fitting two groups of three probit-dosage regression lines, namely, $A, C, A/C$ and $H, C, H/C$.

Table 1 summarizes these combined results.

DISCUSSION

The assumption of parallelism was not contradicted in three of the individual comparisons, but the fourth, which was somewhat erratic, showed significant departure from parallelism. On combining the results as described in (2) and (3) above only (3) A/C showed a deviation from parallelism. In

all four experiments there was a tendency for the regression lines of the mixtures to be steeper than those of their constituents. The inflated variances of (3) H/C are due to a large heterogeneity factor.

The significance of the synergism of a mixture of polysaccharides A and C is undoubtedly shown by these tests. This mixture is estimated to be approximately 70% more active than if its constituents acted similarly: the corresponding figure for the mixture of the sample of heparin and polysaccharide C is approximately 50%.

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The Virulence-enhancing Factor of Mucins

6. IDENTIFICATION OF A HEPARIN AS THE MAIN COMPONENT OF THE THIRD FACTOR INVOLVED IN VIRULENCE ENHANCEMENT

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The third factor involved in the virulence-enhancing action of hog gastric mucin has been shown to be of predominantly carbohydrate nature, and has been split into two quite different active parts named polysaccharides A and C, which form a synergic combination (Smith, Gallop & Stanley, 1952). This paper describes the purification of polysaccharide A, the evidence that it is essentially homogeneous, its identification as a heparin, and its crystallization as an acid barium salt.

EXPERIMENTAL

The same remarks made in the previous paper (Smith *et al.* (1952) regarding the activities of virulence-enhancing samples, and analyses for hexosamine and hexuronic acid, are applicable here.

Purification of polysaccharide A

Polysaccharide A (4.3 g.), prepared as described by Smith *et al.* (1952), was dissolved in water (86 ml.). Solid barium acetate (3%, w/v) was added, the pH was adjusted to 6.9–7.1 and the mixture was left at 0–2° for several days. The precipitate was collected in a refrigerated centrifuge at 0–2° and washed twice (2 × 9 ml.) with ice-cold barium acetate solution (3%, w/v). The neutral Ba salt was dissolved in water (100 ml.) at pH 7.0 and Na_2SO_4 (1 g.) was added. The BaSO_4 was removed by centrifugation and the solution was dialysed against distilled water at 0°, in a

cellophan tube strengthened by an outer 'tyre' of calico, until free from sulphate. A trace of colloidal BaSO_4 was removed by filtering through well washed 'Celite' filter aid, and the solution was freeze-dried; yield 3.2 g., activity 3.9 (2.9–5.1) virulence-enhancing units (v.e.u.)/g. The pH of an aqueous solution was approximately 6, indicating that a mixture of acid and neutral Na salt (see below) was produced owing to a slight loss of sodium ions during dialysis.

When the polysaccharide had been identified as a heparin, it was found that the acid Ba salt could be crystallized directly from the neutral Ba salt, prepared as described above. Neutral Ba salt (50 mg.) was dissolved in warm water (2 ml.) and a trace of insoluble matter was removed by centrifugation. The solution was heated to 65° and glacial acetic acid (0.3 ml.) added; the mixture was cooled slowly to room temperature and then in a refrigerator. The sheaves of crystals (30 mg.) were removed by centrifugation, washed with 50% acetic acid, glacial acetic acid, absolute ethanol and ether, and dried in a vacuum desiccator over P_2O_5 .

Tests for homogeneity

Electrophoresis. An investigation carried out by our colleague, Dr B. R. Record, is described in the addendum to this paper; it showed polysaccharide A to be electrophoretically homogeneous at pH's 8.0 and 4.5.

Analyses carried out by us on the material migrating as the single peak, and on that in the stationary boundary, are given in Table 1; these analyses are proof that the latter was an artifact.

Ultracentrifugal analysis. Polysaccharide A was examined in a Spinco ultracentrifuge by Dr J. M. Creeth of the Courtauld Institute of Biochemistry, Middlesex Hospital, London, who writes in the addendum to this paper.

A 1% (w/v) solution in phosphate buffer at pH 8.0 and in acetate buffer at pH 4.5 gave sedimentation diagrams which were characteristic of a single homogeneous substance.

Fractional solubility test. The modification by Aminoff, Morgan & Watkins (1950) of the classical equilibrium method of solubility test was used because of the extreme solubility in water of polysaccharide A.

