carrying out measurements in dilute buffers, e.g. 0.1 M. In the present work, sedimentation measurements have been made down to such low concentrations that, even in 0.1 M salt, the error due to the primary charge effect would probably have been small. Diffusion measurements could not, however, be made at such low concentrations, and the linear extrapolation would be in some doubt (see, for example, Mandelkern & Flory, 1951).

The pronounced artificial-boundary sharpening observed in sedimentation and the marked dependence of S upon c are consistent with a particle having a large effective volume, and this is reflected in the high values for the frictional ratio. It appears likely from viscosity studies (to be reported elsewhere) that these high values of the frictional ratio are not associated with any marked asymmetry of shape.

The boundary-sharpening effect precludes any assessment of the degree of polydispersity of the specimens from the sedimentation and diffusion data.

SUMMARY

1. Electrophoresis, sedimentation and diffusion studies have been made on two samples of polyglutamic acid prepared from *Bacillus anthracis* grown *in vivo* and isolated from (a) the extracellular material in the blood of the animal and (b) from the intracellular material of the organisms.

2. Both specimens are shown to give only single boundaries in electrophoresis and sedimentation

and have molecular weights of (a) 216 000 and (b) 75 000, expressed in terms of the free acid.

3. The specimens are typical polyelectrolytes with particles of large effective volume exhibiting large frictional ratios.

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The Polysaccharide from *Bacillus anthracis* Grown *in vivo*

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Bacillus anthracis contains polysaccharide material some of which appears to be part of the cell wall (Tomesik, 1951; Chu, 1953). Ivanovics (1940a) first isolated a polysaccharide from *B. anthracis* which contained galactose 34%, glucosamine 34%, N 4.2% and α -carboxyl amino N 0.8%. Cave-Browne-Cave, Fry, El Khadem & Rydon (1954) obtained a polysaccharide preparation from *B. anthracis* which contained galactose 62%, glucosamine 32% and N 3.3%; they suggested that glucosamine residues may have been lost during their repeated treatment of this material with baryta. These authors also reported the isolation of a phosphorylated mannan from culture filtrates and from unwashed *B. anthracis*; this mannan could

not be isolated from washed organisms. During the purification of a protective antigen from filtrates of cultures of *B. anthracis*, Strange & Belton (1954) obtained an inactive polysaccharide fraction which contained galactose 39–43%, glucosamine 34%, N 3.9% and α -carboxyl amino N 1.26%.

The differences between the total nitrogen and glucosamine nitrogen values, together with the figures for α -carboxyl amino nitrogen in these glucosamine-containing polysaccharides, indicated the presence of 5–10% of amino acid residues. Since the various preparations were not subjected to rigorous investigations of their purity, it was still undecided whether the amino acid residues were attached to the polysaccharide or were an impurity.

All the preparations described above were obtained from cultures of *B. anthracis* grown *in vitro*. This paper describes the isolation of a polysaccharide containing glucosamine from *B. anthracis* grown *in vivo*. A thorough investigation of the purity of this product has been made to establish the status of the amino acid residues. A summary of this work has been reported (Smith & Zwartouw, 1954).

MATERIALS AND METHODS

Crude polysaccharide from B. anthracis grown in vivo. This was the material which remained in solution when barium acetate (1%) and ethanol (33%, v/v) was added to a dialysed solution of the ammonium carbonate extract of *B. anthracis* isolated from infected guinea pigs (cf. Smith, Zwartouw & Harris-Smith, 1956). It contained N, 7.6; galactose, 30.0; glucosamine 29.0%.

Anthrax antiserum. This is described by Zwartouw & Smith (1956).

