The Virulence-Enhancing Factor of Mucins

4. THE ROLE OF PARTICULATE INSOLUBLE MATTER AND A VISCOUS MEDIUM IN VIRULENCE ENHANCEMENT, WITH A REVISED ASSAY OF THE THIRD FACTOR INVOLVED

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In Part 3 (Smith, 1951) of this series a virulence-enhancing fraction of hog gastric mucin was separated from the insoluble particulate residue. This residue had an adjuvant action on the enhancement of the virulence by the active factor comparable with that already demonstrated (Smith, 1950*b*) for the viscosity of the original mucin. However, using a biological assay involving a medium of constant viscosity for injection, no residue was obtained completely free from virulence-enhancing activity, even after autoclaving and washing with hot water. The suggestion was made, therefore, that a viscous medium, together with the particulate residue, could impede the host defence mechanisms by themselves, quite apart from their probable action in delaying the absorption by the host of the active factor. This suggestion is supported by the work of Olitzki & Koch (1945), who reported the adjuvant action of kaolin, fuller's earth, Norit A and talcum on the small virulence-enhancing activity of 0.3% agar for *Shigella dysenteriae*, *Escherichia coli* and *Bacterium typhosum* (*Salmonella typhosa*), and of Olitzki, Shelubsky & Hestrin (1946), who noted a similar action of kaolin on the activity of a number of carbohydrates, although the viscosities of the carbohydrate preparations used were not noted or measured. The work described in this paper shows that the suggestion is in fact true.

Thus one can no longer refer to the virulence-enhancing factor of hog gastric mucin; the activity is due to a combination of three factors, all of which are slightly active alone, and none of which can be singled out as having a much greater share in the combined activity than the other two. Nevertheless, in contrast with the particulate insoluble matter and the viscous medium, the third factor is more specific. Knowledge of its chemical nature would be extremely interesting, especially for comparison with that of various bacterial products which have been shown to have virulence-enhancing activity (Olitzki, 1948).

It was essential for further chemical fractionation to obtain a reliable assessment of this third factor, samples of which are still called (somewhat inaccurately) virulence-enhancing samples. Although

an assay involving a simple injection of the sample, together with *Bact. typhosum*, would be desirable, this was found to be impossible; too large a quantity is needed to inject adequate batches of mice with the relatively high concentrations required (approximately 1%, w/v). A constant amount of particulate residue in a medium of constant viscosity is, therefore, combined with the third factor, which then need be used in concentrations of the order of only 0.025% (w/v). The necessary alterations in the technique of the assay are described in this paper.

EXPERIMENTAL

General details for the biological testing of samples have been given (Smith, 1950*a*). Only the modified preparation of the suspensions for injection is described here.

The effect of insoluble particulate matter and of a viscous medium in enhancing the virulence of Bact. typhosum for mice

Insoluble particulate matter. Aqueous suspensions of charcoal, kaolin, fuller's earth, talc, a sample of the residue separated from the virulence-enhancing sample at pH 10.5 as previously recorded (Smith, 1950*b*), and a sample of the same residue which had been autoclaved in aqueous suspension and washed with hot water, were used. Serial dilutions of these suspensions were made and mixed with the requisite number of *Bact. typhosum*. The suspensions were thoroughly shaken before each dose was removed for injection. The results are given in Table 1.

After heating to 60° for 0.75 hr. the unautoclaved residue from hog gastric mucin was grossly contaminated with sporing Gram-positive organisms. This probably accounts for the death of some mice after injection of this residue alone. The other samples tested were sterile or contained only one or two organisms in 0.2 ml.

Viscous media. Gum tragacanth, gum acacia, agar, Cellofas (a methylcellulose), and a sample of semi-pure blood group substance obtained from hog gastric mucin by a combination of the methods of Morgan & King (1943) and Smith (1950*b*) were used.