The chemistry of polysaccharide A, its identification as a heparin

The chemistry of polysaccharide A outlined below leaves no doubt that it is a heparin. Heparins are polymeric mucoitin sulphuric acids in which the number of sulphate groups can vary considerably (Jorpes & Gardell, 1948); details of the chemistry are given by Reinert & Winterstein (1939), Charles & Todd (1940), Wolfrom, Weisblat, Karabinos,

Table 1. *Analysis and virulence-enhancing activities of polysaccharide A and preparations obtained on its electrophoresis*

(A 2% (w/v) solution of polysaccharide A was used for electrophoresis and the samples (approx. 3 ml.) collected from the apparatus by Dr B. R. Record.)

Preparation	Electrophoretic fraction (%, w/v, from refractive index difference)	Wt. of material recovered* (mg.)	Analyses†			
			Hexuronic acid (Dische method)	After 16 hr. hydrolysis with 3N-HCl		Virulence enhancing activity (v.e.u./g.)
				Hexosamine (%)	SO ₄	
Polysaccharide A	.	.	34.6	25.2	+	3.9 (2.9-5.1)
Acetate buffer, pH 4.5:						
Migrating component	1.0	27	35.2	24.1	+	4.1 (2.0-7.6)
Stationary boundary	0.4	Less than 1	Nil	Nil	Nil	.‡
Phosphate buffer, pH 8.0:						
Migrating component	0.9	25	35.2	25.0	+	4.1 (2.0-7.6)
Stationary boundary	0.4	Less than 1	Nil	Nil	Nil	.‡

* After dialysis and freeze-drying. For the stationary boundary only half (1.5 ml.) dialysed.

† For migrating fractions, on material recovered after dialysis; for stationary boundary, on the solution from electrophoresis.

‡ There was insufficient material for an animal test in this experiment, but in a similar experiment, the material from the stationary boundary had negligible activity.

Table 2. *Fractional solubility test on polysaccharide A*

Analysis	Fraction				
	1	2	3	4	5
$[\alpha]_D^{30} \pm 10^\circ$	+50	+50	+52.5	+55	+52.5
Hexosamine (%)	25.3	24.0	23.8	23.6	25.2
Hexuronic acid (%) (Dische, 1947)	34.6	36.0	34.9	36.4	37.0
S (as acid-hydrolysable sulphate %)	10.6	10.8	11.0	11.2	11.2

A small quantity (150 mg.) of polysaccharide A was shaken vigorously with successive quantities (8-10 ml.) of 50% (v/v) aqueous ethanol at room temperature for 0.5 hr. After each addition of aqueous ethanol and subsequent shaking, the suspension was left at 0-2° overnight and centrifuged at this temperature. The material (approx. 20 mg.) was recovered from each supernatant by drying over P₂O₅ at 0.1 mm. Hg and was dissolved to give solutions of known concentration. These solutions were analysed for optical rotation, hexosamine liberated by 16 hr. hydrolysis at 100° with 3N-HCl, hexuronic acid by the Dische method (1947), and S as sulphate hydrolysable by 6N-HCl in 16 hr. at 100°. The results are given in Table 2, and bearing in mind the limits of the assays on such small quantities of material, no heterogeneity is indicated by these figures.

McNeely & McLean (1943), Wolfrom & Rice (1946, 1947), Jorpes, Boström & Mutt (1950), Wolfrom, Montgomery, Karabinos & Rathger (1950) and Meyer & Schwartz (1950). Samples of heparin used by these workers were, in the main, obtained from liver and lung tissue; the analyses indicated the presence of an average of two to three sulphate groups for each hexosamine-uronic acid unit in the polymer chain.

Elementary analysis, hexosamine and hexuronic acid analysis and optical rotation. Table 3 shows the analytical figures for polysaccharide A together with those published by Reinert & Winterstein (1939) and Wolfrom *et al.* (1943)

Table 3. Analytical figures for polysaccharide A and the neutral sodium salt of heparin