Analytical methods. Total nitrogen and α -carboxyl amino nitrogen were determined as described by Zwartouw & Smith (1956). Galactose was determined by the method of Kabat & Mayer (1948) and by that of Trevelyan & Harrison (1952). Glucosamine was estimated by (a) the method of Elson & Morgan (1933) with the Na_2CO_3 - NaHCO_3 buffer of Immers & Vasseur (1950), and (b) by the method of Tracey (1952), which was modified to eliminate from the hydrolysate of the polysaccharide the volatile products, which produced a turbidity with Nessler's reagent and hence interfered with the final estimation of ammonia; the ammoniacal distillate (9 ml.) was collected in 0.1N-HCl (1 ml.) and the mixture evaporated to 3 ml.; the cooled residue was diluted to 10 ml. for nesslerization; glucosamine standards were treated similarly. *N*-Acetylglucosamine was estimated by the method of Morgan & Elson (1934), acetyl by the method of Kuhn & Roth (1933), and reducing sugar by the method of Hagedorn & Jensen (1923). Ammonia in the presence of glucosamine was determined by a method based on that of Tracey (1952). Acid hydrolysates of the polysaccharide were neutralized with K_2CO_3 and mixed with an equal volume of saturated K_2CO_3 solution in a Conway unit. The ammonia was collected in 0.1N- H_2SO_4 and determined by nesslerization. These alkaline conditions did not liberate ammonia from glucosamine. Sugars and amino acids were identified by the paper chromatographic methods of Partridge (1948, 1949) and Consden, Gordon & Martin (1944).

EXPERIMENTAL AND RESULTS

Purification of the polysaccharide

An aqueous solution of crude polysaccharide (1%) and sodium acetate (1%) at pH 7 was cooled to 0° and ethanol added to 50% (v/v). After standing overnight, the precipitated impurity was removed by centrifuging and the ethanol concentration of the supernatant raised to 70% (v/v). After standing overnight, the precipitate of polysaccharide (yield approx. 60%) was collected and dissolved in 0.01N-HCl at 0° to produce a 1% solution (pH 2.5). A quantity of fuller's earth powder (B.D.H. Ltd., 'For adsorption purposes'), equal to the weight of partially purified polysaccharide, was washed several times with 0.01N-HCl and added to the solution. The mixture was shaken for 30 min. at 0° and then the clear solution obtained after centrifuging was adjusted to pH 7, dialysed and freeze-dried (yield 45%).

Criteria of purity

Ultracentrifuging and electrophoresis. The polysaccharide was examined in the Tiselius electrophoresis apparatus and in the Spinco ultracentrifuge at pH 4.5 and 7.8 (see addendum by Dr B. R. Record and Dr R. G. Wallis). No evidence for heterogeneity was detected.

The polysaccharide was also examined by electrophoresis on paper at pH 8.6 by the technique of Flynn & De Mayo (1951). The strips of paper were treated with Naphthalene Black 12B (I.C.I.), which stains protein, and a single band was detected. Similar paper strips were cut into portions and aqueous eluates of these were tested for carbohydrate by the anthrone method. Only the portion corresponding to that stained by the protein reagent contained carbohydrate. Although the polysaccharide migrated towards the anode in the Tiselius apparatus (see Addendum), the endosmotic flow of the buffer on the filter paper resulted in a small net displacement of the single band towards the cathode.

Table 1. *Analysis of fractions obtained from the polysaccharide in a partial-solubility test with aqueous ethanol (53%, v/v, ethanol)*

Galactose was determined by the method of Trevelyan & Harrison (1952). Glucosamine was determined by the method of Elson & Morgan (1933) after hydrolysis with 3N-HCl at 100° for 16 hr.

Fraction	Weight (mg.)	Galactose (%)	Glucosamine (%)	Nitrogen (%)
Original	90	43	37	4.0
1	5.8	45	38	4.1
2	28.7	43	36	4.0
3	30.7	40	36	4.0
4	15.8	43	36	4.0
Residue	3.8	43	37	3.9

Partial solubility. The modification by Aminoff, Morgan & Watkins (1950) of the classical equilibrium method of solubility test was used.

Polysaccharide (90 mg.) was shaken for 5 hr. at 0° with successive portions (0.5 ml.) of aqueous ethanol (53 %, v/v ethanol). After each extraction, the mixture was left at 0° for 16 hr., centrifuged and the supernatant removed. It was then evaporated and the residue analysed. Table 1 shows the constancy of the analysis of the fractions obtained. The low yield in fraction 1 was probably due to hydration of the polysaccharide (the undissolved material separated as an oil on centrifuging). This would reduce the water content of the solvent, which was known to have a pronounced effect on the solubility of the polysaccharide.

Repeated adsorption with fuller's earth. Preliminary experiments on the method of purification of the polysaccharide had shown that the fuller's earth removed protein preferentially from the impure polysaccharide in slightly acid solution. Attempts were made to remove further protein by repeated adsorption.

