work. Hirooka (1942) reported that hog gastric mucin prepared by alkaline extraction of the mucosa and precipitation with acetic acid was toxic to mice. Samples obtained at all stages in the chemical fractionation described in the following paper were appreciably toxic at concentrations two to three times those which demonstrated virulence enhancement; the same was true of the 'standard' mucin. Mouse post-mortems and the histological examination of various tissues have not revealed the nature of this toxicity, the elucidation of which needs further experimental data.

SUMMARY

1. A biological technique has been established for the assay of the virulence-enhancing factor of mucins and chemical fractions thereof.

2. Unknown mucin samples are compared with a 'standard' mucin preparation. A constant number of *Bacterium typhosum* (strain Ty_2) is injected intraperitoneally into batches of twenty mice in the presence of falling concentrations of the test materials. Comparisons of death rates and concentrations are made by probit analysis.

3. The assay can detect twofold differences in virulence-enhancing activity with probability $P = 0{\cdot}95$.

4. The toxicity of mucin preparations at high concentrations is noted.

It is a pleasure to record my gratitude to Dr D. W. Henderson for his helpful advice. I am indebted to Miss J. Richley and Mr P. Peacock for their excellent technical assistance in this large-scale animal work, both during the development of the assay, and in its routine use. My thanks are also due to Miss B. H. Westcombe for the statistical analysis of results.

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

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The Virulence-enhancing Factor of Mucins

2. FRACTIONATION STUDIES ON HOG GASTRIC MUCIN

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(*Received* 3 *October* 1949)

Almost the whole of the extensive published work on the virulence-enhancing action of mucin deals with the bacteriological study and application of the phenomenon, rather than with the chemical nature of an agent causing the effect. It is evident from this literature, however, that batches of commercial mucin vary greatly in virulence-enhancing power, and for any particular mucin, activity is proportional to the concentration.

A few recorded attempts have been made to isolate a possible virulence-enhancing factor from hog gastric mucin. These attempts which are summarized below suffer from two serious disadvantages. First, the details of a statistically adequate biological assay, capable of a reasonably accurate measurement of virulence-enhancing activity, have never been published. Secondly, all workers have used dried commercial hog gastric mucin, of unknown history.

Anderson & Oag (1939) used the hog gastric mucin prepared by L. Light and Co., London, and as a qualitative test for activity, injected mice intraperitoneally with a strain of *Meningococcus* (*Neisseria meningitidis*). After extracting inactive matter with cold water, an active fraction giving protein and carbohydrate reactions was obtained by extraction with 0·33 N-KOH solution and pre-

cipitation with acetic acid. Hydrolysis of this material with NaOH yielded an active product showing protein reactions only.

Olitzki & Koch (1945) repeated and confirmed the work of Anderson & Oag (1939). Two different samples of mucin were used, and the test for activity involved the intraperitoneal injection of *Shigella dysenteriae* (*Shiga*) into mice. In the case of one mucin (Nordemark-Werke) the material insoluble in alkali was intensely active; it contained 40·5% of inorganic matter, a large proportion of which was aluminium.

Gould & King (1947) fractionated Wilson's 'Granular mucin' by differential centrifugation, and used a test involving the intraperitoneal injection of a strain of Meningococcus into mice; no details of this test were given. Centrifuging an aqueous suspension at 15,000–20,000 rev./min. separated an active precipitate from an inactive viscous supernatant liquor. This precipitate was dissolved in Na_2CO_3 solution and reprecipitated with acetic acid at pH 3. A concentration of twenty times was claimed, and colour reactions showed this active residue to be of a protein rather than carbohydrate nature.

King & MacCabe (1949), after stating that the above method was difficult to repeat, described a slightly different procedure. After high-speed centrifugation of the aqueous suspension, the dried active precipitate was extracted with formamide. Removal of the formamide by dialysis, and freeze drying yielded an active product insoluble in water at pH 7 and giving both protein and carbohydrate reactions. A concentration of twelve times was claimed.

Morgan & King (1943) stated that completely soluble blood-group **A** substance from Wilson's 'Granular mucin', still retained the virulence-enhancing effect of the original mucin, although heat sterilization destroyed the effect, together with the viscosity of the solution. Electrophoresis showed the preparations to contain 90% of a single component, and 10% of an ill-defined contaminant.

Recently, Landy & Batson (1949) obtained blood-group **A** substance from Wilson's 'Granular mucin' in 22% yield by the method of Morgan & King (1943), and in 15·6% yield by the method of Zittle (1948). These samples in 3% concentration had approximately the same virulence-enhancing activity for *Bacterium typhosum* (*Salmonella typhosa*) in mice as the original 'Granular mucin' had in 5% concentration.

