The Virulence-enhancing Factor of Mucins

7. THE REMAINING COMPONENTS OF THE 'THIRD FACTOR' INVOLVED IN VIRULENCE ENHANCEMENT

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In preceding papers of this series (Smith, 1950*a, b*, 1951*a*; Smith, Harris-Smith & Stanley, 1951) the virulence-enhancing action of mucin has been shown to be a synergic combination of a viscous medium, a particulate residue and a more specific 'third factor'. The activity of the latter, in a biological assay in which the other two factors were kept constant (Smith *et al.* 1951), was shown to reside in the carbohydrate moiety (Smith, Gallop & Stanley, 1952) which has been separated into several components. Polysaccharide A, the most active component, was purified and identified as a heparin (Smith, Gallop, Harris-Smith & Stanley, 1952). Impure polysaccharide B, a sulphated compound, had a low activity, a proportion of which could be attributed to contamination with polysaccharide A. The latter has now been removed (see preceding paper); as was expected the activity of polysaccharide B (see below) then dropped to a value which ruled out this polysaccharide as a significant participant in the virulence enhancement. Polysaccharide C, also impure, was a fairly active neutral polysaccharide, and formed a synergic combination with polysaccharide A in the biological assay (Smith, Gallop & Stanley, 1952). The preceding paper described the discovery and purification of a further polysaccharide, polysaccharide D; it has a significant virulence-enhancing activity (see below) similar to that of polysaccharide C.

This paper deals mainly with the identification of polysaccharide C as the blood-group mucoid of both A and H activity, which has been previously isolated by Morgan & King (1943) and Bendich, Kabat & Bezer (1946). Proof is also provided that the virulence-enhancing activity in the biological assay for the third factor is not specifically connected with either blood-group A or H activity (see also Smith, 1950*a*).

In addition, as befits the concluding paper of the series, various observations are made on the virulence-enhancing action of whole 'mucin', now that the chief factors operating in this complex system have been identified.

EXPERIMENTAL AND RESULTS

The remarks on the determination of uronic acid and hexosamine and the meaning of 'Virulence-Enhancing Units' (v.e.u.) made by Smith, Gallop & Stanley (1952) apply here.

The relative blood-group activities (A and H) of various samples were determined by comparing the highest dilution of them which inhibits the agglutinating action of the same batch of anti-A or anti-H agglutinin on A and O cells, respectively. We are indebted to Prof. W. T. J. Morgan for gifts of the requisite sera (anti-A human serum, anti-H rabbit serum) and instruction on the technique of these agglutinations. Only the relative values of samples from one fractionation are given here, but a few samples tested by, or under the supervision of, Prof. W. T. J. Morgan showed that absolute activities were high and of the same order as those of materials which were isolated by him.

Virulence-enhancing activities of polysaccharides B and D

Several batches of polysaccharides B and D, prepared as described in the previous paper, were assayed. Weighted means of these activity assays gave: polysaccharide B, 0.5 (0.3-0.7) v.e.u./g.; polysaccharide D, 1.0 (0.8-1.2) v.e.u./g.

Examination of polysaccharide C isolated as described by Smith, Gallop & Stanley (1952)

The following analysis of a typical batch of polysaccharide C (activity 1.4 (0.9-2.0) v.e.u./g.) clearly indicated its similarity to the blood-group mucoid of Morgan & King (1943), Bendich *et al.* (1946) and Aminoff, Morgan & Watkins (1950), in spite of the contamination with sulphated polysaccharides (S, approx. 1%; ash, 4.5%); N, 5%; reducing substances as glucose (0.5N-HCl at 100°, Somogyi method), 46%; acetyl, 8.5%; hexosamine (0.5N-HCl at 100°), 28.5%. Paper chromatography indicated the presence of galactose and fucose, and of the following amino-acids: lysine, arginine, aspartic acid, glutamic acid, glycine, serine, alanine, threonine, valine, leucine and proline.

This chemical similarity between polysaccharide C and the blood-group mucoid was confirmed by positive tests for high blood-group A and H activity.

