first and second moments about the centroidal vertical. The measurements were made in the Lamm cell at 20°, and the calculated diffusion constants corrected to diffusion in pure water. The results are shown in Table III. The diffusion constants show a considerable increase with increasing

FIG. 2.

Sedimentation velocity diagram.

Pneumococcus Type II Polysaccharide.

0·5% *Solution in* 0·2M-NaCl.

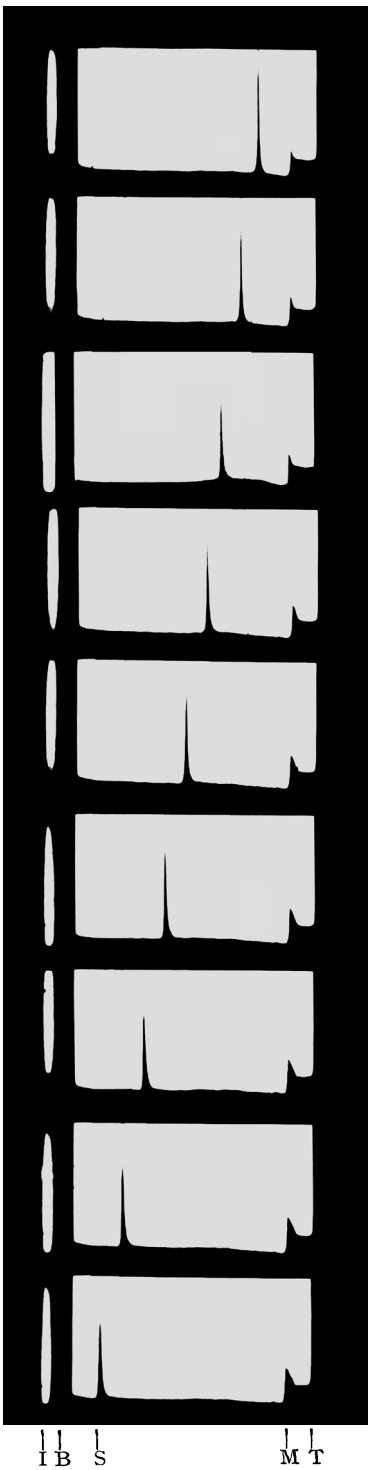
Diagonal Schlieren Optical System.

Speed = 60,000 r.p.m.

Centrifugal Force = 250,000 × g.

Interval between Exposures: 30 mins.

Cell Magnification: 2·03.



Abscissæ: Distance from centre of rotor (x).

Ordinates: Concentration gradient (dc/dx).

T = *top of cell.*
M = *meniscus.*
S = *sedimentation boundary.*
B = *bottom of cell.*
I = *cell index.*

[*To face p.* 1564.

concentration and the relationship appears to be linear over the range examined (Fig. 3). The extrapolated values of D_{20} ($c \longrightarrow 0$) are given in the table.

The Partial Specific Volume.—This was determined at 20° for the Type I polysaccharide only. The values obtained were $V = 0{\cdot}538$ for a 1% solution and $V = 0{\cdot}536$ for a 0·5% solution. A mean value $V = 0{\cdot}537$ was used for all three polysaccharides.

The Molecular Weight.—The molecular weights of the three polysaccharides have been calculated from the Svedberg formula $M = \frac{\boldsymbol{R}Ts}{(1 - V\rho)D}$ using the above values of s and D extrapolated to infinite dilution. The results are shown in Table IV.

The Frictional Ratio.—The value of f/f_0, of between 3·2 and 6·0 (see Table IV) points to considerable asymmetry in the shape of the molecules, a conclusion in conformity with their observed properties, *e.g.*, high viscosity and large deviations from the ideal solution laws as seen in their behaviour in sedimentation and diffusion. On the assumption that there is no appreciable hydration of the particles, the actual values of the axial ratios of each of the polysaccharides have been deduced from the values of the frictional ratio using the equation of Perrin as described by Svedberg and Pedersen (" The Ultracentrifuge ", p. 41), though, as there pointed out, such figures may not have precise significance and are to be treated with reserve.

TABLE III.

Diffusion of pneumococcus polysaccharides in 0·2M-NaCl.

Type I.

Concn., g./100 g. of 0·2M-NaCl.	Mean concn.	Time, hours.	$D_{20} \times 10^7$.	Mean $D_{20} \times 10^7$.
0·10	0·05	36	1·87	2·02
0·10	0·05	35¾	2·16	
0·226	0·113	24	2·29	2·22
		36	2·14	
0·401	0·50	23	2·99	2·84
0·604		34	2·68	
0·53 *	0·26	52½	—	2·19
1·104	1·00	13¼	3·89	3·67
0·905		24½	3·46	

Limit D_{20} ($s \longrightarrow 0$) = $2{\cdot}0 \times 10^{-7}$

* Asymmetrical curve.

Type II.