A solution of polysaccharide (40 mg.) in 0.01 N-HCl (2 ml.) was shaken at 0° for 30 min. with successive quantities of 40, 18 and 7 mg. of fuller's earth powder. After each treatment, the fuller's earth was removed by centrifuging and a portion of the solution reserved for analysis. The results of this analysis (Table 2) on small amounts of material show that although adsorption of the polysaccharide occurred, there was no preferential adsorption of material with high nitrogen content.

Recovery of the polysaccharide from its benzoyl derivative. Polysaccharide (70 mg.) in water (1 ml.) was shaken vigorously with successive small quantities of benzoyl chloride; the mixture was maintained at pH 7 by adding NaOH throughout the reaction. The insoluble derivative was collected and suspended in acetone containing 10 % (v/v) of water (3 ml.). A small insoluble residue was discarded and ether (20 ml.) added to the solution. The precipitated benzoyl derivative was collected, washed with ether and dried; yield 16 mg.; $[\alpha]_D^{20}$ in 80 % (v/v) acetone (C, 0.5) + 61°.

The derivative was hydrolysed as follows. It was dissolved in 80 % (v/v) acetone (3 ml.), and 2N-NaOH (3 ml.) was added. The mixture was maintained at 37° for 21 hr., at which time a test sample was completely soluble in water. The polysaccharide which separated from the aqueous acetone as an oil was collected and dissolved in water (0.5 ml.). The solution was clarified and the polysaccharide precipitated with ethanol (2.5 ml.). The product was dissolved, reprecipitated in the same way, washed with ethanol and dried; yield 7 mg. It contained galactose (Trevelyan & Harrison, 1952) 41; glucosamine (Elson & Morgan, 1933) 37 %. The amino acid

Table 2. *Analysis of the polysaccharide after repeated adsorption with fuller's earth powder*

Galactose (Trevelyan & Harrison, 1952) and nitrogen analyses were made on a solution (2 %, w/v) of the polysaccharide (in 0.01 N-HCl) after each of three adsorptions with fuller's earth powder.

No. of adsorptions	Galactose (mg./ml.)	Nitrogen (mg./ml.)	Galactose/nitrogen
0	8.6	0.79	10.9
1	4.9	0.49	10.0
2	3.3	0.32	10.3
3	2.4	0.23	10.4

Table 3. *Analysis of the polysaccharide from B. anthracis*

Analysis	Result
Paper chromatography*	Galactose and glucosamine
Galactose (Trevelyan & Harrison, 1952)	43 %
(Kabat & Mayer, 1948)	38 %
Glucosamine† (Elson & Morgan, 1933)	38 %
(Tracey, 1952)	44 %
Nitrogen	4.0 %
α -Carboxylamino N	0.3 %
Ash	0.7 %
CH ₃ CO-‡	14.5 %
Molecular weight§	27 200
$[\alpha]_D^{20}$ in water (c, 3)	+ 79°

* After hydrolysis with 0.5N- and 3N-HCl (100° for 3 hr.).

† After hydrolysis with 3N-HCl at 100° for 16 hr.; see later for corrected figures.

‡ The only fatty acid detected by Dr A. J. P. Martin in a distillate from the *p*-toluenesulphonic acid hydrolysate was acetic acid; the material was examined by gas-liquid partition chromatography (James & Martin, 1952).

§ From a combination of sedimentation and diffusion data (see Addendum).

content of this material was compared with that of the original polysaccharide by the paper electrophoretic method described below. Both materials contained aspartic acid, glutamic acid and neutral amino acids.

Analysis and properties

Table 3 shows the average of similar analytical results from four different batches of polysaccharide. The material was dried *in vacuo* (0.1 mm. Hg) at 60° over P₂O₅ before analysis. Two-dimensional paper chromatography on hydrolysates (3N-HCl at 100° for 16 hr.) of the polysaccharide (2 mg.) revealed the presence of the following amino acids: glycine, alanine, aspartic acid and glutamic acid. In addition to these amino acids and a large spot due to glucosamine, several faint unidentified ninhydrin-positive spots were consistently found on chromatograms. When the hydrolysates were examined by the paper-electrophoretic method of Wade & Morgan (1954) with ninhydrin as a spraying reagent,

aspartic and glutamic acids moved towards the anode. Towards the cathode, a spot corresponding to neutral amino acids was evident and beyond this a large area of glucosamine obscured any basic amino acids which might have been present.

