Studies on Diffusing Factors

2. METHODS OF ASSAY OF HYALURONIDASE AND THEIR CORRELATION WITH SKIN DIFFUSING ACTIVITY

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Chain & Duthie [1940], who first described the association between diffusion in the tissues and the mucolytic activity of testicular extracts, reported good agreement in quantitative comparisons of the diffusing and viscosity-reducing activities of enzymes from many sources; they, as well as other workers, isolated a substance closely resembling hyaluronic acid from rabbit skin, which substance was acted on by these enzymes. On the other hand, Madinaveitia, Todd, Bacharach & Chance [1940] failed to obtain satisfactory correlation between diffusing and viscosity-reducing activities and suggested that it was not justifiable to assay diffusing factors in terms of their viscosity-reducing power. Hobby, Dawson, Meyer & Chaffee [1941] and Meyer, Chaffee, Hobby & Dawson [1941] studied the relationship between diffusing factors and hyaluronidase, and the character of these enzymes which were of bacterial and animal origin. The evidence these authors present indicates, that whereas hyaluronidase plays a part in the diffusion in tissues, the phenomenon is a complex one and cannot be explained on the basis of a simple chemical reaction. McClean & Hale [1941] pointed out that the environmental conditions of the test in the skin and those in the viscosimeter differ profoundly, and that such variable factors as salt concentration and pH markedly affect the apparent viscosimetric titre of these enzymes. They concluded that although at present it is certainly unjustifiable to assay skin-diffusing activity in terms of viscosity-reducing activity, it does not follow that the two activities are due to separate agents. Moreover, they reported that the inclusion of purified hyaluronic acid in the culture medium of *Cl. welchii* caused a marked increase, not only in the viscosimetric titre of the culture filtrate, but also in its diffusing activity. The same result was obtained with streptococcal cultures [McClean, 1941]. In view of the recognized specificity of such 'adaptive enzyme' effects, the fact that the presence of hyaluronic acid in the culture medium increases the production of diffusing factors is evidence for the view that this phenomenon is due to the activity of hyaluronidase.

Hyaluronic acid, when mixed with serum protein, gives a typical mucin clot in the presence of acetic acid [Seastone, 1939]; after treatment of the mucin

with mucinase, however, this reaction no longer takes place. This phenomenon was used by Robertson, Ropes & Bauer [1940] to estimate the activity of the *Cl. welchii* mucinase, by observing the rate at which this primary degradation took place. It appeared that a comparative study of the activity of enzyme preparations from various sources with regard to three factors—inhibition of the precipitation of a hyaluronic acid-protein complex; reduction in viscosity of hyaluronic acid; and diffusing power in the skin—would be useful in elucidating the relationship between the successive stages in the degradation of the substrate. This paper includes a study of the mucin-clot prevention (M.C.P.) test, and the correlation between three methods of assay based on the above factors.

METHODS

Preparation of substrate. In the earlier experiments hyaluronic acid was obtained from umbilical cords by the method described by McClean & Hale [1941], which depends upon precipitation of an aqueous extract with 3 vol. of ice-cold ethanol containing a trace of Na acetate. The final product contains about 7% total N. It was found, however, that if the alcohol is saturated with K acetate and brought to pH 9–10 with KOH, the hyaluronic acid can be precipitated by the addition of only 1.25 vol. This precipitate, which forms a characteristic stringy clot, is broken up and thoroughly washed with alcohol to remove the residual K acetate and KOH. It is then washed with ether and dried over P_2O_5 *in vacuo*. This product contains considerably less protein than that derived by the original method and contains 4.8% total N, 2% P and 12% glucosamine. Its aqueous solution does not form a clot on the addition of acetic acid.

A 1% solution of twice-crystallized serum albumen in physiological saline was used for the preparation of the mucin-protein complex, since serum may contain variable amounts of an inhibitor or even traces of hyaluronidase [McClean, 1942]. The albumen was kindly supplied by Dr R. A. Kekwick and prepared by his method [Kekwick, 1938].

