Two Serologically Active Polysaccharides from Bacillus anthracis.

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Two polysaccharides, designated I and II, have been isolated from *B. anthracis*. Polysaccharide I, which is precipitated by rabbit anti-anthrax sera, is a lightly phosphorylated mannan. Polysaccharide II, which is precipitated by horse anti-anthrax sera, is made up of D-galactose and *N*-acetyl-D-glucosamine in the molar ratio 2 : 1. The relation of these polysaccharides to those previously isolated from *B. anthracis* is discussed briefly.

In addition to the capsular polypeptide (Ivánovics and Brückner, *Z. Immunitäts.*, 1937, **90**, 304; 1938, **93**, 119; Hanby and Rydon, *Biochem. J.*, 1946, **40**, 297), *Bacillus anthracis* has long been known to elaborate other antigenic or haptenic substances one, at least, of which is a polysaccharide (Tomcsik, *Magyar Orv. Arch.*, 1927, **28**, 572; Schockaert, *Compt. rend. Soc. Biol.*, 1928, **99**, 1242; Combiesco, Soru and Stamatesco, *Arch. Roum. Path. exp. Microbiol.*, 1929, **2**, 291; Sordelli, Deulofeu and Ferrari, *Folia biol.*, 1932, **1**, 94; Tomcsik and Szongott, *Z. Immunitäts.*, 1932, **76**, 214). A well-defined polysaccharide was first isolated from *B. anthracis* by Ivánovics (*Zentr. Bakt., Abt. I., Orig.*, 1939, **144**, 244; Rep. Proc. 3rd Intern. Congr. Microbiol., 1939, p. 785), who later showed it to be composed mainly of D-galactose and *N*-acetyl-D-glucosamine (Ivánovics, *Z. Immunitäts.*, 1940, **97**, 402). There is, however, abundant and convincing serological evidence for the existence of a second polysaccharide (Zozaya, *J. Exp. Med.*, 1931, **54**, 725; Staub and Grabar, *Ann. Inst. Pasteur*, 1944, **70**, 16; Grabar and Staub, *ibid.*, p. 129) and our work had as its objective the isolation of both polysaccharides in a state of purity with a view to determining their structure.

The crude mixture of polysaccharides, obtained by ethanol precipitation of bacterial autolysates or, more conveniently, culture filtrates, was fractionated by precipitation with barium hydroxide (cf. Ivánovics, *loc. cit.*), the fractionation being followed serologically. By a fortunate chance, we, unlike most previous workers, used a rabbit, rather than a horse, anti-anthrax serum for controlling our purification procedure and our isolation of two serologically active polysaccharides is probably due to this. Ivánovics (*loc. cit.*) found the polysaccharide precipitated by barium hydroxide to be serologically inactive towards his horse anti-serum and accordingly discarded it as a non-specific impurity; we, on the other hand, found that, with our rabbit anti-serum, it was the baryta-precipitated polysaccharide, which we term " polysaccharide I," which was serologically active, the fraction not so precipitated (" polysaccharide II ") being inactive although clearly chemically similar to Ivánovics's polysaccharide; Ivánovics (*Z. Immunitäts.*, 1940, **98**, 373) had previously observed that rabbit anti-anthrax serum differed from the horse anti-serum in not agglutinating type XIV pneumococci.

Polysaccharide I had $[\alpha]_D^{19}$ $+72 \cdot 6°$ and gave a precipitate with the rabbit anti-serum at

dilutions down to 1 in 512 × 10^4; it gave only a slight precipitate, indicating the presence of less than 0·2% of polysaccharide II, with the horse anti-serum. Examined in the Tiselius electrophoresis apparatus in phosphate buffer of pH 8·0, it migrated very rapidly towards the anode as a broad single peak. The reducing power, determined by the method of Hagedorn and Jensen (*Biochem. Z.*, 1923, **135**, 46; **137**, 92), was 2·6%, corresponding to an average molecular weight of 6500 (38·5 hexose residues); the reducing value after hydrolysis reached a maximum value of 91% after 6 hours in 0·5N-hydrochloric acid at 100°.

Colour reactions indicated the presence of aldohexoses, and absence of hexoseamines; paper chromatograms of acid hydrolysates of polysaccharide I showed only a single spot, corresponding to mannose, and the presence of D-mannose was finally established by isolation of its *N*-methyl-*N*-phenylhydrazone. Colorimetric estimation, by Dische's carbazole–sulphuric acid reagent (*Mikrochem.*, 1930, **8**, 4; cf. Gurin and Hood, *J. Biol. Chem.*, 1939, **131**, 211; Siebert and Atno, *ibid.*, 1946, **163**, 511; Knight, *J. Exp. Med.*, 1947, **85**, 99), showed 98·5% of mannose after hydrolysis with 0·5N-sulphuric acid. at 100° for 6 hours.

On oxidation of polysaccharide I with periodic acid a maximum consumption of 1·14 moles of reagent per hexose residue was attained in 46 hours. Methylation (Haworth, *J.*, 1915, **107**, 8) and hydrolysis, followed by paper chromatography, showed the presence of a di-*O*-methyl- and a tri-*O*-methyl-mannose; the latter was identified as 2 : 3 : 6-tri-*O*-methyl-D-mannose by direct isolation and by conversion into the aniline derivative and comparison with an authentic specimen. These findings can be reconciled with various possible structures for polysaccharide I.

