38. The Preparation and Properties of a Specific Polysaccharide from *Bact. typhosum* Ty₂

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The observation of Dochez & Avery [1917] that culture filtrates from the three main types of *Pneumococcus* contain a soluble substance which gives specific precipitation with the homologous immune serum has led to studies on many types of *Pneumococcus* which have established the importance of polysaccharides in determining the type specificity of certain bacterial antigens [Heidelberger & Avery, 1923; Heidelberger *et al.* 1925; Avery & Goebel, 1933]. Artificial carbohydrate-protein antigens have been synthesized by Goebel [1939; 1940], in which the specificity of the antigenic complex may be determined not only by a complex polysaccharide but by a relatively simple specific pattern unit such as an aldobionic acid. This observation has stimulated inquiry into the nature of the component carbohydrate residues and structure of the polysaccharide haptens of bacterial antigens.

Morgan [1936; Morgan & Partridge, 1940; 1941] has shown that the dominant smooth antigen of *B. dysenteriae* (Shiga) consists of a complex of a polysaccharide, a phospholipin and a conjugated protein, in which the polysaccharide, which appears to determine the specificity of the complete antigen, is composed of *d*-galactose, *l*-rhamnose and *N*-acetylglucosamine units [Morgan, 1938].

The antigens of *Bact. typhosum* and the closely related *Bact. typhi-murium* were first isolated and studied by Boivin *et al.* [1933], Raistrick & Topley [1934] and Topley *et al.* [1937] and appeared to consist predominantly of a polysaccharide linked to an insoluble nitrogen- and phosphorus-containing substance believed to be a phosphatide. Freeman *et al.* [1940] obtained the antigens of these organisms in a state approaching chemical homogeneity by modifications of the methods of Boivin and Raistrick & Topley, and in the case of *Bact. typhosum* the antigen was shown to exist as a complex of a polysaccharide, a polypeptide and a small amount of a lipid component [Freeman & Anderson, 1941].

In the present paper the preparation in a pure state, and properties of the polysaccharide component of *Bact. typhosum* Ty₂ are described in detail. Degradation of the polysaccharide and identification of its component hexose residues have resulted in characterization of about 78% of the component carbohydrate residues of the complex substance. In view of the observation of Avery & Goebel [1933] that the specific polysaccharide of *Pneumococcus* Type I exists in the bacterial cell as an acetyl polysaccharide which may be isolated as such and has the power, not possessed by the deacetylated compound, of stimulating the production of specific antibodies on injection into mice, the influence of acetyl groups introduced into the polysaccharide molecule on its precipitability by *Bact. typhosum* O-antiserum has been studied.

EXPERIMENTAL

The dried bacterial cells of *Bact. typhosum* Ty₂ used in this investigation were grown on a synthetic medium as described by Freeman *et al.* [1940].

Preparation of the specific polysaccharide

The specific polysaccharide of *Bact. typhosum* Ty₂ may be prepared either from the complex bacterial antigen, isolated from the bacterial cells by tryptic digestion [Freeman *et al.* 1940], by means of hydrolysis with dilute acetic acid, or directly from the dried bacterial cells by extraction with dilute acetic acid. The bulk of the crude polysaccharide used in these experiments was obtained by hydrolysis of the tryptic digest antigen in the course of investigation of the nature of the latter [Freeman & Anderson, 1941].

(a) *Preparation from tryptic digest antigen.* The tryptic digest antigen, usually in batches of about 5 g., was dispersed by grinding in a mortar with sufficient 0.1 *N* acetic acid to give a 1 % solution which was shaken mechanically until a homogeneous opalescent solution was obtained. This was heated on the boiling water bath, under reflux for 12 hr.; during the hydrolysis the solution first clarified a little (30 min.), but after 2 hr. it became very opalescent and the insoluble polypeptide component of the antigen began to flocculate out. After about 8 hr. heating the flocculation appeared to be complete and there was no further deposition of precipitate and no further change of optical rotation during the last 2 hr. of hydrolysis. The insoluble polypeptide fraction was removed by centrifuging in an angle centrifuge for about 4 hr. at 2000–3000 r.p.m. giving a slightly opalescent brownish coloured supernatant solution, which was concentrated *in vacuo* at low temperature to one-third of the original volume. Recentrifuging at this stage gave a further small precipitate of insoluble polypeptide. The crude polysaccharide was precipitated from the supernatant solution by addition of absolute alcohol to 86 % by weight. Addition of alcohol to 48 % gave slight precipitation, alcohol to 60 % produced a bulky flocculent precipitate and after increasing the alcoholic content to 86 % by weight flocculation of the crude polysaccharide appeared to be complete. The crude polysaccharide ('Polysaccharide F 86 %') was centrifuged off, washed twice with absolute alcohol, twice with ether and dried *in vacuo* over phosphorus pentoxide.

The crude polysaccharide was a water-soluble, almost colourless amorphous substance which corresponded to about 57 % of the antigenic complex. It contained 3.6 % N, 1.6 % organic P, and gave 55 % of reducing sugars on hydrolysis with 2 *N* HCl for 2 hr. at 100° in a sealed tube [Hagedorn & Jensen, 1923], non-volatile ash = 20.2 %. On electro dialysis for 48 hr. against distilled water through cellophane membranes a sample of the crude polysaccharide yielded 39 % of alcohol-precipitated polysaccharide F 86 % containing 1.0 % N and 3.2 % non-volatile ash. In a further experiment after 120 hr. electro dialysis 33 % of a partially purified polysaccharide F 86 %, containing 0.5 % N and 2.6 % non-volatile ash, was obtained.

Alcoholic fractionation of polysaccharide. A mixture of 0.196 g. of two samples of electro dialysed polysaccharide was dissolved in 10 ml. water ($[\alpha]_{5461}^{17} = +134^\circ$, uncorrected for ash) and fractionally precipitated with absolute alcohol. Addition of alcohol to 45 % by weight produced a small amount of precipitate which was centrifuged off and the alcoholic content of the clear supernatant solution increased to 54 %. The process was repeated until fractions corresponding to alcoholic concentrations of 0–45, 45–54, 54–60, 60–70, 70–80 and 80–99 % were obtained, these were washed twice with alcohol, twice with ether and dried *in vacuo* over phosphorus pentoxide. The yields and properties of the fractions obtained are summarized in Table 1.

N, P, ash, reducing sugar value and specific rotation determinations, were carried out as described by Freeman & Anderson [1941], and other analyses were by Dr G. Weiler. Except where otherwise stated analytical results are quoted on an ash-free basis. Melting-points are uncorrected.