The substrate-protein mixture for use in the test is prepared as follows: 1 vol. hyaluronic acid solution is mixed with 1 vol. albumen solution; 2 vol. of distilled water are then added. The required strength of the hyaluronic acid solution for each batch of hyaluronic acid is determined by titrating representative enzymes with substrate-protein mixtures, prepared with both the original and the new substrate. The concentration of hyaluronic acid, which

gives the same end-point as that obtained with the original preparations, is used for all subsequent tests. It is found that the required concentration varies from 0.15 to 0.25% in distilled water. Solution of the hyaluronic acid is facilitated if the bottle is placed at 37° for 2–3 hr. and shaken occasionally. Stock solutions of hyaluronic acid and of albumen are kept in the refrigerator under toluene. The viscosity of hyaluronic acid solutions falls slowly on storage; solutions in which this fall has exceeded 5% of the original viscosity should not be used for the test, since it is known that the partially depolymerized substrate inhibits the activity of the enzyme.

Technique of the test. The test is performed at 100, 50 or 20% differences in dilution of the enzyme, but it is usual to do a preliminary test at a tenfold dilution to ascertain the approximate end-point of activity. The appropriate dilutions of the enzyme sample to be tested are made in a final vol. of 0.5 ml. distilled water. In order to obtain reproducible results if a final test at 20% differences is made, it is necessary to make these dilutions in bulk, say 10 ml., and to transfer 0.5 ml. amounts of the

present in considerable excess are clear. The highest dilution of the enzyme showing no clot or threads is taken as the end-point of activity.

*Influence of concentration of substrate
on apparent titre of enzyme*

A preliminary test with an unpurified culture filtrate of *Cl. welchii* indicated that the apparent potency of the enzyme varies inversely with the concentration of the substrate mixture. This variation depends upon the concentration of the polysaccharide and, within wide limits, is independent of the concentration of protein. Observations were then repeated with purified enzyme derived from testis and from *Cl. welchii*. Substrate mixtures were prepared containing (1) double the usual concentration of polysaccharide and albumen, (2) the usual concentration, and (3) half the usual concentration. The salt content of the mixture was kept constant. The results are shown in Table 1.

Table 1. *The influence of concentration of substrate on apparent potency of purified hyaluronidase*

(a) Purified testis enzyme

	Dilution of enzyme						
	1:500	1:1000	1:2000	1:4000	1:8000	1:16,000	1:32,000
Double conc. substrate	+	+	±	0	0	0	0
Usual conc. substrate	+	+	+	+	±	0	0
Half conc. substrate	+	+	+	+	+	±	0

(b) Purified *Cl. welchii* enzyme

	Dilution of enzyme						
	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200
Double conc. substrate	+	+	+	0	0	0	0
Usual conc. substrate	+	+	+	+	±	0	0
Half conc. substrate	+	+	+	+	+	+	0

+ Complete reaction; no clot or threads. ± Partial reaction; threads only. 0 No reaction; unmodified clot.

dilutions to the tubes for test. 1 ml. of the substrate protein mixture is added to each tube, including the control, starting from that tube containing the highest dilution of enzyme. The test mixtures are incubated for 20 min. at 37°. It is important that the period of incubation should be accurately controlled. Immediately after incubation the tubes are cooled in ice-cold water, to arrest enzymic activity and to assist clot production on the subsequent addition of acetic acid. After 5 min., 0.2 ml. 2*N* acetic acid is added to each tube. The tubes are held up to the light and gently shaken. The control, and those tubes containing little enzyme, show the development of a characteristic stringy clot which contracts on standing. One or two tubes with concentrations near the end-point may show a few threads, or some precipitate. Those tubes in which the enzyme has attacked the substrate show no clot or precipitate, and those in which the enzyme is

It will be seen that, within the limits of accuracy of this particular test, which was done at only two-fold dilutions of the enzyme, the apparent potency of both enzymes is inversely proportional to the concentration of the substrate mixture. This confirms the observation of Robertson *et al.* [1940] as to the influence of substrate concentration upon this manifestation of enzymic activity, and contrasts with the results obtained by Madinaveitia & Quibell [1940] and McClean & Hale [1941], using a viscosimetric technique; the latter workers reported that the reaction constant and the half-life time of the viscous substrate are constant for a fixed amount of enzyme, and independent of the concentration of the substrate, which indicates that, although the time taken to reach half-viscosity level remains constant, the time taken to carry the reaction to completion increases proportionally to the concentration of the substrate.

Rate of the reaction

Table 2 shows the result of a preliminary experiment in which two streptococcal and two testicular enzyme preparations were titrated after incubation periods varying from 5 to 80 min. The diluted enzyme solutions were warmed for 5 min. in the

Table 2. The influence of the period of incubation on the apparent titre of testicular and streptococcal hyaluronidase

The figures indicate the highest dilution of enzyme which prevented the clotting of the substrate.