There is, however, no doubt that polysaccharide I is a D-mannan; of the various mannans which have been described it most closely resembles that recently isolated from yeast by Fischer, Kohtes, and Fellig (*Helv. Chim. Acta*, 1951, **34**, 1132). The small amount of phosphorus (0·9%) found in polysaccharide I would correspond to phosphorylation of one in twenty of the mannose residues; such phosphorylation would be in accord with the observed high electrophoretic mobility.

The relation of polysaccharide I to products obtained from *B. anthracis* by earlier workers is uncertain. Staub and Grabar (*loc. cit.*) obtained both Ivánovics's polysaccharide and another, which they termed polysaccharide "*a*," by fractional precipitation of the glycoproteins of anthrax œdema fluid; our polysaccharide I is clearly not identical with "*a*," since it gives no precipitate with the horse anti-serum, but it may be a degraded form of "*a*."

Polysaccharide II had $[\alpha]_D^{20}$ +61·0° and gave only a trace of precipitate with the rabbit anti-serum at a dilution of 1 in 8 × 10^4, whereas it was highly active in precipitating the horse anti-serum; it caused no inhibition of the precipitation of polysaccharide I by the rabbit anti-serum. In the Tiselius electrophoresis apparatus, in phosphate buffer of pH 8·0, it formed a single sharp, slightly asymmetric peak which migrated very slowly towards the anode, showing no signs of inhomogeneity. The reducing power, determined by the method of Fairbridge, Willis, and Booth (*Biochem. J.*, 1951, **49**, 423), was 2·3%, corresponding to an average molecular weight of 7300. The liberation of reducing sugars on hydrolysis with 0·5N-hydrochloric acid at 100° was followed by several methods; the results indicate a 93% yield after 6 hours' hydrolysis.

Colour reactions indicated the presence of hexoseamine and aldohexose in polysaccharide II. Paper chromatograms of acid hydrolysates showed two spots, one corresponding to glucosamine and the other to galactose. D-Glucosamine was identified by isolation of the hydrochloride and the 2 : 4-dinitrophenyl derivative, and D-galactose by isolation of the *N*-methyl-*N*-phenylhydrazone. The liberation of glucosamine on acid hydrolysis was followed by the methods of Palmer, Smyth, and Meyer (*J. Biol. Chem.*, 1937, **119**, 491) and Tracey (*Biochem. J.*, 1952, **52**, 266); the mean maximum yield was 31·5%. A maximum yield of 62% of galactose was obtained on hydrolysis at 100° with 0·5N-sulphuric acid for 2 hours. The acetyl content of polysaccharide II was 7·0%.

These analytical data point very clearly to the conclusion that polysaccharide II contains D-galactose and *N*-acetyl-D-glucosamine residues in the molar ratio 2 : 1; on the assumption that our freeze-dried material contained 12% of water, all the analytical data, with the exception of the nitrogen content, are in satisfactory agreement with a molecule

containing 26 galactose and 13 *N*-acetylglucosamine residues. Periodic acid oxidation showed a maximal consumption of 0·74 mole of reagent per hexose unit in 40 hours; this is in agreement with oxidative splitting of the galactose residues, the acetylglucosamine residues remaining unaffected, but does not permit any very definite structural conclusion to be reached. Unfortunately, attempts to methylate the polysaccharide failed owing to extensive decomposition (cf. Stacey, *Adv. Carbohydrate Chem.*, 1946, **2**, 205).

Ivánovics's preparation of the hexoseamine-containing polysaccharide of *B. anthracis* was found by him to contain galactose and acetylglucosamine in equimolecular proportion; the same ratio has very recently been reported in a polysaccharide isolated from *B. anthracis* grown *in vivo* (Smith and Zwartouw, *Biochem. J.*, 1954, **56**, viii). The difference between our 2 : 1 molar ratio and Ivánovics's 1 : 1 ratio is striking in view of the fact that both preparations were made by similar methods and had almost identical optical rotations. It is possible that the repeated treatment with barium hydroxide which we employed to remove the last traces of polysaccharide I brings about preferential removal of glucosamine residues from a precursor with a non-uniform distribution of galactose and glucosamine residues; some such non-uniformity in our own material is suggested by our finding that the liberation of galactose on acid hydrolysis is much faster than that of glucosamine.

It is noteworthy that our polysaccharide II, like those prepared by Ivánovics and by Smith and Zwartouw (*locc. cit.*), contains considerably more nitrogen than corresponds to the glucosamine content; in our case only 70%, in Ivánovics's case only 60—65% and in Smith and Zwartouw's case only 73%, of the total nitrogen is accounted for by glucosamine. Direct experiment showed that this "extra" nitrogen was not liberated as ammonia or as elementary nitrogen (cf. macrozamin; Langley, Lythgoe, and Riggs, *J.*, 1951, 2309) on acid hydrolysis. It seems quite probable that this extra nitrogen is polypeptide in nature, as in the human blood-group A substance (Aminoff, Morgan, and Watkins, *Biochem. J.*, 1950, **46**, 426); the discrepancy in our case would be accounted for by 6% of a mixture of amino-acids with a mean nitrogen content of 16% and such a quantity might well have been undetectable in our chromatographic experiments. Confirmation of this view was obtained by repeated reprecipitation of a fresh supply of the crude polysaccharide; in this way we were able to obtain a small amount of a polysaccharide (II_3) containing only galactose and glucosamine, in which 93% of the nitrogen was accounted for as glucosamine; the glucosamine content of this preparation was rather low (27%) and the quantity available was insufficient for further investigation.

