DISCUSSION

The enzyme isolated from ox pancreas is similar in its specificity to the enzyme isolated by Fairbairn (1948) from *Penicillium notatum*. In both cases no activity was discovered against lecithin. The pH optima are different, being 4.0 with the *Penicillium* enzyme and 3.5 for the enzyme found in *Aspergillus oryzae* by Contardi & Ercoli (1933). It was not established in this work whether the enzyme attacks lysocephalin, since no purified substrate was available. Our substrate of lysolecithin contained small amounts of lysocephalin, not in quantities large enough, however, to disturb significantly the equivalence between the ester splitting and the glycerophosphorylcholine formation. In the case of the *Penicillium* enzyme, Fairbairn (1948) showed that some ethanolamine could be demonstrated in the glycerophosphorylcholine fraction liberated by the enzyme. Thus, it is very likely that the enzymes discussed are lysophospholipases, rather than specific lysolecithinases.

It remains to be shown whether the crystalline preparation obtained is a pure lysolecithinase protein. The constancy of its specific activity, in

spite of repeated recrystallizations, tends to show that we are not dealing with a chance contamination. However, adequate proof for its homogeneity must await preparations on a larger scale than could be carried out up to now.

SUMMARY

1. Methods for the determination of lysolecithinase activity are described. These are based on the decrease in ester content, measured by a hydroxamic acid reaction, and on the formation of glycerophosphorylcholine, measured by the choline liberated by acid hydrolysis.

2. A procedure is described, by which the pancreas enzyme is purified 25 times with a reasonable yield (30 %).

3. A crystalline preparation was obtained in low yields, with a specific activity 40 times that of the original extract.

4. Optimum activity was found at pH 6.0 and at a substrate concentration varying from 3–8 μ moles/ml.

5. The enzyme does not attack lecithin and triolein.

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The 'Acid Polysaccharides' of Hog Gastric Mucosa

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Following earlier work by Levene & López-Suárez (1916), Meyer, Smyth & Palmer (1937) described a mucoitin monosulphuric acid as the 'acid polysaccharide' of hog gastric mucosa. They used an alkaline extraction of the commercial hog gastric mucin of Wilson Laboratories, Chicago, which is a preparation from whole gastric mucosal tissue. Wolfrom, Weisblat, Karabinos, McNeely & McLean (1943) prepared from the same source, and by a slightly modified procedure, a mucoitin sulphuric acid differing in rotation and analysis from the

former compound. Meyer & Smyth (1938) and Meyer (1938) described the acid calcium salt of a mucoitin sulphuric acid isolated without alkaline digestion. The sulphur content (9.4 %) indicated more sulphate groups than a mucoitin monosulphuric acid contains, and the optical rotation differed from the above compounds.

No criteria of purity were given for any of these preparations and they were undoubtedly mixtures of compounds. Wolfrom & Rice (1947) showed by electrophoretic analysis that the material produced

by Wolfrom *et al.* (1943) was a mixture of at least two compounds.

Smith, Gallop & Stanley (1952) have recognized two acid polysaccharides during an investigation into the factors involved in the virulence-enhancing action of preparations from fresh hog gastric mucosae. One (polysaccharide A) has been purified and identified as a heparin (Smith, Gallop, Harris-Smith & Stanley, 1952). The second (polysaccharide B) was impure, but approximated in elementary analysis and optical rotation to the acid polysaccharide of former workers.

In further work connected with virulence enhancement, a third sulphated acid polysaccharide (polysaccharide D) has been recognized, purified and found to be homogeneous by ultracentrifugal, electrophoretic and partial solubility determinations. In addition, polysaccharide B, which is different from D and is an insignificant participant in virulence enhancement, has been purified; it is homogeneous by ultracentrifugal and electrophoretic analysis, but some evidence for heterogeneity is revealed by partial solubility studies.

The purification and chemistry of these two polysaccharides is described in this paper. The connexion of polysaccharide D with the virulence-enhancing action of mucin is dealt with in the following paper.

EXPERIMENTAL AND RESULTS

The remarks made by Smith, Gallop & Stanley (1952) on the determination of uronic acid and hexosamine apply here. *N*-acetyl was determined by titrating the distillable acidity after *p*-toluenesulphonic acid hydrolysis (Kuhn & Roth, 1933).