Period of incubation min.	Source of enzyme (dilution)			
	<i>Streptococcus</i> C 21		<i>Streptococcus</i> C 7	
	whole filtrate	Testis	purified	Testis
5	1: 320	1: 10	1: 200	1: 1,000
10	1: 320	1: 20	1: 400	1: 2,000
20	1: 640	1: 20	1: 400	1: 4,000
30	1: 1280	1: 40	—	—
40	1: 1280	1: 40	1: 800	1: 16,000
60	1: 2560	1: 80	—	—
80	—	—	1: 1600	1: 64,000

bath at 37° before the addition of previously warmed substrate mixture. At the end of the period fixed for incubation the racks were transferred to ice-cold water. These tests showed that the reaction was still proceeding rapidly at 20 min. and, in fact, that there was no noticeable lag even after 80 min. It seemed, therefore, that the empirically selected incubation time of 20 min. satisfies the demand that results should be read at a time when the reaction is still proceeding at the initial velocity. This period has the further advantage that it is the same as that used when assaying enzymes viscosimetrically and by diffusion in the skin.

A series of observations were made in which, after preliminary titration, 5 ml. of the appropriate enzyme dilution were mixed with 10 ml. of substrate mixture, both warmed before mixing, and samples of this mixture examined for clotting power at regular intervals. Four dilutions of each enzyme were tested in this way. In addition to the streptococcal and testicular enzyme preparations previously tested, a crude *Cl. welchii* culture filtrate and a purified *Cl. welchii* enzyme were thus examined (see Table 3).

It will be seen that the time taken to destroy the clotting power of the substrate is approximately proportional to the dilution of the enzyme, and this was true for all the enzymes examined. This method of titrating the enzymes did not appear to be as convenient, or as accurate, as that depending upon the determination of the highest effective dilution after a fixed period of incubation; the taking of samples is very time-consuming and, moreover, there is a variable period in which clotting power

is partially destroyed, as evidenced by the appearance of threads in the mixture. It is sometimes difficult to determine exactly when there are no threads present. For these reasons the fixed period of 20 min. incubation is preferred to the method of determining the time taken by one dilution of enzyme, which was used by Robertson *et al.* [1940].

Table 3. The relation between the dilution of the enzyme and the time taken to destroy the clotting power of the substrate mixture at 37°

The times shown in this table are those from the time of mixing enzyme and substrate to that at which the failure to form a typical mucin clot was noticed. Complete destruction of clotting power was preceded by a variable period in which threads appeared in the substrate mixture.

Purified testicular enzyme			<i>Streptococcus</i> C7 culture-filtrate		
Dilution of enzyme	No clot		Dilution of enzyme	No clot	
	Threads only (time min.)	or threads (time min.)		Threads only (time min.)	or threads (time min.)
1: 2,000	5	7	1: 200	4	6 or 10
1: 4,000	—	11	1: 400	12	16
1: 8,000	20	22	1: 800	30	32
1: 16,000	35	45	1: 1600	55-60	60-55

Influence of temperature on the reaction

Robertson *et al.* [1940] observed the influence of temperature on the rate at which a fixed weight of

Fig. 1. Influence of temperature on the activity of purified testicular hyaluronidase.

purified mucin was broken down by the enzyme obtained from *Cl. welchii*. They concluded that the rate of the reaction increased in proportion to the

increase of temperature from 0 to 40°, and that above 50° destruction of the enzyme took place rapidly. The figure obtained by them for K_{10} was 1.75.

A sample of purified testicular hyaluronidase was titrated by the routine M.C.P. test at various temperatures, the period of incubation for all these tests being 20 min. The results are shown in Fig. 1. In addition to these results, a test lasting 40 min. was carried out at 22° and one lasting 1 hr. 50 min. at 4°. It will be seen that there was no activity detectable during 1 hr. 50 min. at 4°, but that the apparent titre of the enzyme increased rapidly as the temperature was raised. The rate of this increase was greater at the higher temperature than would have been necessary to give a figure of about 2 for K_{10} . There was no evidence of destruction of the substrate-protein mixture alone at the higher temperatures.