Purification of polysaccharide C from the viscous constituents of crude mucosal extracts from pooled hog stomachs

Originally polysaccharide C was obtained together with the other polysaccharides from the residue of the crude

mucosal autolysate, after extracting the viscous constituents with water at pH 4.3–4.5 (Smith, Gallop & Stanley, 1952). At the same time it was suggested that a better source of polysaccharide C would be the weakly active viscous constituents (activity 0.08 (0.04–0.15) v.e.u./g.); this proved to be correct. The main object of the fractionation was to obtain the blood-group mucoid free from sulphated polysaccharides. A 90% phenol-ethanol fractionation as described by Morgan & King (1943) was carried out directly on the viscous constituents; the product contained 0.7% S. The latter was removed by introducing the barium acetate-ethanol separation, as in the original preparation of polysaccharide C (Smith, Gallop & Stanley, 1952), before using the 90% phenol-ethanol fractionation.

Preliminary treatment of the viscous constituents. The viscous constituents from 200 stomachs (1000 g.) were dialysed at pH 3 at 0° against distilled water and the precipitate which formed (Smith, 1950b) was removed by centrifugation and discarded. The freeze-dried supernatant was treated with trypsin at pH 8 and 37°, and dialysed as described by Smith, Gallop & Stanley (1952) for the crude third factor.

Barium acetate-ethanol fractionation. (i) 30–50% (v/v) ethanol fraction. The trypsin-treated material (300 g.) (0.88 (0.45–1.8) v.e.u./g.) was dissolved in water to a concentration of 5% (w/v) at pH 7; it was treated with barium acetate and ethanol and the fractions decomposed with Na_2SO_4 as described by Smith, Gallop & Stanley (1952). The yields, virulence-enhancing activities and relative blood-group activities (A and H) are shown in Table 1.

Table 1. *Yields, virulence-enhancing activities and relative blood-group A and H activities of the first barium acetate-ethanol fractionation*

(Initial material, 300 g. (0.88 (0.45–1.8) v.e.u./g.).)

Fraction (ethanol % v/v)	Yield (g.)	Virulence-enhancing activity (v.e.u./g.)	Relative blood-group activity	
			A	H
0–30	51	1.9 (1.3–3.0)	1/8	1/500
30–50	170	1.4 (1.2–1.7)	1	1
Soluble in 50	47	0.2 (0.1–0.3)	1/8	1/8

The main fraction (30–50% (v/v) ethanol) still contained 0.5% S. However, the following extension of the barium acetate-ethanol fractionation reduced the S content to a low figure which dropped to a negligible value after the subsequent ethanol-90% phenol separation.

(ii) 40–55% (v/v) ethanol fraction. The 30–50% (v/v) ethanol fraction (160 g.) above was dissolved in water (3200 ml.) at pH 7, and barium acetate to a final concentration of 3% (w/v) was added. An ethanol fractionation was carried out and the precipitate decomposed with Na_2SO_4 as before. The precipitates at 40% (v/v) ethanol (S, 1%) and 55% (v/v) ethanol (S, 0.18%) were collected. The yields, virulence-enhancing activities and relative blood-group A and H activities are given in Table 2.

90% (w/v) phenol-ethanol fractionation. The 40–55% (v/v) ethanol fraction (10 g.) was shaken with 90% (w/v) phenol solution (1 l.) and then fractionated with ethanol as described by Morgan & King (1943). The material insoluble in 90% (w/v) phenol (2.5 g.) and the 10% (v/v) ethanol fraction (5.7 g.) had virulence-enhancing activities of

1.8 (1.3–2.9) and 1.2 (1.1–1.5) v.e.u./g., respectively, and relative blood-group activities of 1 (A), 1 (H) and 1 (A), 1 (H). The 10% (v/v) ethanol fraction contained S, 0.08%.

Table 2. *Yields, virulence-enhancing activities and relative blood-group A and H activities of the second barium acetate-ethanol fractionation*

(Initial material, 160 g. (1.4 (1.2–1.7) v.e.u./g.).)

Fraction (ethanol % v/v)	Yield (g.)	Virulence-enhancing activity (v.e.u./g.)	Relative blood-group activity	
			A	H
0–40	46	0.8 (0.4–1.4)	1	1
40–55	95	1.4 (0.8–2.3)	1	1
Soluble in 55	4	0.4 (0.2–1.1)	1/16	1/500

Preparation of polysaccharide C from two separate pools of stomach autolysates which had high blood-group A and low blood-group A activity, respectively

To determine whether virulence-enhancing activity was connected with either blood-group A or H activity, two samples of polysaccharide C which showed a large difference in such activity were obtained by extracting individual stomachs (Bendich *et al.* 1946; Annison, Chadwick, Morgan & Smith, 1949).