The analytical data of the fractions isolated by alcoholic fractionation of the electro dialysed polysaccharide afford good evidence that the latter consists predominantly of a single polysaccharide. The mother liquor fraction F 80–99 % contains the bulk of the

Table 1. *Alcoholic fractionation of electrodialysed polysaccharide*

	Polysaccharide					
	0.196 g. 0.072 g.: F 86% (a) N=1.0%, Ash=3.2%					
	0.124 g.: F 86% (b) N=0.5%, Ash=2.6%					
	Dissolved in 10 ml. water, solution centrifuged to remove a trace of residue					
	$[\alpha]_{5461}^{17} = +134^{\circ}$					
	Alcohol added to:					
	45%	54%	60%	70%	86%	99%
	F 0-45%	F 45-54%	F 54-60%	F 60-70%	F 70-80%	F 80-99%
	0.0035 g.	0.0012 g.	0.0342 g.	0.075 g.	0.032 g.	0.0254 g.
Yield (%)	1.8	0.6	17.5	38.2	16.1	13.0
Non-volatile ash %	—	—	1.0	0.8	2.0	11.6
N (%)	—	—	0.2	0.2	0.6	1.5
R.V. (%)	—	—	78	77	76	77
$[\alpha]_{5461}^{17}$	—	—	+149°	+150°	+152°	+166°
Recovery = 87.2 %.						

ash and has a higher nitrogen content than fractions F 54-60%, F 60-70% and F 70-80% which are practically identical and indicate that the purified polysaccharide contains 0.3% N, has a reducing value (on hydrolysis with 2*N* HCl for 2 hr. at 100° in a sealed tube) of 77%, specific rotation $[\alpha]_{5461}^{17}$ = about +150°, non-volatile ash = 1-2%. (The specific optical rotations quoted in Table 1 are based on microdeterminations in a 0.5 dm. tube with readings of 0.3-0.4° and are subject to an error of $\pm 5\%$.) The low and variable nitrogen contents of the fractions isolated by alcoholic fractionation as compared with the constant reducing values suggest that the nitrogen is present as an impurity and does not form an essential molecular constituent, but nitrogen to the extent of 0.2-0.4% has persisted after fractionation with other solvents, e.g. acetic acid and even after acetylation of the polysaccharide and subsequent reconstitution from the acetylated derivative in a virtually unchanged form. In order to eliminate impurities and inorganic salts from the crude polysaccharide repeated alcoholic fractionation between the limits of alcoholic concentration of 45-50 and 86% have been found to be effective as the results of Table 1 clearly show.

Acetic acid fractionation of polysaccharide. 1.15 g. of crude polysaccharide which had previously been twice fractionated between the limits of 42 and 86% of alcoholic concentration (N=2.2%, R.V.=68%, non-volatile ash=6.9%) were dissolved in 100 ml. water and heated on the boiling water bath with a little animal charcoal for about 30 min. to decolorize. The solution was freed from charcoal by filtration and concentrated *in vacuo* to 12 ml. It was then fractionated with alcohol between the limits of 45 and 86% concentration of alcohol by weight, i.e. alcohol was added to 45% and any precipitate produced centrifuged off and rejected, the alcoholic concentration was then increased to 86% and the bulk of the polysaccharide thereby thrown out of solution; this was centrifuged off and redissolved in 12 ml. water and the procedure repeated three times in all. When most of the inorganic salts had been eliminated from the polysaccharide by alcoholic fractionation, it was necessary to assist flocculation by addition of a few drops of glacial acetic acid. At this stage the fractionated polysaccharide gave no precipitate with any of the following reagents: neutral lead acetate, basic lead acetate, 2% phosphotungstic acid in 5% H₂SO₄, and 5% mercuric acetate solution. The polysaccharide was then fractionated with glacial acetic acid; to the solution, 11.3 g. in weight, 60 ml. glacial acetic acid were added and gave no precipitate or opalescence. Addition of 70 ml. glacial acetic acid, i.e. 86.7% by weight, gave an opalescence which flocculated and deposited a precipitate. This was centrifuged off and the acetic acid concentration of the supernatant

solution then increased to 91.8%. This procedure was continued until 94%, 96.2% and finally an acetic acid mother liquor fraction F 96-99% were obtained. The fractionation and analytical data of the fractions are summarized in Table 2.

Table 2. *Alcoholic and acetic acid fractionation of crude polysaccharide*

Polysaccharide					
1.1g. F 42-86%/42-86% N=6.9%, R.V.=68%, Ash=6.9% Three alcoholic fractionations between 45% and 86% alcoholic concentration by weight					
F 42-86% (2)/45-86% (3)					
Redissolved in water and treated with glacial acetic acid					
Alcoholic M.L. residue F 86-99% 0.155 g. (14.1%) N=5.3% Ash=5.2%					
Acetic acid conc.	86.7%	91.8%	94.0%	96.2%	99%
	F 0-86.7%	F 86.7-91.8%	F 91.8-94%	F 94-96.2%	F 96.2-99%
	0.138 g.	0.344 g.	0.082 g.	0.049 g.	0.066 g.
Yield (%)	12.5	31.3	7.5	4.5	6.0
N (%)	0.2	0.3	0.3	0.6	1.4
Non-volatile ash (%)	1.4	1.1	2.9	2.1	6.6
$[\alpha]_{5461}^{19}$	+145	+140	+142	+123	—

The results of the acetic acid fractionation confirm those of the previous alcoholic fractionation. There is again no evidence of the presence of more than one polysaccharide. The fractions F 0-86.7%, F 86.7-91.8%, F 91.8-94% are virtually identical and agree with the corresponding alcoholic fractions in containing 0.2-0.3% N and 1-2% non-volatile ash. As in the case of the alcoholic fractionation the mother liquor fractions F 94-96.2% and F 96-99% contain the bulk of the inorganic impurities as well as much more nitrogen than the main fraction F 0-94% which accounts for the bulk of the polysaccharide.

From the results of the alcoholic and acetic acid fractionations of the crude polysaccharide it was concluded that a satisfactory means of purification of the latter was afforded by repeated alcoholic fractionation (6 times) between the limits of 50-86% alcohol by weight, followed by acetic acid fractionation (3 times) between the limits of 0-94% acetic acid. The purified polysaccharide was then twice reprecipitated from aqueous solution with alcohol to free it from acetic acid and washed twice with absolute alcohol, twice with ether and dried *in vacuo* over phosphorus pentoxide.

The scheme of fractionation of the crude polysaccharide is summarized in Table 3; in a typical fractionation 2.602 g. crude polysaccharide obtained by hydrolysis of 5.07 g. tryptic digest antigen gave 0.657 g. pure polysaccharide or 13.4% of the antigen. Percentage yields are in terms of tryptic digest antigen used as starting material (5.07 g.).

Fractions I-IV (Table 3) were tested by means of the serological precipitation reaction to confirm that the by-product fractions I, II and IV were relatively free from specifically reacting substances as compared with the pure polysaccharide fraction III. A series of dilutions of the substances from 1 in 250 to 1 in 250,000 were mixed with equal quantities (0.15 ml.) of *Bact. typhosum* O-antiserum (for which the author is indebted to Lt.-Col. R. F. Bridges of the Medical Research Council Standards Laboratory, Oxford) and maintained at 37° for 2 hr., room temperature for a further 1 hr. and then observed after standing in a refrigerator for 18-20 hr. Fraction III (Polysaccharide F 50-86% (6)/0-94% (3)) precipitated to a dilution of 1 in 125,000, fraction I (F 50%) gave a slight precipitation to a dilution of 1 in 25,000, whilst fractions II and IV gave no precipitation even at dilutions of 1 in 500. These observations show that the method of fractionation of

Table 3. *Preparation of purified polysaccharide from the crude product*

the crude polysaccharide which has been developed provides a sharp division into non-specific and specific fractions, the bulk of the latter being concentrated in fraction III.