It is well known that there are close serological relations between the glucosamine-containing polysaccharide of *B. anthracis* and those of type XIV pneumococci, the Forsmann antigen, and the various blood-group substances (for references see Stacey, *Adv. Carbohydrate Chem.*, 1946, **2**, 162; Bray and Stacey, *ibid.*, 1949, **4**, 37; Kabat, *Bacteriol. Reviews*, 1949, **13**, 189), but no detailed discussion of the chemical basis of these relations is possible until further structural investigations have been carried out. It does, however, seem desirable at this stage to draw attention to the relatively low glucosamine content of our polysaccharide II, to the fact that this substance differs from the A (Aminoff and Morgan, *Biochem. J.*, 1951, **48**, 74), B (Gibbons and Morgan, *ibid.*, 1954, **57**, 283), Lewis (Annison and Morgan, *ibid.*, 1952, **50**, 460), and H (*idem, ibid.*, 1952, **52**, 247) blood-group substances in being devoid of chondrosamine, and to the very marked difference in optical rotatory power between the anthrax polysaccharide ($[\alpha]_D$ +61°) and the others ($[\alpha]_D$ within the range −40° to +20°).

EXPERIMENTAL

Isolation of Crude Polysaccharide.—Two strains of *B. anthracis* were used, *viz.*, the highly virulent "Vollum" strain, grown under conditions not leading to marked capsule formation, and the avirulent, non-capsulated "Weybridge" strain; further details are given by Gladstone (*Brit. J. Exp. Path.*, 1946, **27**, 394) and by Hanby and Rydon (*loc. cit.*).

The isolation procedures were controlled serologically with anti-sera prepared in rabbits against the two strains; no significant difference was encountered between the two anti-sera in their capacity for precipitating the polysaccharide.

Preliminary experiments showed that little or no serologically active polysaccharide could

be extracted, by a variety of methods, from washed suspensions of cultures grown on CCY-agar (Gladstone and Fildes, *Brit. J. Exp. Path.*, 1940, **21**, 161). It was thought that this failure might be due to liberation, and consequent loss, of the polysaccharide during the extensive autolysis which accompanies washing and this was confirmed by the successful isolation of active material by alcohol precipitation of Seitz filtrates of bacterial suspensions autolysed at 45° for 2 days; it was later found preferable to grow the organisms on liquid CCY (Gladstone and Fildes, *loc. cit.*). It was also found that considerable amounts of active polysaccharide were present in the culture fluid; since the volume of this is so much greater than that of the corresponding amount of autolysate it proved convenient, for bulk preparation, to discard the bacteria and to isolate the polysaccharide from the culture filtrate alone.

Details of the final procedures are given below. The average yield of crude polysaccharide was 500 mg. from 1 l. of autolysate and 200 mg. from 1 l. of culture fluid. The crude material gave precipitates with the rabbit anti-sera at dilutions down to about 1 in 32×10^4; there was no marked difference in serological activity between preparations from the two strains or between preparations from autolysed cells or culture fluid.

(a) *From lysed organisms.* Organisms grown on liquid CCY or on CCY-agar (Gladstone and Fildes, *loc. cit.*) were suspended in water to a creamy consistency and allowed to autolyse at 45° for 2 days. The bacterial bodies were then removed by centrifugation and the supernatant liquid Seitz-filtered; the filtrate was treated with 3 vols. of ethanol and kept at 2° overnight. The precipitate was collected by centrifugation, dissolved in water, and dialysed for 2 days against running tap-water and then for 3 days against frequent changes of distilled water. The product was then concentrated to small bulk under reduced pressure at room temperature; at this stage the biuret test was negative. The solution was treated with 4 vols. of ethanol and kept for some hours; the precipitate was collected by centrifugation, washed with absolute ethanol, and dried *in vacuo* over phosphoric oxide. The product was a light brown powder which was serologically active against the rabbit anti-sera; some properties of materials prepared in this way are given in Table 1 (B1—F16).

(b) *From culture filtrate.* The organisms were grown on liquid CCY, and the bacterial bodies precipitated by the addition of a little acid; the supernatant fluid was decanted through a Seitz filter and the filtrate concentrated under reduced pressure at 35—40° to about one-tenth of its original volume. Crude polysaccharide was precipitated from the concentrate by the addition of 3 vols. of ethanol and collected by centrifugation after being kept overnight at 2°. This product was dissolved in water and dialysed for 2 days against running tap-water and then for 3 days against frequent changes of distilled water. The product was again concentrated under reduced pressure (to about 1/40 of the original volume), precipitated with 3 vols. of ethanol, kept overnight at 2° after the addition of one drop of acetic acid, centrifuged, washed with absolute ethanol, and dried *in vacuo* over phosphoric oxide. The products were serologically active, light brown powders; some of their properties are recorded in Table 1 (F18—F50).

