Appendix. Histological Observations

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Some of the gastric ring sections used in the metabolic experiments were afterwards fixed in Heidenheim's 'Susa' and sent to me in 80% alcohol for histological examination. They were embedded in paraffin, and sections were stained by Ehrlich's haematoxylin and eosin.

Anaerobic experiments with and without glucose (tissue incubated 2-4 hr.). Three specimens from anaerobic experiments without glucose were examined. In two of them the greater part and, in the third, a smaller part, of the epithelium which should line the inner surface of the stomach and the gastric pits were missing. On the other hand, this surface epithelium was intact in all three specimens from comparable experiments in the presence of glucose. Other changes, e.g. separation of the zymogenic cells and focal necrosis in the mucosa, were present in most specimens and appeared unrelated to the presence or absence of glucose.

Aerobic experiments in air and oxygen (2-3 hr. incubation periods). Four specimens from experiments carried out in oxygen were compared with four specimens from comparable experiments in air. Yeast-like organisms were seen on the mucosal surface of all four 'oxygen' specimens, but not in

any of the 'air' specimens. Swelling and rarefaction of the connective tissue immediately beneath the surface epithelium, an abnormality not seen in any anaerobic specimens, was present in three of the 'oxygen' specimens but only in one of the 'air' specimens. On the other hand, the surface epithelium was partially absent in two of the 'air' specimens but not in any of the 'oxygen' specimens. The significance of these differences is doubtful and no others were observed. They are commented upon on p. 25.

Two of the 'oxygen' specimens and two of the 'air' specimens were from experiments in which glucose was present; the other two of each from experiments in which glucose was absent. Swollen empty-looking muscle fibres were present in the muscle coat of all the 'glucose-present' specimens but not in any of the others. No other effect of the presence or absence of glucose in aerobic experiments was recognized.

The specimens from experiments in air or oxygen appeared better preserved on the whole than those from anaerobic experiments. In particular, pyknosis of the nuclei of parietal cells and focal necroses were less common in the 'aerobic' specimens.

A Modified Method for the Viscosimetric Assay of Hyaluronidase

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The method of McClean & Hale (1941) for the viscosimetric assay of hyaluronidase has been in extensive use and is claimed to be accurate to within $\pm 10\%$; in the hands of the present authors, however, it has not proved entirely satisfactory. We have, therefore, been prompted to investigate some of the fundamental principles upon which the method is based and, as a result, have succeeded in devising a simpler and apparently more accurate procedure. The method of McClean & Hale is based on the work of Madinaveitia & Quibell (1940) who calculated the 'half viscosity time' or 'half-life time of the substrate', which they found to be inversely proportional to the concentration of the enzyme and independent of the concentration of the substrate, both of which conclusions were supported

by McClean & Hale and made the basis for their viscosimetric technique. On the other hand, Robertson, Ropes & Bauer (1940) found that the time taken for a fixed amount of enzyme to break down increasing amounts of substrate was proportional to the concentration of the latter. In view of this apparent discrepancy, and since the time taken to reach the half viscosity level of the substrate did not seem to give a satisfactory index of the potency of the enzyme, it was decided to investigate (1) the relationship of the flow-time in Ostwald viscosimeters to the concentration of a simple viscous solution (dextrose); (2) the same relationship for buffered hyaluronate solutions of varying concentration; (3) the curve of flow-times for hyaluronate solutions mixed with hyaluronidase; and (4) the

curve of flow-times of a standard hyaluronate solution incubated for a constant time with varying concentrations of hyaluronidase.

EXPERIMENTAL

Materials and methods

The substrate consisted of a solution of potassium hyaluronate (Glaxo Laboratories), four volumes of which were mixed with one volume of McIlvaine's (1921) citric acid-phosphate buffer M/60, pH 7.0, containing NaCl; (Na_2HPO_4 [anhydrous] 12.75 g.; citric acid 2.02 g.; NaCl 3.50 g.; water to 1 l.). The hyaluronidase consisted of freeze-dried rabbit- and bull-semen filtrates, and all dilutions were made with 0.5% gum arabic solution, since a loss of potency on standing has been observed when distilled water alone is used as the diluent. The Ostwald viscosimeters were of about 1.5 ml. capacity, and all measurements were made in a water bath at 34°. The quantities of buffered substrate (2.5 ml.) and enzyme solution (0.5 ml.) were the same as those employed by McClean & Hale (1941).