It is obvious that the chemical fractionation of hog gastric mucin for a possible virulence-enhancing factor is still in the initial stages. The reported activity of totally different mucin fractions is undoubtedly due to inadequate purification.

This paper describes preliminary steps in the chemical fractionation of a virulence-enhancing factor from hog gastric mucin. It has been proved that, although this is the most important factor in virulence enhancement, it is not the only one involved. A smaller, but nevertheless important, part in the enhancement is due to another substance or substances; this has no inherent virulence-enhancing activity, but, when mixed with the virulence-enhancing factor, greatly increases its activity, probably by providing a viscous medium for its dispersion. A brief summary of this work has already been reported (Smith, 1950).

A biological assay capable of detecting a twofold difference in virulence-enhancing activity has been described in the previous paper. This assay was the keystone for the chemical fractionation of hog gastric mucin described in this paper. Fresh hog stomach linings were used as starting material rather than the dried commercial product.

EXPERIMENTAL AND RESULTS

Each fractionation method described below was repeated at least once, using material from a different initial group of hog stomachs. The virulence-enhancing activity of different fractions is given in arbitrary 'Wilson units', with fiducial limits in brackets, for probability $P=0·95$.

Fresh hog stomach linings. Hog stomachs were obtained from C. T. Harris Ltd., Calne, Wilts. On removal from the freshly killed pigs the unwashed stomachs were packed in ice, and within 3–6 hr. were cut open and washed under a slowly running tap; fast jets of water which would remove the adhering mucus were avoided. The inner mucosae were then separated from the outer muscular layers. Should extraction be postponed for a short time, the mucosae were kept at −20°.

Initial extraction of individual hog stomachs

The literature outlined above suggested the use of two initial extraction procedures on individual hog stomachs; the first involved a pepsin autolysis to extract crude blood-group substances (**A** and **H**), and the second, an Na_2CO_3 extraction, was used to prevent the protein-splitting action of pepsin.

Crude blood-group substances (**A** *and* **H**), *from individual linings.* One minced mucosa was mixed with water (300 ml.), conc. HCl (8 ml.) and toluene (20 ml.), and placed in an incubator at 37° for 1 week; the mixture was shaken at intervals to prevent tissue from rising and rotting on the surface. After the second day the pH was determined daily and maintained at pH $3·0 \pm 0·2$. The bulk of the tissue dissolved, leaving some insoluble matter; most of the latter was removed by centrifuging in an angle centrifuge and straining the centrifugate through lint. The separation was not complete, and the solution remained slightly turbid. It was cooled to 0° and mixed with absolute industrial ethanol (5 vol.) at −18°. After standing overnight at −18° the white solid was centrifuged off, washed once with industrial ethanol (200 ml.), and dried with absolute industrial ethanol and ether; the ether was removed in a vacuum desiccator over P_2O_5. Yield 3–5 g. One hundred and twenty individual mucosae were extracted in this manner and the samples kept separately.

Extraction of individual hog stomach linings with 0·1 N-*sodium carbonate solution.* One minced mucosa was mixed with 0·1 N-Na_2CO_3 solution (1 l.) in a Waring blender. Toluene (20 ml.) and some glass beads were added and the mixture was shaken at room temperature for 36 hr.; the pH during this shaking was between 9 and 10. Residual tissue was removed by spinning in a Laval centrifuge and straining the centrifugate through lint. The solution was cooled to 0° and adjusted with acetic acid to pH $4·2 \pm 0·2$, when a precipitate formed. The crude material was precipitated completely by adding absolute industrial ethanol (5 vol.) at −18°. After standing overnight at −18° the pre-

cipitate was washed once with industrial ethanol (200 ml.), and dried as described for the crude blood-group substance. Yield 8–10 g. Seventy-seven individual mucosae were extracted by this method, and the samples kept separately.

Comparison of blood-group activities with virulence-enhancing activities of samples extracted by the above two methods. Both the crude blood-group substance samples and those got by extraction with sodium carbonate possessed virulence-enhancing activity. It was decided to compare virulence-enhancing activities of different samples with their blood-group activities. A parallelism between these activities would have been a good indication that blood-group substances (**A** and **H**) were responsible for virulence enhancement.

The serological examination of all the individual samples extracted by the two methods was kindly carried out by Dr W. T. J. Morgan and Mr E. F. Annison at the Lister Institute of Preventive Medicine, London. Full details of this examination have been published (Annison, Chadwick, Morgan & Smith, 1949); it entailed the comparison of the unknown samples with 'standard' **A** substance and 'standard' **H** substance, in tests involving the inhibition of agglutination of **A** or **O** red blood cells by their respective antisera. Table 1 shows the virulence-enhancing activities of certain samples which have widely differing blood-group activities.

