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The Virulence-Enhancing Factor of Mucins

3. FURTHER FRACTIONATION OF HOG GASTRIC MUCIN; AN ADDITIONAL FACTOR IN THE ADJUVANT ACTION

By H. SMITH
Microbiological Research Department, Experimental Station, Porton, Wilts

(*Received* 17 *July* 1950)

The first two papers in this series (Smith, 1950*a*, *b*) described a biological assay of the virulence-enhancing activity of mucins for bacteria, and the use of this assay in preliminary fractionation of hog gastric mucin for a factor responsible for such activity. A fraction was obtained which was only sparingly soluble in water at pH 7, and which, although active alone, showed a far greater activity when suspended in an inert viscous solution such as gum tragacanth, agar or semi-pure blood-group substance. It was suggested that the viscosity of the medium probably delays the absorption and inactivation of the active factor by the host at the site of injection.

The present paper describes further purification of the virulence-enhancing factor to a stage where some insight into its chemical nature has been obtained, although it is still not homogeneous. This purification has lead to the recognition of a further factor having an adjuvant action. It is becoming increasingly evident, therefore, that the virulence-enhancing action of hog gastric mucin is due to the specific active factor in association with other non-specific physical factors which are necessary for the full manifestation of its activity.

The difficulty in maintaining the factors having an adjuvant action at a constant value in the biological assay, while studying fractions in the purification of the virulence-enhancing factor proper, is one of the major problems of this work.

EXPERIMENTAL AND RESULTS

Activities are given in 'Wilson units' by direct comparison with a standard mucin, with fiducial limits in brackets for $P = 0{\cdot}95$.

Modification of the biological assay of virulence-enhancing activity

The evidence that the semi-pure blood-group substance, which retains the viscosity of the original mucin, exerts its adjuvant action on the virulence-enhancing factor by virtue of this viscosity is further strengthened by the following experiments.

Semi-pure blood-group substance (40 g.), prepared as described before, was further fractionated with ethanol from 90% phenol solution as described by Morgan & King (1943). The following fractions were obtained: (1) insoluble in 90% phenol, 15 g.; (2) precipitated by 10% (v/v) ethanol, 9·4 g.; (3) precipitated by 25% (v/v) ethanol, 2·9 g.; (4) precipitated by pouring the mother liquor into excess ether, 2·2 g.

The same concentration of a virulence-enhancing fraction was suspended in solutions of the original semi-pure blood-group substance, and of the first three fractions: the viscosities of the suspensions were maintained constant by varying the concentrations of the different fractions. Table 1 shows that the activity of the virulence-enhancing fraction is the same, irrespective of the concentration and nature of the fraction, as long as the viscosity is the same. It was impossible to use fraction 4 because a large proportion of it was insoluble, and a 5% (w/v) suspension had a viscosity scarcely greater than water, and it was toxic to mice.

In view of the adjuvant action on the virulence-enhancing factor of the viscosity of the original mucin, it was essential in future assays that this adjuvant action should remain constant. The viscosity of all test solutions for injection has therefore been maintained at an approximate standard value by adding very dilute tragacanth mucilage. Viscosities were determined at 37° in an Ostwald viscometer. Since only approximate values are needed, the small differences in density of various dilute aqueous solutions were neglected, and viscosities were quoted as times in seconds to flow through the same viscometer. The viscosity chosen as the fixed figure was the mean of those of the 1·75% (w/v) (140 sec.) and 1·25% (w/v) (75 sec.) 'standard mucin'. Workable limits on this figure were 90–125 sec. and the viscosity is about seven

Table 1. *Activities of a sample of virulence-enhancing fraction suspended in different fractions of the semi-pure blood-group substance*

(Controls without organisms ruled out toxicity of any of the samples, and results of these controls and normal results obtained for bacterial counts and 'standard mucin' death rates are not given in the table.)