Influence of concentration of salt on the reaction

In the M.C.P. test already described the solvent is distilled water, except for the albumen portion of the substrate-protein mixture. Thus the final concentration of NaCl in the system is only 0.024 *M*. It was desirable to determine the effect of increasing the salt concentration on the activity of the enzyme and the kinetics of the reaction. A preliminary experiment showed that, in the absence of enzyme, the substrate-protein mixture would not form a typical mucin clot if the concentration of salt was raised above 0.11 *M*. A series of titrations of a purified testicular enzyme were then carried out, in which the NaCl concentration was raised from 0.0024 to 0.1 *M*, with the result shown in Table 4. The trace of NaCl present in the lowest concentration was due to the salt present in the 10% stock albumen solution from which test solutions were made.

Table 4. *The influence of NaCl concentration on the M.C.P. titre of testicular hyaluronidase*

Conc. of NaCl <i>M</i>	Highest effective dilution of enzyme
0.0024	<1: 500
0.017	1: 1,000
0.033	1: 4,000
0.07	? 1: 16,000
0.11	? 1: 32,000

The activity of the enzyme increases with salt concentration up to at least 0.1 *M*, but the determination of the end-point is obscured above 0.033 *M* by the development of a flocculent precipitate, on the addition of acetic acid, in those tubes containing the higher dilutions of enzyme. It appears, therefore, that the salt concentration used in the routine test (0.024 *M*) is very near that at which maximum

activity of the enzyme combined with a clearly defined end-point is obtained. Robertson *et al.* [1940] have already reported that the enzyme from *Cl. welchii* is inactive in the absence of salt, and that the activity increases as the concentration of various salts in the system is raised to 0.08 *M*. It appears from the work of McClean & Hale [1941] and Madinaveitia & Quibell [1941], using a viscosimetric technique, that the activity of the enzyme increases up to an optimum concentration of salt, which varies for the different enzymes examined from 0.1 to 0.3 *M*. A further increase of salt causes a rapid fall in activity. Madinaveitia & Quibell further pointed out, that if the concentration of NaCl is reduced below 0.1 *M* the reaction time is no longer inversely proportional to the concentration of enzyme.

Influence of pH on the reaction

In the routine M.C.P. test, as described, no buffer is added and the reaction is carried out at approximately pH 7.0. Preliminary tests showed that McIlvaine's citric acid-phosphate buffer solutions could not be used in a concentration stronger than 0.5 *M* without themselves inhibiting the clotting power of the substrate mixture. When diluted enzyme solutions are added to the system containing 0.5 *M* buffer, over a range from pH 4.4 to 8.0, it was found that the end-point of activity was obscured by the development of a flocculent precipitate. Attempts to overcome this difficulty by the use of other buffer systems have been unsuccessful for the same reason and, in addition, the apparent titre of the enzyme at the same pH may vary owing to the inhibiting action of the ions in some of the systems employed. Control of the pH by the use of 0.1 *N* HCl and NaOH was unsuccessful, since the substrate mixture clotted spontaneously if the pH was reduced below 5.8.

Limits of accuracy of the M.C.P. test at various dilution levels

Observations were made to determine the standard error of this test at different dilution levels. Repeated tests were made on the same purified and dried testicular enzyme in twofold dilutions, and at 50 and 20% dilution differences. The test in twofold dilutions was repeated 19 times and gave a constant end-point of 1/8000 with no detectable variation. When it was repeated 15 times at 50% differences in dilution, an end-point of 1/9000 was obtained in 11 tests, of 1/6000 in 2 tests and of 1/13,500 in 2 tests. The standard error at this level is therefore approximately 25%. The actual reading of the tests at this level is slightly more difficult than at twofold dilutions; since one or more tubes containing dilutions near the end-point have to be very carefully inspected to exclude the presence of a thread of

precipitate. The tests at 20% differences present the same difficulty in the determination of the end-point, and moreover, occasionally a tube containing a dilution of enzyme below the apparent end-point may show a trace of precipitate. The tests at this level, were, however, repeated 18 times with the following result:

No. of tests	End-point (dilution)	Difference from mean (%)
11	1/8400	—
4	1/6910	20
2	1/10,000	20
1	1/4800	40

Seven out of 18 tests had a doubtful tube below the apparent end-point. The standard error of this test is 18%.

If the results obtained at the 50 and 20% dilution levels are combined we may conclude that the standard error of the test as done here is approximately 22%, and therefore about two-thirds of the results will be within 22% of the true value, and 20 out of 21 will be within 44% of this.