Table 1. *Comparison of blood-group activities and virulence-enhancing activities of the same samples of mucin*

Sample and method of preparation	Blood-group **A** activity (in terms of a standard '**A**' preparation) (%)	Blood-group **H** activity (in terms of a standard '**H**' preparation) (%)	Virulence-enhancing activity ('Wilson units')
Autolysis 1	Nil	200	0·33 (0·25–0·42)
2	Nil	50	0·35 (0·27–0·46)
3	100	3	0·36 (0·27–0·46)
4	25	1·5	0·23 (0·17–0·32)
Sodium carbonate 1	Nil	6	0·39 (0·30–0·51)
2	1·5	Nil	0·29 (0·22–0·38)

It is evident that no parallelism exists between the two activities, and therefore it is unlikely that the virulence-enhancing factor, and blood-group substances (**A** and **H**) are one and the same. In view of this fact, all individual samples of crude blood-group substance were bulked, prior to attempts to separate the virulence-enhancing factor from the blood-group mucoid; the activity of this bulked sample was 0·34 'Wilson unit' (0·26–0·45). Similarly, the sodium carbonate extracts were bulked and had an activity of 0·36 'Wilson unit' (0·28–0·47).

Fractionation of the bulk crude blood-group substance and bulk sodium carbonate extract

Each sample was separated into three main fractions: (1) insoluble at pH 4·2 on prolonged dialysis against distilled water at 0°; (2) soluble at pH 4·2 and precipitated by ethanol; (3) substances passing out of the dialysis bag. From both starting materials the activity was concentrated in the first fraction.

Crude blood-group substance. The crude material (25 g.) was suspended in water (500 ml.) in a Waring blender and the pH adjusted to 4·2±0·2 with 2 N-acetic acid (approx. 12 ml.). The slightly viscous mixture was dialysed against frequent changes of distilled water at 0° for 6 days; the pH remained constant at 4·2±0·2 and a precipitate separated.

The dialysate was adjusted to pH 7·0–7·5, freeze dried, extracted with industrial ethanol (500 ml.) to remove any Na acetate, and finally dried as described for crude blood-group substance. Yield 2·5 g. It was found to be completely inactive.

The contents of the dialysis bag were spun for 1 hr. in the closed bowl of a Sharples centrifuge. The supernatant was filtered through a bacterial candle, cooled to 0° and precipitated with absolute industrial ethanol (5 vol.) at −18°. After leaving overnight at −18° the stringy white solid was washed once by blending with industrial ethanol (1 l.); it was then dried as described for crude blood-group substance. Yield 15 g. From its method of preparation this material, which formed a viscous aqueous solution, contained the blood-group substances (**A** and **H**); this was checked by an inhibition test (Annison, private communication). It had, however, only a trace of virulence-enhancing activity, less than 0·22 'Wilson unit' (0·17–0·29).

The solid remaining in the Sharples bowl was resuspended in water (200 ml.) containing a few drops of 2 N-acetic acid, and recentrifuged. This washing was repeated, and the substance was dried as described for crude blood-group substance. Yield 2·75 g. It was found to have an activity of 1·27 'Wilson units' (0·70–2·05). In a larger scale experiment (300 g. of crude product) this material was not isolated as such, but was separated into two fractions by means of 0·2 N-Na_2CO_3 solution. The solid in the Sharples bowl was stirred with 0·2 N-Na_2CO_3 solution, then spun for 1 hr. in the closed bowl. The supernatant was filtered through a bacterial candle, the pH adjusted to 4·2 with acetic acid, and dialysed for 4 days at 0° against frequent changes of distilled water. The precipitate which formed was spun off, and dried as described for crude blood-group substance. Yield 13·5 g. It was moderately active, 0·43 'Wilson unit' (0·33–0·55). The material insoluble in 0·2 N-Na_2CO_3 solution was washed twice with industrial ethanol and dried as described for crude blood-group substance. Yield 5·0 g. This product was appreciably toxic at 0·3% (w/v), but at 0·2 and 0·15% (w/v) showed virulence-

enhancing activity equivalent to 7·83 'Wilson units' (6·60–9·31).