(b) *Preparation of specific polysaccharide directly from the dried bacterial cells.* A crude polysaccharide was obtained from the dried bacterial cells of *Bact. typhosum* Ty₂ by means of extraction of the cells with dilute acetic acid at 100° (cf. White [1929] and Morgan [1936]). 15.7 g. of *Bact. typhosum* Ty₂ were dispersed in 200 ml. 0.1 *N* acetic acid and the mixture heated on the boiling water bath for 1½ hr. under a reflux condenser. The suspension was then cooled and centrifuged (2000 r.p.m. for 2 hr.) and the supernatant solution passed through a Chamberland *L*₂ candle. The unextracted residue from the centrifuge tubes was redispersed in a further quantity of 0.1 *N* acetic acid and the process repeated 4 times in all. Determinations of optical rotation on the extracts after filtration showed that the 3rd and 4th extracts contained little or no polysaccharide. The combined filtrates were concentrated *in vacuo* to about 1/8th vol. and treated with alcohol to 86% by weight to precipitate the crude polysaccharide. The crude product was submitted to alcoholic fractionation in a manner similar to that described above (Table 1) and from the nitrogen contents and reducing values on hydrolysis of the separated fractions it was again apparent that only a single polysaccharide was present which was probably identical with that obtained by hydrolysis of the isolated antigenic complex; the polysaccharide was purified by repeated alcoholic fractionation (6 times) between concentrations of 50-86% followed by 3 acetic acid fractionations between concentrations of 0-94% as described above.

Properties of the polysaccharide

The polysaccharide, prepared as described, was a water-soluble, colourless amorphous substance. It contained N, 0.2-0.4% and P, 0.4-0.9%, its small reducing value of 1.1%, before hydrolysis, increased to 83% as apparent glucose after hydrolysis with 2 *N* HCl for 2 hr. at 100° in a sealed tube (Hagedorn and Jensen). It was highly dextrorotatory [α]_D¹⁶, +114-115°; [α]₅₄₆₁¹⁶, +135° (uncorr. for ash). Sulphur was absent and the ash content was ca. 2%. On hydrolysis the polysaccharide yielded 3% of volatile fatty acid (calculated as acetic acid) indicating the presence of acetyl residues in the molecule. (Samples of polysaccharide for determination of acetyl were several times reprecipitated from aqueous solution with alcohol and then dried at 1-2 mm. and 100° for 1 hr. to eliminate traces of acetic acid due to fractionation with that solvent.) (Found: C, 44.41; H, 7.12; non-volatile ash, 2.4%, uncorr. for ash. C₆H₁₀O₅ requires C, 44.41; H, 6.22%.) The analytical values of the polysaccharide varied within narrow limits from batch to

batch and according to the source of the substance, i.e. whether isolated from the 'tryptic digest antigen' or directly from the dried organisms. The above results represent a typical sample prepared by hydrolysis of the antigenic complex. A typical sample isolated directly from the dried bacterial cells gave on analysis C, 44.48; H, 7.04; N, 0.4; P, 0.9; reducing sugar value (on hydrolysis), 83; acetyl, 6.0; non-volatile ash, 1.8% (results uncorrected for ash).

The average yield of purified polysaccharide from the antigenic complex was about 13%, equivalent to 0.6–0.7% of the dried bacterial cells, although in one experiment in which the antigenic complex was hydrolysed for only 2 hr. with 0.1 *N* acetic acid at 100°, the yield reached 21% of the antigen. The polysaccharide was obtained directly from the dried bacterial cells in a yield of 0.65–0.75%. The preparation of the polysaccharide directly from the bacterial cells is obviously much quicker and less expensive than that involving intermediate isolation of the antigenic complex, but much of the material used in this investigation was isolated by hydrolysis of the antigen in the course of a study of its chemical nature [Freeman & Anderson, 1941].

The polysaccharide dissolved readily in water, giving water-clear, colourless solutions; they failed to give positive Millon's or α -nitroso- β -naphthol tests for tyrosine and the biuret test was also negative. Ninhydrin tests and the Sakaguchi reaction for arginine were negative. The polysaccharide gave a very strongly positive Molisch's reaction, but gave no coloration with iodine and failed to reduce Fehling's solution. There was no evolution of furfuraldehyde on heating with 12% HCl. Bial's reaction and Tollen's phloroglucinol test for pentoses were also negative as were the Seliwanoff test and Foulger's test for ketoses. Tollen's naphthoresorcinol test was negative, indicating the absence of uronic acid residues. 5 mg. polysaccharide gave no appreciable development of colour on heating with skatole and conc. HCl for 30 min. at 38–40°, under which conditions 5 mg. fructose gave an intense 'permanganate' purple colour [Jordan & Pryde, 1938]. On heating at 80° for 15 min. an intense 'permanganate' purple colour was obtained indicative of hexoses. The above qualitative tests indicate the complete absence or presence only in very small amounts of ketose, pentose and uronic acid residues in the polysaccharide molecule, which consists predominantly of hexose units.

Serological properties of polysaccharide

Samples of the *Bact. typhosum* Ty₂ polysaccharide prepared both from the tryptic digest antigen and directly from the dried organisms precipitated with *Bact. typhosum* 'pure' O-antiserum to dilutions of 1 in 300,000 of the polysaccharide; optimal precipitation was obtained at dilutions of 1 in 300,000 and 1 in 150,000 respectively. No precipitation was observed with mixtures of *Bact. typhosum* 'pure' Vi-antiserum and the specific polysaccharide prepared from either source. In this respect the polysaccharide differs markedly from the intact antigenic complex from which it was derived, which precipitated with both O and Vi antisera to dilutions of 1 in 150,000 and 1 in 15,000 respectively, with optimal precipitation at a dilution of 1 in 300 of the antigen in mixtures with O-antiserum. The author is indebted to Dr A. Felix, who kindly carried out the above series of precipitin reactions.

Times of formation of visible precipitates in mixtures of Bact. typhosum Ty₂ antigen and polysaccharide with O- and Vi-antisera

	<i>Bact. typhosum</i> Ty ₂ 'tryptic digest antigen'		<i>Bact. typhosum</i> Ty ₂ polysaccharide	
	0.5 mg.	0.25 mg.	0.5 mg.	0.25 mg.
'Pure' O-antiserum	+	+	+	+
	10 min.	10 min.	5 min.	3 min.
'Pure' Vi-antiserum	+	+	- ve	- ve
	15 min.	15 min.		

Similar results were obtained in a number of experiments by Dr Joyce Wilson, who observed the times of formation of a visible precipitate when given quantities of polysaccharide and of antigen were mixed with equal volumes of antisera, diluted 1 in 10.