Smith, Gallop & Stanley (1952) used a fractionation involving barium acetate and ethanol in order to separate the different polysaccharides from autolysates of whole hog gastric mucosae. Polysaccharide A was precipitated by barium acetate without the addition of ethanol, and the fraction between 30–50% (v/v) ethanol constituted a neutral compound (polysaccharide C) which is described in the following paper. This paper deals with the purification and properties of the 0–10% (v/v) ethanol fraction (crude polysaccharide B, yield from 200 stomachs approx. 4 g.), and the 10–30% (v/v) ethanol fraction (crude polysaccharide D, yield from 200 stomachs approx. 3 g.).

Purification of polysaccharide B

Electrophoresis and preliminary analysis showed that the chief contaminants were nucleic acid, peptide material and polysaccharide A (heparin); the latter was present to the extent of 7.5–10%. This and similar figures for samples in the fractionations below were obtained by an assay of anticoagulant activity. The system of Foster (1941) was used, i.e. serial dilutions of solutions of the compounds were treated with citrated sheep plasma and then with CaCl_2 . The concentration of the compound under test, in the tube first showing signs of clotting, was compared with that of the

corresponding tube of a similar series containing pure polysaccharide A (heparin).

Barium acetate and ethanol fractionation. Crude polysaccharide B (9.5 g.), dissolved in water (190 ml.) at pH 7, was treated with 3% (w/v) barium acetate. After standing overnight at 0° a small amount of precipitate (0.4 g.) was removed by centrifugation and discarded. Ethanol was added to the supernatant to give a concentration of 5% (v/v), and after standing at 0° overnight, the precipitate (6.7 g.) was removed in the batch bowl of a refrigerated Sharples centrifuge, freeze-dried and reserved. Fractions at 7.5% (v/v) ethanol (2.0 g.) and 10% (v/v) ethanol (1.3 g.) were collected similarly and freeze-dried. The material (0.4 g.) in the supernatant was discarded. Barium and some nucleic acid were precipitated from the 5, 7.5 and 10% (v/v) ethanol precipitates by dissolving in water to make a 5% (w/v) solution, adding Na_2SO_4 (15% of the weight of initial material), $\text{N-H}_2\text{SO}_4$ to pH 1.8–2.0 and ethanol to give a final concentration of 10% (v/v). The precipitates were removed by centrifuging at 0°, and the polysaccharide was precipitated from the supernatants by adding ethanol to 50% (v/v) and sodium acetate to 1% (w/v). The flocculent precipitates were removed at 0°, dissolved in water at pH 7, dialysed and freeze-dried. The yields, and contamination with polysaccharide A (heparin), were 0–5% (v/v) ethanol fraction, 4.5 g.; 7.5% A: 5–7.5% (v/v) ethanol fraction, 1.0 g.; 3% A: 7.5–10% (v/v) ethanol fraction, 0.5 g.; 2% A.

Treatment with fuller's earth. In addition to polysaccharide A, the 5–7.5% (v/v) ethanol fraction contained traces of nucleic acid ($P=0.12\%$) and peptide material (approx. 2% by paper chromatography). Treatment with fuller's earth removed these impurities and reduced the anticoagulant activity of the sample to a negligible figure (see below).

Fuller's earth powder was washed thoroughly with cold 0.1N-HCl before use. A solution of the 5–7.5% (v/v) ethanol fraction (1.6 g.) in 0.1N-HCl (80 ml.) was shaken with fuller's earth (8 g.) for 5 min. The adsorbent was removed by centrifugation, and the supernatant neutralized and dialysed against distilled water; the pH was adjusted to 6.5–7.0 and the solution freeze-dried; yield 1.2 g.

Purification of polysaccharide D

Preliminary analysis showed that crude polysaccharide D contained nucleic acid and peptide material. Experiments in which analyses for uronic acid, hydrolysable sulphate and hexosamine were made on fractions taken at 2.5% (v/v) intervals from 10 to 30% (v/v) ethanol in the 3% (w/v) barium acetate ethanol system, indicated the presence of the new polysaccharide D; this was purified in the following way.