The relationship of flow-time to the concentration of viscous solutions

The curves of flow-time plotted against concentration of dextrose and buffered hyaluronate solutions are shown in Fig. 1. (In Figs. 1 and 2 each

Fig. 1. Relationship between concentration of dextrose ○—○ and buffered hyaluronate ●—● solutions and flow-time in an Ostwald viscosimeter.

point represents one determination on the same viscosimeter.) It will be seen that they resemble one another in so far as flow-time is not directly

proportional to concentration, increasing at a progressively greater rate as the concentration is increased, but they differ markedly in that, over the range of flow-times 60–110 sec. the curve for hyaluronate is practically a straight line, while that for dextrose is not. Within the above-mentioned range, therefore, it is permissible to regard the flow-time of a hyaluronate solution as being directly proportional to its concentration, and for this reason the concentration of hyaluronate used for viscosimetric assays has been adjusted so as to fall within this range.

The curve of flow-times of hyaluronate solutions mixed with hyaluronidase

It became apparent, as an important practical point, that the method of mixing the hyaluronidase solution with the substrate had a marked effect on the subsequent rate of fall of the flow-time of the mixture. Because of the viscosity of the substrate, and of the small volumes of fluid employed, diffusion is minimal and it is therefore imperative that mixing be thorough from the outset. Accordingly, a uniform method of adding the enzyme to the substrate (both solutions being at 34°) was adopted, namely: vigorously squirting the former into the latter from a 1 ml. all-glass syringe through a No. 26 needle. Scrupulous attention to this detail, as well as to ensuring the viscosimeter's being at 34°, was found to be essential for obtaining reproducible results. (The viscosimeter should have stood for at least 10 min. in the water-bath before adding the enzyme-substrate mixture.)

The effect of variation of substrate concentration on the curve of flow-times is seen in Fig. 2, where the 'relative viscosity' (McClean & Hale, 1941) is plotted against time after mixture of enzyme and substrate, the concentration of enzyme remaining constant. Contrary to the findings of Madinaveitia & Quibell, and of McClean & Hale, it is seen that the time to the half viscosity is not independent of the concentration of the substrate, but becomes progressively less as the latter is decreased. Although the discrepancy is not great with more concentrated substrate solutions, i.e. containing about 0.08% hyaluronate or more (such as the above authors have, for the most part, used), it is very prominent with more dilute substrates and is certainly appreciable within relatively narrow limits if the range of concentrations which we advocate (wherein flow-time is proportional to concentration) is used. It is clear that, although with more concentrated substrates the amount of substrate attacked per unit time by a given enzyme preparation may be roughly proportional to the original substrate concentration, with more dilute substrates this relationship fails to hold good.

The curve of flow-times of a standard hyaluronate solution incubated for a constant time with varying concentrations of hyaluronidase

In view of the above theoretical objection to the use of the time to the half-viscosity as a measure of the potency of hyaluronidase, as well as the practical one of the tediousness of its determination, it was decided to investigate the possibility of employing the flow-time of the enzyme-substrate mixture after incubation for a fixed period as the index of hyaluronidase concentration. It can be seen from the curve of flow-times (Fig. 2) that the rapidity of fall decreases progressively, the curve having flattened considerably by 20 min. This time, therefore, was considered to be a suitable and convenient one for practical use.

Fig. 2. Curves of flow-times of mixtures of a fixed concentration of hyaluronidase with varying concentrations of buffered hyaluronate, showing variation of 'time to the half-viscosity' with concentration of substrate. The concentrations of hyaluronate are A, 0.103%; B, 0.066%; C, 0.062%; and D, 0.053%.