Solution injected with 5000 *Bact. typhosum*				
Virulence-enhancing factor (concentration %, w/v)	Semi-pure blood-group substance fraction (concentration %, w/v)	Viscosity of suspension relative to the original semi-pure blood-group substance suspension	No. of deaths in a batch of twenty mice	Activity in 'Wilson units'
0·25	Original sample, 2·5	1·00	14	4·7 (2·1–8·4)
Nil	Original sample, 2·5		Nil	
0·25	Fraction 1, 1·5	1·05	11	4·0 (1·6–6·7)
Nil	Fraction 1, 1·5		Nil	
0·25	Fraction 2, 2·0	1·00	11	4·0 (1·6–6·7)
Nil	Fraction 2, 2·0		Nil	
0·25	Fraction 3, 3·0	0·85	13	5·3 (2·7–9·5)
Nil	Fraction 3, 3·0		2	

times that of water, and about half that of the original crude mucin.

The solution or suspension of the mucin fraction was made up at double the strength required for injection, the pH was adjusted to 7, and after heating to 60° for 0·75 hr., it was cooled and mixed with an equal quantity of a similarly treated tragacanth solution of double the strength required to give the correct viscosity in the final solution. The viscosity of aqueous tragacanth is decreased on adding the sample of virulence-enhancing factor, and this decrease depends on the nature and strength of the particular mucin fraction. As a result of preliminary tests the tragacanth concentration was varied from 0·11 to 0·16 % (w/v), according to the mucin, in order to maintain the viscosity within the above limits. Adequate tests on several batches of twenty mice showed that 5000 *Bacterium typhosum* in 0·16 % (w/v) tragacanth did not kill any mice.

Nine parts of the final solution were added to one part of tryptic meat broth suspension of organisms, before injection. To avoid routine determination of viscosities on solutions containing virulent *Bact. typhosum*, it was assumed that when the same proportion of the same organism suspension was added to each solution, the slight change in viscosity will be the same for all solutions.

The activity of important fractions was also checked using the limited supply of semi-pure blood-group substance to maintain the standard viscosity.

Purification of the virulence-enhancing factor

Essential steps in the preliminary separation of the virulence-enhancing factor are as follows: (1) autolysis of fresh hog stomach mucosae at pH 3 for 1 week; (2) precipitation of the whole autolysate by ethanol; (3) extraction of fat with dichlorethylene; (4) extraction of extraneous material by water at pH 6; (5) solution in saturated aqueous sodium salicylate and precipitation with ethanol. The material obtained was practically insoluble in water at pH 7, and by no means completely soluble at pH 12.

Attempts to use organic solvents for solution and fractionation of the virulence-enhancing fraction. Many solvents were examined; 90 % phenol, *m*-cresol, and saturated aqueous solutions of guanidine hydrochloride, chloral hydrate, urea and resorcinol all dissolved most of the material. However, attempts to fractionate at 0° from these solvents were unsuccessful because the fractions did not separate cleanly, and the residue after the initial solution always retained a high proportion of activity.

Extraction of the active material by stirring at 37° with sodium bicarbonate, followed by sodium carbonate. As suggested before (Smith, 1950*b*), the apparently sparing solubility of the active material in water at pH 7, or in organic solvents; might be due to strong adsorption on to insoluble protein-like material. Experiments were therefore carried out to remove the insoluble protein by tryptic hydrolysis. However, the recovery of activity was higher in blanks containing no trypsin, and it was concluded that a slightly alkaline medium at 37° extracts the active material, which is then largely destroyed by the trypsin. Further experiments established the following process for alkaline extraction, which was carried out on material obtained after stage 4 in the preliminary purification.