When a probit/log-dose curve was fitted to the results from the 18 tests at 20% dilution differences, by taking the 18 tubes at any one dilution as a group with a percentage response, it was found that the departure from linearity was significant in that higher dilutions gave a much steeper slope than lower ones. From this, it is clear that if, for example, the test were repeated 6 times on small groups of tubes, and the correct end-point taken as that of 50% of the tests with readings restricted to below 60%, the method of assay would be very delicate indeed.

Substrate solutions showing any evidence of loss of viscosity (as described under 'Methods', p. 170), were not used for this test, and standard preparations of the substrate and of the albumen were kept in the dry state for comparative tests. Under these conditions there was no evidence of variation in the sensitivity of the substrate mixture. Systematic observations have not been made to determine whether any change in the apparent titre of enzymes is found, when these are retested after varying intervals of time, but chance observations on some of the dried purified enzymes tested at intervals up to 3 months do not indicate any significant variation. I am indebted to Dr C. W. Emmens of the National Institute for Medical Research, Hampstead, for the statistical examination of these results and for much valuable advice.

The neutralization of the M.C.P. reaction by anti-sera

The spread of bacterial diffusing factors in the skin can be inhibited by sera from rabbits immunized with purified diffusing factors, which are free from detectable bacterial toxin [McClellan, 1936].

The anti-sera used contained no detectable anti-toxin. The serum prepared against the diffusing factor obtained from *Cl. welchii* inhibited the spread of the homologous factor, but not that of the factor obtained from *Vibrio septique* (*Cl. septicum*), and *vice versa*. More recently [McClellan & Hale, 1941] it was confirmed that these sera also inhibit the viscosity-reducing, and hydrolytic, activity of the hyaluronidase obtained from the homologous organisms. It was of interest to investigate the influence of these sera on the M.C.P. activity of these enzymes, and to study the activity of sera prepared against enzymes from other sources.

Technique. A fixed dilution of the anti-serum was tested against increasing dilutions of the enzyme. This was found to give a clearer end-point than the reverse method of titrating increasing dilutions of serum against a fixed dose of enzyme.

Since the serum dilutions (1:10) were made with physiological saline the substrate-protein mixture was made up entirely with distilled water, in order to keep the salt content of the system the same as that of the routine M.C.P. test. Enzyme solutions were made at twice the concentration to be tested and then mixed with an equal volume of the diluted serum. Thus in the final mixture there was a constant 1:20 dilution of serum, mixed with increasing dilution of enzyme. The enzyme-serum mixtures were left at room temperature for 30 min. before the addition of the substrate-protein mixture and subsequently incubated at 37°.

In each test controls were set up in which the enzyme was titrated in the absence of any serum, and also in the presence of correspondingly dilute normal serum. The activity of the sera was expressed as the number of minimal clot-preventing doses of enzyme which would be neutralized by the undiluted serum.

Gas gangrene anti-hyaluronidase sera. The sera prepared against the diffusing factor, which had been shown [McClellan, 1936; McClellan & Hale, 1941] to neutralize the diffusing, viscosity-reducing and hydrolytic activity of the extracts of *Cl. welchii* and *Vibrio septique*, were tested by the M.C.P. method. Of the two *Cl. welchii* anti-sera, one neutralized from 80 to 160 minimal M.C.P. doses of the homologous enzyme, and the other 160 to 200 doses. Of the two *Vibrio septique* anti-sera, one neutralized more than 100 doses of enzyme, and the other between 40 and 100 doses. It was not possible to test these *Vibrio septique* sera more accurately as no sufficiently potent enzyme from this source was available.

There was no cross-neutralization of the *Cl. welchii* enzyme by *Vibrio septique* anti-serum or *vice versa*.

Streptococcal anti-hyaluronidase sera. Groups of six rabbits were immunized with purified enzyme

obtained from group A type 4 and group C type 7 streptococci. The culture filtrates were purified by adsorption on to alumina C γ and elution with Na $_2$ HPO $_4$ solution according to the method described by Madinaveitia [1939]. One of these crude filtrates, and the corresponding purified preparation, was kindly examined by Dr E. W. Todd, of the L.C.C. Antitoxin Laboratories for streptolysin O. He reported that, whereas the crude filtrate contained considerable quantities of this haemolysin, none was detectable in the purified preparation. The group A and group C anti-sera were also tested by Dr Todd for antitoxin both before and after immunization. Unfortunately, the tests on the group C anti-sera did not give completely clear-cut results owing to deterioration of the sera during an interval elapsing before they were tested. There was, however, evidence of a slight increase in anti-haemolysin titre after the second course of injections over the figure to be expected for a similar group of normal rabbits. This result suggests that there may have been a small amount of haemolysin toxoid in the purified enzyme preparation. The sera from the group A rabbits all showed less than 1 unit of anti-streptolysin O both before and after immunization. These results indicate that the group A purified enzyme preparations did not contain significant amounts of streptolysin O or the corresponding toxoid, and that their anti-hyaluronidase activity could not be ascribed to any increase in anti-haemolysin.