The autolysis of 300 individual stomach mucosae was carried out as described by Smith (1950a). After a few days samples of the autolysates were neutralized in phosphate buffer and examined in a rough quantitative test for high A and low A blood-group activity. The appropriate autolysates (193 high A, 107 low A) were then bulked into two carboys; treating each carboy separately, viscous constituents were separated as before (Smith, Gallop & Stanley, 1952), and then fractionated as described above.

Table 3. *Yields, sulphur values, virulence-enhancing activities and relative blood-group A and H activities of samples obtained from two separate pools of stomach autolysates*

	30–50% (v/v) barium acetate-ethanol fraction	10% (v/v) ethanol-90% phenol fraction
(a) Autolysates of high blood-group A activity		
Yield (%)	58	58
Virulence-enhancing activity (v.e.u./g.)	1.4 (1.0–1.8)	1.1 (0.8–1.4)
Blood-group activity { A	1	1
{ H	1	1
S (%)	0.4	0.09
(b) Autolysates of low blood-group A activity		
Yield (%)	55	55
Virulence-enhancing activity (v.e.u./g.)	1.3 (0.9–1.7)	1.2 (0.9–1.6)
Blood-group activity { A	1/256	1/256–1/512
{ H	16	16–32
S (%)	0.5	0.07

Table 3 shows the yields, S values, virulence-enhancing activities and relative blood-group activities (A and H) of samples taken at two stages in the fractionation. It is

Table 4. *Analytical results on two samples of polysaccharide C of high blood-group A and H activity, respectively*

	High blood-group A activity	High blood-group H activity
S (%)	0.09	0.07
P (%)	Nil	Nil
Anticoagulation activity	Nil at 1% (w/v)	Nil at 1% (w/v)
Uronic acid	Nil	Nil
Hexosamine (3N-HCl, 100°, 16 hr.) (%)	37.6	36.7
Reducing value as glucose (0.5N-HCl, 100°, 5 hr.) by Hagedorn-Jensen method (%)	59.5	61.8
N (%)	5.6	5.6
Acetyl (%)	10.1	10.7
Ash (sulphated) (%)	0.9	0.7
Paper chromatography:		
(a) Hexosamine	Glucosamine Chondrosamine	Glucosamine Chondrosamine
(b) Sugars (0.5N-HCl, 100°, 24 hr.)	Fucose Galactose	Fucose Galactose
(c) Amino-acids (3N-HCl, 100°, 16 hr.)	Lysine Arginine Aspartic acid Glutamic acid Glycine Serine Alanine Threonine Valine Leucine Isoleucine Proline	Lysine Arginine Aspartic acid Glutamic acid Glycine Serine Alanine Threonine Valine Leucine Isoleucine Proline

obvious that there is no specific connexion between blood-group A or H activity, and virulence-enhancing activity, in the assay for the third factor.

Chemistry of the two samples of polysaccharide C

Table 4 summarizes the analysis on these two samples of polysaccharide C; any contamination with sulphated polysaccharides is now negligible. The results agree with those quoted by Morgan & King (1943), Bendich *et al.* (1946) and Aminoff *et al.* (1950) for the blood-group mucoids, and with the well-known fact that there is no significant difference in analysis between the blood-group A and H mucoids.

Our colleague Dr B. R. Record, who writes an addendum to this paper, states that electrophoretic and ultracentrifugal diagrams showed no evidence for heterogeneity.

The difference in activity of polysaccharide C when used alone to maintain the standard viscosity of the assay and when tragacanth is used

We now know that polysaccharide C is a component of the third factor, as well as a contributor to the viscosity effect of the whole mucin. A concentration of 0.1% (w/v) of polysaccharide C is necessary to kill approximately 50% of the mice in the standard assay of the third factor involving an injection with 0.4% (w/v) charcoal in a tragacanth mucilage of constant viscosity (Smith *et al.* 1951). In past work, before the identification of polysaccharide C as a lesser component of the third factor, high concentrations (1.75–

3.5%) of it, at various stages of purity, were used to maintain constant the initial viscosity of the injection solutions (Smith, 1950a; Smith, 1951a; Smith *et al.* 1951). It is true that we should not have expected any virulence-enhancing effect in those experiments involving injection without particulate residue and which result in no mouse deaths. However, in those experiments involving some particulate residue, and in certain cases (Table 3, Smith *et al.* 1951) a relatively large amount, the death rates were not excessive, and certainly not what one would have expected after injecting seventeen or more times the quantity of third factor needed to give approximately 50% mouse deaths in the normal assay.