The active material from two hundred hog stomach mucosae (170–190 g.) in 2 % (w/v) concentration was stirred with 1 % $NaHCO_3$ and 0·1 % toluene at 37° for 24 hr., the initial pH being approximately 6·5–7·0 and the final pH 8·5–9·0. The suspension was passed through a Sharples centrifuge. To the cooled supernatant 95 % of the theoretical quantity of 5 N-H_2SO_4 necessary to neutralize the bicarbonate was added, and the whole freeze-dried. The residue was homogenized in a volume of 1 % Na_2CO_3, containing 0·1 % toluene, equal to the volume of the $NaHCO_3$ solution first used, and stirred at 37° for a further 24 hr. (pH 10·0–10·5). The suspension was passed through a Sharples centrifuge and the supernatant neutralized and freeze-dried. The combined freeze-dried materials were mixed with water (1·5 l.) and dialysed against distilled water until free from sulphate. Tests showed that the active material did not dialyse through the bag. The contents of the dialysis bag were then freeze-dried. Yield 75–80 g. In six separations carried out by this method the recovery of activity (calculated from most probable activities) in this fraction (fraction I) varied from 48 to 92 % of the original; in a typical separation from an original crude mucin of activity 5·5 (4·6–6·6) 'Wilson units', the activity of fraction I was 8·0 (6·9–9·3) 'Wilson units'. The residue was homogenized with water and freeze-dried. (Yield 90–100 g.) Only rarely was slight virulence-enhancing activity detectable in these residues before toxic concentrations were reached.

Purification of the virulence-enhancing factor by solution at pH 9 *and precipitation at* pH 5. Fraction I contained material insoluble at pH 9, some of which was relatively easy to remove by centrifugation; the remainder was very finely divided material which could only be removed by prolonged centrifugation at very high speed. The separation of the fraction soluble at pH 9·0 and insoluble at pH 4·9–5·0 (fraction II*c*), was therefore carried out in two stages: first a bulk separation involving one solution and precipitation (fraction II), and then a final purification involving high-speed centrifugation at pH 9.

In 2·5% (w/v) concentration fraction I (75–80 g.) was stirred with water and the pH adjusted to 9·0 (±0·05) with 2 N-NaOH. The bulk of the material went into solution, and the mixture was left in the refrigerator overnight. It was then passed through the clarifying bowl of the Sharples centrifuge at an approximate rate of 2 l./hr. The residue in the centrifuge bowl was homogenized with water, and freeze-dried. (Fraction III. Yield 9–12 g.) The pH of the supernatant was adjusted to 4·9–5·0 with 2 N-acetic acid and the mixture left in the refrigerator overnight. The precipitate was collected in the clarifying bowl of the Sharples centrifuge, homogenized with water and freeze-dried. (Fraction II. Yield 30–35 g.) The supernatant at pH 5 was freeze-dried. (Fraction IV. Yield 28–32 g.) The activities of these fractions for several such separations are given in Table 2.

Fraction II above (30–35 g.) in 5% concentration at pH 9 was centrifuged at 45,000–50,000 rev./min. (approx. 50,000 *g*) for 2 hr. in the batch bowl of the Sharples centrifuge, which had been refrigerated so that the liquid in the bowl was at 0–5°. The supernatant was adjusted to pH 4·9–5·0 and the precipitate collected as described above. The process of solution and centrifugation at pH 9 and precipitation at pH 4·9–5·0 was repeated twice, but each time the concentration was increased to 7·5% (on the original solid) and the time of centrifugation to 3 hr. Table 3 shows the activities of the following freeze-dried fractions: (1) Fraction II*a*, residue at pH 9 after washing twice with water; yield 1·5–2·5 g. (2) Fraction II*b*, soluble at pH 5; yield 11–14 g. (3) Fraction II*c*, soluble at pH 9·0, insoluble at pH 4·9–5·0; yield 9·5–11 g. (4) A second crop (fraction II*d*) obtained from the washings of the residue at pH 9 by precipitation at pH 4·9–5·0; yield 3–5 g. In routine use of this method fractions II*b* and II*d*, which inevitably retain some activity, are not discarded but added to the next batch at the stage of the separation of fraction II.

Table 2. *Activities of fractions in the separation of the fraction II*

(Some fractions have been assayed by two methods. Method 1, by injection in a medium of constant viscosity alone: Method 2, with addition of a constant amount (0·2%, w/v) of the same sample of well washed residue insoluble at pH 9. Assay results by method 1 on fraction III are comparable to those by method 2 on the rest of the samples.)