Since the flow-times, not only of water, but also of the substrate varied somewhat in different viscosimeters, it seemed desirable to devise, if possible, a correction factor which would eliminate these differences and so enable one to make comparable readings on different tubes without always having to arrange tests so that differences between

tubes could be eliminated statistically. The 'relative viscosity' of McClean & Hale (1941) is not entirely satisfactory in this respect, and the following considerations led to the adoption of the index which is advocated:

If f_s be the flow-time of the substrate + 0.5 ml. gum arabic solution in place of enzyme, f_o be the flow-time of the solvents without hyaluronate, and f_e be the flow-time of the enzyme-substrate mixture after incubation for 20 min., then $f_s - f_o$ represents the viscosity due to the substrate, while $f_s - f_e$ represents the amount of substrate rendered non-viscous by the action of the enzyme, and, within the limits described above, is directly proportional to the amount of substrate so attacked. It is

reasonable to assume that the ratio $1000 \left(\frac{f_s - f_e}{f_s - f_o} \right) = v$

(the constant 1000 avoids decimal points) will be constant for a given substrate-enzyme mixture in spite of variations (within limits) in the actual values of f_s and f_o , that is, in spite of slight tube-

Fig. 3. Relationship between flow-time index (v) and relative concentration of enzyme for mixtures of hyaluronidase and buffered hyaluronate (the latter remaining constant) incubated for 20 min. at 34°.

differences. This ratio, then, has been used as the flow-time index, and it will be shown by statistical analysis of test assays that the above assumption is true within the limits of experimental error.

Fig. 3 shows the relationship between concentration of enzyme and the flow-time index (v) for a standard hyaluronate solution incubated for 20 min. Although there is an asymptote to the curve at $v = 1000$, it is sufficiently close to the exponential form over the range $v = 0 - 800$ to warrant the

working assumption that the flow-time index is proportional to the logarithm of the enzyme concentration over this range (Fig. 4). The justification for this assumption will be shown later. The advantage of this relationship lies in the fact that it enables one to assay samples of enzyme in terms of a standard preparation as described by Bliss & Marks (1939), and for this purpose a laboratory standard

Fig. 4. Relationship between flow-time index (v) and logarithm of concentration of enzyme for the same conditions as in Fig. 3.

preparation has been set up. This consists of diluted bull-semen filtrate which has been ampouled and freeze-dried, each ampoule containing a constant quantity (about 0.1 ml. of the original semen filtrate). It is proposed to regard the contents of each ampoule as 10 units. The freeze-dried material has been found to weigh 8.5 mg. per ampoule and comparison with a Glaxo Laboratories enzyme preparation as used by McClean (1 mg./ml. $\equiv$ 100 M.P.C. units; McClean, 1943) shows that 1 mg. of the latter is equivalent to 0.475 mg. of our freeze-dried material or 0.56 unit.

In practice, the method of assay consists in incubating the appropriate quantities of buffered hyaluronate with enzyme in two dilutions (one usually half that of the other) and determining the flow-times at the end of 20 min. This is done for each dilution in three tubes, and the process is

repeated with two similar dilutions of standard preparation, so that in all, twelve readings are obtained. One ampoule of the standard made up to 40 ml. is a convenient higher dilution. From these readings one can calculate the relative potencies and estimate any departure from parallelism of the slopes of the dose-effect curves after the method of Bliss & Marks, while from the analysis of variance it is possible to estimate the standard error and the significance, if any, of differences between tubes.

Test and statistical analysis of the method

Following the above considerations, a test was set up in which four different concentrations of a preparation of rabbit semen filtrate were compared in three different viscosimeters. The test was completed in one day, and the order of testing concentrations in different tubes was at random. The results are shown in Table 1. The balance of the

Table 1. *Results of a test of hyaluronidase at four concentrations*

Concentration of hyaluronidase (dilution of standard)	Viscosimeter A		Viscosimeter B		Viscosimeter C	
	Flow-time	v	Flow-time	v	Flow-time	v
1/300	86	316	88	342	95	259
	86.5	303	87	366	92.5	318
	85	342	90	293	93.5	294
1/200	82	420	86.5	378	86	471
	81	447	82	488	87	447
	79	500	82.5	475	86	471
1/100	75.5	593	76	635	79.5	625
	76	580	76.5	622	79	635
	74.5	620	76	635	78.5	647
1/75	70.5	725	72.5	720	77.5	670
	71.5	700	74	685	76.5	695
	71	710	75	660	76	705

test is such that each concentration was tested three times in each viscosimeter, so that variation due to differences between instruments can be segregated in the analysis of variance, set out in Tables 2 and 3.