TABLE 1. *Crude polysaccharide from B. anthracis.*

Prep. no.	Strain	Source	N (%)	$[\alpha]_D$	Titre against rabbit anti-serum	Yield (mg./l.)
B1	Weybridge	Autolysate	—	—	—	5280
F11A	Vollum	,,	3·3	+63°	—	380
F13	Weybridge	,,	—	+47	$1:16 \times 10^4$	520
F16	Vollum	,,	—	—	$1:16 \times 10^4$	445
F18	Vollum	Culture filtrate	2·6	+22	$1:64 \times 10^4$	83
F19	Weybridge	,,	4·0	+32	$1:32 \times 10^4$	119
F28X	Weybridge	,,	—	—	—	123
F29X	Vollum	,,	—	—	—	288
F30	Vollum	,,	—	—	$1:32 \times 10^4$	60
F39X	Vollum	,,	—	—	—	224
F48	Vollum	,,	—	—	$1:32 \times 10^4$	144
F50	Weybridge	,,	—	—	$1:16 \times 10^4$	124

Separation of the Two Polysaccharides.—The following are the details of a typical preparation: The crude polysaccharide (F50) (14·89 g.) was dissolved in water (300 ml.), and a small insoluble residue filtered off and rejected. To the clear brown solution saturated barium hydroxide solution (260 ml.) was added, this amount being sufficient to produce complete precipitation. After 30 min. at 5°, the precipitate was collected by centrifugation and dissolved in 4% acetic acid (125 ml.), and a small insoluble residue was removed by centrifugation. The clear brown solution was dialysed at 2° against frequent changes of distilled water until free

from barium and acetate ions (3 days). The solution was then concentrated under reduced pressure at room temperature and finally dried from the frozen state. The product (F50A) was a pale buff solid; the yield of polysaccharide I was 2·250 g.

The supernatant liquid from the barium hydroxide precipitation was treated with carbon dioxide to remove barium ions, the barium carbonate filtered off, and the filtrate made 5% with respect to sodium acetate (trihydrate) and 2% with respect to acetic acid and then treated with 3 vols. of ethanol. After being kept overnight at 2°, the supernatant liquid was decanted off and the precipitate dissolved in water and dialysed at 2° against frequent changes of distilled water until free from acetate ion (4 days). Concentration and drying from the frozen state gave 2·918 g. of polysaccharide II (F50B).

The results of a number of separations by this procedure are summarised in Table 2.

TABLE 2. *Separation of polysaccharides I and II.*

	Polysaccharide I					Polysaccharide II				
Starting material	Prep. no.	N (%)	$[\alpha]_D$	Titre against rabbit anti-serum	Yield (%)	Prep. no.	N (%)	$[\alpha]_D$	Titre against rabbit anti-serum	Yield (%)
F13	F13A	1·9	+45°	1 : 128 × 10⁴	2·1	F14	4·1	+66°	0	38·5
F16	F16A	3·85	+44	1 : 32 × 10⁴	9·9	F17	4·2	+69	0	23·0
F18	F18A	2·1	+38	1 : 256 × 10⁴	15·5	F18C	4·5	+30	1 : 32 × 10⁴	15·0
F19	F19A	2·6	+41	1 : 256 × 10⁴	11·4	F19C	4·5	+51	0	28·0
F28X	F28A	—	—	1 : 256 × 10⁴	8·0	F28C	—	—	1 : 32 × 10⁴	20·5
F29X	F29A	—	—	1 : 256 × 10⁴	2·9	F29C	—	—	0	26·7
F48	F48A	—	—	1 : 256 × 10⁴	11·1	F48B	—	—	0	14·5
F50	F50A	—	—	1 : 256 × 10⁴	15·1	F50B	—	—	0	19·6
F30	F40	—	—	1 : 128 × 10⁴	11·0	—	—	—	—	—

Polysaccharide I.—(a) *Purification.* The partly purified polysaccharide F50A (2·24 g.) was dissolved in water (70 ml.) and treated with saturated barium hydroxide solution (70 ml.). After 45 min. at 2°, the precipitate was collected by centrifugation, dissolved in 4% acetic acid (20 ml.), and dialysed against distilled water until free from barium and acetate ions. The solution was then concentrated at room temperature under reduced pressure and finally dried from the frozen state. The product, F50C (720 mg.), was an almost colourless porous mass which dissolved readily and completely in water; it had $[\alpha]_D^{19}$ +72·6° (*c* 1·5 in H_2O) and gave a precipitate with the Weybridge rabbit anti-serum at dilutions down to 1 : 512 × 10^4; this material was used for most of the analytical work. A similar purification of F48A (960 mg.) gave F48C (500 mg.) with $[\alpha]_D^{23}$ +69·7° (*c* 1·5 in H_2O), active at 1 : 256 × 10^4. Elementary analyses of these two materials gave the following results :