Viscosity determinations were made on the aqueous solutions in an Ostwald viscometer at 37° as

Table 1. *The effect of aqueous suspensions of insoluble particulate matter in enhancing the virulence of Bact. typhosum for mice*

(General details of the method of testing are given by Smith (1950*a*). Routine experiments on a large number of mice have shown that 5×10^6 *Bact. typhosum* suspended in tryptic meat broth kill 2-8 of a batch of 10 mice. 5×10^5 or less kill no mice. Details of bacterial counts which were always 0.8-1.2 $\times$ 5000 organisms are not included in the table.)

Nature of particulate matter	No. of <i>Bact. typhosum</i>	Death rate (%) in batches of 10 mice injected with the following strengths (% w/v)						Repeat experiments using batches of 20 mice		
								Death rate (%)		
		20	10	5	2.5	2	1	Strength (% w/v) injected	No. <i>Bact. typhosum</i>	5000 <i>Bact. typhosum</i>
Charcoal	5000	90	80	50	10	—	—	7.5	5	95
	Nil	20	Nil	Nil	—	—	—			
Kaolin	5000	90	70	20	Nil	—	—	7.5	Nil	60
	Nil	20	Nil	10	—	—	—			
Fuller's earth	5000	100	90	70	10	—	—	4	Nil	40
	Nil	90	90	10	—	—	—			
Talc	5000	30	40	Nil	Nil	—	—	20	Nil	25
	Nil	10	Nil	Nil	—	—	—			
Residue insoluble at pH 10.5 from hog gastric mucin	5000	—	—	100	70	80	10	1.5	15	50
	Nil	—	—	100	80	30	Nil			
Same residue autoclaved and washed with hot water	5000	—	—	100	70	70	10	3	5	95
	Nil	—	—	50	10	Nil	Nil			

Table 2. *The effect of a viscous medium in enhancing the virulence of Bact. typhosum for mice*

(General remarks as in Table 1.)

Nature of material producing the viscous aqueous solution	Solutions A, of low viscosity*		Solutions B, of high viscosity†		
	Approximate strength (% w/v) of solution injected with 5000 <i>Bact. typhosum</i>		Approximate strength (% w/v) of solution injected	Death rate (%) in batches of 20 mice	
		Death rate (%) in batches of 20 mice		No. <i>Bact. typhosum</i>	5000 <i>Bact. typhosum</i>
Tragacanth	0.1	Nil	0.3	Nil	25, 35
Blood group substance from hog gastric mucin	1.75	Nil	6.0	Nil	15, 55
Acacia	10	Nil	30	Nil	10, 40
Agar	0.16	Nil	0.35‡	Nil	10, 10
Cellofas	0.4	5, nil	1.2	Nil	10, 25

* Viscosity 6-9 times that of water, compared in an Ostwald viscometer, B.S.S. 2, vol. 8 ml. at 37°.

† Viscosity 8-12 times that of solutions A, compared in an Ostwald viscometer B.S.S. 3, vol. 23 ml. at 37°.

‡ The viscosity of agar solutions near the gelling concentration is difficult to control for a given concentration and one batch of mice was injected with 0.35% (w/v) agar of viscosity higher than that stated.

described by Smith (1951), before mixing with the *Bact. typhosum* suspension. The viscosities of the more concentrated solutions were not compared directly with that of water, but with those of the dilute solutions, as the viscometer necessary for the determination of these very viscid solutions was too large to give a reasonably accurate figure for water. Results are given in Table 2.

Insoluble particulate matter combined with viscous media. Suspensions of the six insoluble materials noted above, in concentrations which were inactive alone in aqueous suspension, were made in aqueous solutions of the five viscous materials used, at concentrations which had already been proved to be

inactive alone. The suspensions of the insoluble matter and solutions of the viscous materials were heated to 60° for 0.75 hr. in double strength before mixing. As before, viscosities were determined before adding the *Bact. typhosum* suspensions. Results are given in Table 3.