The polysaccharide, which reacts specifically with O-antiserum but not with Vi-antiserum is to be regarded as the O-specific haptén of *Bact. typhosum*.

Homogeneity of polysaccharide

Before undertaking a detailed examination of the nature and properties of the specific polysaccharide it was submitted to a further fractionation with alcohol to determine whether it was possible by this means to separate the material into components which were chemically or serologically distinct. A sample was also examined in the ultracentrifuge for any evidence of more than a single component.

Alcoholic fractionation. A sample (1.84 g.) of polysaccharide was dissolved in water, 50 ml., giving a clear, colourless solution free from any undissolved residue. Absolute alcohol was added to 50 % by weight but no precipitate or opalescence was obtained. When the alcoholic concentration was raised to 55 % a small precipitate was obtained which was removed by centrifuging. Further increments of alcohol were added until the material had finally been divided into seven fractions. The bulk of the substance (88 %) was precipitated between alcoholic concentrations of 55 % and 70 %. Only 1 % was insoluble in 55 % alcohol, and 11 % remained soluble when the concentration had reached 70 %.

The seven fractions isolated were compared by determination of specific optical rotations and reducing values on hydrolysis with 2*N* HCl for 2 hr. at 100° in a sealed tube (Hagedorn and Jensen), and the results obtained are summarized in Table 4.

Table 4. *Alcoholic fractionation of O-specific haptén*

Fraction	Wt. g.	Yield %	$[\alpha]_D^{19}$	R.V. on hydrolysis (as glucose (%))
Unfractionated polysaccharide	1.84	—	+114°	—
(1) F 50–55 %	0.0216	1.1	+108°	67
(2) F 55–57.6 %	0.3987	21.7	+111°	77
(3) F 57.6–59.6 %	0.3431	18.6	+112°	79
(4) F 59.6–63.2 %	0.5286	28.7	+110°	77
(5) F 63.2–70 %	0.3557	19.3	+112°	79
(6) F 70–85 %	0.1590	8.6	+113°	69
(7) F 85–99 %	0.0463	2.5	+113°	—

(Results uncorrected for ash.)

Fractions of the polysaccharide differing slightly in solubility in alcohol-water mixtures have been shown by specific rotation and reducing value (on hydrolysis) determinations to be practically identical and it may be concluded that the O-polysaccharide haptén prepared as described above represents a chemical entity.

Ultracentrifuge data. The above conclusions were confirmed by examination of a sample of the polysaccharide in the ultracentrifuge by J. St L. Philpot, who reports that the substance appeared to be practically homogeneous, 79 % being accounted for as a single molecular entity (see Addendum to this paper). The minimum molecular weight is of the order of 10,000, i.e. about 60 anhydrohexose units, and the polysaccharide molecules are probably not spherical.

Acetylation of polysaccharide. A number of preliminary experiments were made on crude samples of the specific polysaccharide to determine the optimal conditions for acetylation. 0.507 g. of the pure substance was dissolved in water (2 ml.) and the solution treated with pyridine, redistilled over KOH, B.P. 115° (25 ml.), and acetic anhydride (25 ml.), the latter being added gradually so that the temperature of the mixture did not

exceed 35° [Challinor *et al.* 1934]. The clear, pale yellow mixture was kept at room temperature for 48 hr., and then poured into ice-cold water (400 ml.) giving an immediate colourless precipitate of acetyl polysaccharide. The crude product was separated off, washed with ice-cold water until substantially free from acetic acid, and dried *in vacuo* over phosphorus pentoxide, giving 0.477 g. crude material (94 %).

The crude acetyl derivative was extracted with about 100 ml. boiling absolute ethyl alcohol in which it dissolved and was thrown down again on cooling. The cooled mixture was kept at 0° overnight and the reprecipitated acetyl polysaccharide centrifuged off and the process repeated. The alcohol-extracted residue was taken up in 15 ml. chloroform and the solution heated on the water bath with a little animal charcoal for about 15 min. The charcoal was removed by centrifuging and the clear supernatant solution treated with excess (6 vol.) of light petroleum B.P. 40–60°. The precipitated acetyl derivative was again taken up in chloroform (15 ml.) and reprecipitated with light petroleum. By addition first of light petroleum to 48 % by weight (32 ml.) followed by centrifuging and raising of the petroleum concentration to 70 % conc. by weight (82 ml.) and again removal of the precipitate by centrifuging, fractions of the acetylated polysaccharide were obtained and analysis showed that the derivative was homogeneous.

The acetyl polysaccharide was a colourless amorphous solid; it was insoluble in cold ethyl alcohol, ether and water, but was readily soluble in chloroform, pyridine and acetone; $[\alpha]_D^{20}$, +94–96° (in chloroform, $c=1$); organic P, 0.3 %; C, 50.8; H, 5.8; acetyl, 40.0 %. $C_6H_7O_5(OC.CH_3)_3$ requires C, 50.0; H, 5.6; $CH_3.CO$, 44.8 %. Pyridine solutions of the acetyl polysaccharide did not precipitate with *Bact. typhosum* O-antiserum and chloroform solutions of the substance gave no specific precipitation at the interface when O-antiserum was layered upon the chloroform solutions. It is concluded that the polysaccharide hapten loses its property of specifically reacting with the homologous antibody on conversion into the triacetyl compound.

Regeneration of the polysaccharide. After a series of preliminary experiments in which the acetyl polysaccharide dissolved in acetone or pyridine was treated with *N* KOH for various periods of time at 37°, 50°, etc., a product identical with the original specific polysaccharide prepared from the tryptic digest antigen was obtained. Intermediate acetyl derivatives containing 15 and 5.3 % acetyl have also been isolated. In a typical saponification of the acetyl polysaccharide, 0.27 g. of the substance dissolved in 8 ml. pyridine was treated with 2 ml. *N* KOH at 37° for 4 hr. At the end of this period the mixture was diluted with an equal volume of water and dialysed in a cellophane sac for 2 hr. to eliminate the bulk of the excess alkali and pyridine. The dialysed solution was acidified by addition of 2 ml. *N* acetic acid and poured into absolute alcohol (10 vol.) to precipitate the regenerated polysaccharide. The precipitated polysaccharide was taken up in water (10 ml.) and three times fractionated between the limits of 50 and 86 % concentrations of alcohol by weight. Finally the precipitated polysaccharide was washed twice with absolute ethyl alcohol, twice with ether and dried *in vacuo* over phosphorus pentoxide; obtained 0.12 g. or 44 % yield; acetyl, 14.9 %.

The regenerated polysaccharide and the partially deacetylated products give precipitates with *Bact. typhosum* O-antiserum to high dilutions, which however differ according to the acetyl content, but no precipitates were obtained with Vi-antiserum. The properties of several partially acetylated polysaccharides are summarized (Table 5) together with details of the conditions of their preparation.