	C (%)	H (%)	N (%)	P (%)	Ash (%)
F48C	35·7	7·8	0·7	—	4·43
F50C	36·8	7·5	0·5	—	3·3
	37·6	6·8	1·1	0·87	3·3

(b) *Reducing power before and after hydrolysis.* Solutions of the polysaccharide in 0·5N-hydrochloric acid (*ca.* 1 mg./ml.) were heated at 100° in sealed tubes for various times. The tubes were cooled and the contents accurately neutralised with sodium carbonate and made up to 10 ml. with water. The reducing power was then determined by Hagedorn and Jensen's method (*loc. cit.*) with the following results :

Time (hr.)		0	1	2	3	3·5	4	5	6	18	21	72
Reducing value as glucose, %	F48C	2·6	26·1	38·5	72·4	81·5	86·1	90·5	90·5, 91·3	86·8	84·5	48·0
	F50C	—	—	—	—	—	—	—	91·3	—	—	—

(c) *Paper chromatography of hydrolysate.* The polysaccharide F50C (50 mg.) was hydrolysed for 6 hr. at 100° in a sealed tube with 0·5N-sulphuric acid. Sulphate ions were removed with barium hydroxide and the supernatant liquid from the barium sulphate was evaporated to dryness and made up to 2·5 ml. with water.

One-dimensional chromatograms were run on Whatman No. 1 filter paper, with both phenol and collidine for development and ammoniacal silver nitrate as the spraying reagent (Partridge, *Biochem. J.*, 1948, **42**, 238). Only one spot appeared with either solvent. That obtained with phenol had R_F 0·48, controls on the same paper giving the following R_F values: galactose, 0·44; mannose, 0·48; fructose, 0·56; glucosamine, 0·69 (Partridge, *loc. cit.*, gives 0·44, 0·45, 0·51, and 0·62 respectively). With collidine, the spot given by the hydrolysate had R_F 0·58;

controls gave the following R_F values: galactose, 0·52; fructose, 0·55; mannose, 0·58 (Partridge, *loc. cit.*, gives 0·34, 0·42, and 0·46 respectively, but the variability of commercial collidine is well-known, cf. Dent, *Biochem. J.*, 1948, **43**, 169). Control experiments showed the monosaccharide from the hydrolysate to be easily separable from added fructose but inseparable from mannose.

(d) *Estimation of mannose.* The following procedure was used: 1 ml. of solution, containing 0·1—0·2 mg. of mannose, was added to 9 ml. of 8 : 1 (by vol.) concentrated sulphuric acid–water, with ice-cooling; 0·3 ml. of a 0·5% solution of carbazole (purified as described by Gurin and Hood, *loc. cit.*) in ethanol was then added. After thorough mixing, the solution was heated in a boiling-water bath for 10 min., then cooled and the colour read on the Spekker absorptiometer, a 1-cm. cell and Ilford Spectrum Green Filter No. 604 being used. The reference curves was linear, 0·2 mg. of mannose giving a reading of 0·230.

F50C was hydrolysed with 0·5N-sulphuric acid at 100° for 6 hr.; duplicate analyses gave mannose yields of 98·5 and 98·6%.

(e) *Isolation of mannose methylphenylhydrazone.*—F50C (20 mg.) was hydrolysed for 6 hr. at 100° in a sealed tube with 0·5N-sulphuric acid. The cooled product was exactly neutralised with barium hydroxide solution, barium sulphate removed by centrifugation, and the supernatant liquid concentrated under reduced pressure to a syrup. 1 ml. of a 25% solution of *N*-methyl-*N*-phenylhydrazine in ethanol containing 3% of acetic acid was added and the mixture kept at 30° for 1 hr. and then at 0° for 20 min. The crystals which separated on scratching were filtered off, washed, and recrystallised from 70% ethanol; the product (10 mg.) had m. p. 179°, not depressed on admixture with an authentic specimen of D-mannose *N*-methyl-*N*-phenylhydrazone, m. p. 179° (lit., m. p. 181°).

(f) *Oxidation with periodic acid.* F50C (16·2 mg.) was dissolved in water (10 ml.); 1-ml. portions of this solution were kept at room temperature with 0·00548N-sodium periodate (5 ml.) and acetate buffer of pH 5·2 (0·5 ml.). The results, expressed in terms of monosaccharide units of mol. wt. 197, being hexose units corrected * for hydration on the basis of the observed reducing power, were as follows:

Time (hr.)	1	3·5	6	1·5	23·5	4·6
Mols. periodate consumed per monosaccharide unit	0·13	0·59	0·85	1·02	1·11	1·14

(g) *Methylation.* F50C (100 mg.), dissolved in water (2 ml.) and 35% sodium hydroxide solution (3 ml.), was stirred under nitrogen while methyl sulphate (1·5 ml.) was added dropwise (1 drop per hr.). After the addition was complete the temperature was raised to 100° and stirring continued for 1 hr. Extraction of the acidified solution with chloroform yielded only 15 mg. of an incompletely methylated product (Found: OMe, 22·7%). This was dissolved in acetone (5 ml.) and added to the solution previously extracted with chloroform. Sodium hydroxide solution (35%; 14 ml.) was added and the mixture shaken under nitrogen while methyl sulphate (8·5 ml.) was added dropwise (2 drops per min.). When the addition was complete, acetone (10 ml.) was added and the solution was refluxed for 1 hr. The cooled solution was acidified with 0·5N-sulphuric acid and cooled to 0°; the sodium sulphate which separated was removed by filtration and washed with acetone (10 ml.). The washings were added to the filtrate which was then again subjected to the methylation procedure. After fifteen methylations the final acidified solution was evaporated under reduced pressure to 200 ml. and kept for some hours at 0°. Sodium sulphate was removed by filtration, and both it and the filtrate were thoroughly extracted with chloroform. Evaporation of the dried extract and washing of the residue with light petroleum (b. p. 40—60°) yielded 20 mg. of methylation product.