Acid hydrolysis of the polysaccharide. Solutions of the polysaccharide (0.25%) in 0.1N- and 0.5N-HCl were heated at 100°. Samples were removed at intervals and analysed for reducing sugar, *N*-acetylglucosamine and glucosamine (Elson & Morgan, 1933). Figs. 1 and 2 show the amount of these constituents liberated by hydrolysis with 0.1N- and 0.5N-HCl. Liberation of reducing groups occurred rapidly; in 0.5N-HCl the maximum amount (71% as glucose) was set free in 2 hr. This value agrees with the calculated reducing value for 41% glucosamine plus 41% galactose. The maximum quantity of *N*-acetylglucosamine detected during either hydrolysis was 10%.

Fig. 3 shows the liberation of glucosamine by hydrolysis at 100° in 0.1N-, 0.5N-, 3N- and 6N-HCl.

Fig. 1. Hydrolysis with 0.1N-HCl (100°). ×, Glucosamine (Elson & Morgan, 1933); ●, reducing sugar; ○, *N*-acetylglucosamine.

Fig. 2. Hydrolysis with 0.5N-HCl (100°). ×, Glucosamine (Elson & Morgan, 1933); ●, reducing sugar; ○, *N*-acetylglucosamine.

The maximum amount of glucosamine liberated by hydrolysis with 0.5N-HCl was 28%, whereas 38% was set free by 3N-HCl. This seems to indicate two types of glucosamine linkages within the molecule. It will be noted that the initial rapid liberation of glucosamine by 3N-HCl ceased at a level of approx. 30%; the remainder of the glucosamine was liberated at a slower rate. Furthermore, hydrolysis with 3N-HCl of a sample from which no further glucosamine could be liberated by the action of 0.5N-HCl released an additional quantity of glucosamine.

The following evidence shows that the higher value given by the Elson & Morgan (1933) method for the liberation of glucosamine by 3N-HCl was not due to substances other than hexosamines and particularly not due to those formed from sugar-amino acid mixtures (Immers & Vasseur, 1950).

(1) The value obtained with glucosamine in the Elson & Morgan (1933) assay after heating with 0.5N- or 3N-HCl was unaltered by including in the treatment with acid an equal quantity of galactose and 25% of the protein from *B. anthracis*.

(2) The polysaccharide hydrolysates produced no colour in the assay when acetylacetone was omitted from the reagents. Sugar-amino acid mixtures produce faint colours under these conditions (Immers & Vasseur, 1950; Gottschalk & Partridge, 1950; Aminoff, Morgan & Watkins, 1952).

(3) The absorption spectra of the colours obtained from glucosamine and the polysaccharide after heating in 3N-HCl were identical and had one maximum (530–535 mμ.). The colour produced by sugar-amino acid mixtures has two maxima at 530 and 560–570 mμ. (Boas, 1953).

(4) Glucosamine and acetylglucosamine were heated with 0.5N- and 3N-HCl for 16 hr. at 100°; the same quantity of glucosamine was detected after either acid treatment.

Fig. 3. Liberation of glucosamine, as measured by the Elson & Morgan (1933) technique, from the polysaccharide during hydrolysis at 100° with different strengths of acid. ×, 0.1N-HCl; ●, 0.5N-HCl; ○, 3N-HCl; ▽, 6N-HCl.

Total nitrogen and the glucosamine content. The α -carboxyl amino nitrogen value (0.3 %) indicates that the amino acid residues do not account for more than 0.4 % of the polysaccharide nitrogen content. The remaining nitrogen (3.6 %) could correspond to a glucosamine content of 46 %.

After hydrolysing the polysaccharide with 3N-HCl, the glucosamine value found by the Tracey (1952) method (see Table 1) was close to this calculated figure, but the Elson & Morgan (1933) method produced a significantly lower result. One possible explanation of this is that glucosamine deaminated during the hydrolysis has been included in the former value but not in the latter. However, this is not so. The difference between the two results [16 % of the value obtained by the Tracey (1952) method] could not be accounted for by deamination, since glucosamine liberated only 4 % and the polysaccharide 6.5 % of its total nitrogen as ammonia when heated for 16 hr. at 100° in 3N-HCl (cf. Boas, 1953). Hence, hydrolysis with 3N-HCl does not appear to liberate all the glucosamine residues in a form which reacts in the Elson & Morgan (1933) assay, although they are detectable by the Tracey (1952) method.