The intermediate acetyl derivatives containing 15 and 5.3 % acetyl resemble the polysaccharide rather than the fully acetylated compound; they are freely soluble in water and precipitate with homologous antiserum in contrast to the acetyl polysaccharide. Loss of acetyl groups is accompanied by a gradual rise in specific optical rotation from +95° to +122°. The precipitation titres of the partially acetylated polysaccharides increase with decreasing acetyl content until a region of maximum precipitability is

Table 5. *Properties of acetylated derivatives of O-specific polysaccharide*

	Method of preparation	C %	H %	Non- volatile ash %	Acetyl %	P %	$[\alpha]_D$ [18° C.]	Limiting dilution of precipitation with O-antiserum
I. Fully acetylated polysaccharide: insoluble in water	Acetylation of polysaccharide as described	50.8	5.8	—	40	0.3	+ 95° (18° C.)	Does not precipitate
II. Partially acetylated polysaccharide: water-soluble	0.27 g. I in 8 ml. pyridine + 2 ml. <i>N</i> KOH at 37° for 4 hr. Obtained 0.12 g. II (44%)	45.9	6.7	Nil	15	—	+ 110° (18° C.)	1 in 2500
III. Polysaccharide: water-soluble	2g. I in 50 ml. pyridine + 50 ml. acetone + 50 ml. water heated with 20 ml. <i>N</i> KOH at 50° for 3/4 hr., gave 0.6 g. III (30%)	43.8	6.9	1.9	5.3	0.4	+ 114° (18° C.)	1 in 125,000
IV. Specific polysaccharide	From 'tryptic digest antigen' as described	44.41	7.12	2.4	3.5	0.4	+ 115° (16° C.)	1 in 125,000
V. Polysaccharide: water-soluble	0.44 g. III in 20 ml. water + 20 ml. <i>N</i> KOH at 50° for 2 hr. obtained 0.318 g. V (73%)	—	—	—	2.1*	—	+ 122° (17° C.)	1 in 125,000
VI. Deacetylated polysaccharide: water-soluble	0.44 g. I in 20 ml. pyridine + 20 ml. water heated with 20 ml. <i>N</i> KOH at 100° for 1 hr. gave 0.144 g. VI (33%)	43.9	6.8	2.2	Nil	—	+ 121° (23° C.)	1 in 125,000

* N = 0.6%.

Results uncorrected for ash.

reached at acetyl contents of 0–5%. Polysaccharides III and V are practically identical, in chemical properties and serological precipitation, with the original specific polysaccharide, IV, prepared from the 'tryptic digest antigen'. This affords further strong evidence that the specific properties of the latter are a function of the polysaccharide and not of small amounts of concomitant impurities [cf. Heidelberger & Avery, 1924].

Benzoylation of the polysaccharide

0.5 g. of the polysaccharide was dissolved in 20 ml. water; benzoyl chloride (2.5 ml.) was gradually added with vigorous shaking the reaction mixture being kept alkaline by addition of 2*N* NaOH. The benzoylated polysaccharide separated almost immediately from the solution as a heavy, white precipitate. This was filtered off, washed successively with 0.01*N* NaOH, water and alcohol and dried *in vacuo* over phosphorus pentoxide. The crude benzoyl polysaccharide (0.70 g.) was insoluble in ether, slightly soluble in chloroform and acetone, but freely soluble in pyridine. The crude material was dissolved in about 5 ml. of pyridine and reprecipitated by addition of excess ether, after the pyridine solution had been centrifuged to remove traces of insoluble matter. This process was twice repeated and the benzoyl derivative finally washed with ether until free from pyridine and dried *in vacuo* over phosphorus pentoxide. The benzoylated polysaccharide was a light cream-coloured amorphous solid, $[\alpha]_D^{16.5}$, + 93° (in pyridine, *c* = 1); organic P, 0.4; C, 59.2; H, 5.2; non-volatile ash, 1.6%.

Acid hydrolysis of the specific polysaccharide

The optimal conditions of liberation of reducing sugars by hydrolysis of the polysaccharide with mineral acid were investigated as a preliminary to identification of the sugars present. 0.0143 g. of the polysaccharide (dried *in vacuo* at 100° for 1 hr.) was dissolved in 25 ml. $N H_2SO_4$ and the clear solution hydrolysed under reflux on the boiling water bath. 1 ml. samples were withdrawn at intervals, neutralized and the reducing sugars determined by the Hagedorn and Jensen method. A determination was also made on the unhydrolysed polysaccharide (5 mg.) in 2 ml. water. The results (Table 6)

Table 6. *Liberation of reducing groups from the polysaccharide by hydrolysis with $N H_2SO_4$*

Time hr.	Apparent glucose liberated mg.	Reducing value %	Time hr.	Apparent glucose liberated mg.	Reducing value %
0*	0.054	1.1	3.5	0.219	76.6
0†	0.024	8.5	5	0.225	78.7
0.5	0.128	45.0	6	0.226	79.0
1	0.169	59.3	8	0.223	78.0
2	0.206	72.0			

* From 4.95 mg. polysaccharide in neutral solution.

† In $N H_2SO_4$ solution.

show that hydrolysis proceeds rapidly for the first hour and then becomes markedly slower until after 5–6 hr. the liberation of reducing groups reaches a maximum of 79 % and thereafter falls gradually owing to destruction of the liberated sugars. The addition of $N H_2SO_4$ (which was shown to be free from reducing substances) produced an appreciable increase (7 %) of reducing power in the cold; this may be due to an effect on associated polysaccharide chains but is not fully understood. The author is indebted to Dr T. H. Anderson for the above reducing value determinations.

Nature of component reducing sugars

Evidence that the specific polysaccharide consists predominantly of anhydrohexose units has already been described. The presence of *d*-galactose in the antigenic complex from which the polysaccharide was derived has been demonstrated by Freeman *et al.* [1940]. The presence of this sugar in the specific polysaccharide was therefore sought at the outset; it was, however, necessary to test for the presence of *d*-mannose as the latter, if present, forms an insoluble methylphenylhydrazone under the optimal conditions of precipitation of galactose methylphenylhydrazone [Müther & Tollens, 1904].

0.4–0.6 g. quantities of the dried specific polysaccharide were hydrolysed by heating on the water bath at 100° for 5 hr. with 60 ml. $N H_2SO_4$. The acid was removed from the cooled solution after hydrolysis by addition of a slight excess of hot saturated baryta to the hydrolysate cooled in ice, the precipitated Ba salts were removed by centrifuging and the supernatant solution and washings made just acid by addition of $2N H_2SO_4$ and the excess acid removed by addition of an excess of pure $BaCO_3$. The clear, colourless, neutral filtrate was concentrated *in vacuo* at low temperature to 10–20 ml. bulk. The optical rotation and reducing value (Hagedorn and Jensen) of the solution were determined at this stage: $\alpha_D^{16} = +0.85^\circ$, 1 dm. (volume of solution, 17.8 ml.); total apparent glucose = 0.3066 g. (or 75 % of the original polysaccharide). On the assumption that the reducing constituents of the solution have a reducing value approximating to that of glucose, the 'apparent specific rotation' of the contents of the solution is $[\alpha]_D^{16} = +49^\circ$. (Specific rotation, $[\alpha]_D$, glucose, $+52.8^\circ$; galactose, $+81^\circ$; and mannose, $+14.2^\circ$.)