This was refluxed overnight with 1% methanolic hydrogen chloride (10 ml.); the solution was evaporated to dryness under reduced pressure and then heated at 100° for 6 hr with 0·5N-hydrochloric acid (10 ml.). Silver carbonate (1 g.) was added and the solution filtered; some residual silver ions were removed by hydrogen sulphide and filtration. Finally, the solution was neutralised with barium carbonate and evaporated to dryness under reduced pressure. The crude product, on paper chromatography by the method of Hirst, Hough, and Jones (*J.*, 1949, 931), with butan-1-ol–ethanol–water as developing liquid and tetra-*O*-methyl-D-glucose as reference sugar, gave two spots of R_G 0·895 and 0·745 respectively.

Distillation gave a crystalline (b. p. 80°/0·5 mm.) and an amorphous (b. p. 100°/0·5 mm.) product; the former had m. p. 93—97°.

* The correction is made as follows. A polyhexose should give 111% of hexose on hydrolysis; F50C gave only 91%. The discrepancy is ascribed to hydration and the mol. wt. of the hydrated hexose unit is $162 \times 111/91 = 197$.

The hydrolysis product was boiled under reflux for 1 hr. with aniline (10 mg.) in ethanol (10 ml.); on evaporation followed by addition of one drop of ether, the product crystallised in needles, m. p. 118°. Two sublimations at 110°/0·005 mm. yielded 2 : 3 : 6-tri-*O*-methyl-D-mannosylaniline, m. p. 127°, mixed m. p. with an authentic specimen (m. p. 129°) 128°.

Polysaccharide II.—(a) *Purification.* Mixed, weakly serologically active fractions (5·27 g.) from both Vollum and Weybridge strains were dissolved in water (76 ml.) and treated with sufficient saturated barium hydroxide solution (40 ml.) to give complete precipitation. The precipitate was removed by centrifugation and the supernatant liquid freed from barium ions with carbon dioxide. The barium carbonate was filtered off and the completely inactive preparations F50B and F39 (3·39 g. in all) were dissolved in the filtrate. Polysaccharide was precipitated from this solution, which had been made 5% with respect to sodium acetate and 2% with respect to acetic acid, by addition of 3 vols. of ethanol. The precipitate was collected by centrifugation after being kept at 2° overnight, then dissolved in water (50 ml.), and the whole procedure was twice repeated. The final product was dissolved in water, dialysed, concentrated, and dried from the frozen state. The product, F51 (5·295 g.), gave only a trace of precipitate with the Weybridge rabbit anti-serum at a dilution of 1 : 16 × 10^4 and had $[\alpha]_D^{22}$ +61·5° (*c* 1·5 in H_2O).

This material was further purified by dissolving it (4·5 g.) in water (45 ml.) and treating this with saturated barium hydroxide solution (20 ml.); no precipitate appeared even overnight at 2°. The bulk of the barium ions were removed by carbon dioxide, and the remainder, after removal of the barium carbonate by filtration, by the addition of the necessary amount of 2N-sulphuric acid (0·3 ml.). Barium sulphate was removed by centrifugation and sodium acetate trihydrate (2·0 g.) and acetic acid (0·8 ml.) were dissolved in the supernatant liquid; ethanol (120 ml.) was added and the mixture kept at 2° overnight. The supernatant liquid was poured off and the sticky residue dissolved in water (40 ml.) and again precipitated with ethanol (120 ml.). The final precipitate was dissolved in water and dialysed at 2° against frequent changes of distilled water. The dialysed solution (71 ml.) was diluted to 200 ml. and dried from the frozen state. The product, R926D (3·6 g.), was a pale cream-coloured spongy mass which was readily and completely soluble in water; it gave only a trace of precipitate with the Weybridge rabbit anti-serum at a dilution of 1 : 8 × 10^4 and had $[\alpha]_D^{20}$ +61·0° (*c* 1·0 in H_2O). The results of elementary analyses of F51 and R926D were as follows:

	C (%)	H (%)	N (%) *	Acetyl (%)	Loss of wt. at 100° (%)	Ash (%)
F51	40·0	6·8	4·0 (K)	—	—	0·9
R926D ...	39·0	7·1	3·0 (D), 3·4 (K)	7·3	—	0·0
	39·9	7·0	3·2 (D)	6·6	1·2	0·0
	38·4	7·3	3·6, 3·4 (K)	—	2·2	0·0

* D = Dumas; K = Kjeldahl.

Repeated application of the baryta precipitation procedure to a further 10 g. of crude polysaccharide yielded 80 mg. of a specimen (II_3) of polysaccharide II in which 93% of the nitrogen was accounted for as glucosamine. This product had $[\alpha]_D^{20}$ +62·0° (*c* = 1 in H_2O) and a reducing value after hydrolysis (method of Fairbridge, Willis, and Booth, *loc. cit.*) of 100%, and gave 27·0% of glucosamine (estimated by the method of Palmer, Smythe, and Mayer, *loc. cit.*) on hydrolysis.