Sodium carbonate extract. By a similar method the crude material (100 g.) was separated into fractions: (1) Insoluble at pH 4·2; yield 39 g.; activity, 0·42 'Wilson unit' (0·37–0·49). (2) Soluble at pH 4·2 and precipitated by ethanol; yield 15 g.; only a trace of activity, less than 0·20 'Wilson unit' (0·16–0·26). (3) Dialysable material; yield 25 g.; completely inactive.

The direct extraction from fresh stomach linings of material insoluble at pH 4·2*: further purification of this product*

Sodium carbonate extraction was abandoned in favour of autolysis. In view of the sparing solubility of the active material, the substance which remained insoluble after autolysis, and which was to a very large extent discarded in the method of extraction for crude blood-group substances, was retained in the product.

Material insoluble at pH 4·2 *obtained direct from fresh mucosae.* Minced linings from thirty stomachs were autolysed under exactly the same conditions as described for individual mucosae. The whole of the autolysate was strained and precipitated with industrial ethanol (5 vol.). The precipitate was blended with water, brought to pH 4·2 and dialysed at 0° for 1 week. The precipitate was removed and freeze dried. Yield 210 g. A large amount of fat was removed by washing four times with ether (2 l.) and the product was finally dried in a vacuum desiccator over P_2O_5. Yield 100 g. Activity, 0·73 'Wilson unit' (0·46–0·98). The fat recovered from the ether extracts was completely inactive.

Extraction with water at pH 6·0. The above material (100 g.; activity, 0·73 'Wilson unit'), was blended with water (1·5 l.), the pH adjusted to 6 and the mixture left at 0° overnight. The precipitate was spun off in a Laval centrifuge, washed twice at 0° by water at pH 6, and freeze dried. Yield 45 g. Activity, 1·20 'Wilson units' (0·81–1·55). The combined supernatants were filtered through a bacterial candle and freeze dried. Yield 51 g. This product was only very slightly active, 0·30 'Wilson unit' (0·18–0·41).

Extraction with 0·1 N-Na_2CO_3 *solution.* The above product (5 g.; activity, 1·20 'Wilson units') was blended with 0·1 N-Na_2CO_3 solution (100 ml.) and left at 0° overnight; the pH was 10·0–10·5. After spinning at 4000 rev./min. in a refrigerated centrifuge at 0° for 8 hr., the slightly turbid supernatant liquor was removed, the pH adjusted to 6 with acetic acid, and set aside. The solid material was washed a further three times at 0° with 0·1 N-Na_2CO_3 solution (50 ml.) and finally was suspended in water, dialysed at 0° for 2 days to remove Na_2CO_3, and freeze dried. Yield 2·4 g. Activity, 1·82 'Wilson units' (1·01–2·9). The combined supernatants were dialysed at 0° for several days, then freeze dried. Yield 2·1 g. It was moderately active, 0·68 'Wilson unit' (0·38–1·25).

The method finally adopted for the preparation of a reasonably active product direct from hog stomach linings

In spite of the increased activity of the residue after extraction with 0·1 N-Na_2CO_3 solution, this method was precluded from the general procedure by the relatively poor separation effected and the moderate activity of the extract. Non-inflammable dichlorethylene was substituted for ether as a fat solvent.

The whole autolysate, prepared as above, from thirty stomachs (approx. 10 l.) was strained to remove gross particles, and poured into absolute industrial ethanol (40 l.). The precipitate was dried by stirring with absolute industrial ethanol (10 l.) and defatted by stirring three times with dichlorethylene (9 and 2 × 6 l.). After evaporating the dichlorethylene, the white powder was extracted at 0° overnight four times with water (4·5 and 3 × 3 l.) at pH 6·0 ± 0·2. The washed precipitate was freeze dried. Yield 25–40 g. The activities of three typical batches of product prepared in this manner were 1·71 'Wilson units' (1·14–2·55), 2·55 'Wilson units' (1·96–3·32) and 2·53 'Wilson units' (2·09–3·08).

Removal of foreign matter. To remove small amounts of foreign matter a large number of solvents were examined for dissolving the active material, which hitherto had been obtained only as a precipitate. Those containing a phenolic group were the most promising, and success was achieved using saturated Na salicylate solution. Before describing the use of this solvent, it is relevant to report the failure of attempts to use formamide as a solvent as described by King & MacCabe (1949). The formamide was purified by repeated recrystallization before use. One of the above mucin fractions (5 g.; activity, 1·2 'Wilson unit') was extracted four times with formamide (50 ml.). The combined formamide solutions were dialysed against frequent changes of distilled water until free of formamide, when a precipitate formed. The suspension in the dialysis bag was then freeze dried. Yield 0·6 g. Activity, 0·85 'Wilson unit' (0·68–2·13).

