The Virulence-enhancing Factor of Mucins

5. THE DIFFERENT COMPONENTS OF THE 'THIRD FACTOR' INVOLVED IN VIRULENCE ENHANCEMENT

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Previous work (Smith, 1950*a*, *b*, 1951; Smith, Harris-Smith & Stanley, 1951) has shown that the virulence-enhancing action of hog gastric mucin is due to a synergic action between two non-specific factors (a viscous medium and a particulate residue) and a more specific soluble 'third factor'. Partial purification of the latter (Smith, 1951) yielded a heterogeneous product which was predominantly peptide in nature, but which contained 5–10% of carbohydrate residues.

This paper describes further purification of the third factor using a revised assay (Smith *et al.* 1951) in which the two non-specific factors are maintained constant. It has been shown that the carbohydrate moiety is responsible for the activity. Furthermore, this moiety has been separated into at least two active fractions, which have different chemical and biological properties, and which, when combined together, show a synergic effect in the system of the revised assay. This is an important result affecting investigations on the mode of action of mucin.

In the light of this work, various bacterial polysaccharides have been examined for virulence-enhancing activity in the assay for the third factor: they are active and the results are included in this paper, together with a report on their other relevant biological properties.

EXPERIMENTAL AND RESULTS

Methods

Activities are given in 'Virulence-enhancing units' (V.E.U.) (Smith *et al.* 1951) by direct comparison with a standard mucin, with fiducial limits shown in brackets for $P=0{\cdot}95$. The yields and activities of samples obtained from a particular fractionation are quoted for a single experiment. The activities of the various fractions may not be significantly different when taken alone, but become so when coupled with similar results in a number of such fractionations on different batches of the third factor. The inclusion of all the latter results would be superfluous.

Hexosamine, which had been liberated from the various polysaccharides by hydrolysis with HCl at 100° in sealed tubes, was estimated by the method of Elson & Morgan (1933) using the $NaHCO_3$-Na_2CO_3 buffer of Immers & Vasseur (1950) for the acetonylacetone condensation. Replicate assays on different hydrolysates showed the method to have an error of ±5% for most of the compounds used (see also Wolfrom, Weisblat, Karabinos, McNeely & McLean, 1943; Meyer, 1945).

Uronic acid was determined by the carbazole method of Dische (1947), and by the modification due to Tracey (1948) of the classical Lefèvre & Tollens (1907) method. Discrepancies between the two methods for heparin and chondroitin sulphuric acids have been noted by Dische (1947). Replicate assays showed that the error of the Dische method was ±5%, and of the classical method ±3% for the compounds used.

Large osmotic pressures developed during the dialysis of the majority of products, probably due to their highly ionic nature; the cellophan dialysis tubes were therefore protected by calico 'tyres'.

Preparation of crude third factor from fresh hog gastric mucosae

When the full role of a viscous medium and particulate insoluble matter in enhancing virulence was appreciated, and a more accurate biological assay for the third factor was available (Smith *et al.* 1951), the methods already in use (Smith, 1950*b*, 1951) for removing the two non-specific factors from the crude third factor were re-examined. It became apparent that these methods, which were established using an assay (Smith, 1950*a*) now known to be obsolete for determination of the third factor, were essentially sound, and only slight modification was needed to improve yields. The procedure which has been used is as follows:

(1) Autolysis of 200 fresh hog gastric mucosae, precipitation with industrial ethanol, and defatting with trichloroethylene (not dichloroethylene) were as described by Smith (1950*b*).

(2) The viscous constituents were removed at 0° by four aqueous extractions (30, 10, 10 and 10 l.) at pH 4·3–4·5, yielding 800–1000 g. dry matter. This material was assayed for the third factor in two ways. At a concentration of 0·4% (w/v) it was combined with charcoal (0·4%, w/v) and dilute tragacanth mucilage as in the normal procedure for virulence-enhancing samples (Smith *et al.* 1951). At a concentration of 6·5% (w/v) it had an inherent viscosity equal to that used in the normal assay: 0·4% (w/v) charcoal was added and the normal assay procedure was then carried out without the addition of tragacanth mucilage. The former method gave an activity of 0·08 (0·04–0·15) V.E.U./g., and the latter 0·02 (0·01–0·04) V.E.U./g. and a weighted mean of these figures gave 0·05 (0·03–0·07) V.E.U./g.