The following figures confirm that polysaccharide C gives a much lower activity if its inherent viscosity is maintained at the constant value, than if tragacanth is used. The 30–50% (v/v) barium acetate-ethanol fractions of the two samples (high A activity, high H activity) of polysaccharide C, prepared as described above, had activities, by the normal assay procedure, of 1.4 (1.0–1.8) and 1.3 (0.9–1.7) v.e.u./g., respectively. Solutions of these samples at 1.5% (w/v) concentration had viscosities equal to the constant value used in the normal assay procedure. The usual batch of charcoal was suspended in the solutions at 0.4% (w/v) concentration and these suspensions were used in the normal assay without the addition of tragacanth. Death rates in a total of 300 mice for each sample were compared with those of the standard third factor in the usual manner. Results of 0.04 (0.03–0.06) and 0.02 (0.01–0.03) v.e.u./g., respectively, were obtained.

Table 5. *Change in viscosity of solutions of tragacanth and polysaccharide C after being in the peritonea of live mice for several hours*

(The time quoted is that taken for the ball to fall from a Michell's ball and cup viscosimeter; this is a measure of the viscosity of the solution. Normal peritoneal fluid of mice gave a figure of 1.6 sec. Temp., 23–30°.)

Sample	Concn. (%, w/v)	Time in mouse peritoneum (hr.)	Average time for ball to fall from Michell's viscosimeter	
			Before injection (sec.)	After the time interval in the mouse peritoneum (sec.)
30–50% (v/v) barium acetate- ethanol fraction	1.5*	6	1.6	1.6
	3.0	6	7.8	2.8
Tragacanth	0.13*	6	1.0	3.1
	0.26	6	6.6	11.5

* Viscosity, determined in an Ostwald viscosimeter, approximately equal to that used in the biological assay.

The change of viscosity of solutions of tragacanth and polysaccharide C after being present in the peritonea of mice for several hours

When the viscosity effect was discovered, it was suggested (Smith, 1950*b*) that not only was the initial viscosity of the injected solution important, but also the degree to which it is maintained under the influence of the body fluids of the host. The following experiments show that the probable explanation of the different results outlined above, lies in the fact that the viscosity of solutions of polysaccharide C is destroyed in the peritonea of mice far more rapidly than that of tragacanth suspensions.

Since the amount of material harvested from the peritoneum of a mouse is small, a Michell's ball and cup viscosimeter yielded results sufficiently accurate and was used for this work.

Various solutions of tragacanth and polysaccharide C were prepared at pH 7 and heated to 60° for 45 min. The viscosity was determined in an Ostwald viscosimeter at 37° as for the normal assay, and then the average of ten readings on the Michell's ball and cup viscosimeter was taken. Each solution (0.5 ml.) was then injected intraperitoneally into 25–50 mice and after several hours the mice were killed and the peritoneal fluid removed. Usually the contents of the peritonea of five mice were bulked for viscosity measurements, giving 5–10 readings for each batch of mice and appropriate solution.

Table 5 shows the results obtained in one of four similar experiments. As well as solutions with viscosities equal to that used in the normal assay procedure, solutions of higher viscosity were used, since the former gave such low, and therefore inaccurate, results with the ball and cup viscosimeter.

Reconstitution of a 'virulence-enhancing mucin' by combining the main factors involved

According to the work described in this series of papers, the main factors involved in the virulence-enhancing action of mucin are a viscous medium, a particulate residue and a third factor; the latter is composed of polysaccharide A (a heparin), polysaccharide D (a chondroitin sulphuric acid) and polysaccharide C, which is present in an amount sufficient to contribute materially to the viscosity of the original mucin. These factors were now added together one

by one and the virulence-enhancing effect noted. Only the composition of the injected solutions is given here. These solutions were treated as in the normal assay procedure, and injected with 5000 *Bacterium typhosum* (*Salmonella typhosa*) cells, i.e. one-thousandth of the LD₅₀ if the organisms were injected in tryptic meat broth. Adequate controls ruled out the possibility of toxicity in any of these injection solutions. Table 6 shows the details of these experiments.