Method of assay	Activity in 'Wilson units'				Percentage activity recovered (calculated from yields and most probable activities)	
	Fraction I	Fraction III	Fraction IV	Fraction II	Total in all fractions	In fraction II
1	8·0 (6·9–9·3)	Less than 6·2 (5·1–7·5)	Less than 4·1 (3·4–5·0)	10·1 (8·9–11·5)	—	52
2			—	10·8 (8·3–14·2)	—	55
1	6·8 (5·4–8·6)	4·9 (3·5–6·7)	4·0 (2·8–5·5)	9·2 (6·5–12·9)	81	54
2			—	10·7 (8·4–13·8)	—	63
1	6·9 (5·4–8·8)	6·2 (4·7–8·3)	4·2 (2·4–5·9)	8·0 (6·1–10·4)	81	49
2			8·8 (6·8–11·4)	11·1 (8·5–14·5)	118	63
1	6·5 (4·9–8·6)	4·7 (3·6–6·2)	—	—	—	—
2			8·5 (6·7–10·9)	10·8 (8·5–13·9)	127	68

Table 3. *Activities of fractions in the purification of the fraction II by high-speed centrifugation*

(Remarks as in Table 2.)

Method of assay	Activity in 'Wilson units'					Percentage activity recovered (calculated from yields and most probable activities)	
	Fraction II	Fraction II*a*	Fraction II*b*	Fraction II*c*	Fraction II*d*	Total in all fractions	In fractions II*c* and II*d*
1	10·1 (8·9–11·5)	4·2 (2·9–6·1)	Less than 3·2 (2·2–4·7)	9·3 (8·1–10·6)	—	—	41
2	10·8 (8·3–14·2)		—	22·0 (16·4–30·0)	—	—	92
1	9·2 (6·5–12·9)	5·0 (3·4–6·4)	4·7 (3·6–6·0)	7·8 (6·5–9·3)	6·5 (3·4–8·7)	53	37
2	10·7 (8·4–13·8)		—	16·7 (14·5–19·2)	—	—	Over 57
1	8·0 (6·1–10·4)	3·2 (1·6–4·2)	3·1 (1·6–4·3)	5·7 (4·5–7·1)	6·1 (3·1–8·3)	41	20
2	11·1 (8·5–14·5)		9·7 (7·5–12·5)	20·5 (17·2–24·5)	16·1 (12·4–21·0)	87	47
1	—	4·2 (3·1–5·6)	—	—	—	—	—
2	10·8 (8·5–13·9)		7·8 (6·1–10·0)	16·8 (7·6–25·8)	12·4 (9·4–16·3)	94	64

The adjuvant action of the residue insoluble in water at pH 9

When samples of the fractions II and IIc were assayed by method 1 (Tables 2 and 3), in which the normal procedure of injection alone in a medium of constant viscosity was adopted, two important points emerge. (i) The recovery in fraction II of activity originally present in fraction I was satisfactory (about 50%). This fraction still contained some finely divided residue insoluble at pH 9. (ii) After further purification of fraction II by high-speed centrifugation, both the total recovery of activity, and the activity recovered in fraction IIc, was very low.

The following experiments show, however, that the residue at pH 9 has an adjuvant effect on the activity of fraction IIc. Redetermination of activities with the addition of a constant amount of the washed residue, insoluble at pH 9 (method 2), eliminated the above discrepancies in recovery. The small activities of these residues shown in Tables 2 and 3 (fractions III and II*a*) could be reduced to very low figures (1·3–1·7 'Wilson units') by repeated washing in large volumes of water at pH 9. It was impossible to remove the activity entirely by as many as eight washings, or even by autoclaving and washing twice with hot water.