These two analyses of variance show that the highly significant differences between the flow-times of different viscosimeters were completely eliminated in this particular test by conversion to the index, v , and that no significant interaction of concentration with instruments occurred. A most satisfactory degree of significance of the slope of the line relating concentration to flow-time was obtained, the slope for the data of Table 3 being 620 and its standard error 6.6 (using the relative indices, 1, 1.5, 3 and 4, for the four concentrations).

Tests of goodness of fit show that the points do not deviate significantly from a straight line for the data of either Table 2 or 3, but that the fit for Table 3 is the better ($F=2.81$). If the first and

Table 2. *Analysis of variance for a test of hyaluronidase at four concentrations, using actual flow-times*

Source of variation	Degrees of freedom	Mean square	F
Concentrations	3	431.1	312*
Viscosimeters	2	99.0	72*
1st order interaction	6	2.76	2.0
Error	24	1.38	—

* $P > 0.99$.Table 3. *Analysis of variance for a test of hyaluronidase at four concentrations, using the index v*

Source of variation	Degrees of freedom	Mean square	F
Concentrations	3	263327	293*
Viscosimeters	2	84.1	0.10
1st order interaction	6	1125	1.3
Error	24	842	—

* $P > 0.99$.

third concentrations are assayed against the second and fourth, the logarithm of the relative potency and its standard error are:

(a) from actual flow-times, 0.0221 ± 0.0181 (b) from the index v , 0.0237 ± 0.0162 .

By all criteria, therefore, it is best to employ v as a criterion of response. The fiducial limits of error, using v ($P = 0.95$), for an assay of the first and third concentrations against the other two are 97.9–114.0%, mean estimate 105.6%. (The correct answer is, of course, 100%.)

Further tests were made in which known dilutions (but unknown to the operator) of preparations of hyaluronidase were tested against the parent solutions. Since no significant mean square was associated with interaction, each concentration was tested only once with each viscosimeter, in order to determine the accuracy of assays using a small number of individual determinations of flow-time. The results of these tests are shown in Tables 4 and 5.

There is rather a large difference between the mean squares for interaction, used as error, in these two tests. The difference is significant at the 5% level, but not at the 1% level. The apparent failure completely to eliminate differences between instruments in the first test (Table 4) is more likely due to a depressed error mean square in this test than to a real failure of elimination, as the error mean square is lower than in the other similar tests here reported. There is also a significantly better agreement between the slopes of the two dose/response lines in Table 4 than could be expected by chance; again the significance is not high ($P > 0.95$, < 0.99). Otherwise, these two further tests are completely

satisfactory; the internal evidence from each shows that a valid assay with quite low limits of error has been performed. From the trial test reported in Tables 1–3, it would have been predicted that an assay with three observations at two concentrations of each of two substances should have an error of about $\pm 14\%$ ($P = 0.95$), whereas we find errors ($P = 0.95$) of about ± 10 and $\pm 20\%$ in the two trials, and deviations from the known potency ratio of 3 and 4%.

Table 4. *Analysis of variance for a test of two samples of hyaluronidase with a potency ratio of 0.292*

Source of variation	Degrees of freedom	Mean square	F
Differences between samples*	1	60066	341†
Slope of combined dose/response line	1	89960	505†
Departure from parallelism	1	0.083	0.00047‡
Differences between viscosimeters	2	1146	6.4‡
Interaction (error)	6	178.4	—

* Estimated potency ratio = 0.284; limits ($P = 0.95$) 0.257–0.313, or 90.7–110.3% of the mean estimate.