Barium acetate and ethanol fractionation. Crude polysaccharide D (10.1 g.) in water (202 ml.) was fractionated with barium acetate and ethanol as described for polysaccharide B. The following fractions were collected: 0–12.5% (v/v) ethanol, 1.6 g. (after decomposition with Na_2SO_4 , removal of BaSO_4 , dialysis and freeze-drying); 12.5–20% (v/v) ethanol, 6.6 g. (treated as described below); material soluble in 20% (v/v) ethanol, 1.4 g. (after decomposition, etc.).

Barium and nucleic acid were removed from the 12.5–20% (v/v) ethanol fraction exactly as described for polysaccharide B, except for two points: (i) ethanol was added to the mixture at pH 1.8–2.0 to 20% (v/v), and (ii) sodium acetate was added to a concentration of 2% (w/v) to pre-

precipitate the polysaccharide from 50% (v/v) ethanol. The yield (2.8 g.) could be increased by a further 1.3 g. by raising the ethanol concentration for the final precipitation of polysaccharide D to 80% (v/v).

Treatment with fuller's earth. The material from the 12.5 to 20% (v/v) ethanol fraction contained a negligible amount of polysaccharide A, but appreciable quantities of nucleic acid ($P=0.3\%$) and peptide material (approx. 5% by paper chromatography) were present; 1.8 g. of this fraction gave 1.3 g. of product after removing these impurities by a treatment with fuller's earth as described for polysaccharide B.

Criteria of purity

(1) Electrophoresis and ultracentrifuge studies. Our colleague, Dr B. R. Record, describes in an addendum to this paper that by these methods the preparations show no evidence of heterogeneity.

(2) Assuming no inherent anticoagulant activity, anti-coagulation assay showed that the maximum content of polysaccharide A in B and D was 0.6% and less than 0.07%, respectively.

(3) The complete absence of blood group A or H activity at 2% (w/v) concentration rules out any contamination with the neutral polysaccharide C (see following paper).

(4) A modification of the classical solubility test due to Aminoff, Morgan & Watkins (1950) and used by Smith, Gallop, Harris-Smith & Stanley (1952) was carried out on polysaccharides D and B. The Dische (1947) method for hexuronic acid, which can be carried out on small quantities and gives very different results for the two polysaccharides (see below), was used as the analytical method.

Polysaccharide D (50 mg.; 31.6% hexuronic acid by the Dische (1947) method) was shaken with successive quantities (2 ml.) of 65% (v/v) aqueous ethanol for 1 hr. After centrifugation the successive supernatants were evaporated and dried over P_2O_5 at 0.1 mm. and 60°. A weighed quantity of each of the five fractions was then analysed for hexuronic acid by the Dische (1947) method. The following results were obtained on successive fractions: 29.6, 33.4, 33.5, 32.7 and 31.8% hexuronic acid. No heterogeneity is indicated by these figures. Polysaccharide B (100 mg.; 16.6% hexuronic

acid by the Dische (1947) method) was treated as for polysaccharide D, but 55% (v/v) aqueous ethanol (1 ml.) was used for each successive solvent. The following results were obtained on successive fractions: 14.5, 15.1, 19.4, 21.9 and 17.6% hexuronic acid. Several experiments gave similar results, and it must be concluded, in spite of the evidence from ultracentrifugation and electrophoresis, that polysaccharide B is to some extent heterogeneous.

Chemistry of polysaccharides B and D

Similarities

(1) *Elementary and other analyses.* Table 1 shows the close similarity of the main analytical figures for polysaccharides B and D; they are in fair agreement with those calculated for the Na salt of a polymer containing equimolar amounts of acetyl hexosamine, uronic acid and H_2SO_4 as a statistical unit, taking into consideration the difficulties and inaccuracies expected in the elementary analysis of polymeric compounds. The sulphur of both polysaccharides is present as acid-hydrolysable sulphate, and after hydrolysis of an appreciable quantity of each compound a distinct odour of acetic acid is apparent. Emission spectroscopy showed the ash of both compounds to contain large amounts of Na and only traces of other metals.

(2) *Absence of amino-acids.* Paper chromatography of polysaccharides B and D using 3N-HCl (16 hr., 100°) hydrolysates, phenol and collidine as solvents and ninhydrin as developer, indicates the absence of amino-acids in conditions which would detect 0.1% of any amino-acid.