Comparison of activities of fraction IIc when injected alone in a medium of standard viscosity, and when residues insoluble at pH 9 *was added.* The sample of washed residue (0·8%, w/v) was mixed with an equal volume of a solution of fraction IIc of four times the concentration needed for final injection, and adjusted to pH 7·0–7·2. After shaking together overnight so that any adsorption could take place, the mixture was heated at 60° for 0·75 hr., cooled and mixed with an equal volume of tragacanth mucilage to give the standard viscosity, and tested as usual. Batches of mice were injected with the residue alone and with fraction IIc, and adequate controls without organisms were also injected.

Table 4 shows the results of the combination of two different samples of fractions IIc with three different residues.

It will be noted that 0·2% (w/v) of the residues alone, in a medium of constant viscosity, may kill a very small number of mice.

Comparison of activities of fraction IIc when injected alone in a medium of standard viscosity, and when different samples of known adsorbents are added. The residue insoluble at pH 9 used in the previous experiments was replaced by four adsorbents: activated charcoal, kaolin, fuller's earth and talc. Exactly the same experimental procedure was used, and it is obvious from Table 5 that these substances have a similar adjuvant action.

Adsorption of the virulence-enhancing factor by the residue insoluble at pH 9. A solution containing 0·4% (w/v) of washed residue and twice the concentration of virulence-enhancing factor needed in the final solution was shaken at room temperature overnight and heated at 60° as described before. In addition, a 0·4% (w/v) suspension of residue in water was treated likewise. Taking aseptic precautions, some of each suspension was centrifuged in an angle centrifuge. The supernatant of one was added to the residue of the other, and the two mixtures were thoroughly homogenized by vigorous shaking with glass beads. All four solutions were

Table 4. *Comparison of the activities of fraction IIc injected alone in a medium of standard viscosity, and with residue insoluble at* pH 9

(Controls without organisms ruled out toxicity of any of the samples, and results of these and normal results obtained for bacterial counts and 'standard mucin' death rates are not given in the table.)

Solution injected with 5000 *Bact. typhosum* in a medium of standard viscosity					
Fraction IIc		Residue		No. of deaths in a batch of twenty mice	Activity of fraction IIc in 'Wilson units'
Sample	% (w/v)	Sample	% (w/v)		
Mucin 258	0·15	Nil	Nil	5	7·8 (6·5–9·3)
	0·075	Nil	Nil	Nil, 1	
	0·075	Residue 1	0·2	12	20·5 (15·1–27·7)
	0·075	Residue 2	0·2	9, 5, 8	15·9 (13·2–19·0)
	0·075	Residue 3	0·2	7	15·7 (11·5–21·5)
Mucin 268	0·15	Nil	Nil	6	
	0·075	Nil	Nil	1, 3, 2	5·7 (4·5–7·1)
	0·06	Nil	Nil	Nil, nil, 2	
	0·075	Residue 1	0·2	12	18·8 (13·9–25·4)
	0·06	Residue 2	0·2	7	19·6 (14·3–26·9)
	0·06	Residue 3	0·2	11	23·3 (17·2–31·7)
Nil		Residue 1	0·2	2	—
Nil		Residue 2	0·2	2, nil, 2, nil	—
Nil		Residue 3	0·2	Nil, 1	—

Table 5. *Comparison of the activities of fraction IIc injected alone in a medium of standard viscosity, and with different adsorbents*

(Remarks as in Table 4.)