(3) The crude third factor (130–150 g.) was then extracted from the insoluble residue (120–130 g.) by the method described by Smith (1951) except that the second alkaline

extraction was also made with 1% (w/v) $NaHCO_3$ (48 hr.) instead of with 1% (w/v) Na_2CO_3 (24 hr.). A typical batch had an activity of 0·43 (0·19–0·82) v.e.u./g.

Fractionation between pH's 9 and 5 (Smith, 1951) has been omitted from the process because the above substitution of $NaHCO_3$ for Na_2CO_3 leaves only a small amount of residue at pH 9 in the preparation. This was easily removed, together with the material precipitated by 15% $(NH_4)_2SO_4$, at the next stage of purification: also a relatively large amount of activity remained in the material soluble at pH 5.

Precipitation of extraneous matter with 15% (w/v) ammonium sulphate

Crude third factor (150 g., activity 0·43 (0·19–0·82) v.e.u./g.) was dissolved in water (6%, w/v) at pH 6·9–7·1. Solid $(NH_4)_2SO_4$ was added with stirring to 15% (w/v), the pH was readjusted to 6·9–7·1 and the mixture left at 0–2° for several days. The precipitate was removed by centrifuging at 45 000–50 000 rev./min. in the batch bowl of a Sharples centrifuge which had been refrigerated at 0–5°. The precipitate was dissolved in water and, together with the supernatant, was dialysed at 0° against distilled water until free from $(NH_4)_2SO_4$. To stop bacterial action, a little toluene was added to the material in the bag. Finally, both fractions were freeze-dried. The 15% $(NH_4)_2SO_4$ precipitate, yield 34 g., had activity 0·22 (0·08–0·39) v.e.u./g.; the material soluble in 15% $(NH_4)_2SO_4$ solution, yield 88 g., had activity 0·53 (0·30–0·95) v.e.u./g.

Experiments showed that no further fractionation was possible with higher $(NH_4)_2SO_4$ concentrations: even after saturation with $(NH_4)_2SO_4$ the supernatant contained appreciable activity.

Treatment with trypsin followed by dialysis

Treatment with trypsin resulted in no loss of activity. Subsequent dialysis removed from the active fraction a large proportion of the peptide material (biuret test) and some nucleic acid residues (phosphorus analysis and absorption at 2600 A.).

The material soluble in 15% (w/v) $(NH_4)_2SO_4$ solution (88 g., activity 0·53 (0·30–0·95) v.e.u./g.) was dissolved in water (5%, w/v) containing 1% (v/v) $CHCl_3$ as a preservative; the pH of the mixture was adjusted to 8·0 and the temperature to 37°. Crystalline trypsin (0·125%, calculated on the initial quantity of material soluble in 15% $(NH_4)_2SO_4$ solution) (Armour and Co. Ltd.) was added, and the mixture allowed to stand for 4 hr. at 37°. The large amount of nucleic acid in the mixture provided an effective buffer and the pH dropped very little. The pH was readjusted to 8·0, a further quantity of trypsin (0·125%) was added and the mixture left at 37° for 16 hr.

The action of the trypsin was followed by measuring the liberated α-amino groups using the method of Pope & Stevens (1939). The titration figures showed that the action of the trypsin was almost complete after 1 hr. The fact that maximum action had occurred after 20 hr. was checked by taking a small sample and adding 0·5% trypsin; after a further 1 hr. at 37° no increase in the number of α-amino groups was discernible.

The mixture was then dialysed at 0° against distilled water for several days. The contents of the dialysis bag were freeze-dried. Yield 55 g.; activity 1·0 (0·65–1·5) v.e.u./g. The material passing through the membrane had negligible activity.

Separation of different polysaccharides by fractionating with barium acetate and ethanol, followed by removal of nucleic acid by precipitating with acid

Analysis of barium acetate/ethanol fractions for nucleic acid, peptides and hexosamine; virulence-enhancing activities of selected fractions. The material (2·5 g.), after treatment with trypsin, was dissolved in water (5%, w/v), solid barium acetate was added to 3% (w/v) and the pH adjusted to 6·9–7·1. After standing in the refrigerator overnight, the precipitate that formed was removed by centrifugation at 0°. The supernatant was fractionated by adding various concentrations of ethanol, and, after standing overnight, removing the precipitates at 0°. Each precipitate was dissolved in water (20 ml.) and decomposed with Na_2SO_4 (0·5 g.); $BaSO_4$ was removed by centrifugation and the excess Na_2SO_4 by dialysis, and finally the solution was freeze-dried. The following determinations were made on each fraction: (1) total P; (2) absorption of light of wavelength 2600 A. by the same depth of 0·005% (w/v) aqueous solution; (3) biuret test of Kingsley (1939) on a 0·2% (w/v) aqueous solution; (4) a determination of the hexosamine set free by 16 hr. hydrolysis with 0·5 N-HCl at 100°.