After 4 and 16 hr. hydrolysis with 6N-HCl, the Elson & Morgan (1933) method gave a result (39 %) almost identical with that obtained after 3N-HCl hydrolysis, although the stronger acid would produce considerably more decomposition of the glucosamine. Thus glucosamine heated with 6N-HCl for 16 hr. at 100° liberated 15.3 % of its total nitrogen as ammonia. Boas (1953), using the Elson & Morgan (1933) method, showed that 20.4 % decomposition of glucosamine occurred under similar acid conditions. If the glucosamine value obtained after hydrolysis of the polysaccharide with 6N-HCl is corrected for 15.3 % decomposition, a figure (45 %) agreeing with that calculated from the nitrogen content is obtained. Furthermore, the ammonia nitrogen (0.75 %) found to be present in the polysaccharide after hydrolysis with 6N-HCl is equivalent to 9.5 % of glucosamine. When this quantity of glucosamine is added to that detected by the Elson & Morgan (1933) method it shows that a total of 47.5 % may be present in the polysaccharide.

Acetyl residues. *N*-Acetylglucosamine was detected during acid hydrolysis, and it is probable that all the glucosamine residues of the polysaccharide occur in this form. The acetyl content of the polysaccharide was greater than that required on this basis. Hence, the following experiments were carried out to detect *O*-acetyl groups by means of their lability to cold alkali. Three samples of polysaccharide (10 mg.) in 2 % KOH (1 ml.) were allowed to stand at 0° for 2 and 120 min. and at 37° for 120 min. Cold ethanol (10 ml.) was added and

Table 4. *Acetyl content of polysaccharide after treatment with cold alkali*

Polysaccharide (10 mg.) was treated with 2 % KOH (1 ml.) and then recovered by precipitation with ethanol.

Hydrolytic conditions		Recovered polysaccharide	
Temp. (°)	Time (min.)	Yield (%)	Acetyl content (%)
Untreated control	—	—	14.5
0	2	82*	12.4
0	120	98	12.0
37	120	94	12.3

* Some was lost by accident.

the precipitated polysaccharide washed twice with ethanol (5 ml.) to complete the removal of potassium acetate. Table 4 shows the acetyl contents of the recovered polysaccharide samples. They indicate that a rapid loss of acetyl groups occurred in the first 2 min., which was not increased by extending the time of treatment with alkali. The average acetyl content (12.2 %) of the recovered samples corresponds to a loss of 2.3 % of acetyl. This figure was confirmed by direct measurement of the acetic acid set free.

A solution of polysaccharide (40.2 mg.) in water (1 ml.) with one drop of phenol red and one drop of phenolphthalein added was adjusted to a chosen indicator end-point (about pH 8). Sodium hydroxide (1 ml. of 0.1N) was added at 0° and after 105 min. the mixture was back-titrated with 0.01N-HCl to the original indicator colour. The acid liberated by the alkali treatment was equivalent to 2.35 % of alkali-labile acetyl in the polysaccharide. *N*-Acetylglucosamine liberated no acid when treated similarly. The polysaccharide recovered from the alkaline treatment by dialysis and freeze-drying the solution had an acetyl content of 11.9 %.

Stability of the glucosamine residues to cold alkali. A solution of the polysaccharide in half-saturated baryta was placed in a Conway unit at 20° for 94 hr. After this treatment, no free glucosamine or acetylglucosamine was present and only 1.5 % of the total nitrogen was liberated as ammonia.

Biological properties. The purified polysaccharide from *B. anthracis* grown *in vivo*, like the crude preparation, was inactive at 1 % in tests for virulence-enhancing, antiphagocytic and anticomplementary activity (see Smith *et al.* 1956). A dilution of $1:5 \times 10^6$ precipitated with anthrax antiserum. Professor W. T. J. Morgan (private communication) found the polysaccharide to have no blood group A, B or O activity in serological tests. Professor M. Heidelberger (private communication) obtained a precipitate when antipneumococcus Type XIV serum was added to the polysaccharide solution.