	Heparin					
	Reinert & Winterstein (1939).		Wolf from <i>et al.</i> (1943)		Calc. for mucoitin disulphuric acid	
	Polysaccharide A. Mixture of neutral and acid Na salt	Neutral sodium salt from crystalline barium salt	'Roche' heparin neutral sodium salt	Neutral sodium salt from crystalline barium salt	Neutral salt, C ₁₅ H ₁₆ O ₁₀ NS ₂ Na ₃	Acid salt, C ₁₂ H ₁₇ O ₁₀ NS ₂ Na ₃
C (%)	25.3	23.4	.	.	25.6	26.6
H (%)	3.7	3.5	.	.	2.8	3.2
N (%)	2.5 (Dumas)	2.6	2.6	.	2.5	2.6
	2.7 (Kjeldahl)					
S (%)	11.7	11.9	11.7	.	11.3	11.8
Na (from sulphated ash, %)	10.5	11.6	12.0	12.4	12.2	8.5
P (%)	Nil	Nil	0.1	.	Nil	Nil
Hexosamine (%)	.	.	.	.	31.7	33.0
(1) Elson & Morgan (1933) after 3N- or 4N-HCl hydrolysis	25.2	.	24.7	23.8	.	.
(2) Van Slyke after 3N-HCl hydrolysis (100°, 24 hr.)	30.0	.	.	.	.	.
Hexuronic acid (%)	.	.	.	.	34.4	35.8
(a) Lefèvre & Tollens method	26.6	.	24.5	.	.	.
(b) Dische method (1947)	34.6	.	.	.	.	.
[α] _D ^{20-25°} in water	+53° (c, 1) (±2)	+45° (c, 1) corrected for 6.9% water)	+42° (c, 2)	+55° (c, 2)	.	.

for the neutral Na salts of different samples of heparin, and those calculated for an average polymer unit composed of a mucoitin disulphuric acid. Hydrolysis with 3N-HCl at 100° was needed to liberate the maximum quantity of hexosamine; only 7.5% was set free by 16 hr. hydrolysis with 0.5N-HCl: some resistance to acid hydrolysis has been noted for heparin (Jorpes, 1946).

The sample contained no inorganic sulphate. After hydrolysis with 3N-HCl for 6 hr., sulphate was recovered equivalent to 11.3% S: this shows that all the S was present as ester sulphate.

Amino N. (a) *Van Slyke method.* After hydrolysis with 3N-HCl at 100° for 24 hr. polysaccharide A gave 2.35% amino N: glucosamine hydrochloride gave a recovery of 99.4% after the same hydrolysis. With 6N-HCl at 100° for 24 hr. the corresponding figures were: polysaccharide A, 2.31%, glucosamine hydrochloride, 87.8% recovery.

On applying the Van Slyke method to polysaccharide A without previous hydrolysis, the amino N figure increased with the time of reaction with HNO₃ at 25° (2 min. 0.47%, 30 min. 1.03%, 60 min. 1.34%, 90 min. 1.97%). Extrapolation to zero time gave a strong indication that the hexosamine amino group is fully substituted. The same result has been reported for heparin (Wolfson *et al.* 1943).

(b) *Ninhydrin method.* No α -amino N was detectable in polysaccharide A by the ninhydrin method after 24 hr. hydrolysis with 6N- and 3N-HCl. This indicates the absence of amino-acid residues, and it is confirmed by evidence from paper chromatography and absorption of ultraviolet light (see below).

Paper chromatography. (a) *D-Glucosamine.* 24 hr. (100°) hydrolysates of polysaccharide A with 3N- and 6N-HCl were examined on one-dimensional paper chromatograms using a mixture of collidine and lutidine as solvent. On developing with alkaline acetonylacetone followed by *p*-dimethylaminobenzaldehyde (Partridge, 1948), the presence of D-glucosamine and the absence of chondrosamine was indicated.

(b) *Absence of amino-acids.* The same hydrolysates were examined on a two-dimensional paper chromatogram using phenol and collidine as solvents and ninhydrin as developer. No amino-acid spot was visible.

(c) *Absence of various sugars.* 1, 5 and 24 hr. hydrolysates (100°) of polysaccharide A with 0.1N-, 0.5N- and 3N-HCl were examined on one-dimensional paper chromatograms, using butanol and a mixture of collidine and lutidine as solvents and aniline phthalate as developer. Glucose, ribose, galactose, fructose, mannose, xylose and galacturonic acid were also placed on the same paper. No sugar spot, but only pink smears, were apparent in the hydrolysates. Furfural, which is formed readily from D-glucuronic acid, gives a pink colour with the developer, but is too volatile to give a definite spot on the paper (see also Partridge, 1950).

Absence of N-acetyl groups. After much controversy, it is now generally agreed that heparins do not contain any appreciable quantity of N-acetyl groups (Wolfson *et al.* 1943; Jorpes *et al.* 1950). On hydrolysis with 25% (w/v) *p*-toluenesulphonic acid, both under reflux for 2 hr. and in a sealed tube at 100° for 16 hr., the maximum amount of distillable acid from several different quantities of polysaccharide A was equivalent to less than 0.5% N-acetyl (CH₃CO—). The same method gave consistent results for the appreciable N-acetyl groups in polysaccharide B (see Smith *et al.* 1952), and in a sample of crude blood group A substance.