(3) *Absorption of ultraviolet light.* The curves for aqueous solutions of polysaccharides B (0.05%, w/v) and D (0.2%, w/v) showed no significant absorption maxima.

(4) *Potentiometric titration.* The curves (Fig. 1) for the titration of the Na salts of polysaccharides B and D (20 mg.) with 0.1N-HCl are identical, and show that the carboxyl groups of their uronic acid groups are free. An approximate equiv. wt. of 600 is indicated for both polysaccharides. A statistical polymer unit, containing one molecular equivalent each of acetylhexosamine, uronic acid and H_2SO_4 , requires an equivalent weight under these conditions of 503.

Table 1. *The similar analysis of polysaccharides B and D*

	Polysaccharide B	Polysaccharide D	Sodium salt of a statistical polymer unit of acetyl-hexosamine, uronic acid and H_2SO_4 in 1:1:1 ratio
	(%)	(%)	
C	30.6	33.0	33.4
H	4.4	4.3	3.8
N (Dumas)	2.4	2.6	2.8
N (Kjeldahl)	2.6	2.8	—
Ash	26.3	23.5	—
Sulphated ash	27.5	26.0	28.2
S	6.1	5.7	6.4
P	Nil (less than 0.05%)	Nil (less than 0.05%)	
Hexuronic acid (Lefèvre-Tollens)	34.6	36.2	38.4
Van Slyke amino N (3N-HCl hydrolysis, 100°, 16 hr.)	2.6	2.8	2.8
Hexosamine (3N-HCl hydrolysis, 100°, 16 hr.)	33.6	34.0	35.5
N-acetyl (CH_3CO)	8.6	9.2	8.6

(5) *Reducing values after hydrolysis with 0.5N-HCl.* Table 2 shows the reducing values at various time intervals for solutions of polysaccharides B and D (0.1%, w/v) when hydrolysed at 100° with 0.5N-HCl in sealed tubes.

Fig. 1. Potentiometric titration of polysaccharides B (x) and D (o). 20 mg. material used in each case.

(6) *Presence of chondrosamine.* Paper chromatography of 3N-HCl hydrolysates (100°, 16 hr.) was carried out using collidine as solvent and the reagent of Elson & Morgan (1933) as developer. Many experiments on different batches of polysaccharides B and D, and in which chondrosamine, glucosamine and similarly hydrolysed samples of polysaccharide A (contains glucosamine only) and polysac-

(1937) give $[\alpha]_D^{20} + 95^\circ$, and Stacey (1944) gives $[\alpha]_D^{20} + 96^\circ$ for chondrosamine hydrochloride isolated from cartilage chondroitin sulphuric acid.

Polysaccharide D when treated similarly gave a crude product (40 mg.) having N, 5.3%; hexosamine hydrochloride, 87%. The recrystallized product had N, 6.6% and $[\alpha]_D^{20} + 92^\circ \pm 8^\circ$ in water (c, 0.5) after 24 hr.

A final check on the identity of these two compounds was given by the fact that their X-ray powder photographs were identical with that of chondrosamine hydrochloride, and not with that of glucosamine hydrochloride; we are indebted to Prof. A. R. Todd and Mr A. R. Gilson of the University Chemical Laboratory, Cambridge, for these photographs and to Prof. W. T. J. Morgan for a gift of authentic chondrosamine hydrochloride.

(7) *Paper chromatography for sugars and uronic acids.* Polysaccharides B and D were hydrolysed by 0.5N-HCl at 100° for 24 hr. Paper chromatograms of these hydrolysates using collidine and also butanol-acetic acid as solvent and aniline phthalate as developer indicated the absence of glucose, galactose and the more common hexoses and pentoses. Using butanol-acetic acid as solvent and aniline phthalate as developer, these hydrolysates were compared with similarly hydrolysed mixtures of chondrosamine hydrochloride, with D-glucuronolactone, D-galacturonic acid and alginic acid (as a source of D-mannuronic acid). D-Galacturonic acid and D-mannuronic acid are reasonably stable to acid hydrolysis (Link & Nedden, 1931-2; Link & Shoeffel, 1932), whereas D-glucuronic acid is easily degraded; this was apparent on the chromatogram. The results from paper chromatography favoured the presence of D-glucuronic acid and the absence of the other two uronic acids in both polysaccharide B and D, although extensive degradation products prevent a definite conclusion being reached.