Specificity of the third factor involved in virulence enhancement

Using the revised method of assay described below, virulence-enhancing samples from hog gastric mucin show definite activity at 0.0125% (w/v). Undegraded thymus deoxypentose nucleic acid, yeast pentose nucleic acid, crystalline pepsin,

Table 3. *The effect of a combination of insoluble particulate matter and a viscous medium in enhancing the virulence of Bact. typhosum for mice*

(General remarks as in Table 1. The death rates of adequate control batches injected without *Bact. typhosum* are not included in the table: these death rates were nil in nearly all cases and never more than 10% except for the unautoclaved residue from hog gastric mucin which showed a consistent death rate of 10–20% irrespective of the viscous medium in which it was injected. The viscosity of all suspensions was 6–9 times that of water compared in an Ostwald viscometer (B.S.S. 2, vol. 8 ml.) at 37°.)

Death rates (%) in batches of 20 mice injected with 5000 *Bact. typhosum* and the following strengths (w/v) of the stated particulate matter in the various viscous media

Nature and approximate strength (% w/v) of the material producing the viscous suspension	None	Charcoal (2.5 %)	Kaolin (2.5 %)	Fuller's earth (2.5 %)	Talc (5 %)	Autoclaved residue from hog gastric mucin (1.5 %)	Residue from hog gastric mucin (0.75 %)
None	—	10	Nil	10	Nil	10	Nil
Tragacanth, 0.12	Nil	55	45	50	55	45	50
Blood group substance from hog gastric mucin, 1.75	Nil	100	65	65	30	90	70
Acacia, 10	Nil	95	30	40	20, 15	60	45
Agar, 0.16	Nil	80	70	80	60*	55	55
Cellofas, 0.4	5, nil	95	10, 25	20, 15	55	45	45

* Viscosity 5.1 times that of water at 37°.

crystalline trypsin, bovine albumen, human globulin, dialysed and undialysed acid and alkaline hydrolysates of virulence-enhancing samples showed no activity at 0.05% (w/v). In addition, various polysaccharides and mucopolysaccharides have been shown to act only by virtue of their viscosities.

The third factor involved in virulence enhancement is, therefore, to some extent specific. However, it would be interesting to test several bacterial products under the conditions of the assay.

Revision of the biological assay

In the original assay of virulence-enhancing activity (Smith, 1950a) samples were compared with a 'standard' mucin preparation consisting of a well homogenized batch of crude hog gastric mucin (Wilson's Granular Mucin; Smith, 1950a). This preparation gave a viscous solution and contained insoluble particulate matter as well as the more specific factor. Hence, the slope of the regression line of the probit of the death rate with dose, used for each comparison of 'standard' with unknown mucin, was a function of the variation of all the three factors which are now known to have virulence-enhancing activity. In the assay described below the unknown sample is compared in the same test with a new 'standard' preparation freed from the two non-specific factors, each being injected in the presence of a constant amount of particulate residue and in a suspension of constant viscosity. As was expected from the above considerations, the slope of the regression line of both the new 'standard' preparation and unknown samples (1.5, 1.0–2.0) was different from, and smaller than, that for the original 'standard' preparation (5.8, 5.1–6.3).

Hence more batches of mice than in the original assay are needed to yield the accuracy aimed at—namely the detection with probability, $P = 0.95$, of approximately a twofold difference in virulence-enhancing activity.

New 'standard' virulence-enhancing preparation. A batch (100 g.) of crude virulence-enhancing mucin was freed from materials yielding a viscous solution and from particulate residue by a modification of the methods given by Smith (1950b, 1951). It is kept over P_2O_5 at 0° and contains 5% moisture (loss at 0.1 mm. Hg and 60° over P_2O_5). It is injected into batches of 20 mice at concentrations of 0.4, 0.2, 0.1 and 0.05% (w/v), which, under the conditions of the assay, cover the death rate range 20–80%.

Unknown virulence-enhancing samples. The material is injected into batches of 20 mice at four concentrations (calc. for the substance dried at 0.1 mm. Hg at 60° over P_2O_5), at twofold intervals, which have been shown by preliminary experiments to cover the death rate range 20–80% under the conditions of the assay.