The results, which are shown in Table 1, suggested a procedure in which the following fractions were separated at 0°, decomposed with Na_2SO_4, dialysed and freeze-dried as described above: (1) precipitate on adding 3% (w/v) barium acetate; (2) precipitate on adding ethanol to 10% (v/v); (3) precipitate on adding ethanol to 50% (v/v); (4) supernatant from fraction 3. The yields, analyses and virulence-enhancing activities of these fractions are given in Table 2.

Combination of the barium acetate/ethanol fractionation with removal of nucleic acid by precipitating at pH 2. The fractionation outlined in Table 2, and assays for virulence enhancement on various electrophoretic samples, indicated that neither the nucleic acid nor the peptide material was connected with virulence enhancement. Preliminary experiments on the individual fractions 1, 2 and 3 above showed that the nucleic acid could be removed as a precipitate of low activity by adjusting the reaction of an aqueous solution (5%, w/v) to pH 2, combined with, in the case of fractions 2 and 3, the addition of ethanol to 10 and 20% (v/v) respectively. The active material was then precipitated from the supernatant by adding ethanol to 50% (v/v) and a little sodium acetate. This procedure has now been coupled with the barium acetate/ethanol fractionation. In the process which follows it is imperative that close attention be paid to the temperature of fractionation and centrifuging, and the concentration of constituents, otherwise undue mixing of fractions results.

An aqueous solution (5%, w/v) of trypsin-treated third factor (55 g., activity 1·0 (0·65–1·5) v.e.u./g.) containing barium acetate (3%, w/v) was adjusted to pH 6·9–7·1 and left at 0–2° for several days. The precipitate which formed was removed at 0–2° at 4000 rev./min. in the angle head of a refrigerated centrifuge, washed once with ice-cold 3% (w/v) aqueous barium acetate (100 ml.) and freeze-dried. Fraction *A* (7·5 g.). If the mixture froze at any stage in this process fraction *A* was contaminated with fraction *B*. If this occurred, the mixture was stirred for several hours at room temperature and then cooled again to 0–2°.

Ethanol was added to the supernatant and washings to 10% (v/v) and the mixture was left at 0–2° overnight. The precipitate was removed and washed twice (2 × 100 ml.) as described above with 3% (w/v) barium acetate solution to which ethanol had been added to 10% (v/v). It was then freeze-dried. Fraction *B* (24·9 g.).

The ethanol content of the supernatant and washings was raised to 50% (v/v) and after standing at 0–2°, the precipitate was removed, washed twice (2 × 100 ml.) with 3% (w/v) barium acetate/50% (v/v) ethanol as described above, and freeze-dried. Fraction *C* (19·1 g.). The supernatant was reserved.

Fraction *B* was treated as described for fraction *A* except for two points. After adjusting the pH of the mixture to 1·8–2·0, ethanol was added to 10% (v/v). The final product was precipitated in a rather gelatinous state and was best removed in the batch bowl of the Sharples centrifuge. (Polysaccharide B (5·0 g.); activity 0·74 (0·44–1·3) v.e.u./g.)

Fraction *C* also was treated as described for fraction *A* except for two points. A concentration of 20% (v/v) ethanol was used in the nucleic acid precipitation mixture and 2% (w/v) sodium acetate was added to the 50% (v/v) ethanolic precipitation of the active material (3·6 g.); activity 1·9 (1·3–2·9) v.e.u./g. This material contained hexuronic acid and ester sulphate residues (see below under polysaccharides A and B). Most of these residues could be removed in a precipitate (1·7 g., activity 1·5 (1·2–1·9) v.e.u./g. after decomposing with Na_2SO_4) obtained by adding ethanol to 30% (v/v) to a 5% (w/v) solution at pH 7 containing 3% (w/v) barium acetate. Polysaccharide C was precipitated by raising the ethanol concentration to 50% (v/v). After reaction with Na_2SO_4 and centrifugation to remove $BaSO_4$, polysaccharide C was dialysed against distilled water and freeze-dried. (Yield 1·1 g.; activity 2·1 (1·7–2·6) v.e.u./g.)