† $P > 0.99$.‡ $P > 0.95$, < 0.99 .Table 5. *Analysis of variance for a test of two samples of hyaluronidase with a potency ratio of 0.70*

Source of variation	Degrees of freedom	Mean square	F
Differences between samples*	1	27075	30.6†
Slope of combined dose/response line	1	82336	92.7†
Departure from parallelism	1	33.3	0.038
Differences between viscosimeters	2	506.3	0.57
Interaction (error)	6	886.8	—

* Estimated potency ratio = 0.674; limits ($P = 0.95$) 0.551–0.823, or 82.0–122.4% of the mean estimate.

† $P > 0.99$.

Analysis of several subsequent assays has yielded results fully in agreement with the above findings, the errors ranging from ± 3 to $\pm 16\%$ ($P = 0.95$).

SUMMARY

1. Some of the fundamental principles involved in the viscosimetric assay of hyaluronidase have been investigated.

2. As a result, a method of assay has been devised depending upon the fact that, within certain prescribed limits, the fall in the flow-time through an Ostwald viscosimeter of a buffered hyaluronate-hyaluronidase mixture incubated for a fixed time is proportional to the logarithm of the concentration of the enzyme.

3. Satisfactory assays which have standard errors

of less than 10 % can be made with a total of twelve readings.

Our thanks are due to Dr S. J. Folley for providing the bull semen from which the standard hyaluronidase preparation has been made. We are also indebted to Dr H. J. Rogers and Dr I. W. Rowlands for invaluable advice.

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The Action of Sulphites on the Cystine Disulphide Linkages of Wool

5. THE EFFECT OF SOME CHEMICAL MODIFICATIONS OF THE WOOL ON THE MAGNITUDE OF THE BISULPHITE-REACTIVE FRACTION (*A* + *B*) AND THE RELATIVE MAGNITUDES OF SUBFRACTIONS *A* AND *B*

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Elsworth & Phillips (1938, 1941) showed that approximately half of the cystine disulphide-S of wool reacted with solutions of NaHSO_3 to give thiol and *S*-cysteine-sulphonate groups according to the reaction:

where *R'* and *R''* represent polypeptide chains. Middlebrook & Phillips (1942) found that this reactive fraction of the disulphide-S could be divided into two subfractions: subfraction *A* which was readily reversed to disulphide on rinsing the bisulphited wool with water, and subfraction *B* which gave thiol and *S*-cysteine-sulphonate groups which were stable to water-rinsing.

In an endeavour to elucidate the cause of this difference in the behaviour of subfractions *A* and *B*, we have examined a number of chemically modified wools. Whilst we have not discovered any method of modifying the wool which causes more of the total cystine-S to react with NaHSO_3 , we have found that any treatment which blocks the free carboxyl groups of the combined glutamic and aspartic acids, produces an increase in the number of thiol and *S*-cysteinesulphonate groups stable to water rinsing (subfraction *B*) and a corresponding decrease in the water-labile subfraction *A*.

METHODS

The chemically modified wools were prepared, as described below, from the sample of wool (64's combed merino) used by Lindley & Phillips (1945). All analyses are calculated on the weight of the anhydrous wool.

Deaminated wool. The wool (10 g.) was immersed for 2 days in a solution prepared by mixing 80% (w/v) NaNO_2 (2 vol.) with glacial acetic acid (1 vol.), washed for 2 days in running water, dried and conditioned by exposure to a controlled atmosphere of 21° and 70% relative humidity until it attained constant weight. This method deaminates the lysine ϵ -amino-N and in addition an indefinite number of terminal arginine amino-N groups (Speakman & Stott, 1934; Rutherford, Harris & Smith, 1937).

Acetylated wools. These were obtained by (a) boiling, under reflux, for 30 min. with excess acetic anhydride, (b) by immersion overnight in acetic anhydride at room temperature.

Methylated wools. These were prepared by the methods described by Blackburn, Carter & Phillips (1941), Blackburn & Phillips (1944). Prior to conditioning, all the methylated wools were rinsed thoroughly in distilled water to remove buffer salts.

O-Methyl was determined by Baernstein's method (1932, 1936). Pregl's method (1924) was used for *N*-methyl, but the methyl iodide evolved was determined as in the *O*-methyl determinations. Neither the use of gold chloride nor fuming HI was found advantageous (Edlbacher, 1918). Preliminary investigations showed that in agreement with