TABLE 3. *Hydrolysis of polysaccharide II.*

(a) *Analysis by Hagedorn and Jensen's method*; F51.

Time (hr.)	4	5	6	16	21	48
Reducing value as glucose (%)	65·5	66·3	68·4	67·5	37·0	32·0
Yield of 2 : 1 galactose : glucosamine (%)	76·7	77·6	80·1	79·1	43·3	37·5

(b) *Analysis by Jendrassik and Polgar's method.*

Time (hr.)		2	4	6
Reducing value as glucose (%)	R926D	54·5	60·8	64·5
	Galactose	77·5	69·5	59·2
	Glucosamine	97·8	94·0	90·5
Yield of 2 : 1 galactose : glucosamine (%)		64·7	78·3	92·7

(b) *Reducing power before and after hydrolysis.* In the first series of experiments the hydrolysis was conducted as described (p. 3870) for polysaccharide I and the reducing power determined by Hagedorn and Jensen's method (*loc. cit.*); the results, corrected for the known Hagedorn–Jensen reducing powers of galactose (132 mg. equiv. to 100 mg. of glucose; Sobotka and Reiner, *Biochem. J.*, 1930, **24**, 394) and glucosamine (95 mg. equiv. to 100 mg. of glucose; Ivánovics, *Z. Immunitäts.*, 1940, **97**, 402), are given in Table 3(a).

In the second series of experiments the hydrolyses were conducted similarly and the reducing power determined by Jendrassik and Polgar's method (*Biochem. Z.*, 1940, **304**, 271); correction was made for the not inconsiderable decomposition of galactose and glucosamine by carrying out simultaneous " hydrolyses " with these two monosaccharides; the results are shown in Table 3(b).

In another series of experiments the reducing value was determined colorimetrically with triphenyltetrazolium chloride (cf. Fairbridge, Willis, and Booth, *loc. cit.*). Solutions (1 ml.) of the polysaccharide and of galactose and glucosamine (all 0·50 mg./ml.) were mixed with 2N-hydrochloric acid (0·5 ml.) and heated at 100° in sealed tubes for 6 hr.; the tubes were then opened, treated with N-sodium hydroxide (2 ml.) and 1% aqueous triphenyltetrazolium chloride (0·5 ml.), and heated in a vigorously boiling water-bath for 3 min. 1·1N-Acetic acid (1 ml.) was added to the cooled solutions, and the mixture diluted to 10 ml. with aldehyde-free *iso*propyl alcohol (distilled from *m*-phenylenediamine); 1 ml. of this solution was made up to 10 ml. with the same *iso*propyl alcohol and the colour read on the Spekker absorptiometer, an Ilford Spectrum Blue Filter No. 602 and a 1-cm. cell being used. Under these conditions both galactose and glucosamine give identical linear reference curves; the actual colour value is very sensitive to slight changes in conditions and it was for this reason that parallel experiments were performed with the two sugars. Another similar series of tubes was set up with the hydrochloric acid replaced by water and without heating. The results indicated a reducing value of 2·3% (as hexose) before hydrolysis and 93·5% after hydrolysis.

(c) *Paper chromatography of hydrolysate.* The polysaccharide, R926D (50 mg.), was hydrolysed for 6 hr. at 100° with 0·5N-sulphuric acid. Sulphate ions were removed with aqueous barium hydroxide, and the supernatant liquid from the centrifuged sulphate was evaporated to dryness and dissolved in water (2·5 ml.). One-dimensional paper chromatograms were run on Whatman No. 1 filter paper, with both phenol and collidine as developing solvents, with ammoniacal silver nitrate as the spraying reagent (Partridge, *loc. cit.*). With phenol, two spots were obtained with R_F values of 0·42 and 0·65; reference specimens of galactose and glucosamine run simultaneously on the same sheet gave R_F values of 0·42 and 0·65 (Partridge, *loc. cit.*, gives 0·44 and 0·62). Our sample of collidine failed to separate the two sugars, giving only a single spot with R_F 0·52—0·53; Partridge (*loc cit.*) gives R_F values of 0·30 and 0·43 for glucosamine and galactose, respectively, but the variability of commercial collidine is well known (cf. Dent, *loc. cit.*).

In experiments designed to identify the hexosamine, the polysaccharide was hydrolysed, as usual, with 0·5N-hydrochloric acid at 100° for 6 hr. and the hydrolysate evaporated to dryness *in vacuo* over sodium hydroxide. Chromatograms were run one-dimensionally with phenol and collidine on Whatman No. 1 paper and sprayed with Moore and Stein's ninhydrin reagent (*J. Biol. Chem.*, 1948, **176**, 367); only a single spot was obtained, showing the absence of amino-acids and other primary amines. Comparative runs with the hydrolysate and authentic specimens of glucosamine and chondrosamine hydrochloride showed the hexosamine in the hydrolysate to be chromatographically identical with glucosamine; glucosamine moves faster than chondrosamine in collidine and more slowly in phenol, and the hexosamine in the hydrolysate was readily separated on the paper from added chondrosamine but inseparable from added glucosamine.