Table 2. Reducing power of polysaccharides B and D after hydrolysis with 0.5N-HCl at 100°

(Estimations by method of Hagedorn-Jensen, and results calculated as % glucose.)

Hydrolysis (hr.) ...	Nil	1	2	4	5	7	16	24
Polysaccharide B	0.9	36.4	39.5	40.3	41.2	42.3	41.0	39.9
Polysaccharide D	1.6	38.9	43.1	45.2	46.7	46.2	44.9	43.6

charide C (contains both glucosamine and chondrosamine) were run on the same paper, showed that both the polysaccharides contained chondrosamine and no glucosamine.

Attempts to isolate chondrosamine hydrochloride directly from the Na salts of polysaccharides B and D failed, due to the extreme solubility of chondrosamine hydrochloride in aqueous solvents, and co-precipitation of NaCl from methanol and acetone mixtures. The Na salts were, therefore, converted into well washed Ba salts by the addition of barium acetate and ethanol. After hydrolysis with 3N-HCl (100°, 16 hr.) and removal of excess HCl by distillation and Ba quantitatively by H_2SO_4 , the chondrosamine hydrochloride was crystallized from methanol-acetone containing a drop of conc. HCl.

The crystals (150 mg.) were collected by centrifugation, washed with methanol containing 30% (v/v) acetone, then with acetone. Analysis: N, 6.3%; hexosamine hydrochloride, 95% by the method of Elson & Morgan (1933). They were recrystallized by dissolving in two drops of water, adding warm methanol until a slight opalescence was obtained, and leaving in the refrigerator. The recrystallized product had N, 6.6%; $[\alpha]_D^{24} + 94^\circ \pm 3^\circ$ in water (c, 2) after 24 hr. Calc. for $C_6H_{13}O_5N \cdot HCl$: N, 6.5%. Meyer & Smyth

Differences

In view of the close similarities of the two polysaccharides, the differences between B and D quoted here were confirmed on two further batches of each polysaccharide from different hog stomach pools.

(1) *Optical rotation.* $[\alpha]_D^{24}$ in water at pH 6.5-7.0 (c, 1): B, $-52^\circ \pm 3^\circ$; D, $-28^\circ \pm 3^\circ$.

(2) *Infrared spectra.* Mr L. C. Thomas, Chemical Defence Research Establishment, Porton, Wilts., examined the infrared spectra of polysaccharides B and D in the Perkin-Elmer (Model 21) recording infrared spectrophotometer, after evaporating aqueous solutions on an AgCl plate. Fig. 2 shows the absorption curves obtained. They are similar as one would expect, but B shows two significant peaks (X and Y) which are not shown by D, and D shows one peak (Z) which is not shown by B.

(3) *Uronic acid determination by the Dische carbazole method.* Uronic acid (%) calculated as glucuronic acid: B, 16.6; D, 31.6. The difference between these figures for D and those obtained by the Lefèvre-Tollens (1907) method, checks with the difference quoted by Dische (1947) for chondroitin sulphuric acid. The large difference between the

two values for samples of polysaccharide B is notable. We confirmed the facts quoted by Dische that D-galacturonic acid gives 20–25% lower results than D-glucuronic acid, and the D-mannuronic acid in alginic acid (for a sample of which we are indebted to Prof. E. L. Hirst) gives only approximately 20% of the value yielded by the classical method, but neither behaviour fits that of polysaccharide B.

Fig. 2. Infrared absorption spectra of polysaccharides B and D. Points at which there is a significant difference are X and Y for polysaccharide B and Z for polysaccharide D.

(4) *Rate of liberation of hexosamine by acid hydrolysis.* The total quantity of hexosamine in each compound is almost the same (see Table 1). Table 3 shows that the rate of hydrolysis in 0.5 N-HCl at 100° of the hexosamine groups reacting in the Elson & Morgan (1933) assay for polysaccharide B, is greater than the rate for D. Figures for the hydrolysis with 3 N-HCl at 100° confirm this result.