Solution injected with 5000 *Bact. typhosum* in a medium of standard viscosity					
Fraction IIc		Adsorbent		No. of deaths in a batch of twenty mice	Activity of fraction IIc in 'Wilson units'
Sample	% (w/v)	Sample	% (w/v)		
Mucin 258	0·15	Nil	Nil	5	7·8 (6·5–9·3)
	0·075	Nil	Nil	1, nil	
	0·075	Activated charcoal	1·0	11	17·8 (13·0–24·3)
	0·075	Kaolin	1·5	16	30·4 (21·3–43·3)
	0·075	Fuller's earth	1·0	5	13·5 (9·6–19·0)
	0·075	Talc	2·0	10	23·5 (17·0–32·3)
Mucin 268	0·15	Nil	Nil	6	5·7 (4·5–7·1)
	0·06	Nil	Nil	Nil, nil, 2	
	0·06	Activated charcoal	1·0	10	28·6 (21·0–39·0)
	0·06	Kaolin	1·0	6	18·3 (13·3–25·2)
	0·06	Fuller's earth	1·0	4	21·5 (15·3–30·1)
	0·06	Talc	2·0	6	23·9 (17·4–32·9)
Nil		Activated charcoal	1·0	Nil	—
Nil		Kaolin	1·5	Nil	—
Nil		Fuller's earth	1·0	1	—
Nil		Talc	2·0	1	—

Table 6. *Adsorption of the virulence-enhancing factor by the residue insoluble at* pH 9

(General remarks as in Table 4. The numbers quoted are the numbers of deaths in batches of twenty mice. Exps. 1–3 were done with mucin 258 (0·075%, w/v) and residue 2 (0·2%, w/v) and Exp. 4 with mucin 268 (0·06%, w/v) and residue 3 (0·2%, w/v).)

	Solutions injected with 500 *Bact. typhosum* in a medium of standard viscosity			
Exp. no.	*A* Virulence-enhancing factor and residue	*B* Residue alone	*C* Supernatant *A* and residue *B*	*D* Residue *A* and supernatant from *B*
1	9	2	5	4
2	8	Nil	3	2
3	5	2	7	3
4	11	1	10	4

then diluted with an equal quantity of tragacanth mucilage to give the standard viscosity, and injected together with five thousand *Bact. typhosum*.

Table 6 shows the result of four such experiments, three using the same residue and fraction IIc, and one using a different residue and fraction IIc. The results of individual experiments are not significant, but statistical analysis by my colleague Mr S. Peto, using probits of the death rates, of a combination of all four experiments shows that: (i) the test for the occurrence of a drop in activity of the supernatant combined with a gain in activity by the residue, is highly significant (greater than 100–1); (ii) the loss in activity from the supernatant to the residue is somewhere between 15 and 56% of the whole, with a most probable figure of 37%. In the removal of the supernatant from the residue no washing of the latter was carried out, but the amount of supernatant solution adhering mechanically to the residue could not have exceeded 5%.

Chemical investigation of material soluble at pH 9 *and insoluble at* pH 5

The quantitative results are for one sample, but several samples have been examined by the methods described with similar results.

Physical form and solubility in water. The material was a grey amorphous powder sparingly soluble in water at pH values lower than 5. It was practically insoluble in dil. HCl and H_2SO_4, and in 1 in 5000 solution it was easily detected by the turbidity produced on acidification. Above pH 5 the solubility increased with rise of pH, being 0·5–1% (w/v) at pH 7 and greater than 10% (w/v) at pH 9. When the pH of an alkaline solution was lowered there was a strong tendency to form a supersaturated solution.

Elementary analysis. C, 50·7; H, 7·5; N (Dumas) 14·8; N (Kjeldahl) 13·8; S, 1·96; ash, 0·66%. Emission spectroscopy in a carbon arc on the original sample (not the ash) showed the presence of 0·1% of Mg and P, and faint traces of Fe, Si, Sn, Cu, Pb, Al, Ag and Ca.

Protein-peptide reactions and analysis. (i) *α-Amino nitrogen.* After hydrolysis with 6 N-HCl overnight at 100° in

a sealed tube, the method of Van Slyke detected 10·8% of α-amino-N and the ninhydrin method 10·3%. Before hydrolysis the method of Van Slyke detected 1·10% of α-amino-N (and/or lysine ε-amino-group) and the ninhydrin method indicated the absence of α-amino-N.

(ii) *Colour reactions.* The Millon, xanthoproteic, Ehrlich, Sakaguchi, Hopkins-Cole and biuret reactions were positive.