Table 1. *Yields and analyses of samples from the barium acetate/ethanol fractionation.*

	Fractions precipitated									In supernatant after adding 50% (v/v) ethanol
	By 3% (w/v) barium acetate solution alone	With ethanol added to 3% (w/v) barium acetate solution to give the following % (v/v)								
		2·5	5·0	7·5	10	15	25	35	50	
Yield (%)	10	12	16	8	4	2	6	2	6	10
Absorption of light of wavelength 2600 A. by 0·005% (w/v) solution (1 cm. depth) (%)	47	42	79	88	88	68	55	10	6	7
P (%)	1·8	1·4	4·2	5·1	5·8	3·2	1·0	0·5	0·6	0·25
Biuret test on 0·2% (w/v) solution	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Trace	Strong
Hexosamine liberated by 0·5 N-HCl at 100° for 16 hr. (%)	5·0	22	12·5	4·5	6·3	14·5	15·3	22·0	24·5	7·5

Neg. = negligible.

Table 2. *Yields, analyses and virulence-enhancing activities of selected barium acetate/ethanol fractions*

(Initial trypsin-treated material 0·87 (0·45–1·7) v.e.u./g.)

Fraction no.	Yield (%)	Virulence-enhancing activity (v.e.u./g.)	*P* (%)	Hexosamine set free by 0·5 N-HCl at 100° for 16 hr. (%)	Biuret test
1 (3% Barium acetate, no ethanol)	9	2·7 (1·9–4·0)	1·4	5·5	Negligible
2 (3% Barium acetate, and ethanol added to 10%, v/v)	28	0·84 (0·53–1·3)	4·0	10·3	Negligible
3 (3% Barium acetate and ethanol added to 50%, v/v)	16	1·1 (0·64–1·9)	1·7	12·0	Trace
4 (Supernatant from 3)	8	0·73 (0·49–1·3)	0·4	8·7	Strong reaction

Fraction *A* was suspended in water (6%, w/v), warmed to about 37° and solid Na_2SO_4 (15% of the weight of fraction *A*) was added, and the mixture shaken at room temperature for 0·5 hr. The pH was adjusted to 1·8–2·0 with 12 N-H_2SO_4, and the mixture immediately cooled to 0–2° and left at this temperature overnight. The precipitate of nucleic acid and $BaSO_4$ was removed at 0–2° at 45 000–50 000 rev./min. in the batch bowl of a refrigerated Sharples centrifuge, and reserved. Ethanol was added to the supernatant to 50% (v/v), then 1% (w/v) sodium acetate was added and the mixture was left at −10° overnight. The precipitate was removed at −10 to 0° in the angle head of a refrigerated centrifuge, dissolved in water at pH 7, dialysed for several days against frequent changes of distilled water and freeze-dried. (Polysaccharide A (2·6 g.); activity 4·5 (2·8–7·3)

The nucleic acid precipitates and the 50% (v/v) ethanolic supernatants from all three fractions were combined with the supernatant containing peptide material from the barium acetate/ethanol fractionation, which had been decomposed with Na_2SO_4. The pH was adjusted to 7 and $BaSO_4$ removed by centrifugation. After dialysis for several days against distilled water it was freeze-dried (30·5 g.). (Activity 0·22 (0·13–0·35) v.e.u./g.)

The chemical nature and biological properties of polysaccharides A, B and C

Table 3 summarizes the important distinguishing properties of these compounds. It includes a determination of anticomplementary activity in a system involving sheep cell haemolysis: our colleague, Dr H. P. Lambert, has shown that such activity is a distinct property of polysaccharide A.

Polysaccharide A has been further purified and obtained essentially homogeneous; it has been identified as a heparin and crystallized as the acid barium salt. The following paper describes its properties in detail, but relevant data are included in Table 3 for comparison with the other polysaccharides.