Emission spectroscopy for metals. This was carried out by Mr L. C. Thomas of the Chemical Defence Experimental Station, Porton, Wilts. A solution of polysaccharide A (not the ash) was sprayed into a flame. The emission spectrum produced indicated the presence of 9.4% Na, 0.2% Ca and traces of Al, Si, Mg, Cu, Ag and Fe.

Absorption of ultraviolet light. The ultraviolet light absorption of polysaccharide A was examined in a Unicam quartz spectrophotometer in a cell 1 cm. thick. A 0.05% (w/v) solution of polysaccharide A in water at pH 7 gave an absorption curve which is shown in Fig. 1. There is no significant increase in absorption at 2600 and 2800 Å. Nucleic acids and aromatic amino-acids, respectively, absorb ultraviolet light at these wavelengths.

Fig. 1. The ultraviolet absorption spectrum of polysaccharide A (0.05% (w/v) in water, pH 7).

Fig. 2. Potentiometric titration of polysaccharide A (20 mg.).

Potentiometric titration. Polysaccharide A (20 mg.) was dissolved in water (2.5 ml.); one drop of 0.5N-NaOH was added and the pH rose from 6.1 to 10.4. This solution was then titrated against 0.01245N-HCl, the pH being measured by a glass electrode. The curve (Fig. 2) for this titration shows that the uronic acid carboxyl group is free, as is that in heparin (Wolfson *et al.* 1943; Jorpes, 1946). The amount of acid (2.7 ml.) required to carry the titration from pH 6.5 to 3.0 indicates an approximate equivalent weight of 595. Di- and tri-sodium salts of mucoitin disulphuric acid would have equivalent weights of 541 and 563 respectively.

Isolation of D-glucosamine hydrochloride. The Ba salt of polysaccharide A (0.5 g.) was refluxed for 16 hr. with 3N-HCl (50 ml.). Approximately the correct quantity of extra Ba needed to precipitate the excess sulphate was added as BaCl₂. After removing the BaSO₄ by centrifugation, the liquid was concentrated to a syrup under reduced pressure to remove excess HCl. Water (50 ml.) was added, the solution was freed from barium and sulphate ions and evaporated to a syrup. After standing in a refrigerator overnight crystals formed, the quantity being increased by cautiously adding acetone. These crystals (90 mg., $[\alpha]_D^{25}$ in water = +66.2° (c, 0.4), 96.4% hexosamine hydrochloride by the method of

and in polarized light. We are indebted to our colleague Mr E. O. Powell for these photographs.

Found on material dried at 60° over P₂O₅ at a pressure of 0.1 mm. Hg, C, 22.3; H, 3.4; N, 2.4; S, 9.9; Ba, 22.3; ash (sulphated), 36.7%; hexosamine liberated by 16 hr. hydrolysis with 3N-HCl, 22.8%; hexuronic acid by the method of Tollens & Lefèvre, 23.3%, by the method of Dische (1947) 29.9%. Calc. for the acid Ba salt of a polymer with an average unit of mucoitin disulphuric acid, i.e. C₁₈H₁₇O₁₆NS₂Ba, C, 22.8; H, 2.7; N, 2.2; S, 10.1; Ba, 21.7; ash (sulphated), 36.8; hexosamine, 28.2; hexuronic acid, 30.4%.

Fig. 3. Crystals of the acid barium salt of polysaccharide A in ordinary (a) and polarized (b) light. Magnification, $\times 500$.

Elson & Morgan, 1933) were recrystallized from a mixture of water, methanol and acetone. Found: N, 6.5. Calc. for C₆H₁₃O₅N, HCl: N, 6.5%; $[\alpha]_D^{25}$ in water = +71° $\pm$ 2° (c, 1), after 24 hr.; the generally accepted value for glucosamine hydrochloride is $[\alpha]_D^{25}$ = +72.5°. As a final check on the identity, an X-ray powder photograph gave a diffraction diagram which was the same as that for an authentic specimen of D-glucosamine hydrochloride and not that for an authentic specimen of chondrosamine hydrochloride. We are indebted to Prof. A. R. Todd, F.R.S., and Mr A. R. Gilson of the Chemical Laboratory, Cambridge, for these X-ray powder photographs, and to Dr W. T. J. Morgan, F.R.S., for the gift of the chondrosamine hydrochloride.

Molecular weight. Dr B. R. Record and Dr J. M. Creeth, in their addendum to this paper, give the approximate molecular weight of polysaccharide A as 16 500 from a combination of ultracentrifuge and diffusion data.