(iii) *Precipitation reactions.* A dilute solution of the material at pH 7 gave precipitates with solutions of $HgCl_2$, $AgNO_3$, lead acetate, trichloroacetic acid, phosphomolybdic acid and phosphotungstic acid.

(iv) *Two-dimensional paper chromotography.* The material was hydrolysed as described above, and the excess HCl removed under reduced pressure. A two-dimensional paper chromatogram was made using phenol and collidine as solvents and ninhydrin as developer. Comparison with a known mixture of amino-acids showed the following to be present: aspartic acid, glutamic acid, serine, glycine, threonine, alanine, cystein, histidine, proline, valine, phenylalanine, tyrosine, arginine and/or lysine, and leucine and/or isoleucine and/or methionine.

Carbohydrate reactions and analysis. (i) Analysis by the quantitative Molisch reaction of Krainick (1941) gave a total carbohydrate content of 6% calculated as glucose, and by the orcinol method of Sørensen & Haugaard (1933) 5%. Glucosamine gave no reaction in these methods.

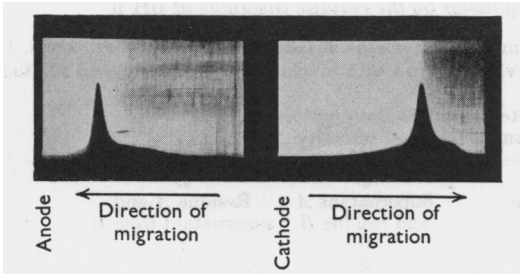

Fig. 1. Electrophoresis patterns of a 1% solution of fraction IIc in a glycine (0·2 M), sodium chloride (0·2 M) solution adjusted to pH 9·0 with 0·2 N-NaOH. Current = 10 ma. Exposure 202 min. after starting the current.

(ii) *Hexosamine reaction.* After hydrolysis by acid the method of Elson & Morgan (1933) gave a figure of 3% calculated as glucosamine. Immers & Vasseur (1950) report that sugar-amino-acid mixtures give false hexosamine colours in the above reaction. These false colours can, however, be distinguished by two methods. They are produced if the acetonyl acetone is left out of the method above, and if it is included, increasing its concentration in the original reagent solution above 0·8% markedly decreases the colour formed. No such behaviour was observed in the above reaction.

Electrophoresis. Fraction IIc was examined by my colleague Dr B. R. Record in the Tiselius electrophoresis apparatus at 0° in a glycine-sodium chloride buffer at pH 9·0. Fig. 1 shows that the migration is anodic and occurs as one peak, except for a small amount of material which shows on the descending boundary only. A similar result was obtained at 0·5% concentration in a phosphate buffer of $I = 0·2$ at pH 8·0. The sparing solubility of fraction IIc below pH 7 precluded an examination on the acid side.

Ultracentrifuge. Through the kindness of Dr J. M. Creeth of the Courtauld Institute of Biochemistry, fraction IIc was examined in the Spinco ultracentrifuge at 60,000 rev./min. (approx. 210,000 *g*). A 0·5% (w/v) solution in glycine-sodium chloride buffer at pH 9 was used. About 75% of the material was of low molecular weight and the peak corresponding to this component did not separate completely from the meniscus. The remainder was of considerably higher molecular weight, and soon settled as a yellow deposit on the bottom of the cell.

DISCUSSION

It was reported earlier (Smith, 1950*b*) that the virulence-enhancing fraction could be separated from materials giving rise to the viscosity in the original mucin, by extraction of the latter with water at a slightly acid pH. This paper describes the separation from contaminating insoluble matter of the virulence-enhancing fraction, in a form fairly soluble at pH 7, by extraction at a slightly alkaline pH.

It has been shown that, in addition to the adjuvant action due to the non-specific physical effect of the viscosity of the original mucin, an additional adjuvant action is exerted by the insoluble residue. The activity of the purified virulence-enhancing fraction in a medium of constant viscosity is increased approximately three times by the inclusion of a small amount of residue in the solution injected. Activities of approximately 20 'Wilson units' have been obtained consistently, indicating a concentration of activity of at least sixty times that of the original crude mucin (0·34 'Wilson units'—Smith, 1950*b*), because the standard viscosity used in the assay is about half that of the original mucin.