0·100	0·05	23⅓	0·749	0·73
		35½	0·712	
0·102	0·195	21	1·104	1·10
0·287		32	1·096	
0·406	0·495	23	1·829	1·65
0·584		35	1·668	
		47½	1·451	
0·85	1·01	22	2·490	2·56
1·17		36	2·633	
0·069	0·122	16	1·241	1·06
0·175		28½	1·103	
		41	0·826	

Limit D_{20} ($s \longrightarrow 0$) = $0{\cdot}75 \times 10^{-7}$

Type III.

0·145	0·220	21⅓	2·386	2·18
0·295		33½	1·964	
0·390	0·501	23⅓	2·543	2·66
0·613		35½	2·773	
0·105	0·052	12	1·727	
		23	1·533	1·63
		36	1·624	

Limit D_{20} ($s \longrightarrow 0$) = $1{\cdot}60 \times 10^{-7}$.

TABLE IV.

Molecular weight and frictional ratio of pneumococcus polysaccharides.

Type.	$s_{20} \times 10^{13}$ ($c \longrightarrow 0$).	$D_{20} \times 10^7$ ($c \longrightarrow 0$).	M.	f/f_0.	Axial ratio b/a.
I	6·5	2·00	171,000	3·2	60
II	7·2	0·75	504,000	6·0	200
III	4·3	1·60	141,000	4·3	110

The Degree of Polydispersity.—As already pointed out, the sharpness of the sedimentation boundary in concentrations of 0·5% or more (see Fig. 2) may lead one to conclude that the three pneumococcus polysaccharides examined possess a high degree of molecular homogeneity. Bearing in mind their low diffusion constants, however, the boundary spreading observed at the lowest concentrations examined, *ca.* 0·1%, leaves no doubt as to the polydisperse nature of all three polysaccharides. Owing to the practical difficulties in the way of carrying out sedimentation-velocity runs in a sufficiently dilute region where mutual interference between the molecules is no longer significant, no attempt has been made to assess the degree of polydispersity. For a similar reason the equilibrium centrifuge cannot be directly applied to determine the molecular weight of such asymmetric molecules. Further consideration of the results of this paper suggests that even in the absence of any boundary sharpening effect, *i.e.*, in solutions sufficiently dilute to reduce the sharpening to negligible proportions, it may yet be possible to have polydispersity without boundary spreading. Thus, the Types I and II polysaccharides, although of widely different molecular weight, have almost identical sedimentation–concentration curves, so that a mixture of these two substances would sediment as one component. In such a case, the differences in molecular weight will be revealed almost entirely by differences in the diffusion constant, and with long-chain molecules this may prove to be a far more sensitive index of polydispersity than sedimentation velocity. In general, therefore, the behaviour in both sedimentation and diffusion must be taken into account in assessing the extent of the polydispersity.

How far the values quoted in Table IV for the molecular weight and shape of the three polysaccharides are truly representative of intrinsic differences between them and how far the result of differences in their treatment, purification, etc., can be decided only by extended investigation. It is at least clear, however, that the pneumococcus polysaccharide consists of large molecules with average molecular weights of the order 10^5—10^6 possessing greatly extended length in relation to their breadth.

Experimental.

Preparation and Properties of the Polysaccharides.—Tap water (20 l.) was heated to 80°; "Difco" Bacto-peptone (20 g. per litre) was added, the liquid boiled for about 10 minutes and cooled, and the pH adjusted to 7·8 by the addition of 3—3·5 c.c. of 2N-sodium hydroxide per l.

The filtered liquid was distributed in 1400 c.c. amounts in sterile two-litre Erlenmeyer flasks. A "Swift–Hodge" buffer solution was made up as follows: glucose, 5 g.; sodium bicarbonate, 2 g.; sodium chloride, 2 g.; hydrated disodium hydrogen phosphate, 1 g.; all dissolved in 1 l. of distilled water.

The phosphate was dissolved first and then the other salts to make 2 l. of solution which was sterilised by filtration through Chamberland L.2 or L.3 candles into sterile flasks. The broth solution was sterilised in an autoclave at 15 lbs. pressure for 15 minutes. To each flask was added the buffer solution (100 c.c.) under sterile conditions. The flasks were incubated at 37° for a few days in order to ascertain whether they were completely sterile. In the meanwhile a Type I pneumococcus organism was put through a "mouse passage" in order to increase its virulence.

After incubation overnight the tubes showed a healthy growth, and a loopful of the organisms was examined by the Gram- and capsule-staining methods. Sterile meat broth (300 c.c.) in 3 flasks was inoculated with 1 c.c. of the live suspension from the tubes and incubated overnight. There was a good growth, and this was used for inoculating the 20 l. of medium, each flask being inoculated with 10 c.c. of the prepared virulent culture of pneumococcus Type I and then incubated at 37° for 72 hours. Each flask showed a thick turbidity and a heavy deposit of the bacterial cells. The organisms were killed by the addition of phenol (10 c.c. of a solution of 10 g. per l.). After 3 hours, agar plates were made of the organism; later inspection showed that 3 hours with phenol was sufficient to kill all the organisms. The flasks were left for 24 hours before the contents were emptied. After being well mixed the solution was evaporated at room temperature in a vacuum. The volume of the concentrate was made to 2·75 l. with the washings, and in this syrupy liquid was dissolved 200 g. of sodium acetate and 20 c.c. of gacial acetic acid. The polysaccharide was precipitated as a white curdy mass by the addition of 95% ethanol (2·75 l.). After standing overnight the clear yellow supernatant liquid was siphoned off. The polysaccharide was isolated on the centrifuge. Previous experiments in Professor M. Heidelberger's laboratory showed that this polysaccharide material was very impure but that it probably contained about 1 g. of pure Type I polysaccharide.