Use of saturated aqueous sodium salicylate for solution and filtration of the active material, followed by precipitation with ethanol. The crude active material (15 g.), prepared as described above, was slowly added to stirred saturated aqueous Na salicylate (750 ml.). Foaming was reduced to a minimum by stirring slowly and adding a few drops of capryl alcohol. The mixture was stirred for 30 min. at room temperature and a slightly turbid, dark-red solution, pH 6·5–7·5, was formed. On spinning the solution at 2000 rev./min. for 15 min. in an angle centrifuge, a deposit, which was obviously foreign matter, separated. The yield of this material, after washing free from salicylate with ethanol and freeze drying, was 0·1–0·2 g. In the biological assay different samples did not behave consistently. Some had no action, others were very toxic, and still others showed some virulence enhancement, but this was never greater than that of the original crude active product. To make certain all foreign matter was removed, the centrifugate was filtered through a battery of six no. 54 Whatman filter papers (11 cm.) into flasks immersed in an ice and salt freezing mixture. Filtration took about 30 min. by which time the filtrate had cooled to 0°. Absolute industrial ethanol (5 vol.) at −18° was added and the mixture kept at −18° overnight. The precipitate was collected and washed with successive quantities (400 ml.) of cold 90% industrial ethanol until free from salicylate ($FeCl_3$) and freeze dried. Yield 7·5–9·0 g. Larger quantities of crude active material were dealt with in 15 g. batches up to the precipitation with ethanol; batches were then combined for washing free from

salicylate, using the Sharples centrifuge. Table 2 shows the activities of a few different batches prepared in this manner. These results, and those of the fractionations below, indicate the absence of any large-scale destruction of activity in saturated aqueous Na salicylate under these conditions.

Table 2. *Activities of some fractionation samples before and after filtration in saturated aqueous sodium salicylate and precipitation with ethanol*

Exp. no.	Activity of initial crude mucin ('Wilson units')	Yield of filtered material (%)	Activity of filtered material ('Wilson units')
1	2·53 (2·09–3·08)	58	2·77 (2·20–3·50)
2	1·71 (1·14–2·55)	60	1·55 (0·77–2·88)
3	1·71 (1·14–2·55)	58	1·60 (1·03–3·46)

Properties of the salicylate filtered products. These were grey powders almost insoluble in water at pH 7; the aqueous suspensions were non-viscous, and contained the suspended material in a very fine state of subdivision. Two different samples had respectively, N (Dumas), 14·5 and 13·5, and sulphated ash, 5·4 and 6·9%, calculated with reference to the substance dried over P_2O_5 at 0·1 mm. Hg at 60°. The outside chance of chemical combination between active material and Na salicylate during the removal of foreign matter was ruled out by the following experiments. Three different samples (0·1 g.) were hydrolysed with 5 ml. of N-HCl for 1 hr. at 100°, followed by cooling and neutralizing to phenol red. The same procedure was carried out using 5 ml. of N-NaOH on 0·1 g. samples. After standing overnight, the clear supernatant fluids gave no colour with $FeCl_3$ solution. A deep violet colour was obtained when Na salicylate (2 mg.) was included in identical tests.

Attempted fractionations of the active product in aqueous sodium salicylate solutions

Exhaustive attempts were made to fractionate the active material from aqueous Na salicylate. The fractionations effected a degree of separation, but this was not sufficient to warrant their inclusion in any general process of purification.

Fractionation of the crude active material with different strengths of aqueous sodium salicylate. High-speed centrifugation of saturated salicylate solutions deposited a small amount of slime; the amount of slime could be increased by lowering the salicylate concentration. 5 g. batches of crude active product (activity, 1·31 (1·14–1·57) 'Wilson units') were dissolved in 250 ml. each of saturated, 90% saturated and 80% saturated Na salicylate solutions, and spun at 2000 rev./min. The supernatants were not filtered, but spun for 1 hr. at 26,000 rev./min. in the closed bowl of the Sharples. The clear fluid was cooled to 0° and mixed with absolute industrial ethanol (10 vol.) at −18°. After remaining overnight at −18° the precipitate was collected, washed free from salicylate and freeze dried. The slime left in the centrifuge bowl was cooled to 0° and mixed with absolute industrial ethanol (500 ml.) at −18°. The precipitate was collected and dried as above. Table 3 shows the results of these experiments. A tendency for insoluble fractions to have a greater activity than either the original sample or the soluble fractions was indicated.