Crystallization of polysaccharide A as an acid barium salt

Polysaccharide A (0.5 g.) was dissolved in water (20 ml.) and 5% (w/v) barium acetate (7 ml.) was added. After standing 4 days at room temperature, a trace of insoluble matter was removed by centrifugation. To the supernatant at 65° glacial acetic acid (5 ml.) at the same temperature was added; the mixture was allowed to cool slowly to room temperature and then in a refrigerator. The sheaves of crystals (0.35 g.) were collected by centrifugation, washed with 50% acetic acid, glacial acetic acid, absolute ethanol and ether, and dried in a vacuum desiccator over P₂O₅. Fig. 3 shows photomicrographs of the crystals in ordinary

The acid Ba salt of polysaccharide A had $[\alpha]_D^{25}$ in water = +47° ($\pm$ 2°) (c, 1). Wolfrem *et al.* (1943) give $[\alpha]_D^{25}$ in water = +44° and +47.5° (c, 2) for two separate samples of the acid Ba salt of heparins.

After decomposing with sodium sulphate and removing the BaSO₄ by centrifugation, the crystals of the acid Ba salt of polysaccharide A had a virulence-enhancing activity of 3.5 (2.2–5.3) v.E.U./g.

The anticoagulation activity of polysaccharide A and the virulence-enhancing activities of authentic samples of heparin

Table 4 shows the anticoagulation activity of polysaccharide A compared with that of three different, highly active, samples of heparin from Boots Pure Drug Co. Ltd. (102 i.u./mg.), Evans, Lescher and Webb Ltd., (93 i.u./mg.) and Roche Products Ltd. (120 i.u./mg.). Fresh ox blood (3.75 ml.) was mixed with solutions (0.3 ml.) of different strengths of the samples under test and the times for complete coagulation were noted, using the technique described by Jorpes (1935, 1942). Polysaccharide A has a slightly greater activity than that of the three authentic heparin samples.

Corresponding virulence-enhancing activities are: polysaccharide A 3.9 (2.9–5.1), Boots heparin 3.2 (2.1–4.8), Evans, Lescher & Webb heparin 2.2 (1.4–3.3), Roche heparin 3.5 (2.3–5.1) v.E.U./g., determined by the assay used for the third factor

involved in virulence enhancement (Smith, Harris-Smith & Stanley, 1951). Adequate controls ruled out any possibility of the toxicity of these samples being a factor in the virulence enhancement at the concentration used for the test.

In addition to these commercial samples of heparin, Dr Bergel of Roche Products Ltd. kindly supplied us with a purified sample of sodium heparinate with the following analytical figures: S, 13.3, ash 40.2%, $[\alpha]_D = +42.5^\circ$ for the anhydrous material. The material, which contained 8.1% of water, had an anticoagulation activity 130 i.u./mg. This sample had a virulence-enhancing activity of 3.5 (2.3–5.4) v.e.u./g.

In addition to the similar virulence-enhancing activities and anticoagulation activities of polysaccharide A and heparin, our colleague Dr H. P. Lambert had found that polysaccharide A inhibits sheep cell haemolysis, and that this effect is due to an anticomplementary action. Heparin has already been shown to be anticomplementary (Ecker & Cross, 1929).

sulphuric acid, and had a small positive optical rotation ($+2^\circ$) and an appreciable acetyl content (6.4%). It was tested for heparin activity, but it was reported that it contained only 1% of the activity of the crystalline barium salt of heparin. No criteria of purity were given for any of these preparations, and they were undoubtedly mixtures of compounds. We would point out here that our crude hog gastric mucin and commercial samples are made from the whole mucosae. The excreted mucus itself, however, has appreciable virulence-enhancing activity (Smith 1951b), and tests on the 'peptic' extract (see Smith, 1951b) of this mucus showed it to have appreciable anticoagulant activity.

It is well known that, owing to their highly ionic nature, polysaccharide sulphates resemble nucleic acid in readily forming complexes with proteins, these complexes being sparingly soluble at pH values on the acid side (Meyer, 1938). It is not surprising, therefore, that impure samples of the third factor (Smith, 1950b, 1951a) contained much protein and were sparingly soluble below pH 5. This strong

Table 4. *Anticoagulation test on polysaccharide A and three different heparin samples*

(Technique using fresh ox blood as Jorpes, 1935, 1942.)