d-Mannose phenylhydrazone. The presence of *d*-mannose in the polysaccharide hydrolysate was proved by the formation of *d*-mannose phenylhydrazone on treatment with phenylhydrazine in acetic acid solution. The neutral hydrolysate corresponding to 0.385 g. specific polysaccharide (25 ml. volume) was treated with a mixture of 0.2 g. freshly distilled phenylhydrazine (B.P. 120–124°/15 mm.) and 0.4 g. of 25 % acetic acid [van der Haar, 1920]. The mixture was allowed to crystallize overnight at 0° giving a mass of crystals of *d*-mannose phenylhydrazone, which were filtered off, washed with a little ice-cold water, then with ice-cold alcohol and ether and dried *in vacuo* over phosphorus pentoxide. The mother liquor and aqueous washings were concentrated to 9 ml. and treated with 0.1 g. phenylhydrazine and 0.2 g. 25 % acetic acid when a further quantity of crystals appeared after 24 hr. at 0°. The crude product was recrystallized from boiling 60 % alcohol:40 % water mixtures to constant M.P.; *d*-mannose phenylhydrazone, colourless microcrystalline plates, M.P. 193–195°, unchanged on admixture with an authentic specimen was obtained. (Found: C, 53.3; H, 6.7; N, 10.3 %. $C_{12}H_{18}N_2O_5$ requires C, 53.3; H, 6.7; N, 10.4 %.) The yield of crude material was 0.1156 g., corresponding to a mannose content of 20.0 % of the specific polysaccharide.

The excess phenylhydrazine was removed as benzaldehyde phenylhydrazone [Morgan, 1938]. Treatment with benzaldehyde also liberates any hexoses that have formed soluble phenylhydrazones. The mother liquors and aqueous washings from the mannose phenylhydrazone crystallizations were concentrated *in vacuo* to 15 ml. and heated with 0.8 g. freshly distilled benzaldehyde and 0.02 g. benzoic acid for 1 hr. under reflux on the boiling water bath in an atmosphere of CO_2 . The mixture was then cooled to 0°, filtered and the oily residue washed twice with small quantities of ice-cold water; the combined filtrate and washings were then immediately extracted several times with ether and the slightly coloured aqueous solution concentrated *in vacuo* to about 10 ml. The solution was heated at 100° for about 10 min. with a small quantity of norit, which had previously been extracted with conc. HCl and well washed with water. The almost colourless filtrate containing the remaining sugars was concentrated *in vacuo* to 10 ml.

d-Galactose methylphenylhydrazone. The methylphenylhydrazone of *d*-galactose was isolated under the conditions described by Neuberg [1907] and Lüttke [1929]; the aqueous sugar-containing solution was treated with an equal volume of absolute ethyl alcohol, 0.2 g. methylphenylhydrazine (asym.), redistilled B.P. 104° at 14 mm., and 0.1 g. 50 % acetic acid. The mixture was kept at 37° for 7 hr. and then at 0° for a further 17 hr. Galactose methylphenylhydrazone crystallized as colourless, microcrystalline plates which were filtered off on to a small filter paper in a tared Gooch crucible, washed with 10 ml. ice-cold absolute alcohol and dried to constant weight *in vacuo* over phosphorus pentoxide. The mother liquors and washings were concentrated *in vacuo* at low temperature to 2–3 ml. volume, 10 ml. absolute methyl alcohol added and the mixture kept at 0° for 24 hr.; usually no further crystallization of the hydrazone occurred. The crude product was recrystallized from hot 50 % pyridine:50 % ethyl alcohol mixtures; after 1–2 crystallizations pure *d*-galactose methylphenylhydrazone, M.P. 190°, was obtained. The M.P. was unchanged on admixture with authentic *d*-galactose methylphenylhydrazone (M.P. 190°). (Found: C, 55.1; H, 7.2; N, 10.2 %. $C_{13}H_{20}N_2O_5$ requires C, 54.9; H, 7.1; N, 9.9 %.) The yield of crude hydrazone from 0.385 g. specific polysaccharide was 0.0688 g., or 11.3 % galactose in terms of the original polysaccharide.

Excess methylphenylhydrazine was eliminated as described above for phenylhydrazine and the aqueous solution concentrated *in vacuo* at low temperature to 10 ml. At this stage the solution had α_D^{19} , +0.33°, 1 dm., equivalent to 0.0625 g. apparent glucose in 10 ml.

Glucosazone. The aqueous solution was treated with 0.5 g. phenylhydrazine hydrochloride (decolorized with norit and recrystallized) and 0.75 g. sodium acetate crystals

and the pale yellow solution heated at 100° on the water bath for 1 hr. Glucosazone crystals appeared after about 30 min. in the hot solution, and crystallization was complete after 17 hr. at 0°. The osazone was filtered off, washed with 2–4 ml. ice-cold water, a few ml. cold alcohol and finally with ether and dried *in vacuo* over phosphorus pentoxide. The yield of crude phenylglucosazone was 0.0174 g. (0.1 g. pure glucose treated with phenylhydrazine HCl and sodium acetate under the conditions described gave 0.033 g. glucosazone). The crude product was recrystallized several times from dilute aqueous ethyl alcohol finally giving an osazone which had a typical wheatsheaf microscopical appearance and m.p. 203°; on admixture with an authentic specimen of glucosazone (m.p. 205°) gave mixed m.p. 203°. The identity of the osazone was confirmed by determination of its optical rotation in alcoholic pyridine solution, and comparison with that of an authentic specimen [Neuberg, 1899].

d-Glucose p-nitrophenylhydrazone. The presence of glucose as a unit in the molecule of the specific polysaccharide was confirmed and its amounts determined by isolation of the sugar as the *p*-nitrophenylhydrazone from a further batch of neutralized polysaccharide hydrolysate [van Ekenstein & Blanksma, 1903; Reclaire, 1908]. The neutral aqueous solution equivalent to 0.5887 g. of specific polysaccharide was concentrated *in vacuo* at low temperature to a syrup which was taken up in methyl alcohol (5 ml.), and the solution heated with 0.6 g. pure *p*-nitrophenylhydrazine at 100° on the water bath under reflux for 10 min. After 24–48 hr. at 0° a yellow, crystalline mass of *d*-glucose *p*-nitrophenylhydrazone had separated; the product was filtered off, washed with ice-cold alcohol and with ether and dried *in vacuo* over phosphorus pentoxide. The mother liquors and washings were evaporated almost to dryness *in vacuo*, diluted to 2–3 ml. with methyl alcohol and allowed to crystallize at 0°; further crops of hydrazone were obtained in this way. The total yield of crude *p*-nitrophenylhydrazone was 0.3338 g., equivalent to a glucose content of 32.4 % of the specific polysaccharide. The hydrazone was recrystallized from hot ethyl alcohol until a constant m.p. of 184° was obtained. The mixed m.p. with an authentic specimen of *d*-glucose *p*-nitrophenylhydrazone (m.p. 184°) was 183°. (Found: C, 45.9; H, 5.5; N, 13.2 %. $C_{12}H_{17}O_7N_3$ requires C, 45.7; H, 5.4; N, 13.3 %.)