Fractionation with ethanol of saturated aqueous sodium salicylate solutions of the filtered active material. Filtered active material (2·5 g., activity, 1·55 (0·84–2·61) 'Wilson units') was dissolved in saturated aqueous Na salicylate (50 ml.). The solution was cooled to 0° and ethanol (1 vol.) at −18° slowly added. After standing for 2 hr. at 0°, the bulky precipitate was spun off at 0° and washed twice with 1 part saturated aqueous Na salicylate and 1 part ethanol (25 ml.). When washed free from Na salicylate and freeze dried the yield was 1·5 g. Activity, 1·91 (1·35–2·68) 'Wilson units'. A further volume of ethanol was added under the same conditions and the precipitate (0·35 g.) had an activity of 2·43 (1·54–5·43) 'Wilson units'. Final precipitation was made using 10 vol. ethanol; this material (0·3 g.) had activity 0·99 (0·32–1·99) 'Wilson unit'.

Table 3. *Yields and activities of products fractionated in different strengths of aqueous sodium salicylate*

(Initial crude sample 1·31 'Wilson units' (1·14–1·57).)

Sodium salicylate solution (% saturation)	Insoluble material: Yield (%)	Insoluble material: Activity ('Wilson units')	Soluble material: Yield (%)	Soluble material: Activity ('Wilson units')
100	12	2·29 (1·43–2·58)	54	1·84 (1·33–2·87)
90	18	2·47 (1·71–3·59)	46	1·34 (0·96–1·77)
80	23	2·10 (1·34–2·99)	42	1·35 (0·90–1·92)

The marked increase of activity of purified fractions when suspended in inactive solutions of semi-pure blood-group substance

If active fractions obtained by the above procedure were suspended in an inactive solution of semi-pure blood-group substance, a three- to four-fold increase in activity resulted. The semi-pure blood-group substance was the material soluble at pH 4·2 which was precipitated with ethanol in earlier work. Full details of these results, which involve different combinations of two different batches of inert semi-pure blood-group substance with two purified virulence-enhancing fractions are given in Table 4.

The same marked increase of the activity of purified fractions when suspended in solutions of tragacanth and agar to give approximately the same viscosity as with semi-pure blood-group substance

The high viscosity of suspensions with semi-pure blood-group substance, as compared with aqueous suspensions, suggested the use of other viscous

Table 4. *Comparison of the activities of two virulence-enhancing fractions when suspended (a) in water and (b) in inert semi-pure blood-group substance solutions*

(The results were obtained using the normal biological assay procedure (see previous paper) combining the injection solutions with a constant number of *Bact. typhosum*. Controls without organisms were carried out on batches of twenty mice for all injection solutions; not more than one mouse died in any of these controls. Normal results were obtained for the 'standard' mucin death rates, organisms control death rates and bacterial counts; these results are not included in the table. Two virulence-enhancing fractions Mucin 100 and Mucin 131 were used and two different samples of semi-pure blood-group substance, abbreviated Bgs. *A* and Bgs. *B*.)

Solution injected with 5000 *Bact. typhosum*: Nature and strength (w/v) of the virulence-enhancing fraction used	Suspending medium	Death rates in batches of 20 mice in one or more experiments (%)	Activities of virulence-enhancing fractions 'Wilson units' obtained by direct comparison with the 'standard' preparation
Mucin 100 0·75%	Water	15, 25, 30	1·53 (1·28–1·83)
Mucin 100 0·25%	Water	Nil	
Mucin 100 0·75%	Bgs. *A*, 3·5% (w/v)	80, 95	5·91 (5·06–6·90)
Mucin 100 0·25%	Bgs. *A*, 3·5% (w/v)	40, 45, 20	
Mucin 100 0·25%	Bgs. *B*, 3·5% (w/v)	35	5·11 (4·04–6·43)
Mucin 131 0·6%	Water	45, 60	2·77 (2·20–3·50)
Mucin 131 0·2%	Water	Nil	
Mucin 131 0·2%	Bgs. *A*, 3·5% (w/v)	40, 45	7·99 (5·60–11·97)
Nil	Bgs. *A*, 3·5% (w/v)	Nil	—
Nil	Bgs. *B*, 3·5% (w/v)	5	—

Table 5. *Comparison of the activities of two virulence-enhancing fractions when in suspensions of approximately the same viscosity using semi-pure blood-group substance, tragacanth and agar*

(Experimental arrangements and materials: see Table 4.)