Polysaccharide B is the sodium salt of a polysaccharide sulphuric ester. The pH (6·0–6·5) of the aqueous solution indicates a mixed acid and neutral salt. It is contaminated slightly with nucleic acid as shown by a phosphorus content of 0·4% and a slight peak at 2600 A. in the absorption of ultraviolet light in solution. Although it is still impure, the following analysis, and that given in Table 3, indicate that it is predominantly mucoitin monosulphuric acid, or the acid polysaccharide of Meyer, Smyth & Palmer (1937), and Meyer (1938), which was also prepared by Wolfrom *et al.* (1943), and later shown to be a mixture of substances by Wolfrom & Rice (1947). Found: *N*-acetyl (CH_3CO), determined by hydrolysing with 25% (w/v) *p*-toluenesulphonic acid at 100° overnight and titrating the distilled acetic acid, 5·2; total S (fusion) 5·5; ash 21·7; sulphated ash 22·7; N, 5·2%. $[\alpha]_D^{20°}$ in water $= -15·5°$ (*c*, 1). Meyer *et al.* (1937) give $[\alpha]_D^{25°} = -20·2$ and $-22·2°$ for two preparations of acid salt and $[\alpha]_D^{24°} = -35·7$ for a neutral preparation. Meyer (1938) gives $[\alpha]_D = -8·1°$ for a neutral sodium calcium salt; Wolfrom *et al.* (1943) give $[\alpha]_D^{25°}$ in water $= -15°$ (*c*, 2) for the neutral sodium salt and $-7·4°$ for the acid sodium salt.

The virulence-enhancing activity of polysaccharide B is low compared with the other polysaccharides, especially with polysaccharide A. It is known that polysaccharide A remains slightly soluble at 0° on the addition of 3% (w/v) barium acetate at pH 6·9–7·1, and that addition of ethanol to a final concentration of 10% (v/v) precipitates a large amount of this material. Polysaccharide B, therefore, contains some A, and a rough estimation of the proportion of the latter can be obtained from the figures on the liberation of hexosamine by hydrolysis at 100° with 0·5N- and 3N-hydrochloric acid. Hydrolysis of polysaccharide A at 100° for 16 hr. liberates 7·5% hexosamine if 0·5N-hydrochloric acid is used, and 25·2% if 3N-hydrochloric acid is substituted; the latter figure is not increased by further hydrolysis. Hydrolysis of polysaccharide B with 3N-hydrochloric acid for 16 hr. liberates 25·1% hexosamine, and this is not increased by longer hydrolysis. Fig. 1 shows that the hydrolysis with 0·5N-hydrochloric acid liberates 20% hexosamine in 8 hr. and this figure is only increased slowly by longer hydrolysis. It is probable that the difference between these values for polysaccharide B is due to the amount of polysaccharide A present. A short calculation shows that the virulence-enhancing activity of polysaccharide B can be largely attributed to the presence of polysaccharide A, and if B has any inherent activity it is very low.

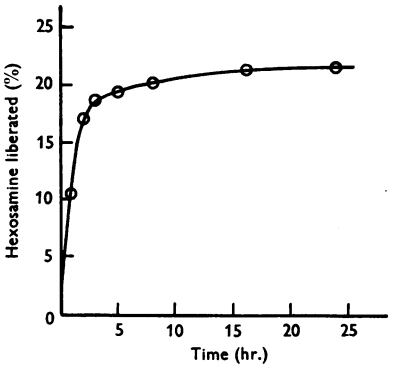

Fig. 1. The liberation of hexosamine from polysaccharide B by 0·5N-HCl at 100°.

Polysaccharide C. Table 3 shows that this compound is different from polysaccharide A. It is still impure (P = 0·2%, slight positive biuret reaction), but its relatively high activity, coupled with the difference in chemical and biological properties from polysaccharide A, warrant further purification and investigation of this compound or mixture of compounds.

Polysaccharide C must form some complex with protein just as do the sulphated polysaccharides, since it remains insoluble at pH 4·3–4·5, when the crude third factor is washed with water at that pH. Nevertheless, the relatively weak ionic nature of polysaccharide C would tend to make this complex less stable than that of polysaccharides A or B. It is possible, therefore, that the low activity (0·05 (0·03–0·07) v.e.u./g.) of material extracted at pH 4·3–4·5 is due more to polysaccharide C than to A. Investigation of this material as an additional source of polysaccharide C is being carried out.