Table 6. *Reconstitution of an active 'mucin' by combining the main factors involved in virulence enhancement*

(General details of the test are given by Smith *et al.* (1951). Adequate controls (20 mice) injected without *Bact. typhosum* showed the complete absence of toxicity in any of the solutions. Further controls in each experiment checked the fact that the LD₅₀ for *Bact. typhosum* (Ty2) is 5×10^6 when injected in tryptic meat broth. The viscosities of the injected solutions were determined, and all were the same and equal to that used in the biological assay for virulence enhancement (Smith *et al.* 1951).)

Injection solution		Deaths in batches of 50 mice when injected with 5000 <i>Bact. typhosum</i>		
Nature	Concn. (% w/v)	Exp. 1	Exp. 2	Exp. 3
Polysaccharide C	1.5	0	2	2
Polysaccharide C	1.5	11	7	12
Particulate residue	0.25			
Polysaccharide C	1.5	30	21	24
Particulate residue	0.25			
Polysaccharide A	0.05			
Polysaccharide C	1.5	—	38	29
Particulate residue	0.25			
Polysaccharide A	0.05			
Polysaccharide D	0.1			

Polysaccharide C (a mixture of the two final samples of high blood-group A and high blood-group H activity) at 1.5% (w/v) concentration has a viscosity equivalent to that used in the standard biological assay, but produces practically no deaths if injected alone (see also Smith, 1950*b*, 1951*a*; Smith *et al.* 1951). The addition of 0.25% (w/v) of autoclaved particulate residue prepared as described by

Smith (1951*a*) from crude mucin, results in a few deaths. A notable increase in deaths is produced by adding 0.05% (w/v) of polysaccharide A. This death rate is further increased by adding finally 0.1% (w/v) of polysaccharide D. Our statistician colleague, Mr S. Peto, tells us that the increase in death rate for each step in the reconstitution is significant in individual experiments, except for two cases (Exp. 2, 2-7 and Exp. 3, 24-29), and becomes highly significant when the data of all three experiments are combined.

DISCUSSION

In this paper the remaining components of the third factor involved in the virulence-enhancing action of mucin have been identified as polysaccharide D (a chondroitin sulphuric acid) and polysaccharide C, the blood-group mucoid originally isolated by Morgan & King (1943) and Bendich *et al.* (1946). It has been proved that the third factor activity of the latter is not specifically connected with either blood-group A or H activity. The activity of both these remaining components of the third factor is approximately of the same order, and much less than that of polysaccharide A. Polysaccharide C is present in an amount sufficient to contribute materially to the viscosity of the original mucin, and therefore probably has two functions. Its activity as part of the third factor is much lower if its inherent viscosity is used to maintain the standard value, which is normally given by a tragacanth mucilage, in the assay for virulence enhancement. This is almost certainly due to the more rapid reduction of the viscosity of solutions of polysaccharide C, than that of tragacanth solutions, by the tissues of the host. As was emphasized when it was initiated (Smith *et al.* 1951), the biological assay for the third factor was intended for use merely as an instrument for fractionation of the latter, and in fact has resulted in the separation of the three components; it gives results which cannot be related, quantitatively, directly to the activity of the original mucin. The effect of various samples of 'blood-group substance' used in previous work, and attributed at the time solely to the viscosity effect, is in reality a combination of a smaller viscosity effect and a weak third factor effect.

Having established the nature of the main factors involved in virulence enhancement, the final experiment of this work was a successful attempt to reconstitute a virulence-enhancing 'mucin' from these factors step by step; the final mixture was powerfully virulence-enhancing. However, it would be unwise to claim that this 'mucin' is identical with the original mucin for a number of reasons. Many substances contribute to the viscosity of mucin in addition to polysaccharide C; the residue used above is that insoluble at pH 9 and has been

autoclaved, whereas the residue in the original mucin is that insoluble at pH 7. Although the amounts of the materials used are roughly of the same order as their yields in the above processes, the same proportions would not be present in the original mucin, due to unequal losses in the fractionations.