(d) *Isolation of glucosamine derivatives.* The polysaccharide, R926D (120 mg.), was refluxed for 5 hr. with 5N-hydrochloric acid (10 ml.); humin was removed by filtration through asbestos and the filtrate steam-distilled to remove volatile decomposition products. The residual solution was boiled with charocoal, filtered, concentrated to 1 ml., and kept overnight at 0°. Large crystals of D-glucosamine hydrochloride (40 mg.; 28% as glucosamine) separated and were recrystallised from hot water (0·5 ml.). The product had $[\alpha]_D^{20}$ + 76·0° (*c* 1·0 in H_2O) (lit., +72·5°) and gave an *X*-ray powder photograph indistinguishable from that given by authentic D-glucosamine and quite different from that given by authentic D-chondrosamine. Paper chromatography of the crude hydrochloride and of the filtrate revealed no ninhydrin-reacting material other than glucosamine.

A portion of the crude hydrochloride (15 mg.) was dissolved in water (1 ml.), and the solution thoroughly mixed with 2 : 4-dinitrofluorobenzene (0·01 ml.) in ethanol (2 ml.); N-sodium carbonate (0·01 ml.) was added and the mixture shaken for 1 hr. The ethanol was then removed under reduced pressure and the aqueous residue extracted continuously with ether for 12 hr. The extract was dried (Na_2SO_4) and evaporated; addition of a few drops of chloroform to a solution of the residue in acetone (0·5 ml.) gave a crystalline precipitate of *N*-2 : 4-dinitrophenyl-D-glucosamine, m. p. 197—200° (Found: C, 41·75; H, 4·9. Calc. for $C_{12}H_{15}O_9N_3$: C, 41·7;

H, 4·3%); there was no depression of m. p. on admixture with an authentic specimen, m. p. 200° [Annison, James, and Morgan, *Biochem. J.*, 1951, **48**, 477, give m. p. 202—204° (corr.)].

(e) *Isolation of galactose methylphenylhydrazone.* The polysaccharide, R926D (80 mg.), was heated with 0·5N-hydrochloric acid (2 ml.) at 100° in a sealed tube for 6 hr. The hydrolysate was cautiously neutralised with sodium carbonate (53 mg.) and warmed to expel carbon dioxide. A 25% alcoholic solution (2 ml.) of *N*-methyl-*N*-phenylhydrazine, containing a few drops of acetic acid, was added and the mixture kept at 30° for 1 hr. The product was cooled in ice and filtered and the crystals were washed with ethanol and recrystallised from the same solvent. The product (50 mg., 40% as galactose) had m. p. 188°, not depressed on admixture with authentic D-galactose *N*-methyl-*N*-phenylhydrazone, m. p. 189° (lit., m. p. 191°).

(f) *Estimation of galactose.* The procedure was identical with that used for the estimation of mannose (p. 3871). The reference curve was linear and passed through the origin; 0·40 mg. of galactose gave a Spekker reading of 0·490; glucosamine gave a negligible colour. Hydrolyses were carried out in the usual manner with 0·5N-sulphuric acid; the results were as follows:

Time (hr.)	1·17	2	3	4	6	25
Galactose (%)	60	62	60	59	57	37

(g) *Estimation of glucosamine.* (i) Method of Palmer, Smyth, and Meyer. The hydrolyses were carried out at 100° in the usual manner. The colours were measured on the Spekker absorptiometer with 1 cm. cells and Ilford Spectrum Green Filter No. 604; the reference curve was linear and passed through the origin, 100 g. of glucosamine giving a reading of 0·400. The results are recorded in Table 4; no detectable glucosamine was liberated after 22·5 hours' heating with N-acetic acid at 100°.

(ii) Tracey's method. The hydrolysate (*ca.* 1 mg./ml.) was distilled with saturated trisodium phosphate solution, saturated with sodium borate, in a Markham apparatus (*Biochem. J.*, 1942, **36**, 790) and the ammonia collected in 2% boric acid and estimated colorimetrically with Nessler's reagent (Ilford Spectrum Violet Filter No. 601). The results are recorded in Table 4.

TABLE 4. *Liberation of glucosamine from polysaccharide II on hydrolysis.*

Time (hr.)			1·25	1·5	2·5	3·75	4·0	4·5	6·0	8·0	22
Glucosamine liberated (%)	with 0·5N-HCl	(a)	12·7	—	24·3	27·3	—	—	29·3	29·0	28·3
		(b)	—	16·6	17·6	—	25·6	—	33·4	—	33·2
	with 6N-HCl	(a)	—	—	28·9	32·7	—	—	31·0	29·2	—

(*a*) Glucosamine determined by the method of Palmer, Smyth, and Meyer. (*b*) Glucosamine determined by Tracey's method.

(h) *Oxidation with periodic acid.* R926D was oxidised as described above for F50C (p. 3871). The results, calculated for a monosaccharide unit of mol. wt. 193 (cf. p. 3871), were as follows:

Time (hr.)	1	4	8	17	24	40
Periodate (mol.) consumed per monosaccharide unit	0·455	0·515	0·61	0·665	0·715	0·74

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