DISCUSSION

A purified polysaccharide has been obtained by mild treatment from *B. anthracis* grown *in vivo*. Analysis has indicated that this preparation is composed of *N*-acetylglucosamine and galactose in approximately equimolecular proportions, together with small amounts of *O*-acetyl (2.3 %) and amino acid residues (2–3 %). The polysaccharide was subjected to a number of tests for homogeneity, in all of which the preparation showed no evidence for heterogeneity. In view of these results, it is considered that a small peptide moiety is firmly attached to the polysaccharide.

The purified polysaccharide is probably identical with the polysaccharide synthesized by *B. anthracis* growing *in vitro*. The analysis of our preparation is generally similar to those of Ivanovics (1940*a*) and Strange & Belton (1954), and the quantitative differences are open to explanation. The preparation of Strange & Belton (1954) had galactose (39–43 %) and acetyl (14.3 %) values almost identical with those obtained by us; its glucosamine value (34 %) obtained after hydrolysis with 3*N*-HCl for 3 hr. may have been low in view of the investigations described above. A similar comment may be made about the preparation of Ivanovics; the glucosamine value (34 %) was obtained after hydrolysis for 2–3 hr. with 5*N*-HCl.

The higher α -carboxyl amino nitrogen values of their preparations, which were not rigidly examined for heterogeneity, indicated the presence of more amino acid residues than in our preparation. A proportion of these may have been present as protein impurity.

In our work no evidence has been found of either of the two polysaccharides described by Cave-Browne-Cave *et al.* (1954). The phosphorylated mannan could not be expected to occur in our bacterial extracts since these were obtained from washed organisms. We have found no evidence that the second of their polysaccharides, containing a 2:1 ratio of galactose to glucosamine, could have been formed by the action of baryta on a precursor having an equal ratio of these constituents. No free glucosamine or acetylglucosamine was formed by the action of cold baryta on our polysaccharide. However, there does appear to be a difference in the linkage of glucosamine residues. This is indicated by the resistance of some of the linkages to hydrolysis by HCl at the lower concentrations.

The polysaccharide isolated in this work, like that obtained by Ivanovics (1940*a, b*), precipitated with an antipneumococcus Type XIV serum. It did not inhibit the agglutination of erythrocytes (A, B or O) by their antisera. Ivanovics (1940*b*) had shown that anthrax antiserum would precipitate with partially hydrolysed blood group A substance,

although it did not agglutinate group A erythrocytes.

Ivanovics (1940*a*) considered that the polysaccharide played no role in the virulence of *B. anthracis* since it was present in both virulent and avirulent strains. The same argument could be used with reference to polyglutamic acid (see Smith & Keppie, 1955), which is, however, connected with virulence (Zwartouw & Smith, 1956). Positive evidence that the polysaccharide is not important in the virulence of the organism has been obtained here; it is completely inactive in tests for aggressive activity.

SUMMARY

1. A purified polysaccharide has been obtained by mild treatment from *Bacillus anthracis* grown *in vivo*. It showed no evidence for heterogeneity in a number of tests.

2. The preparation is composed of *N*-acetylglucosamine and galactose in approximately equimolecular proportions, together with small amounts of *O*-acetyl (2.3 %) and amino acid residues (2–3 %).

3. The polysaccharide plays no important role in the virulence of *B. anthracis*.

We are grateful to Professors W. T. J. Morgan and M. Heidelberger for serological testing and to Dr A. J. P. Martin for the identification of acetic acid. This work was part of a thesis submitted by one of us (H. T. Z.) for the Ph.D. degree of the University of London.

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ADDENDUM

Physicochemical Examination of the Polysaccharide from *Bacillus anthracis* Grown *in vivo*

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The polysaccharide was examined in the electrophoresis apparatus and the ultracentrifuge for evidence of homogeneity. Sedimentation and diffusion coefficients have been determined over a range of concentration, and a value for the molecular weight has been calculated from these results extrapolated to infinite dilution.

The methods employed followed closely those described by Record & Wallis (1956).