Strength of anticoagulant solution (%, w/v)	Anticoagulant (mg./100 ml. blood)	Time for complete coagulation (hr.)							
		Polysaccharide A		Boots heparin		Evans heparin		Roche heparin	
		Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
0.0156	1.25	No clot	96	84	84	24	36	48	36
0.0125	1.0	No clot	48	24	36	18	36	48	36
0.00625	0.5	4	5	5	4	2.5	5	9	4
0.00313	0.25	2	2	1.25	1.25	1.25	1.25	2	1.5
0.00156	0.125	1	1	0.5	0.5	0.5	0.5	0.75	1.25
Nil	Nil (saline control)	0.25		0.25		0.3		0.3	

DISCUSSION

The work described in this paper establishes beyond reasonable doubt that the main component of the third factor involved in the virulence-enhancing action of hog gastric mucin is a heparin: both the chemical and biological properties have been adequately checked.

In addition to its significance in virulence enhancement, the isolation of a crystalline heparin from crude hog gastric mucin has not been reported before. Meyer, Smyth & Palmer (1937) described as the 'acid polysaccharide' of crude hog gastric mucin (Wilson's Laboratories, Chicago) an acetylated mucoitin monosulphuric acid with a negative optical rotation, which was isolated after initial alkaline treatment. Later Meyer & Smyth (1938) and Meyer (1938) described the acid calcium salt of a mucoitin sulphuric acid isolated without alkaline digestion. This contained 9.1% of sulphur, indicating more sulphate groups than a mucoitin mono-

association with protein to form complexes of varying size was demonstrated also by the virulence-enhancing activities of some samples obtained from an impure batch of third factor in the preparative Spinco ultracentrifuge. Four samples were taken from different depths in the ultracentrifuge tube, and there was no significant difference in their virulence-enhancing activities.

The presence of a highly ionic sulphate group in polysaccharide A and in synthetic detergents, e.g. tergitol (Young & Zelle, 1946; Barnes, 1947), which are also virulence-enhancing, is extremely interesting. It is impossible, however, to attribute the virulence-enhancing activity, or for that matter anticomplementary and anticoagulant activity, solely to the presence of sulphate groups in view of the chemical and biological properties of polysaccharide B (see Smith *et al.* 1952). The more recent chemistry of heparin (Meyer & Schwartz, 1950; Wolfrom *et al.* 1950) has shown the presence of glucosamine-*N*-sulphate groups ($-\text{NH}-\text{SO}_3\text{H}$),

and the biological activities above may possibly be related to these particular groups.

The fact that a heparin is the main component in the third factor involved in virulence enhancement may give rise to some thought on the wide anatomical occurrence of heparin, and also on its therapeutic use. It must be remembered, however, that heparin is only really effective in enhancing virulence when coupled with the non-specific factors (a particulate residue and a viscous medium), and needs to be combined with polysaccharide C for a maximum effect (see Smith *et al.* 1952). In addition, as has been pointed out before (Smith, 1951*b*), the method of testing virulence-enhancing samples involves a completely artificial infection of mice with *Bact. typhosum* (*Salmonella typhi*) (Smith, 1950*a*) and in no way resembles natural infection. Nevertheless, the presence of heparin in relatively large amounts in such tissues as the lung, liver and spleen (Jorpes, 1946) is worth bearing in mind in any studies on natural infection.

SUMMARY

1. The main component of the third factor involved in the virulence-enhancing action of hog gastric mucin has been obtained essentially homogeneous as judged by electrophoresis, ultracentrifugation and fractional solubility. It has been identified as a heparin by its chemical and biological properties.

2. This is the first isolation of a heparin mucocitin disulphuric acid from crude hog gastric mucin obtained from whole hog mucosae.

3. The significance of these findings is discussed.

Our thanks are due to Dr D. W. Henderson for his interest in this work. We are also indebted to Mr R. Strange for microanalysis and paper chromatography and to Mr S. Peto for statistical analysis of results.

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ADDENDUM

Physico-chemical Examination of Polysaccharide A

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The polysaccharide, which had been twice purified by precipitation as the neutral barium salt and conversion to the sodium salt, was finally dialysed against water and freeze-dried. It was examined in the electrophoresis apparatus and the ultracentrifuge and diffusion measurements were made at various concentrations by two independent methods. The partial specific volume was also determined. The data obtained lend support to the characterization of the polysaccharide as a heparin. The degree of homogeneity of the material has also been assessed and a value calculated for its molecular weight.