The method of identification of the constituent hexose units of the specific polysaccharide as described above, viz. treatment of the neutralized polysaccharide hydrolysate with substituted phenylhydrazines under conditions in which the derivative of a single hexose is obtained at each stage, provides conclusive proof of the co-existence of *d*-glucose, *d*-mannose and *d*-galactose as constituents of the specific polysaccharide. The losses of sugars in the intermediate removal of excess of substituted hydrazines with benzaldehyde are, however, sufficiently great markedly to lower the yields of hydrazones of the sugars. Thus when mannose, galactose and glucose derivatives were isolated in that order from the same polysaccharide hydrolysate the yields of galactose and glucose derivatives were low. In order to obtain a more accurate determination of the quantities of these hexoses present in the original specific polysaccharide it is therefore necessary to take aliquot portions of a neutralized hydrolysate and treat with the appropriate substituted hydrazines. Under these conditions yields of substituted hydrazones equivalent to 32.4 % glucose, 20.0 % mannose and 15.8 % galactose have been obtained. Control experiments were made under the same conditions with similar concentrations of single pure hexoses, when pure *d*-glucose gave 79.6 % of the theoretical yield of the *p*-nitrophenylhydrazone, pure mannose gave 94.9 % of the theoretical yield of *d*-mannose phenylhydrazone and pure galactose gave 90–95 % of the theoretical quantity of *d*-galactose methylphenylhydrazone (with larger quantities of hexose in more concentrated solutions the yields of hydrazones are practically theoretical). On this basis a rough estimate of the actual hexose contents of the specific polysaccharide has been made (Table 7).

Table 7. *Hexose contents of Bact. typhosum Ty₂ specific polysaccharide*

	Observed yield	Yield from pure hexose under identical conditions	Estimated content of polysaccharide
	%	%	%
<i>d</i> -Glucose	32.4	80	40
<i>d</i> -Mannose	20.0	95	21
<i>d</i> -Galactose	15.8	90-95	17
Total hexose	68.2		78

The total hexose thus accounted for, i.e. 78 %, agrees well with the reducing value of 80-83 % in terms of apparent glucose, obtained when 5 mg. samples of the substance are hydrolysed with 2 ml. quantities of 2*N* HCl in sealed tubes for 2 hr. at 100° (Hagedorn and Jensen). If the specific polysaccharide consisted entirely of anhydrohexose units it would yield about 111 % apparent glucose on complete hydrolysis. Of the 30 % so far unaccounted for, 4-5 % is due to the presence of acetyl groups and the remainder probably consists of non-reducing substances not yet identified. Decomposition of hexose during acidic hydrolysis may account for some of the undetermined fraction; it is unlikely that an appreciable quantity of the polysaccharide remains unhydrolysed under these conditions as the neutralized hydrolysate gives no appreciable precipitate on addition of 10 vol. of absolute alcohol, which completely precipitate the untreated polysaccharide.

Acid equivalent of specific polysaccharide

A sample of the specific polysaccharide (0.78 g.) was three times reprecipitated between the limits of alcoholic concentration of 50 and 86 % by weight, with acid-free alcohol redistilled over KOH. The reprecipitated polysaccharide (0.713 g.) was dried at 100° and 1-2 mm. for 3 hr., dissolved in 10 ml. water and titrated with 0.01*N* NaOH (CO₂-free) using phenolphthalein as indicator. The polysaccharide required 2.30 ml. 0.0093*N* NaOH for neutralization giving an acid equivalent of 30,000.

Action of enzymes

The stability of the polysaccharide towards enzymes capable of splitting α -glucosidic linkages in polysaccharides, viz. α - and β -malt amylases and β -linkages in glucosides (emulsin) was investigated. Malt amylase was used as a source of the mixed α - and β -malt amylases and as β -glucosidase, a commercial sample of emulsin which had been fractionated to remove a part of the inactive proteins, was used. A sample of the specific polysaccharide incubated at 25° and pH 4.63 with malt amylase showed no appreciable liberation of reducing sugars after 24 hr. at which time a parallel conversion of soluble starch with the same enzymic concentration had reached 79 % hydrolysis as apparent maltose. The polysaccharide-emulsin mixtures were incubated at 25° and pH 5.0; there was no detectable liberation of reducing sugars. A parallel emulsin hydrolysis of β -methyl glucoside under the same conditions produced 21.7 % hydrolysis after 23 hr. and 76.3 % after 168 hr.

DISCUSSION

A specific polysaccharide associated with the O-antigen of *Bact. typhosum* has been isolated both directly from the dried bacterial cells and from the soluble antigenic complex of the organism, which has been shown by earlier workers [Raistrick & Topley, 1934; Topley *et al.* 1937] to account for the specific properties of the intact organism. The complete antigenic complex comprises besides the specific polysaccharide, an insoluble polypeptide component, a soluble nitrogenous substance and a small amount of lipid constituents [Freeman & Anderson, 1941], but of these the polysaccharide is probably of outstanding importance and accounts for some, if not all, of the specific properties of the O-antigen.

Although the specific substance precipitates to extreme dilution with *Bact. typhosum* O-antiserum it has not yet been determined whether it accounts for all the specific serological properties of the O-component of the antigenic complex, but work on these lines is proceeding. The polysaccharide probably plays the part of a hapten as defined by Landsteiner, but it has not yet been possible to determine whether or not it is capable of exhibiting antigenic properties.

The O-specific substance belongs to the class of polysaccharides and exists as a colloidal compound of high molecular weight. The usual chemical criteria of purity cannot be applied in determining its integrity; several attempts to divide it into chemically distinct components by means of fractionation from aqueous solution by means of solvents such as ethyl alcohol and acetic acid have so far failed to produce any components which differed significantly in chemical composition and properties. This evidence, coupled with the facts that the polysaccharide behaves as a single molecular entity in the ultra-centrifuge and that it may be reconstituted in a virtually unchanged form from its triacetyl derivative, provides reasonable justification for the claim that it is a single substance. The possibility of admixture with non-specific polysaccharides derived from agar or other constituents of the culture medium was eliminated by the use of a 'synthetic' medium for the growth of the organisms from which the specific polysaccharide was derived.