Particulate matter. This is a large batch of charcoal (SS 14) which has been thoroughly mixed and passed through a fine sieve (160 mesh). The constant concentration in suspension in all injected fluids is 0.4% (w/v).

Constant viscosity. The viscosity is maintained at seven to nine times that of water (Ostwald viscometer B.S.S. 2, 37°) by means of tragacanth solutions (0.13–0.18% (w/v) according to the particular mucin solution and its strength) as previously described. The finely divided charcoal does not interfere with the viscosity determination.

Preparation of the material for injection. The new 'standard' third factor, the unknown virulence-enhancing samples and the charcoal are made up at four times the strength ultimately required and adjusted to pH 7.0–7.2. The tragacanth solution is prepared at double strength and adjusted to the same pH. Equal quantities of virulence-enhancing sample and charcoal suspension are mixed and shaken with glass beads overnight to allow adsorption to take place. These

mixtures and the tragacanth solutions are heated separately to 60° for 0.75 hr., cooled and mixed in equal quantities. After determining the viscosities at 37° on separate samples, the requisite quantity of *Bact. typhosum* is added and the mixtures injected into batches of 20 mice. Particular care is taken that the suspensions have been well mixed before doses are removed.

In addition to the usual controls previously described (Smith, 1950*a*), in 18 experiments the suspension of charcoal in the medium of constant viscosity was injected into mice together with 5000 *Bact. typhosum*. Only 14 of 360 mice died, and in no batch of 20 mice did more than two die. This shows that the effect of the two non-specific factors had been sufficiently reduced.

Comparison of the death rates produced by the four concentrations of 'standard' and unknown virulence-enhancing samples. This was done by probit analysis. The activity of the unknown sample in terms of the 'standard' is expressed in units/g., the strength of the 'standard' being 1 unit/g. These units are arbitrarily called 'Virulence-enhancing units' as distinct from 'Wilson units' (Smith, 1950*a*). Mr S. Peto writes a note on the statistical treatment of the data as an addendum to this paper.

Table 4 shows some typical results, together with weighted means of duplicate assays. The fiducial limits show that with probability, $P=0.95$, a difference of virulence-enhancing power of 3.0-3.5 times is detected by one assay, and of 2.0-2.5 times by a combination of duplicate assays.

DISCUSSION

The results described by Smith (1950*b*, 1951), together with those in the first part of this paper, make it clear that no single chemical substance is responsible for the virulence-enhancing action of mucin. The activity is due to a combination of one factor which is relatively specific, with two that are non-specific. Each factor has some activity when used alone, but a much higher dose is needed than when it is combined with the other two. The differences between the minimum concentrations showing an effect for each agent acting alone (charcoal 7.5% (w/v), viscosity ten to twelve times that in the active combination, third factor 1%, w/v) and those for the agents in combination (charcoal 0.4% (w/v) viscosity unity, third factor 0.025%, w/v) show that the effect is synergic and not additive.

It is true that no one agent has a much greater share in the combined activity than the other two. Nevertheless, consideration of the yields and activities of the three agents from the crude mucin indicates that the relative effects are in the descending order third factor, particulate residue, and viscous medium. The difference between the relative effects of the first two is less than that between the last two.

Table 4. *The results of duplicate assays on various virulence-enhancing fractions together with their weighted means*

Virulence-enhancing fraction	Assay	Most probable result in 'Virulence-enhancing units'/g.	Fiducial limits ($P=0.95$)	
			Minimum	Maximum
588	1	2.5	1.2	4.3
	2	2.8	1.4	4.9
	Weighted mean	2.7	1.7	4.1
589	1	3.7	2.0	6.5
	2	8.0	4.8	14.7
	Weighted mean	5.6	3.7	8.4
590	1	4.5	2.6	7.6
	2	4.2	2.3	7.1
	Weighted mean	4.3	3.0	6.4
553	1	2.7	1.3	4.9
	2	1.4	0.7	2.4
	Weighted mean	1.9	1.2	3.0
554	1	3.9	2.2	7.2
	2	3.5	2.0	6.1
	Weighted mean	3.7	2.4	5.5
641	1	2.3	1.5	3.6
	2	3.7	2.3	5.8
	Weighted mean	2.9	2.1	3.9
642	1	3.4	2.2	5.4
	2	3.6	2.2	5.9
	Weighted mean	3.5	2.5	4.8

