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The Virulence-enhancing Factor of Mucins

1. A BIOLOGICAL ASSAY OF VIRULENCE-ENHANCING ACTIVITY

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Nungester, Wolf & Jourdonais (1932) discovered that a marked increase in the virulence for mice of strains of *Staphylococcus aureus*, *Streptococcus pneumoniae* (Type II) and *Strep. haemolyticus* could be effected by injecting intraperitoneally a suspension of the organisms in 5 % (w/v) hog gastric mucin. About the same time Miller (1933) found that the same procedure would reduce the minimum lethal dose for mice of strains of *Neisseria meningitidis* by 10^{-6}.

Since that time the virulence-enhancing power of mucin (review: Olitzki, 1948) has been used extensively, particularly in serum and chemotherapeutic testing, for the production of experimental infection in several different test animals; relatively small numbers of bacteria of many different species have been used. The intratracheal route of infection for the production of pneumonias has been used with a success equal to that of the more widely exploited intraperitoneal route. Most workers have used dried commercial hog gastric mucin, mainly but not solely the 'Granular Mucin 1701W' of Wilson Laboratories, Chicago, U.S.A. Anderson & Oag (1939) and Tunnicliffe (1940) are in agreement on the activity of

human salivary mucin; human gastric mucin (Sindbjerg-Hansen, 1943), hog intestinal mucin (Levaditi & Vaisman, 1937) and guinea-pig intestinal mucin (Cantacuzène & Marie, 1919) have also been reported active, but these observations have yet to be confirmed. It must be pointed out that mucin enhances the virulence of pathogenic or potentially pathogenic (e.g. *Escherichia coli*) organisms, but has no effect on non-pathogens such as *Bacillus subtilis* and Sarcinae.

The method by which pathogenic bacteria attack intact epithelial surfaces and eventually cross them in order to invade other tissues is almost unknown. Pronounced specific antibacterial action of mucin lining the alimentary tract, due to the presence of lysozyme, has been reported to be absent by Goldsworth & Florey (1930); these authors suggest that any protective function against bacteria lies in the mechanical properties of the mucin. However, the well-established phenomenon described above suggests that mucins coating these epithelial surfaces may well help bacteria in their initial attack. Hill, Huffer & Nell (1945) have obtained more direct evidence on this point. They found that strains of *Neisseria intracellularis*, when mixed with mucin, survived far more easily on the mouse vaginal surface than when mixed with saline.

Thus the investigation of mucins for a chemical entity producing the virulence-enhancing effect is an interesting problem, and papers in this series will describe researches to this end.

An essential prerequisite was the development of a statistically adequate method of biological assay. This necessarily involves large-scale animal work, but facilities are available for such work and this communication describes the successful establishment of the assay. In essence it consists of a comparison of unknown mucin fractions with a 'standard' mucin preparation. A constant number of *Bacterium typhosum* (*Salmonella typhosa*), which is far below the normal lethal dose, is injected intraperitoneally into mice in the presence of falling concentrations of the two mucins. Comparisons of death rates and mucin concentrations are made using probit analysis.

EXPERIMENTAL

The 'standard' mucin. A large quantity (10 lb.) of one batch of 'Granular Mucin 1701 W' of Wilson Laboratories, Chicago, U.S.A., was finely powdered in a drug disintegrator, passed through a No. 80 sieve and thoroughly homogenized by rolling in a ball mill for 2 days. It is kept over P_2O_5 and NaOH at 0° and contains 6% moisture (loss at 0·1 mm. Hg and 60° over P_2O_5). The density of this mucin was found to be approximately unity. A 1·75% (w/v) suspension needed for the assay is made by adding 0·93 g. of the solid to 49·1 ml. of sterile water containing enough 0·1 N-NaOH to give a final pH 7·0–7·2. The mixture is homogenized in a special homogenizer described by Folley & Watson (1948). If the homogenizer is not used the mucin is difficult to disperse efficiently.

Unknown mucin samples. The densities are taken as unity. Suitable dilutions for injection are made by adding a quantity of sample (calculated with reference to the substance dried at 0·1 mm. Hg at 60° over P_2O_5) to the necessary quantity of sterile water containing sufficient 0·1 N-NaOH to give a final pH 7·0–7·2. The mixture is homogenized in the special homogenizer, or if foaming is excessive, in a bottle with glass beads.

The killing of vegetative organisms in mucin suspensions. Filtration of the mucin samples is impossible because a large part of the material remains in suspension. Autoclaving definitely reduces the virulence-enhancing activity (see Discussion), and produces variable amounts of deposit. To avoid the effects of excessive heat on the virulence-enhancing factor, the mucins are placed in a water bath at 60° for exactly 0·75 hr. in order to kill vegetative pathogens. The degree of contamination with heat-resistant organisms is noted by plating out 0·1 ml. samples of all 'standard' and unknown mucins on tryptic meat agar, incubating aerobically and anerobically, and counting and examining the colonies. Thus prepared, the 'standard' mucin, contains about thirty sporing organisms per injection dose (0·45 ml.). The same result has been obtained with a very large number of mucin fractions, which, during their chemical preparation, have been filtered through a bacterial candle when in solution in a suitable solvent. In some cases, however, the unknown mucin fractions were precipitates, and at this stage of the fractionation, filtration was not possible; such mucin samples had a greater contamination with Gram-positive rods. In all the bioassay tests, groups of control mice received mucin only, and there is no evidence that any of the organisms present in the samples are pathogenic.