Table 3. *Rate of hydrolysis in 0.5 N-HCl at 100° of the hexosamine residues in polysaccharides B and D*

Hydrolysis (hr.)	Hexosamine (%)	
	Polysaccharide B	Polysaccharide D
1	11.6	10.3
2	21.1	16.1
3	23.3	16.4
5	25.4	18.9
7	28.7	18.1
16	30.1	23.8

(5) *Periodate oxidation.* Samples of the polysaccharides (5 mg. in 0.5 ml.) were mixed with 0.02 M-sodium metaperiodate (2.0 ml.) and allowed to stand at room temperature. After 24 and 48 hr. the excess periodate was estimated by the method of Barnaby (1916). The amounts of periodate reacting with samples of polysaccharides B and D, expressed as the number of periodate molecules per statistical polymer unit of uronic acid, acetylhexosamine and sulphate (1:1:1 ratio; mol.wt. 503), were as follows: 24 hr., B 0.8, D 0.4; 48 hr., B 1.1, D 0.6. In both cases no titratable acid was set free by the periodate treatment.

(6) Polysaccharide D is more soluble than polysaccharide B in aqueous ethanol alone, and in the presence of barium or sodium acetate.

(7) There is a significant difference between polysaccharides B and D in virulence-enhancing activity (see following paper).

DISCUSSION

The work described in this paper makes it quite clear that there are at least three different sulphated acid polysaccharides in preparations from whole hog gastric mucosae, which are somewhat inaccurately called hog gastric mucins. One has been identified as a heparin (Smith, Gallop, Harris-Smith & Stanley, 1952). The other two differ from the first, and from the mucoitin sulphuric acids of former workers, in that their hexosamine unit is chondrosamine; this fact has been adequately checked.

It is true that the products of other workers were probably mixtures of compounds, but the fact that we find no mucoitin monosulphuric acid in the preparations, whereas former workers have found no chondrosamine-containing compounds is confusing. There are two possible explanations for the former case; we started from fresh hog gastric mucosae, whereas other workers have used the commercial mucin of relatively unknown history. Former workers used alkaline conditions for extraction, and these may have degraded the mucoitin disulphuric acid to the monosulphuric acid; the compound reported by Meyer & Smyth (1938) and Meyer (1938) had a high sulphur content when isolated without alkaline treatment. It is relevant to point out here that a number of Japanese workers have recently been casting doubt on the existence of a mucoitin monosulphuric acid in hog gastric mucosae and in other animal tissues (Sasaki & Yasuoka, 1947; Takahashi & Yasuoka, 1947; Asagi, Masuma, Takahashi & Yasuoka, 1947; Masuma, 1949).

Working with tissues other than hog gastric mucosae, Meyer & Rapport (1951) and Meyer (1953) have recently described three chondroitin sulphuric acids, namely A, B and C; A is prepared from cartilage and the other two are present in connective tissue. The compounds were distinguished by their rotations and by enzymic data. Polysaccharide D agrees in rotation and analysis with the chondroitin sulphuric acid from cartilage (see also Meyer & Smyth, 1937; Meyer, 1938; Wolfrom *et al.* 1943). The difference between the Lefèvre-Tollens value for uronic acid, and that obtained by the Dische method, is also similar to that recorded by Dische (1947) for this chondroitin sulphuric acid. Polysaccharide B is also a chondroitin sulphuric acid but it is different from polysaccharide D. The preparation is homogeneous by electrophoresis and ultracentrifugation, but in view of some evidence for heterogeneity given by the partial solubility test, it would be unwise to name it as a new compound. Its rotation (-50°) agrees with that of the chondroitin sulphuric acid B described above, but further

comparison is difficult since our data are chemical whereas those of the above workers are enzymic. Work on this compound has been discontinued, since the main object of the research was the elucidation of factors involved in the virulence-enhancing action of mucin, in which polysaccharide B is an insignificant participant.

SUMMARY

1. There are at least three acid polysaccharides in preparations from whole hog gastric mucosae.

2. In addition to a heparin (Smith, Gallop, Harris-Smith & Stanley, 1952), there are two different chondroitin sulphuric acids (polysaccharides B and D). Polysaccharide D is probably the chondroitin sulphuric acid formerly isolated from cartilage.

3. These polysaccharides have been obtained homogeneous as evidenced by electrophoresis and ultracentrifugation; partial solubility studies yield no evidence for heterogeneity in polysaccharide D, but show polysaccharide B to be to some extent heterogeneous.