Knowledge of the combined activity of these three agents in hog gastric mucin helps to explain the results recorded in the literature (Olitzki, 1948) dealing with the bacteriological use of mucin and its mode of action. The effect of the viscosity of the granules of 'whole' mucin (Macleod, 1941), and of the inorganic residue (Olitzki & Koch, 1945; Landy & Batson, 1949) have all been noticed, together with the inadequacy of each alone to account completely for the activity. The small virulence-enhancing activity which has been reported for many substances could be attributed, probably, to the possession of one or two but not all three of the factors contained in crude hog gastric mucin. A clearer picture of the mode of action of mucin should result from further bacteriological and physiological work in which the above relationship is recognized.

The purification of the third, more specific, factor involved in the enhancement of virulence is being continued using the revised biological assay described in this paper, in which an inactive suspension of a constant amount of particulate residue in a medium of constant viscosity is combined with the third factor. In this way only low concentrations of the latter factor are needed and the limited supply of fractionation samples is sufficient to inject adequate batches of mice. The mixture of charcoal and tragacanth was chosen after preliminary experiments had been carried out to find the maximum sensitivity of the assay for the virulence-enhancing samples. Charcoal is used as the particulate residue instead of the residue obtained from the hog gastric

mucin, because the latter material is heavily contaminated with sporing organisms which are not destroyed by heating to 60° for 0.75 hr.; the charcoal suspension is sterile, or contains only one or two organisms in 0.2 ml.

It must be emphasized that there is no direct relationship between the activities of purified virulence-enhancing samples, and the activity of the original crude mucin. The former are measured in 'Virulence-enhancing units'/g. which refer to a new 'standard' preparation containing the third factor only, whereas the latter was measured in 'Wilson units' which refer to a crude 'standard' preparation containing all three of the factors involved in the virulence enhancement.

SUMMARY

1. Insoluble particulate matter and a viscous medium, alone and combined, enhance the virulence of *Bacterium typhosum* for mice.

2. The activity of hog gastric mucin is due to a synergic action between a particulate residue, a viscous medium and a more specific third factor.

3. The biological assay of samples of the third factor has been revised in the light of this knowledge. It can detect with probability, $P=0.95$, a difference in activity of two to two and a half times, using the weighted mean of duplicate assays.

It is a pleasure to record our gratitude to Dr D. W. Henderson for his helpful criticism.

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ADDENDUM

Statistical Analysis of the Biological Assay of Virulence-enhancing Activity

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About four tests are carried out weekly, each involving a comparison of the 'standard' preparation with unknown virulence-enhancing samples. The death rates of the 'standard' preparation in four to eight tests are investigated every week or fortnight for homogeneity of response in each dose group and for parallelism of the respective probit regression lines. Over such a short period of time a mean regression line for the 'standard' preparation should be a more reliable basis for comparison with the unknown sample than that of the individual 'standard' in the test and the fiducial limits are considerably narrower when using a 'mean standard'. The results quoted in Table 4 are ob-

tained by this method. It may well happen that the three factors which should remain constant in the assay (viscosity, particulate matter and the inherent virulence of the *Bact. typhosum*) do in fact vary, so that the regression lines of the various standard tests are not parallel. In this case, an individual comparison with wider fiducial limits is made; such assays are regarded as pilot experiments only. The relative potencies thus obtained are expressed in units/g. ('Virulence-enhancing units') different from 'Wilson units' (Smith, 1950a), since the latter refer to a crude 'standard' preparation which contains all three factors affecting the virulence enhancement.