The test organism. The 'Ty_2' strain of *Bact. typhosum* was selected as the test organism. This strain, originally isolated in 1918, is the classical strain of maximum virulence and O inagglutinability, widely employed as a vaccine strain and for the preparation of the 'test dose' in mouse-protection tests (Felix, 1938; private communication). It was used at the Lister Institute of Preventive Medicine, London, by Henderson (1939). The organism was kept at low temperatures in dried gelatine-ascorbic acid culture as described by Stamp (1947). A fresh tube of dried culture is used every week; three progressive subcultures are made during the week for three assays. The dried culture is dissolved in a small amount of warm tryptic meat broth and spread on four tryptic meat agar slopes which are incubated overnight at 37°. At the same time a tryptic meat agar plate is streaked to make certain the colonies are uniformly smooth. The four slopes are washed off with tryptic meat broth, diluted to opacity no. 2 (Brown's tubes) equivalent to 10^9 organisms/ml. Tenfold dilutions in tryptic meat broth are made to 10^5 organisms/ml. and the dilutions stored for a short time in ice water ready for mixing with the mucin. The mixing is done immediately prior to injection. A viable count is made by the method of Miles, Misra & Irwin (1938) on the 10^5/ml. dilution. This method consists of placing drops of known volume of a suitable dilution on a tryptic meat agar plate and spreading by rotation. The plates are incubated overnight, and colonies (roughly 1000) are counted. Since a constant number of organisms are needed for the assay, on the very few occasions when this count is not within the limits $0·8–1·2 \times 10^5$ organisms/ml. the assay is repeated.

The test animal. A strain of white mouse is bred for the Microbiological Research Department by the Ministry of Supply Animal Farm. Weight limits are 18–20 g.

The route of infection. Each mixture (0·5 ml.) is injected intraperitoneally.

The form of the assay. The composition of the injection solutions and the form of the assay is shown in Table 1.

The dilutions x and y% of the unknown mucin fraction are obtained from a preliminary test in which more and wider mucin dilutions are injected with 5×10^3 organisms into batches of ten mice. Mucin and organism controls, together with the 'standard' mucin, are omitted from this preliminary test. Occasionally, if little material is available, only one dilution of the unknown mucin is used.

To avoid inaccuracy in pipetting viscous mucins they are added to the suspensions of organisms from a pipette calibrated to contain; the mixing is then done with this same pipette.

The duration of observations. The majority of deaths occur on the second and third days, but the animals are kept until the fifth day after injection.

Deaths of mucin controls. Occasional deaths in mucin controls are disregarded, but should the death rate be more than two in a batch of twenty mice, then the assay is repeated using lower mucin dilutions.

Table 1. *Form of the virulence-enhancing assay*

Injection solution (0·5 ml./mouse)		No. of *Bact. typhosum* (Ty_2) per injection	No. of mice	Expected death rate (%)
Nature	Composition			
Organism control	9 vol. tryptic meat broth 1 vol. 1×10^7 dilution Ty_2	5×10^5	10	Nil
Organism control	9 vol. tryptic meat broth 1 vol 1×10^8 dilution Ty_2	5×10^6	10	20–80
Standard mucin 1·75% (w/v) control	9 vol. mucin 1·75% (w/v) 1 vol. tryptic meat broth	Nil	10	Nil
Standard mucin 1·75% (w/v) + organisms	9 vol. mucin 1·75% (w/v) 1 vol. 1×10^5 dilution Ty_2	5000	20	50–75
Standard mucin 1·25% (w/v) + organisms	9 vol. mucin 1·25% (w/v) 1 vol. 1×10^5 dilution Ty_2	5000	20	25–50
Unknown mucin x% (w/v) control	9 vol. mucin x% (w/v) 1 vol. tryptic meat broth	Nil	20	Nil
Unknown mucin x% (w/v) + organisms	9 vol. mucin x% (w/v) 1 vol. 1×10^5 dilution Ty_2	5000	20	50–75
Unknown mucin y% (w/v) + organisms	9 vol. mucin y% (w/v) 1 vol. 1×10^5 dilution Ty_2	5000	20	25–50

RESULTS

Comparison of the unknown mucin with the 'standard' mucin preparation is made using probit analysis. The slope of the regression of probit mortality on log dose, necessary for this analysis of one particular assay, is obtained from a regression line constructed from twelve observations on the 'standard' mucin, made over the current period of roughly 3 weeks. This regression line is reassessed every 3 weeks, and therefore takes into account seasonal variations of mice, etc.

The virulence-enhancing strength of the unknown mucin in terms of the 'standard' mucin preparation is expressed in units which are arbitrarily called 'Wilson units'.