Tests for anti-hyaluronidase activity with the sera from the six rabbits immunized with the group C enzyme showed that after the first course of 7 injections they neutralized from 160 to 500 minimal M.C.P. doses of the homologous enzyme; after the second course of 4 injections the sera from all these rabbits neutralized 750 or more doses of enzyme. The sera obtained from the animals immunized with the group A enzyme neutralized from 40 to 160 doses of enzyme after the first course of injection. Cross-neutralization tests showed that there is no neutralization of group A enzymes by group C sera or *vice versa*. On the other hand, neutralization tests carried out with enzymes from group C types 20 and 21 strains and the serum prepared against the enzyme derived from group C type 7 strain showed that this serum was equally potent against these enzymes. It appears, therefore, that in their present state of purification, these streptococcal enzymes are group- but not type-specific.

Testicular anti-hyaluronidase sera. Through the courtesy of Dr Ungar of the Glaxo Laboratories a sample of rabbit serum, prepared against partially purified enzyme from bull's testis, was obtained. This serum had been shown by Dr Ungar and his colleagues to inhibit the spreading activity in the

skin of the homologous testicular enzyme (private communication). Neutralization tests by the M.C.P. method were set up with this, against a purified enzyme derived from bull's testis, a crude mouse testis enzyme, and bacterial enzymes derived from *Cl. welchii*, *Vibrio septique*, group A and group C streptococci. This serum neutralized 200 minimal M.C.P. doses of the homologous bull's testicular enzyme, but showed no neutralizing activity against the enzyme from mouse testis nor any of the bacterial enzymes. This anti-testicular serum was also tested viscosimetrically with the homologous enzyme. A 1:10 dilution of the serum neutralized the activity of at least 7 viscosity-reducing units of this enzyme.

It should be borne in mind that the apparent specificity of these anti-hyaluronidase sera, prepared from various bacterial enzymes and from testis, may be due to the partial purification of the enzymes used as antigens, and to the fact that these enzymes were still linked to some other bacterial or tissue component, which determined the specificity of the anti-sera.

The relation between the M.C.P. reaction and viscosity reduction by the enzyme

It was known that the highest dilution of a purified testicular enzyme which would destroy the clotting power of a substrate protein mixture in 20 min. would reduce the relative viscosity of the substrate only from 153 to 148 in that time. The dilution of this sample was 1:100. When this sample of enzyme was tested viscosimetrically in a system containing the same amount of NaCl as the M.C.P. system, it was found to contain 16 viscosity-reducing units (v.r.u.); by definition [McClellan & Hale, 1941], 1 v.r.u. is that concentration of enzyme which will reduce the viscosity of a standard substrate preparation to a level halfway between its original figure and that of the solvent employed in 20 min. Thus the clotting power of the substrate mixture was destroyed in 20 min. by a dilution of enzyme containing only 0.16 v.r.u. It was of interest to determine how rapidly a solution containing 1 v.r.u. would prevent the formation of the clot.

When the substrate mixture and a dilution of enzyme containing 1 v.r.u. were warmed separately to 37°, the capacity to form a typical mucin clot was destroyed in less than 1 min., threads only developing in a sample tested after 1 min. A 2 min. sample remained clear after the addition of acetic acid. These observations indicate that mucin-clot prevention is the first stage in the degradation of the substrate, and that this change can be detected before any appreciable fall in viscosity occurs. The fact that diffusion in the tissues can also be detected immediately, and occurs in high dilution

suggests that this phenomenon may be closely related to this primary stage in the degradation of the substrate.