Table 3. *Virulence-enhancing activities and biological properties of polysaccharides A, B and C, and various bacterial and other polysaccharides: distinguishing analyses of the former, and known chemical nature of the latter*

Polysaccharide	Virulence-enhancing activity of a typical batch (V.E.U./g.)	Anticoagulation activity on fresh ox blood using the technique of Jorpes (1935, 1942)		Anticomplementary activity in sheep cell haemolysis		S as acid-hydrolysable sulphate (%)	Hexosamine set free by 16 hr. hydrolysis at 100° (%) with		Hexuronic acid (%)	
		Activity	Amount added to 100 ml. blood (mg.)	Activity	Concentration in the complement mixture (%, w/v)		0·5 N-HCl	3 N-HCl	Dische method	Lefèvre & Tollens method
A	3·9 (2·9–5·1)	Strongly active	0·0125	Strongly active	0·01	11·3	7·5	25·2	34·6	26·6
B	0·69 (0·52–0·90)	Active	1·0	Slightly active	0·025	5·2	20·5	25·1	20·0	27·6
C	1·4 (0·9–2·0)	Inactive	1·0	Inactive	0·025 and 0·075	Approx. 1·0	28·5	28·0	Less than 1·0	Approx. 3
						Known chemical nature of polymer units				
Dextran from *Leuconostoc mesenteroides*	1·6 (1·0–2·7)	Inactive	1·0	Inactive	0·025	Glucose with mainly 1:6 links, branched chain (Evans & Hibbert, 1946)				
Dextran from *Betacoccus arabinosaceus*	1·6 (1·0–2·7)	Inactive	1·0	Inactive	0·025	Glucose with mainly 1:6 links, branched chain (Evans & Hibbert, 1946)				
Dextran from *Leuconostoc dextranicum*	2·0 (1·2–3·3)	Inactive	1·0	Inactive	0·025	Glucose with mainly 1:6 links, straight chain (Evans & Hibbert, 1946)				
Pneumococcus Type I polysaccharide	1·1 (0·6–2·1)	Inactive	1·0	Inactive	0·025	Hexosamine, acetyl residues, D-galacturonic acid (Stacey, 1946)				
Pneumococcus Type V polysaccharide	1·6 (1·0–2·7)	Slightly active	1·0	Slightly active	0·025	Hexosamine, acetyl residues and others (Brown, 1939)				
Polysaccharide from *Azobacter chroöcoccum*	2·4 (1·4–4·3)	Inactive	1·0	Inactive	0·025	D-Glucose, D-galactose, D-glucuronic acid (Lawson & Stacey, 1950)				
Galactogen from trypsin	Negligible activity	Inactive	1·0	Slightly active	0·025	D-Galactose				
Seromucoid	Negligible activity	Inactive	1·0	Inactive	0·025	D-Mannose, D-glucosamine and amino-acids (Stacey, 1946)				

The synergism of a combination of polysaccharides A (or a sample of heparin) and polysaccharide C in the biological assay for the third factor

In consultation with our colleague, Mr S. Peto, experiments were devised to test the synergism of a mixture of polysaccharides A and C in the biological assay for the third factor. In view of the identification of polysaccharide A as a heparin, an authentic sample of heparin was also included in the experiments.

Twenty mice were injected with each concentration in each test and the death rates for all these individual tests were analysed by Mr S. Peto, who has written an addendum to this paper. He found the test for synergism of the combination of polysaccharides A and C to be significant, and the mixture is estimated to be approximately 70% more active than if its constituents acted similarly. This is of the order one would have expected from the recovery of activities mentioned above. In the case of the sample of heparin there is also a strong indication that a combination of it with polysaccharide C is synergic, and it is estimated that the mixture is approximately 50% more active than if its constituents acted similarly.

Table 4. *Synergism of a combination of polysaccharide A (or a sample of heparin) and polysaccharide C in the biological assay for the third factor*

(For the general technique of the biological assay for the third factor see Smith *et al.* (1951). The figures quoted are the average death rates (%) for each concentration in all of four tests, twenty mice being injected with each concentration in each test. The individual tests were analysed by Mr S. Peto, who has written an addendum to this paper. Adequate control experiments rule out any toxicity in the samples injected.)

Injection material	Concn. (%)	Deaths (%)	Concn. (%)	Deaths (%)	Concn. (%)	Deaths (%)
Polysaccharide A	0·05	63	0·025	56	0·0125	50
Polysaccharide C	0·1	77	0·05	65	0·025	50
Polysaccharide A 2 parts / Polysaccharide C 3 parts	0·07	89	0·035	68	0·0175	54
Heparin (Boots Pure Drug Co. Ltd.)	0·05	75	0·025	57	0·0125	42
Heparin (Boots Pure Drug Co. Ltd.) 2 parts / Polysaccharide C 3 parts	0·07	93	0·035	54	0·0175	60

This series of experiments was prompted by two facts. First, the wide difference in chemical and biological properties of the two polysaccharides suggested that they might impede different host defence mechanisms, and some synergic effect would be expected. Secondly, the total yield of activity (based on the most probable figures) of all the fractions recovered in the barium acetate/ethanol fractionation was low (50–70%); this would not be surprising for one fractionation, in view of the fiducial limits of the virulence-enhancing activities, but all such fractionations yielded similar figures. There was a loss of material through dialysis membranes when the complexes of protein with nucleic acid and polysaccharide were split, but we have no evidence of loss of activity through these membranes.