Soru & Combiesco [1940] have isolated a polysaccharide by dilute acetic acid hydrolysis of the O-antigen of *Bact. typhosum*; this substance has apparently not been submitted to extensive fractionation and its state of purity is unknown but its chemical composition shows that it is probably identical with the specific polysaccharide described in the present communication. Soru & Combiesco's polysaccharide is nitrogen-free, gives 72.4 % of reducing sugars on hydrolysis, is highly dextrorotatory ($[\alpha] + 125^\circ$) and contains 11.65 % of ash and 2.88 % of P (results, except that for P, calculated on an ash-free basis) as compared with the present polysaccharide which contains 0.2–0.4 % N, gives 83 % of reducing sugars on hydrolysis, has a specific rotation of $[\alpha]_D^{25} + 114$ – 115° and contains about 2 % of ash and 0.4–0.9 % P. It is not yet clear whether the small nitrogen content of our polysaccharide is due to a small quantity of impurity or whether the nitrogen is an essential component of the polysaccharide. The latter alternative would appear to be more probable as a specifically reacting polysaccharide containing 0.6 % N has been reconstituted from the triacetyl derivative (Table 5). Many of the polysaccharides isolated from bacteria during recent years as well as others which have some specific biological role have been found to contain *N*-acetylhexosamine as an important unit of the polysaccharide molecule, e.g. the polysaccharide hapten of smooth forms of *B. dysenteriae* (Shiga) [Jolles & Morgan, 1940], the specific polysaccharide of *Pneumococcus* Type IV [Heidelberger & Kendall, 1931], Freudenberg & Eichel's [1934], Group A polysaccharide from urine, and the carbohydrate of ovomucoid [Stacey & Woolley, 1940]. On the other hand, the important acetyl polysaccharide of *Pneumococcus* Type I [Avery & Goebel, 1933] contains an unacetylated amino-sugar, the acetyl groups being attached through oxygen directly to a carbon atom. In the present polysaccharide some of the acetyl groups are probably attached to nitrogen and some are in the form of O-acetyl, but in the case of polysaccharide V (Table 5) obtained by partial deacetylation of the fully acetylated derivative, the acetyl groups are probably attached to nitrogen atoms in view of the relatively vigorous conditions required to eliminate them. This interesting problem of the mode of linkage of the acetyl groups in the polysaccharide is receiving further investigation.

The bulk of the constituent reducing sugars which comprise the polysaccharide molecule has been identified and isolated as substituted phenylhydrazones. 40 % *D*-Glucose has been isolated as the *p*-nitrophenylhydrazone, 21 % of *D*-mannose as mannose phenylhydrazone and 17 % *D*-galactose as the methylphenylhydrazone. That it has been possible

to separate the hexoses in this way depends upon the fact that mannose is the only hexose which readily gives an insoluble phenylhydrazone under the experimental conditions. Thus *d*-galactose also forms a phenylhydrazone but only when a very concentrated aqueous solution of the sugar is treated with phenylhydrazine. Most of the hexoses, including mannose, galactose and glucose form *p*-nitrophenylhydrazones under suitable conditions but that of *d*-glucose is the only one which readily crystallizes under the conditions of our experiments [van Ekenstein & Blanksma, 1903; Reclaire, 1908; van der Haar, 1920]. The *p*-nitrophenylhydrazone isolated in this investigation melted at the same temperature, 183–184°, alone or mixed with an authentic sample of *d*-glucose *p*-nitrophenylhydrazone. (The *p*-nitrophenylhydrazones of the hexoses exist in two isomeric forms according to whether they crystallize from acetic acid or alcoholic solutions [van Ekenstein & Blanksma], the lower-melting form from alcoholic solution was isolated in this work.) As a further confirmation of the presence of *d*-glucose in the hydrolysates from the specific polysaccharide, phenylglucosazone was isolated from a hydrolysate from which the mannose had been carefully and completely removed, and in a yield of the same order as that obtained from pure glucose at the same concentration. Glucose, fructose, xylose and glucuronic acid do not interfere with the determination of *d*-galactose by means of methylphenylhydrazine; arabinose, mannose and fucose, according to Muther & Tollens [1904] are precipitated under the same conditions as galactose. The intervention of mannose was avoided by prior precipitation of this sugar as the phenylhydrazone. A useful confirmation of the relative proportions of the three hexoses present in the polysaccharide hydrolysates is afforded by a comparison of the observed optical rotation of a neutralized hydrolysate with that calculated from the reducing value of the solution (Hagedorn and Jensen) in terms of apparent glucose. The hydrolysate described above had $\alpha_D^{16} = +0.85^\circ$ (1 dm.), total volume 17.8 ml. and total apparent glucose, 0.3066 g. (Hagedorn and Jensen). In such a solution containing glucose, mannose and galactose in the ratio of 40 : 21 : 17, the rotation of the *d*-glucose component is $+0.47^\circ$, that of the mannose component $+0.07^\circ$ and that of the galactose $+0.30^\circ$ giving a total calculated rotation, 1 dm., of $+0.84^\circ$, which agrees sufficiently accurately with the observed rotation.

The observation of Goebel [1939; 1940] that relatively simple carbohydrate molecules such as those of aldobionic acids may have specific properties in relation to bacterial antigens, e.g. by specifically inhibiting precipitation between antigen or hapten and homologous antiserum if the latter be initially incubated with an aldobionic acid derived from the complex polysaccharide hapten, or by determining the specificity of artificial carbohydrate-protein antigens, has stimulated search for a simpler pattern unit in the specific carbohydrate of *Bact. typhosum*. Attempts to isolate such a simpler specific carbohydrate unit by graded hydrolysis of the polysaccharide hapten have so far proved unsuccessful.

SUMMARY

1. A method for the extraction and purification of an O-specific polysaccharide from the bacterial cells or isolated antigenic complex of *Bact. typhosum* Ty₂ has been described.
2. The polysaccharide is dextrorotatory, $[\alpha]_D^{16} = +114-115^\circ$; $[\alpha]_{5461}^{16} = +135^\circ$, it contains 0.2–0.4 % nitrogen, and 0.4–0.9 % phosphorus, and yields 83 % of reducing sugars on acidic hydrolysis.
3. The polysaccharide appears to be free from ketose, pentose and uronic acid residues but yields 40 % *d*-glucose, 21 % *d*-mannose and 17 % *d*-galactose on hydrolysis.
4. The specific substance precipitates to a high dilution with *Bact. typhosum* O-antiserum but not with Vi-antiserum. Elimination of the 3 % of acetyl groups in the molecule does not appear to influence the limit of precipitation with O-antiserum.
5. The polysaccharide has a minimum molecular weight of the order of 10,000, i.e. about 60 anhydrohexose units.

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Examination in the Ultracentrifuge

By J. St L. Philpot

The specimen of polysaccharide was examined in the Svedberg ultracentrifuge, the conditions being: concentration, 1%; pH, 7; NaCl concentration, 0.2 M; centrifugal field, 300,000 times gravity. Fig. 1 shows a photograph by the 'diagonal schlieren' method about 2 hr. after the start of sedimentation. The corrected sedimentation constant is 1.2×10^{-13} , which corresponds to a molecular weight of about 10,000 for a spherical

molecule, or more for an aspherical one. The maximum in Fig. 1 is sharper than it would be with a spherical molecule of weight 10,000, so the heavier aspherical molecule is more

Fig. 1. Sedimentation curve of *Bact. typhosum* polysaccharide. x = distance from centre of rotation (arbitrary units). dc/dx = concentration gradient (arbitrary units). A = meniscus, B = sedimentation boundary, C = index.

likely. The refractive increment of the 1 % solution was 0.00127, and 79 % of this value was obtained by integration of the refractive gradient curve in Fig. 1. Hence 79 % $\pm$ about 5 % of the material is homogeneous in sedimentation.