RESULTS

Electrophoresis. (a) in phosphate (*I* 0.1, containing 0.0022M-KH₂PO₄ and 0.0326M-Na₂HPO₄)-NaCl (*I* 0.1) buffer. A 1% solution of the freeze-dried material was made up in this buffer and dialysed for 5 days against daily changes of buffer. After running for 19 hr. at 12 mA in the standard 10 ml. U-tube, one component only was observed. Appreciable spreading of the boundary had occurred but the anode- and cathode-limb boundaries were nearly symmetrical and practically identical, and

δ- and ε-buffer boundaries were hardly apparent. The low anodic mobility of 0.124×10^{-4} cm.²/sec./v was substantially the same in both anode and cathode limbs.

(b) A similar experiment carried out in acetate (pH 4.5, *I* 0.1)-NaCl (*I* 0.1) buffer showed a very similar behaviour, with a still lower mobility (anodic) of 0.056×10^{-4} cm.²/sec./v.

Sedimentation. Both these solutions showed only a single sedimenting boundary in the ultracentrifuge. Fig. 1 shows the appearance of the boundary during the run with the solution of pH 8. The picture obtained with the solution of pH 4.5 was practically identical.

The variation in sedimentation rate with concentration was explored in the phosphate (pH 8, *I* 0.1)-NaCl (*I* 0.1) buffer in order to arrive at the value at zero concentration for calculating the molecular weight. A stock 1% solution of the freeze-dried sample was prepared in this buffer and dilutions were made by weight. The sedimentation coefficients (corrected to water at 20°) plotted

Fig. 1. Ultracentrifuge patterns obtained with a 1% solution of the polysaccharide in phosphate (pH 8, *I* 0.1)-NaCl (*I* 0.1) buffer. Speed = 59 780 rev./min. Interval between exposures = 64 min. (First exposure 53 min. after full speed was attained.)

against concentration are shown in Fig. 2, from which it is seen that the relationship appears to be closely linear, and leads to an extrapolated value for $S_{20,w}$ ($c \rightarrow 0$) = 2.23×10^{-13} .

Partial specific volume. This was determined at 20° pyknometrically, in the same buffer, as 0.656 for a 1.58% solution and 0.656 for a 0.79% solution, and this value, 0.656, has been used in correcting the sedimentation rates to standard conditions and in the calculation of the molecular weight.

Diffusion. Measurements were made in the same buffer (pH 8) in the Gouy diffusimeter over the

range 0.5% down to 0.2%. The values of the diffusion coefficient shown (Fig. 3) were calculated from the movement of the outermost Gouy fringe. The measurements were made at 25° and corrected for temperature and buffer viscosity to water at 20°. The diffusion coefficients so obtained have been plotted against j_m , where j_m is proportional to the polysaccharide concentration and equal to the sum of the integral number of fringes plus the fractional part of a fringe in the Gouy pattern. The straight line drawn in Fig. 3 was calculated by the method of least squares and indicates a very small increase in D with rising concentration. If a linear extrapolation is assumed to be valid at concentrations below the experimental points,

$$D_{20,w} (c \rightarrow 0) = 5.50 \times 10^{-7}.$$

Molecular weight. Applying the Svedberg equation:

$$M = \frac{RTS}{D(1 - V\rho)}$$

to these results, a value of 29 000 is obtained for the molecular weight. The frictional ratio f/f_0 is 2.0.

The broadening of the boundary in sedimentation has not been quantitatively assessed, but does not appear to be excessive for a molecular weight of this order, and the material, if polydisperse, cannot be markedly so.

SUMMARY

1. The polysaccharide from *Bacillus anthracis* shows only one component in prolonged electrophoresis and in sedimentation, both at pH 8 and pH 4.5.

2. The molecular weight, calculated from the diffusion coefficient and the sedimentation coefficient at infinite dilution and the partial specific volume (0.656), is 29 000; the frictional ratio is 2.0.

REFERENCE

Record, B. R. & Wallis, R. G. (1956). *Biochem. J.* **63**, 443.

Fig. 2. Variation of sedimentation coefficient with concentration. Phosphate (pH 8, 1 0.1)-NaCl (1 0.1) buffer.

Fig. 3. Variation of diffusion coefficient with concentration. Phosphate (pH 8, 1 0.1)-NaCl (1 0.1) buffer.