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Amino-acid Requirements of Amylase Synthesis by Pigeon-Pancreas Slices

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In previous publications (Hokin, 1950, 1951a), it was shown that pigeon-pancreas slices synthesize α -amylase when incubated in glucose saline media and that the addition of acid-hydrolysed casein supplemented with tryptophan, or a mixture of twenty amino-acids, increased the rate of synthesis approximately threefold.

The present paper is concerned with the role of the individual amino-acids in α -amylase synthesis. A mixture of ten amino-acids (tryptophan, arginine, threonine, valine, tyrosine, lysine, leucine, histidine, phenylalanine and isoleucine) was found to be necessary and also sufficient for the maximum rate of amylase synthesis.

Part of this work has been communicated to the Biochemical Society (Hokin, 1951b).

EXPERIMENTAL

Media. The medium used for the incubation of slices was the bicarbonate saline of Krebs & Henseleit (1932) gassed with 5% CO₂ in O₂. A similar saline containing only 0.00043 M-bicarbonate not gassed, was used as a suspending medium

for slices before incubation. Glucose (0.2%) was added in all experiments.

Amino-acids. Commercial amino-acids were used. The standard mixtures of amino-acids and the final concentrations in the medium are shown in Table 1. In earlier experiments mixtures I and III, which contained L-amino-acids, with the exception of DL-serine and DL-threonine, were used. Mixture I contained the ten amino-acids found to be essential for the nutrition of the rat (Rose, 1938) and chick (Almquist, 1947), whilst mixture III contained ten 'non-essential' amino-acids. In the later experiments, after it had been established by paper chromatography that three of the L-amino-acids from natural sources; i.e. leucine, isoleucine and tryptophan were not pure, mixtures II and IV, which contained synthetic racemic amino-acids, with the exception of L-arginine, L-histidine and L-hydroxyproline, were used. Stock solutions of mixtures II and IV were made up by dissolving the amino-acids in water and neutralizing with 1N-NaOH. Mixtures I and II were added from stock solutions in which the amino-acids were dissolved in bicarbonate saline.

Ketonic acids. Indolylpyruvic acid was synthesized from indole aldehyde according to the method of Ellinger & Matsuoka (1920). It was light buff coloured; it darkened at 200° and melted at 205° (uncorr.). Indole aldehyde was

Table 1. *Standard amino-acid mixtures*
 (Concentrations are those in incubation vessels in routine experiments.)

Mixture I		Mixture II		Mixture III		Mixture IV	
Amino-acid	Concn. (M)	Amino-acid	Concn. (M)	Amino-acid	Concn. (M)	Amino-acid	Concn. (M)
L-Tryptophan	0.002	DL-Tryptophan	0.002	L-Glutamate	0.002	DL-Glutamate	0.002
L-Arginine	0.002	L-Arginine	0.001	L-Aspartate	0.002	DL-Aspartate	0.002
DL-Threonine	0.004	DL-Threonine	0.002	L-Alanine	0.002	DL-Alanine	0.002
L-Lysine	0.002	DL-Lysine	0.002	L-Glycine	0.002	Glycine	0.001
L-Leucine	0.002	DL-Leucine	0.002	DL-Serine	0.004	DL-Serine	0.002
L-Valine	0.002	DL-Valine	0.002	L-Proline	0.002	DL-Proline	0.002
L-Histidine	0.002	L-Histidine	0.001	L-Hydroxyproline	0.002	L-Hydroxyproline	0.001
L-Isoleucine	0.002	DL-Isoleucine	0.002	L-Tyrosine	0.002	DL-Citrulline	0.001
L-Phenylalanine	0.002	DL-Phenylalanine	0.002	L-Cystine	0.002	DL-Methionine	0.001
L-Methionine	0.002	DL-Tyrosine	0.002	L-Citrulline	0.002	L-Citrulline	0.002