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. In every case the solutions were dialysed to equilibrium with the buffer used (ionic strength, $I=0.2$). Observations were made at two different pH's, 8.0 and 4.5, and at two concentrations, 2.0 and 0.7% at each pH. The 2.0% solutions were examined in order to reveal any traces of contaminating material; those at the lower concentration were used for the calculation of the electrophoretic mobility of the material.

Ultracentrifuge. This part of the investigation was carried out with the 'Spinco' electrically driven ultracentrifuge, fitted with a diagonal schlieren optical system. The experimental technique followed exactly that outlined by Creeth (1952) and Charlwood (1952). The solutions were prepared directly either in phosphate or phosphate-sodium chloride buffer, pH 8.0, total I , 0.2; one run was made at 1.0% concentration in acetate buffer pH 4.5, $I=0.10$. The rotor speed was 59 780 rev./min. in all experiments. As the sedimentation constant is comparatively low, the distances moved by the boundary are rather small; accordingly between seven and ten photographs of the sedimenting boundary position were obtained at regular intervals in each experiment.

Diffusion. Measurements of the diffusion constant were made by two entirely independent methods: (1) By means of the standard Tiselius electrophoresis apparatus using the diagonal schlieren optical system to determine the concentration distribution. The measurements were carried out at 0°. (2) More accurately, especially in the low concentration range, by the Gouy interferometric diffusion apparatus (Coulson, Cox, Ogston & Philpot, 1948; Creeth, 1952). The measurements in this case were made near 25°.

Partial specific volume. This parameter was determined at 25° in the usual pycnometric manner at two concentrations, 2.0 and 1.0%, in phosphate buffer, pH 8.0; I , 0.2.

RESULTS

Electrophoresis. Fig. 1 shows the patterns obtained in each of the four experiments carried out. In every case, there appeared to be present, on casual inspection, two components; a major component migrating rapidly to the anode and a minor non-migrating component. By careful removal of this latter 'component' it was possible to show that it was in fact none other than the result of a δ or ϵ boundary, and consisted only of the buffer used. (See Table 1 of the main part of this paper.) The polysaccharide was therefore electrophoretically a single component. It has a high anodic mobility at pH 8.0 and this is not greatly reduced at pH 4.5. These observations fit in well with the chemistry of the material as outlined in the main part of this paper. The highly ionic nature of the material also explains the abnormally large boundary anomalies observed.

Records of similar measurements made on heparin (see Wolfrom & Rice, 1947) point to a behaviour closely resembling that of polysaccharide A, though it would appear probable that the large proportion of 'stationary component' reported by these workers is none other than an enhanced δ and ϵ boundary.

Since the electrophoretic mobility of the polysaccharide may be an important characteristic of the material, mobility values have been calculated in each case from the rate of movement of the cathode descending boundary. From the cross-sectional area of the U-tube and the current passing, the operative potential gradient was computed using the conductivity as measured on the original solution at 0°. For a 0.7% solution at pH 8.0 the mobility was found to be 1.41×10^{-4} cm.²/sec./V. A slightly lower value, 1.27×10^{-4} cm.²/sec./V., was obtained for a 0.75% solution at pH 4.5 (see Fig. 1). These values fall within the range given for heparin by Wolfrom & Rice (1947).

Sedimentation. At all concentrations and at both pH 4.5 and 8.0, only one, apparently symmetrical, peak was obtained. Fig. 2 shows the experimental record obtained at 1% concentration.

value -0.778 ± 0.068 (s.e.). The extrapolated value of the sedimentation constant at zero concentration is then 2.25 ± 0.11 (95% fiducial limits), the value used in calculating the molecular weight.

Fig. 1. Electrophoresis patterns of the polysaccharide A. The arrows indicate the direction of migration of the polysaccharide towards the anode. A, in phosphate buffer pH 8.0, $I=0.2$; (a) 2.0% solution: exposure after 1 hr. 46 min., current = 9.3 ma., mobility = 1.26×10^{-4} cm.²/sec./V.; (b) 0.7% solution: exposure after 2 hr. 8 min., current = 6.5 ma., mobility = 1.41×10^{-4} cm.²/sec./V. B, in acetate buffer pH 4.5, $I=0.2$; (a) 2.0% solution: exposure after 2 hr., current = 5.0 ma., mobility = 1.18×10^{-4} cm.²/sec./V.; (b) 0.7% solution: exposure after 2 hr., current = 7.4 ma., mobility = 1.27×10^{-4} cm.²/sec./V.

Fig. 2. Sedimentation pattern of polysaccharide A. 1% concentration in phosphate buffer pH 8.0, $I=0.20$. Speed = 59 780 rev./min. Interval between exposures = 32 min. Migration from right to left.