Polysaccharide A (activity 3·9 (2·9–5·1) v.e.u./g.) and a sample of heparin (Boots Pure Drug Co. Ltd., activity 3·2 (2·1–4·8) v.e.u./g.) were tested in the biological assay for the third factor at concentrations of 0·05, 0·025 and 0·0125% (w/v) and a sample of polysaccharide C (activity 2·1 (1·7–2·6) v.e.u./g.) at concentrations of 0·1, 0·05 and 0·025% (w/v). In the same test, mixtures of 2 parts of polysaccharide A or heparin and 3 parts of polysaccharide C were injected at concentrations of 0·07, 0·035 and 0·0175% (w/v), which should give approximately the same death rates as the individual samples if no synergism occurred. This test was repeated four times with the same batch of polysaccharide C.

Table 4 summarizes these experiments. For brevity, the average death rates (%) for each concentration of the materials used in all four tests is quoted instead of the individual values.

The virulence-enhancing activities of various bacterial polysaccharides: their chemical nature and biological properties

The fact that the activity of the third factor lies in the polysaccharide moiety prompted an investigation of several bacterial polysaccharides for virulence enhancement, anticoagulation and anti-complementary activity. Prof. M. Stacey and Dr W. G. Overend of the University of Birmingham kindly supplied us with a number of such polysaccharides. Two of animal origin—a galactogen from trypsin and seromucoid—have also been included in the investigation. The virulence-enhancing activity was determined at concentrations 0·1, 0·05, 0·025 and 0·0125% (w/v) in the normal assay for the third factor (Smith *et al.* 1951), making certain that the inherent viscosity of some of the preparations did not affect unduly the 'standard' viscosity of the solutions used in the assay.

The results of this investigation, together with a brief outline of the known chemical nature of these compounds are included in Table 3 for comparison

with polysaccharides A, B and C. All the bacterial polysaccharides were active in enhancing virulence, yet the two of animal origin had negligible activity at the concentrations used.

DISCUSSION

The fourth paper in this series (Smith *et al.* 1951) made it clear that the virulence-enhancing action of hog gastric mucin was due to a synergic combination of a viscous medium, a particulate residue and a soluble third factor. In the present paper the activity of the crude third factor (Smith, 1951) has been shown to reside in the carbohydrate moiety: furthermore, this moiety has been separated into at least three different parts, two of which—polysaccharides A and C—are involved in the virulence enhancement.

Polysaccharide A has been obtained essentially homogeneous and identified as a heparin (see following paper). Polysaccharide B is impure, but is predominantly mucoitin monosulphuric ester or the 'acid polysaccharide' of Meyer *et al.* (1937). Its laevorotation, ester sulphate analysis and the presence of acetyl residues show that it is different from polysaccharide A and heparin. It has a low activity which can be largely, if not totally, attributed to the presence of polysaccharide A. Purification of this compound with regard to its virulence-enhancing activity, therefore, has been discontinued. Polysaccharide C is as yet impure, but it is clearly different from polysaccharide A. It has a relatively high activity and further fractionation of this compound or mixture of compounds is being continued.

Coupled with the difference in the chemical and especially the ionic nature of polysaccharides A and C is a marked difference in biological properties. Polysaccharide A is a powerful anticoagulant for blood and has a strong anticomplementary effect in a system involving sheep cell haemolysis; polysaccharide C is devoid of any such activity. It is quite possible, therefore, that the two polysaccharides may have different modes of action in helping to enhance virulence; this view is strongly supported by the synergic effect of a combination of the two in the assay for the third factor involved in virulence enhancement. Investigations into the mode of action of mucin in enhancing virulence must take account of this possibility, in addition to the effects of the particulate matter and of a viscous medium. The virulence-enhancing action of hog gastric mucin is an excellent example of biological activity being due to a system of interacting factors, rather than to one factor alone. The relative importance of the various factors operating might well vary with the nature of the organism establishing the infection and the particular host defence mechanisms called into play in combating that infection.