It is probable that, unlike the viscosity, this second factor in the adjuvant action is produced as an 'artifact' during the initial extraction process, but naturally occurring mucus is rarely clear. The adjuvant action of the residue is probably non-specific, since activated charcoal, kaolin, fuller's earth and talc produced a similar effect. It is probable that these absorbents maintain the virulence-enhancing fraction at the site of injection, thus delaying its assimilation by the host.

Experiments showed that in fact the residue can absorb some activity from a solution of the specific factor. The small activity which the residue retained after thorough washing at pH 9, and even after autoclaving and washing with hot water, may be due to strong adsorption of the virulence-enhancing factor. On the other hand, it is possible that the residue itself, in a medium of constant viscosity, may have some slight action on the body defences *in vivo*. It has already been pointed out (Smith, 1950*b*) that if the viscosity is increased to a very large value, e.g. 2, 4 and 10% (w/v) agar (Anderson & Oga, 1939), 6% (w/v) starch paste (Robertson & Fox, 1939),

2·5% (w/v) tragacanth (Steinberg, 1931) and 5% (w/v) tragacanth (Benians, 1924), a small virulence-enhancing action results. It is quite possible, therefore, that both the viscosity and the residue may affect the host defences *in vivo*, quite apart from delaying the adsorption by the host of the virulence-enhancing factor proper.

A further interesting point has emerged in connexion with the adjuvant action of the residue insoluble at pH 9. The toxicity of the crude mucin at concentrations two to three times those needed for enhancing virulence was noted by Smith (1950*a*). A dose of the virulence-enhancing factor about three times greater than that needed to show virulence enhancement when in combination with the residue at pH 9 is non-toxic when injected alone in a medium of standard viscosity. If injected with the residue, which is also completely non-toxic alone, the dose is slightly toxic.

The reactions and analysis of fraction II*c* suggest at present that the virulence-enhancing factor is predominantly peptide in nature; the slightly low nitrogen content and the positive carbohydrate reactions indicate, however, that about 5–10% of carbohydrate residues are also present. The presence of only a trace of phosphorus rules out nucleoproteins, a fact that had already been indicated by negative animal tests on samples of undegraded thymus deoxypentosenucleic acid and nucleoprotein.

Although in the electrophoresis apparatus one peak accounts for the migration of all but a very small proportion of the material, which shows on one side of the U-tube only, the heterogeneity of fraction II*c* is demonstrated by the ultracentrifuge. The molecular weight of the active material is sufficiently high to prevent it passing through cellophan on dialysis.

Purification of the virulence-enhancing factor is being continued.

SUMMARY

1. The biological assay of virulence-enhancing activity (Smith, 1950*a*) has been modified; a medium of standard viscosity is now used for all solutions injected.

2. The virulence-enhancing factor has been further purified from insoluble material, and is of predominantly peptide nature, but a small proportion of carbohydrate residues is present. The material is still heterogeneous by ultracentrifugal analysis.

3. The insoluble residue separated from the virulence-enhancing fraction has an adjuvant action on the activity of the latter, in addition to that due to the viscosity of the original mucin. The same adjuvant action is shown by known adsorbents, namely activated charcoal, kaolin, fuller's earth and talc.

4. The nature of this second adjuvant action is discussed.

5. The activity of the specific virulence-enhancing factor is only fully manifested when in association with other non-specific physical factors.

It is a pleasure to record my gratitude to Mr J. S. Stanley, Miss P. Hollis and Mr R. C. Gallop for excellent technical assistance, without which this work would not have been possible. My thanks are also due to Dr B. R. Record for facilities for large-scale freeze-drying, and to Mr R. Strange for micro-analysis.

Acknowledgement is made to the Chief Scientist, Ministry of Supply, for permission to publish this paper.

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