Solution injected with 5000 *Bact. typhosum*: Nature and strength (w/v) of the virulence-enhancing fraction used	Suspending medium	Experiments on direct comparison of the same concentration of active sample in different suspensions: Death rates in batches of 20 mice (%)	Viscosities at 37° relative to the Bgs. *A*, 3·5% (w/v) suspension	Separate experiments, each suspension compared directly with the 'standard' mucin preparation: Activities ('Wilson units')	Viscosities at 37° relative to the Bgs. *A*, 3·5% (w/v) suspension
Mucin 100 0·2%	Water	Nil	—	—	—
Mucin 100 0·2%	Bgs. *A*, 3·5% (w/v)	60	1·0	5·91 (5·06–6·90)	1
Mucin 100 0·2%	Tragacanth, 0·17% (w/v)	40	0·9	6·64 (5·12–8·44)	1·1
Mucin 100 0·75%	Water	20	—	1·53 (1·28–1·83)	—
Mucin 100 0·75%	Bgs. *A*, 3·5% (w/v)	95	1·0	—	—
Mucin 100 0·75%	Tragacanth, 0·17% (w/v)	95	0·6		
Mucin 131 0·6%	Water	48	—	2·77 (2·20–3·50)	—
Mucin 131 0·2%	Water	Nil	—		
Mucin 131 0·2%	Bgs. *A*, 3·5% (w/v)	45	1·0	7·99 (5·60–11·97)	1
Mucin 131 0·2%	Tragacanth, 0·17% (w/v)	75	1·1	8·46 (5·97–11·05)	0·8
Mucin 131 0·2%	Agar, 0·15% (w/v)	60	0·7	8·28 (6·94–9·87)	0·8
Nil	Bgs. *A*, 3·5% (w/v)	Nil	—	—	—
Nil	Tragacanth, 0·17% (w/v)	Nil	—	—	—
Nil	Agar, 0·15% (w/v)	Nil	—	—	—

solutions as media for carrying the virulence-enhancing fractions. The same marked increase in virulence-enhancing activity was observed with tragacanth, and in smaller scale experiments, with agar.

Preliminary experiments were done to ascertain the concentrations of tragacanth and agar necessary to give suspensions of the active fractions with approximately the same viscosity as the semi-pure blood-group substance suspension. These viscosities were determined at 37° in an Ostwald viscometer, after the suspensions had been heated at 60° for 0·75 hr. (killing vegetative organisms), and just before adding the bacterial suspensions. Full details of the results of this work, which involved two different virulence-enhancing samples, are given in Table 5.

DISCUSSION

The first part of the work described in this paper is conclusive evidence that blood-group substances *per se* are not responsible for the virulence-enhancing activity of hog gastric mucin. This was indicated by the lack of parallelism between the virulence-enhancing activity and the blood-group activity of samples from individual hog stomach linings; it was proved by the separation of an active virulence-enhancing fraction from the blood-group mucoid, by dialysis at pH 4·2. The separation was confirmed by fractionation of the mucin extracted with 0·1N-Na_2CO_3 solution.

After autolysing the minced mucosae at pH 3 some material remains insoluble and is discarded in the preparation of crude blood-group substance. Large yields of a product having double the activity of the original blood-group substance were obtained by a method of preparation which included this insoluble matter in the dialysis precipitate at pH 4·2, while removing the fat present in it. Extraction of this material at pH 6 removed much extraneous material and a more active product was obtained. These results were embodied in a method of preparing a bulk product of relatively high activity, direct from hog stomach linings.

Up to the present, the active fractions have always been obtained as insoluble solids, and consequently were contaminated with foreign matter. To remove this foreign matter, and to assist in further fractionation, many solvents were investigated. Urea (40%), phenol (90%) and cresol were examined in some detail, but always a residue remained which constituted over 20% of the initial material. This residue always proved to be active: it is impossible as yet to say whether this effect is due to the sparing solubility of the active principle, or to its strong adsorption on the residue. Eventually success was achieved using saturated aqueous sodium salicylate, followed by precipitation with ethanol.

The active products thus obtained were practically insoluble in water at pH 7·0–7·2, forming non-viscous suspensions. They were too heterogenous for detailed chemical analysis, but the nitrogen (Dumas) figure suggests a protein or peptide nature.

The most active fractions which could be obtained consistently by the above procedures had most probable activities of 2·5–3·0 'Wilson units'. This was a concentration of seven to nine times the activity of the original crude blood-group substances. In view of the methods described and the yields obtained, this concentration of activity was low. Therefore, the possibility that other factors present in the crude mucin were necessary for a full manifestation of the virulence-enhancing activity, was considered. The material chosen for experiment was the soluble fraction which had been separated from the active substance insoluble at pH 4·2; it had been precipitated with ethanol, and contained blood-group activity, but had negligible virulence-enhancing power. This substance was designated semi-pure blood-group substance. In 3·5% (w/v) solution it had no inherent virulence-enhancing activity, but its viscosity was of the same order as that of the active solutions of crude blood-group substances. The virulence-enhancing activity of salicylate-filtered fractions, in aqueous suspension, was increased three- to fourfold when suspended in the above solution. This was confirmed using different batches of virulence-enhancing fraction, and of semi-pure blood-group substance. The concentration of activity was therefore the more reasonable figure of 25–30 times.