4. As far as the authors are aware, this is the first isolation of chondrosamine-containing polysaccharides from preparations of hog stomach mucosa; no mucoitin monosulphuric acid has been extracted by us from these preparations.

Our thanks are due to Mr R. Strange for analysis, and especially for paper chromatography. We are also indebted to Mrs P. W. Harris-Smith for assays of anticoagulation activity, and to Mr R. C. Hale for large-scale preparation of crude polysaccharides.

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ADDENDUM

Electrophoretic and Ultracentrifugal Examination of Polysaccharides B and D

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Samples of polysaccharides B and D prepared as described above, were examined in the electrophoresis apparatus and in the ultracentrifuge for evidence of heterogeneity.

METHODS

Electrophoresis. The experiments were carried out at 0° in the medium-size (10 ml.) U-tube of the Tiselius electrophoresis apparatus, using the diagonal schlieren optical

system. Approx. 1% (w/v) solutions of polysaccharides B and D were dialysed to equilibrium with the buffers used, observations being made at pH 8.0 and 4.5.

Ultracentrifuge. This part of the investigation was carried out with the 'Spinco' electrically driven ultracentrifuge, fitted with a diagonal schlieren optical system. Observations were made on approx. 1% (w/v) solutions at pH 8.0 in phosphate ($I=0.1$)-sodium chloride ($I=0.1$) buffer and at pH 4.5 in acetate ($I=0.1$)-sodium chloride ($I=0.1$) buffer. The rotor speed was 59750 rev./min. in all experiments.

RESULTS

Electrophoresis. Fig. 1 shows the patterns obtained in each of four experiments carried out. Both the polysaccharides have high anodic mobilities at pH 8 and these are not greatly reduced at pH 4.5.

Fig. 1. Electrophoretic patterns of polysaccharides B and D. The arrows indicate the direction of migration of the polysaccharide towards the anode. (a) In phosphate buffer, pH 8.0, $I=0.2$. (1) Polysaccharide B, 1% (w/v). Exposure after 110 min. Current, 7.6 ma. (2) Polysaccharide D, 0.75% (w/v). Exposure after 101 min. Current, 7.3 ma. (b) In acetate buffer, pH 4.5, $I=0.1$. (1) Polysaccharide B, 1% (w/v). Exposure after 91 min. Current, 8.2 ma. (2) Polysaccharide D, 0.75% (w/v). Exposure after 99 min. Current, 8.3 ma.

With such highly ionic compounds, large γ and ϵ boundaries are to be expected. On the likely assumption that the large stationary boundaries observed are purely buffer gradients, as was conclusively proved to be the case with polysaccharide A (Creeth & Record, 1952), the polysaccharides B

and D are also to be regarded as electrophoretically single components.

The electrophoretic mobilities calculated from the rate of migration of the cathode boundary were as follows:

	pH	Mobility (10^{-4} cm. ² /sec./V.)
Polysaccharide B	8	1.30
	4.5	1.19
Polysaccharide D	8	1.38
	4.5	1.29

Ultracentrifuge. Fig. 2 shows the sedimentation patterns obtained in each of four experiments. In all cases one symmetrical peak was obtained.

Fig. 2. Sedimentation patterns of polysaccharides B and D. Speed 59 750 rev./min. Migration from right to left. (a) In phosphate-sodium chloride buffer, pH 8.0, $I=0.2$. (1) Polysaccharide B, 1.15% (w/v). Exposure after 206 min. at full speed. (2) Polysaccharide D, 1.22% (w/v). Exposure after 215 min. at full speed. (b) In acetate-sodium chloride buffer, pH 4.5, $I=0.2$. (1) Polysaccharide B, 1.02% (w/v). Exposure after 216 min. at full speed. (2) Polysaccharide D, 1.00% (w/v). Exposure after 214 min. at full speed.

DISCUSSION AND SUMMARY

The fact that only one component is apparent both in electrophoresis and sedimentation, at two widely separated pH values, means that these methods show no evidence for heterogeneity in either polysaccharide B or D.

REFERENCE

Creeth, J. M. & Record, B. R. (1952). *Biochem. J.* **52**, 23.