Since this is the last paper of the series, the results of this investigation on the virulence-enhancing activity of so-called hog gastric mucin are summarized as follows. The material examined was prepared from whole hog gastric mucosae, as are all similar commercial preparations (e.g. Wilson's Granular Mucin), and not, as the name 'mucin' implies, solely from the adhering mucus, although the latter is virulence-enhancing (Smith, 1951*b*). The activity is due to a synergic combination of a viscous medium, a particulate residue and a third factor which is composed of a highly active heparin (polysaccharide A), a chondroitin sulphuric acid (polysaccharide D) and the neutral blood-group mucoid (polysaccharide C). The last contributes to the viscosity of the original mucin, and it probably plays a dual role; its activity is not specifically connected with blood-group A or H activity. This complex system has been worked out for the enhancement of the virulence of *Bact. typhosum* in mice. The relative importance of the various factors operating might well vary with the nature of the organisms establishing the infection, and the particular host defence mechanisms called into play in combating that infection.

In conclusion, it is hoped that the results of this research, which has been carried out purely on a chemical basis, will help further investigation on the mode of action of these preparations and the role of mucin in pathogenesis of mucosal infections.

SUMMARY

1. The remaining components of the third factor involved in virulence enhancement are a chondroitin sulphuric acid (polysaccharide D) and the neutral blood-group mucoid (polysaccharide C) of Morgan & King (1943) and Bendich *et al.* (1946); the activity of the latter is not connected specifically with either blood-group A or H activity.

2. The work in this series has been summarized and a virulence-enhancing 'mucin' has been reconstituted from the various factors involved.

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REFERENCES

- Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1950). *Biochem. J.* **46**, 426.
 Annison, F. F., Chadwick, D. W., Morgan, W. T. J. & Smith, H. (1949). *Nature, Lond.*, **164**, 61.
 Bendich, A., Kabat, E. A. & Bezer, A. E. (1946). *J. exp. Med.* **83**, 485.
 Morgan, W. T. J. & King, H. K. (1943). *Biochem. J.* **37**, 640.
 Smith, H. (1950*a*). *Biochem. J.* **46**, 352.
 Smith, H. (1950*b*). *Biochem. J.* **46**, 356.
 Smith, H. (1951*a*). *Biochem. J.* **48**, 441.
 Smith, H. (1951*b*). *J. infect. Dis.* **88**, 207.
 Smith, H., Gallop, R. C., Harris-Smith, P. W. & Stanley, J. L. (1952). *Biochem. J.* **52**, 23.
 Smith, H., Gallop, R. C. & Stanley, J. L. (1952). *Biochem. J.* **52**, 13.
 Smith, H., Harris-Smith, P. W. & Stanley, J. L. (1951). *Biochem. J.* **50**, 211.

ADDENDUM

Electrophoretic and Ultracentrifugal Examination of Polysaccharide C (Blood-Group Mucoïd)

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Two samples of polysaccharide C of high blood-group A (sample 752) and high blood-group H (sample 753) activity, respectively, prepared as described above, were examined in the electrophoresis apparatus and in the ultracentrifuge for evidence of heterogeneity.

The methods used were exactly as described in the addendum to the previous paper (Record & Grinstead, 1953). Approximately 1% (w/v) solutions of each sample in buffers at pH 8.0 and pH 4.5 were examined. Only one component was apparent in both electrophoresis and sedimentation patterns, at two widely separated pH values. The electrophoretic mobilities calculated in each case from the

rate of migration of the cathode boundary were as follows:

Sample	pH	Mobility (10^{-4} cm. ² /sec./V.)
752	8	0.16
	4.5	0.08
753	8	0.16
	4.5	0.06

Fig. 1 shows the electrophoretic and ultracentrifuge patterns for the two samples in acetate buffer at pH 4.5. No evidence for heterogeneity was shown by these methods in the two samples of polysaccharide C.

Fig. 1. Electrophoretic and ultracentrifugal patterns of two samples (752, 753) of polysaccharide C: (a) Electrophoretic patterns in acetate buffer, pH 4.5, $I=0.1$. The arrows indicate the direction of migration of the polysaccharide to the anode. (1) Sample 752, 1% (w/v). Exposure after 322 min. Current, 8.5 ma. (2) Sample 753, 1% (w/v). Exposure after 322 min. Current, 8.5 ma. (b) Ultracentrifugal patterns in acetate-sodium chloride buffer, pH 4.5, $I=0.2$. Speed 59750 rev./min. Migration is from right to left. (1) Sample 752, 1% (w/v). Exposure after 56 min. at full speed. (2) Sample 753, 1% (w/v). Exposure after 56 min. at full speed.

REFERENCE

- Record, B. R. & Grinstead, K. H. (1953). *Biochem. J.* **53**, 671.