Table 1. Sedimentation constants of polysaccharide A

(Solvent: Phosphate buffer pH 8.0, $I=0.20$, unless otherwise stated.)

Concentration (%)	0.13	0.13	0.28	0.30	0.40	0.47	0.60	1.0	1.0*
S_{20}	2.10	2.18	2.08	1.96	1.98	1.94	1.75	1.47	1.58

* In this experiment, the solvent was sodium acetate, pH 4.5, $I=0.10$.

The sedimentation constant of the substance is markedly dependent on concentration, as is shown by the results summarized in Table 1.

All S_{20} values are given in Svedberg units and have been reduced to sedimentation in water.

The S_{20} values in phosphate buffer are approximately linearly related to concentration, the slope of the line expressing the regression of the sedimentation constant on concentration having the

The limits of error of this value, 4.9%, are somewhat higher than the corresponding limits observed with albumins (Creeth, 1952; Charlwood, 1952); this reflects the greater difficulty in dealing with substances of lower sedimentation constant, especially when this shows an appreciable variation with concentration.

Diffusion. All diffusion constants were corrected for viscosity and temperature to diffusion in water

at 20°, and the coefficients are quoted in units of 10^{-7} cm.² sec.⁻¹.

The diagonal schlieren patterns obtained with 1.5 and 0.70 % concentrations of the polysaccharide (undialysed) in pH 8.0 phosphate buffer, $I = 0.20$, were somewhat asymmetric, and yielded D_A values of 7.87 and 7.47 respectively. A dialysed solution of 0.70 % concentration in sodium chloride ($I = 0.10$), phosphate ($I = 0.10$), buffer at pH 8.0 (total $I = 0.20$), gave almost symmetrical concentration distributions, yielding mean values of $D_A = 6.91$ and $D_M = 6.63$. These values are consistent with those obtained with the Gouy diffusimeter (Table 2).

The molecular weight of 16 500 may be compared with the value of about 20 000 for the sodium salt of heparin, quoted by Wolfrom, Montgomery, Karabinos & Rathger (1950), though this figure is not of much significance if it has been calculated from diffusion measurements alone on the assumption that the molecule is spherical. For example, taking our value for D_{20} of 6.66×10^{-7} we find for a spherical molecule a molecular weight of 163 000, a figure nearly ten times too large.

It has not been possible in this brief investigation to give any estimate of the degree of polydispersity of the polysaccharide. In common with its class (see,

Table 2. *Diffusion coefficients for polysaccharide A*

(V = refraction increment of polysaccharide in wavelengths of retardation.)

Concentration (%)	0.28	0.31	0.40	0.51	0.56
V	6.96	7.78	9.88	12.73	13.85
D_{20}	6.63	6.64	6.39	6.63	6.77
	6.68	6.53	6.54	6.75	6.81

There is thus little apparent dependence of the diffusion coefficient on concentration over the lower concentration range. In view of the greater accuracy and consistency of the Gouy method with dilute solutions (Creeth, 1952) the mean value of the results in Table 2, $D_{20} = 6.66 \pm 0.09$ (s.d.) has been used for the subsequent calculation of the molecular weight.

DISCUSSION

The fact that only one component is apparent in both electrophoresis and sedimentation, at two widely separated pH's, adds considerable weight to the evidence that the polysaccharide A consists of a single well defined molecular species.

Combining the values of the sedimentation and diffusion constants with the value of 0.50 obtained for the partial specific volume, the figure of 16 500 is obtained for the molecular weight of the substance.

The frictional ratio, f/f_0 , has a value of 2.15; this corresponds to an axial ratio of 25 if the molecule is assumed to be an unhydrated rod.

for example, Record, 1948; Record & Stacey, 1948) a certain degree of polydispersity is to be expected, but a study of the sedimentation diagrams suggests that in this case it is probably not extensive.

SUMMARY

1. The polysaccharide A is homogeneous both in electrophoresis and in sedimentation.

2. The molecular weight calculated from the diffusion coefficient (6.66×10^{-7}), the sedimentation constant, $C \rightarrow 0$ (2.25×10^{-13}) and the partial specific volume (0.50) is 16 500, the frictional ratio 2.15 pointing to marked molecular asymmetry.

3. The electrophoretic mobility of the material is 1.41×10^{-4} cm.²/sec./V. in pH 8.0 phosphate and 1.27×10^{-4} cm.²/sec./V. in pH 4.5 acetate buffer, being anodic in each case. The electrophoretic behaviour is that of a highly ionized molecule.

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