Hence it was shown that the virulence-enhancing power of hog gastric mucin was not due to one factor alone, as reported or tacitly assumed in previous work, but to a combination of two factors. These were the more important virulence-enhancing factor itself, and also an inert factor or factors present in the crude and semi-pure blood-group substance, which were necessary for the full manifestation of the activity of the virulence-enhancing factor.

Attention was now turned to the nature of this adjuvant action. The most obvious difference between the aqueous suspensions and the semi-pure blood-group substance suspensions was the marked viscosity of the latter (10–15 times greater). In order to discover whether the adjuvant effect was connected with the viscosity, suspensions of the virulence-enhancing factor were made using solutions which would give approximately the same viscosity at 37° as the semi-pure blood-group suspension. Tragacanth, and agar in smaller scale experiments, were used at very low concentrations (0·17% (w/v) and 0·15% (w/v), respectively) to give the required viscosity; these solutions showed no virulence-enhancing activity alone. Again, a marked increase in activity occurred when these solutions were used to suspend the virulence-enhancing factor, and, within the limits of the biological assay, this increased activity was the same as that obtained with the suspensions in semi-pure blood-group substance.

These experiments suggest that the materials causing the viscosity of hog gastric mucin are those necessary for the full manifestation of the activity of the virulence-enhancing factor. The presence of blood-group substances is undoubtedly the cause of some, if not all, of the viscosity. The reported virulence-enhancing activity of some samples of blood-group substances may well have been due to the presence of a small amount of virulence-enhancing factor exerting its maximum effect in the viscous medium.

The viscosity of the medium probably delays the absorption and inactivation of the active factor by

the host at the site of injection. If this is so, not only is the initial viscosity important, but also the degree to which it is maintained under the influence of the body fluids of the host. With reference to this delay in absorption, it is relevant to point out that Olitzki & Koch (1945) have reported that adsorbents such as kaolin increase the action of mucins in enhancing virulence. Also, it is interesting that extremely viscous media such as 2, 4 and 10 % (w/v) agar (Anderson & Oag, 1939), 6 % (w/v) starch paste (Robertson & Fox, 1939), 2·5 % (w/v) tragacanth (Steinberg, 1931) and 5 % (w/v) tragacanth (Benians, 1924), have virulence-enhancing effects alone which are, however, far smaller than that of mucin.

Future work on the virulence-enhancing factor of mucin, therefore, falls into two parts: the more important fractionation of the virulence-enhancing factor itself, and the investigation of the other factors necessary for the full manifestation of its activity. The work is being continued along these lines.

SUMMARY

1. Preliminary fractionations have been carried out on preparations from fresh hog stomach linings for a factor responsible for the virulence-enhancing power for bacteria.

2. Crude blood-group substance prepared by autolysis had virulence-enhancing activity which, in the same samples, did not run parallel with the blood-group activity.

3. Dialysis at 0° at pH 4·2 separated a small amount of active insoluble material from inactive soluble blood-group mucoid; the latter was precipitated in a semi-pure state by ethanol. More of this active material was prepared directly from fresh linings and further purified.

4. The still heterogeneous active material contained nitrogen (Dumas) 14·5, 13·5%; in aqueous suspensions, which were non-viscous, different batches consistently had activities seven to nine times those of the crude blood-group substance.

5. This activity, however, was increased three- to fourfold when suspended in a completely inactive 3·5 % (w/v) solution of semi-pure blood-group substance (see above), which imparts a marked viscosity to the suspension. This viscosity was of the same order as that of the original active crude blood-group substance solution; a concentration of activity of 25–30 times is therefore implied.

6. The same marked increase in activity was produced by suspending the active material in tragacanth and agar solutions to give suspensions with approximately the same viscosity at 37° as the above.

7. The virulence-enhancing power of hog gastric mucin is due to a virulence-enhancing factor which is active alone. But it only manifests its full activity when in combination with a second inert factor or factors, which may well act by providing a viscous medium for its suspension.

My thanks are due to Mr G. M. Hills for his valuable criticism. It is also a pleasure to record my gratitude to Mr D. W. Chadwick for help in the chemical extractions, and to Miss P. Hollis for assistance in the biological assay of mucin fractions.

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

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