Polysaccharides of non-pathogenic bacteria (levans from *Aerobacter levanicum* and *Bacillus subtilis*, dextran from *Leuconostoc mesenteroides* cellulose from *Acetobacter xylinum*) and of pathogenic bacteria (*Escherichia coli*, *Salmonella typhi*, *Salmonella enteritidis*, *Klebsiella capsulata*, *Pneumococci* Types I and II) have been shown to have virulence-enhancing activity at concentrations of 0·2–2·0% (Olitzki, 1948; Olitzki, Shelubsky & Hestrin, 1946; Felton & Bailey, 1926; Ward, 1930). When examined in the biological assay for the third factor, in which samples are injected in the presence of a fixed amount of particulate residue and in a medium of constant viscosity (Smith *et al.* 1951), various bacterial polysaccharides proved active at low concentrations (0·1% (w/v) and lower). The interesting fact is that the activities of these compounds are of the same order as polysaccharide C, which they also resemble in containing no large amounts of highly ionic sulphate residues, and in having no anticoagulatory or anticomplementary action. It is relevant to point out that the virulence-enhancing activity or 'aggressin effect' of Types I, II and III pneumococcus polysaccharides has been attributed to some extent to their antiopsonic and antiphagocytic action (for discussion see Wilson & Miles, 1946).

Although the above compounds have been designated polysaccharides, Stacey (1946) believes that all bacterial polysaccharides retain a small amount of amino-acid residues which are important as regards serology and antigenicity. Mucopolysaccharides is perhaps a better name for these compounds, and for polysaccharide C; the latter still contains amino-acid residues which may have an important bearing on its activity.

SUMMARY

1. The virulence-enhancing activity of the third factor of hog gastric mucin resides in at least two predominantly polysaccharide fractions.

2. These fractions differ in chemical nature and in some biological properties. The first, which has been purified and identified (see following paper), contains highly ionic sulphate residues and has an intense anticoagulatory and anticomplementary activity; the second fraction, which is still impure, is devoid of such residues and activities. When combined together in the biological assay for the third factor the two fractions have a synergic effect.

3. Various bacterial polysaccharides have virulence-enhancing activities and other biological properties similar to the second of these fractions.

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ADDENDUM

Statistical Treatment of Data Regarding the Synergism of Mixtures of Polysaccharides A and C

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METHOD

The general details of the tests involving polysaccharide A (A), a sample of heparin (H), polysaccharide C (C) and mixtures of the latter with the other two (A/C and H/C) are given above.

Assuming similar synergic action five parallel probit-log dose regression lines (A, H, C, A/H, H/C) have been fitted for each of the four replicate experiments. The mean probit difference ($\Delta \pm$ its standard deviation) between the observed probit-dosage relationship of the mixture and that predicted according to the hypothesis of similar action has been calculated as described by Finney (1947) and used as a measure of the synergism. In addition, the logarithm ($M \pm$ its standard deviation) of the ratio of the observed potency of the mixture to that predicted on the above-mentioned hypothesis was calculated.

These results (Δ's and M's) for the four individual experiments have been combined using three different methods:

(1) Taking weighted means with reciprocal variances as weights and the reciprocal of the sum of the weights as the variance of the weighted mean.

(2) Constructing a new set of regression lines with $\Sigma Snwx^2$, $\Sigma Snwxy$, $\Sigma Snwy^2$, etc.

(3) By adding corresponding mortalities and

Table 1. *Mean probit differences* (Δ) *and the logarithms* (M) *of the ratio of observed potency of the mixture to that predicted on the hypothesis of similar action of mixtures* (A/C, H/C) *of polysaccharide A, a sample of heparin* (H) *and polysaccharide C*

Method of calculation	$\Delta\ A/C$	$M\ A/C$	$\Delta\ H/C$	$M\ H/C$
1	$0{\cdot}242 \pm 0{\cdot}134$	$0{\cdot}244 \pm 0{\cdot}108$	$0{\cdot}234 \pm 0{\cdot}136$	$0{\cdot}196 \pm 0{\cdot}102$
2	$0{\cdot}311 \pm 0{\cdot}106$	$0{\cdot}236 \pm 0{\cdot}084$	$0{\cdot}242 \pm 0{\cdot}106$	$0{\cdot}184 \pm 0{\cdot}083$
3	$0{\cdot}305 \pm 0{\cdot}106$	$0{\cdot}261 \pm 0{\cdot}099$	$0{\cdot}245 \pm 0{\cdot}253$	$0{\cdot}168 \pm 0{\cdot}202$

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.