Rapid Method for the Purification of the Type I Polysaccharide.—The precipitate was dissolved in distilled water (500 c.c.), forming a white emulsion. This was shaken on the mechanical shaker for 2 hours with chloroform (100 c.c.) and butanol (20 c.c.). This treatment rendered a large amount of the protein material insoluble, and this could be removed by 2 hours' centrifuging at 2000 revs. per minute. The precipitate was washed thrice by shaking it for ½ hour with water (50 c.c.), and the washings added to the polysaccharide solution (650 c.c.).

This liquid was shaken for 1 hour with chloroform (50 c.c.) and butanol (10 c.c.), centrifuged, and the precipitate washed as before with water (50 c.c.).

Sodium acetate (100 g.) and glacial acetic acid (20 c.c.) were dissolved in the solution and washings (total vol., 700 c.c.). The polysaccharide was precipitated by the cautious addition of 95% alcohol (700 c.c.). It was isolated on the centrifuge as before and the supernatant liquid rejected.

The polysaccharide was dissolved in water (300 c.c.) and shaken for 1 hour with chloroform (25 c.c.) and butanol (5 c.c.). After this solution had been centrifuged for 2 hours at 2000 revs. per minute there was a trace only of precipitate showing that all the protein had been removed. The polysaccharide was isolated as before by precipitation from acid solution, in the presence of sodium acetate, by an equal volume of alcohol. It formed a flocculent white precipitate which was free from glycogen and phosphate and gave no biuret test. It reacted very strongly in extremely dilute neutral solution, *e.g.*, in a dilution of 1 in 3 millions, with a suitable absorbed Type I pneumococcal antisera. It formed frothy opalescent solutions in water.

The polysaccharide was separated into fractions by precipitation from a solution containing 2% of sodium acetate with solvents such as acetone or alcohol. The material imparting the opalescence was first separated at low alcohol concentration and the main bulk of the polysaccharide (2·2 g. from 24 l. of culture medium) was obtained in the form of a white fibrous solid which could not be separated into further fractions by precipitation methods or by adsorption on kieselguhr, etc. {Found, for a typical preparation of the sodium salt : $[\alpha]_D^{20°}$ + 230° (*c*, 0·6 in water) ; $\eta_{sp.}^{18°}$ in water, 13·8 ; $\eta_{sp.}^{18°}$ in 1% sodium chloride, 2·9 ; N, 5·6 ; P, 0·12 ; ash (sodium), 5·8% ; equiv. (free acid), 406}.

Products with similar properties and in similar yields were obtained by growing the pneumococci on a medium containing meat infusion broth instead of peptone. Products made on the medium were more readily separated from extraneous protein material.

Type II Specific Polysaccharide.—This was grown in a manner identical with that described for the Type I material. The polysaccharide was more readily obtained in homogeneous form, and yields were slightly higher, *e.g.*, 2·6 g. from 24 l. of medium. The product was a white fibrous solid, completely water-soluble {Found, for a typical preparation of the sodium salt : $[\alpha]_D^{20°}$ + 59° (*c*, 0·5 in water) ; $\eta_{sp.}^{18°}$ in water, 12·8 ; $\eta_{sp.}^{18°}$ in 1% sodium chloride, 2·5 ; N, 0·34 ; P, 0·18 ; ash (sodium), 3·8% equiv., (free acid), 740}. From an acid hydrolysate of this polysaccharide L-rhamnose was identified as a constituent unit.

Type III Polysaccharide.—This was produced on the above medium in the Birmingham laboratories from a Type III virulent pneumococcus culture provided by Dr. R. M. Jenner of Glaxo Laboratories Ltd. The product was a fibrous white solid which formed highly viscous solutions in water and in buffers, and it was difficult to purify {Found, for a typical sample of the sodium salt : $[\alpha]_D^{20°}$ − 36° (*c*, 0·4 in water) ; $\eta_{sp.}^{18°}$ in water, 18·8 ; $\eta_{sp.}^{18°}$ in 1% sodium chloride, 4·1 ; N, 0·35 ; P, 0·15 ; ash (sodium), 5·9% ; equiv., (free acid), 390}.

Samples of closely similar preparations of the Type III polysaccharide were kindly provided by Dr. Elvin A. Kabat.

No claim is made that any of the preparations described above represent the pneumococcus polysaccharide in a completely undegraded state. All have been made from autolysed cultures, and it may yet be possible to extract from unautolysed cells polysaccharides with higher molecular weights than those described above.

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