Over 100 different unknown mucin samples have been assayed. Table 2 shows the results of duplicate assays on a few samples obtained at important stages in the chemical fractionation of hog gastric mucin (see following paper). The fiducial limits shown in the table are representative of those obtained for other mucin fractions which were only assayed once. Weighted means are included, together with their fiducial limits, showing the increased accuracy obtained by duplicate assays.

DISCUSSION

In establishing a biological assay for virulence-enhancing activity, the effect of two possible variables that could not be eliminated was minimized. These were small changes in inherent virulence that might occur in the chosen test organisms, and also the inaccuracy inherent in counting viable bacteria. For these reasons all unknown mucin samples were compared with a 'standard' mucin preparation in the presence of the same amount of the same dilution of the test organism.

Bact. typhosum (strain Ty_2) was chosen as the test organism, because of its low pathogenicity for mice without mucin. Also it would probably maintain its inherent virulence at a reasonably constant level, provided conditions of growth and dilution were kept standard. Nevertheless, controls which would detect gross changes in the inherent virulence were included in every assay. These controls in fifty or so assays show that the *Bact. typhosum* was constant in killing

two to eight out of ten mice with 5×10^6 organisms, and none, or perhaps an odd one, with 5×10^5 organisms. The workable limits of agreement between the viable count and the initial opacity tube count on which the dilutions for injection are made, are $\pm 20\%$. In view of this fact 5×10^3 organisms were used as the constant test dose with mucin; it was thought that the possible variations on smaller numbers, say 5×10^2 might affect the assay unduly, in spite of the comparison between unknown and standard. The chosen dose, therefore, represents a virulence-enhancing factor of 10^3 if a death point of 50% is obtained with mucin.

The large batches of mice used in this assay are obviously of paramount importance to obtain reasonably accurate results.

sterilize the mucins for vegetative pathogens by standing them for long periods with organic solvents, which were then removed under reduced pressure prior to making the suspensions. The attempts proved unsuccessful and the method described above was instituted. This has yielded excellent results, as evidenced by the constancy of the 'standard' mucin death rates and slopes (5·0–7·5) of the regression lines. Contamination of unknown mucin samples with many sporing organisms is prevented by filtration through a bacterial candle as soon as this is feasible in the fractionation procedure.

It is obvious from Table 2 that agreement between duplicate results is remarkably good for a biological assay of this nature. Consideration of the fiducial limits shows that the assay is capable of detecting a

Table 2. *The results of replicate assays on various mucin fractions together with their weighted means*

Mucin	Assay	Most probable result in 'Wilson units'	Fiducial limits ($P = 0.95$) Minimum	Maximum
31	1	1·11	0·93	1·33
	2	0·90	0·64	1·27
	Weighted mean	**1·06**	**0·91**	**1·24**
53	1	1·88	0·97	3·22
	2	1·52	0·70	2·56
	Weighted mean	**1·71**	**1·14**	**2·55**
98	1	2·43	1·51	3·25
	2	2·66	1·77	3·56
	Weighted mean	**2·55**	**1·96**	**3·32**
100	1	1·41	0·80	2·03
	2	1·41	0·93	1·90
	3	1·62	1·28	2·06
	Weighted mean	**1·53**	**1·28**	**1·83**
102	1	6·69	5·19	8·41
	2	5·22	4·04	6·55
	Weighted mean	**5·91**	**5·06**	**6·90**
115	1	1·31	1·06	1·78
	2	1·37	1·10	1·85
	Weighted mean	**1·34**	**1·14**	**1·57**
131	1	3·03	1·95	4·24
	2	2·46	1·44	3·57
	Weighted mean	**2·77**	**2·20**	**3·50**
A	1	0·77	0·63	0·95
	2	0·63	0·50	0·76
	Weighted mean	**0·69**	**0·61**	**0·79**

The method of sterilizing the mucin suspensions presented some difficulty. Filtration was out of the question. Several workers have mentioned that autoclaving at 15 lb./sq.in. for 15–20 min. reduces the activity, and Anderson & Oag (1939) have recorded that two such autoclavings destroy practically all the activity. A quantitative measure of the loss of activity on autoclaving has been ascertained. The 'standard' mucin at pH 7·0–7·2 was autoclaved at 15 lb./sq.in. for 20 min.; the virulence-enhancing activity (1 'Wilson unit') dropped to 0·69 (0·61–0·79) 'Wilson unit'. Attempts were made to

twofold difference in virulence-enhancing power with probability $P = 0.95$: somewhat smaller differences can be demonstrated with the same probability in some cases. On the occasions when, to economize in material, only one dilution of the unknown is used, the fiducial limits show a slightly wider range.

The assay therefore fulfilled the requirements for the chemical fractionation of mucins for a virulence-enhancing factor. Its use in the initial part of these fractionations is described in the following paper.

The question of the toxicity of hog gastric mucin has arisen in the course of this large-scale animal

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.

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The Virulence-enhancing Factor of Mucins

2. FRACTIONATION STUDIES ON HOG GASTRIC MUCIN

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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-