The combined influence of salt concentration and of pH on the viscosity-reducing activity of the enzyme

It has been shown [McClellan & Hale, 1941; Madinaveitia & Quibell, 1941] that the concentration of salt profoundly affects the viscosity-reducing activity of these enzymes and that this effect varies with enzymes from different sources and with the nature of the ions present. The combined effect of salt concentration and pH was studied. Madinaveitia *et al.* [1940] reported their failure to obtain correlation between skin-diffusing and viscosity-reducing activities of several enzymes from different sources. Their viscosity estimations were, however, carried out at pH 4.6 in a final concentration of *M/6* buffer solution, whereas the diffusion in the skin presumably took place at about pH 7.0 in isotonic salt concentration.

Two samples of testicular enzymes purified by different methods, one purified *Cl. welchii* enzyme and one crude *Cl. welchii* culture filtrate, were titrated viscosimetrically in McIlvaine's citric acid-phosphate buffer at pH 4.6 and 7.0. The titrations were carried out in duplicate with final concentrations of *M/6* and *M/60* buffer in the system. It had already been established that the apparent titre of the enzyme was not materially affected by substituting McIlvaine's buffer at pH 4.6 for the Na citrate usually employed. The results obtained in these titrations are shown in Table 5.

Table 5. *The combined influence of salt concentration and of pH on the viscosity-reducing activity of the enzyme*

Source of enzyme	At pH 7.0		At pH 4.6	
	Buffer <i>M/60</i>	Buffer <i>M/6</i>	Buffer <i>M/60</i>	Buffer <i>M/6</i>
	V.R.U.	V.R.U.	V.R.U.	V.R.U.
Testis Gl. 3	1,280	670	205	830
Testis P.M. 1	125	37	17.6	47
<i>Cl. welchii</i> 74 (purified)	15,150	4000	4000	6100
<i>Cl. welchii</i> (filtrate)	50	15.5	27	27

Note. McIlvaine's citric acid-phosphate buffer used throughout.

V.R.U. viscosity reducing units.

It will be seen that, with all three purified enzymes, there is a marked decrease in the apparent activity at pH 7.0 if the concentration of buffer is increased from *M/60* to *M/6*. On the other hand, at pH 4.6 there is a substantial increase in activity as the concentration of buffer is increased. Thus, if titrations of any of these enzymes were carried out at different pH's in the presence of *M/6* buffer it would appear that their activity would be greater at pH 4.6 than at pH 7.0. If, however, the tests

were done in *M/60* buffer the enzymes would be much more active at pH 7.0 than at pH 4.6. The crude *Cl. welchii* enzyme shows a similar response

Fig. 2. Combined influence of pH and salt concentration. Purified testicular hyaluronidase. ●—● titration in *M/60* buffer. ○—○ titration in *M/6* buffer. V.R.U. viscosity-reducing unit.

Fig. 3. Combined influence of pH and salt concentration. Crude *Cl. welchii* enzyme. ●—● titration in *M/60* buffer. ○—○ titration in *M/6* buffer. V.R.U. viscosity-reducing unit.

to increased concentration of buffer at pH 7.0 but no change in titre at pH 4.6. The reason for this is clear from Fig. 2.

Curves showing variation of activity with change of pH were obtained for one of the purified testicular enzymes and for the crude *Cl. welchii* enzyme in both *M/6* and *M/60* buffer solutions. The pH of the systems in which the observations were made were checked electrometrically. Fig. 2 shows that, in a buffer concentration of *M/60* the pH optimum of the testicular enzyme is about 6.8, whereas in *M/6* buffer the optimum shifts to about pH 5.0. Moreover, around the pH optimum the activity of

The correlation between different methods of assaying enzymic activity

Nine representative enzyme preparations obtained from different sources and showing a wide range of activity were selected for assay, by their diffusion in the skin, their viscosity-reducing activity and by the mucin-clot prevention test. A complete description of the test for diffusing activity is given by J. H. Humphrey [1943].

Table 6. *Tests of enzymic activity by different methods*

Source of enzyme	Diffusing activity (Glaxo units/ml.)	Viscosity reducing activity units	Mucin-clot prevention. Highest effective dilution	Ratios		
				Diffusion V.R.U.	Diffusion M.C.P.	M.C.P. V.R.U.
<i>Streptococcus</i> C 7 (purified)	750	12	208	63	3.6	17.3
Glaxo standard prep. (1 mg./ml.)	2,000	29	100	69	20	3.5
<i>Cl. welchii</i> (purified)	4,000	57	860	70	4.75	15.1
<i>Cl. welchii</i> (filtrate)	8,000	57	750	140	10.6	13.2
Testis (P.M. 1)	8,000	125	1,040	64	7.7	8.3
<i>Streptococcus</i> (A 4 filtrate)	10,000	93	1,040	107	9.6	11.2
<i>Streptococcus</i> (C 7 filtrate)	10,000	225	10,000	44.5	1.0	44.5
Testis (G 3)	200,000	1,280	8,000	156	25	6.2
<i>Cl. welchii</i> (W 74 purified)	800,000	15,150	125,000	52	6.4	8.3
Coefficient of correlation				0.980	0.978	0.998
				$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$

Diffusing activity. Tested in twofold dilutions; see Humphrey (1943). 0.5 μ g. of the Glaxo standard preparation contains 1 diffusing unit.

Viscosity-reducing activity. Estimated at pH 7.0 in *M/60* McIlvaine's buffer solution. Unit defined in text. Results accurate to $\pm 10\%$.

Mucin-clot prevention. Titrated at 20% differences in dilution.

the enzyme is very much greater in the more dilute buffer. Fig. 3 shows a similar picture for the crude *Cl. welchii* enzyme though the shift of pH optimum as the buffer concentration is altered is not as marked as it is for the testicular enzyme; in *M/60* buffer the optimum activity is obtained at about pH 6.0 and in *M/6* buffer at about pH 4.5. This figure shows why the activity of this enzyme at pH 4.6 appeared to be the same in both concentrations of buffer; this is the point at which the two curves cross and the curve of activity in *M/60* buffer is exceedingly steep.

These results cast doubt on the validity of the 'pH optima' for this group of enzymes that have been previously reported by us and many other workers. It is obvious that the pH curves are profoundly modified not only by the concentration of the salts that are present but also by the nature of the ions in the system [McCLean & Hale, 1941; Madinaveitia & Quibell, 1941]. M. G. Macfarlane & H. J. Rogers state (private communication) that with different buffer systems the pH optimum of the lecithinase derived from *Cl. welchii* is different. Similarly, Myrbäck [1926] and Sherman, Caldwell & Adams [1928] found that some ions increase the activity of amylases and change the pH optimum.

In the light of the observations described in the foregoing section, it was decided to carry out viscosimetric titrations in *M/60* buffer at pH 7.0. The error of this method has been shown to be less than $\pm 10\%$. The M.C.P. test was carried out at 20% differences in dilution, and the standard error of this test is approximately 22% (see Table 6). It will be seen that the coefficient of correlation between any two of the three tests is very high, and that the ratio between the individual values for any sample obtained by the skin diffusion, and by the viscosimetric tests, are as good as could be expected, when the limit of accuracy of the former test is taken into consideration. When the ratios between the values obtained by the M.C.P. test, and by either of the other methods, are examined, the result is not so satisfactory. The variation is much larger than can be explained by the internal errors of the different types of test. This difference in the ratios does not show any constant trend as the potency of the enzymes increase, nor does it vary consistently with the source from which the enzyme was obtained. At this stage it is not possible to give any satisfactory explanations for this discrepancy, but the fact that the coefficient of correlation for all three tests, on nine different enzymes from

diverse sources and of a wide range of activity, is so high, is very strong evidence that all three tests are, in fact, measuring the activity of the same agent. In view of the contradictory reports that have appeared in the literature it is interesting to note, that when the viscosimetric assay is carried out at pH 7.0, in approximately physiological salt concentration, very good agreement is obtained with the results of skin-diffusing activity.

SUMMARY

1. A method of assaying hyaluronidase is described, which depends upon the destruction by the enzyme of the power of a substrate-protein complex to form a typical 'mucin clot' on the addition of acetic acid.

2. It appears that the destruction of the clotting power of the substrate is an early stage in its degradation, which can be detected before any appreciable fall in viscosity occurs.

3. Sera which inhibit the diffusing and viscosity-reducing activity of these enzymes also inhibit the mucin-clot prevention reaction. Sera prepared against enzymes obtained from *Cl. welchii* and *Vibrio septique* are species- but not type-specific. Those obtained against streptococcal enzymes are group- but not type-specific. A serum prepared against diffusing factor from bull's testis inhibited this enzyme, but did not inhibit a testicular enzyme from the mouse, or any of the bacterial enzymes.

4. Both the apparent pH optimum of viscosity-reducing activity, and the titre of the enzyme, are markedly affected by the concentration and nature of salts in the system.

5. Nine enzymes from various sources have been examined for diffusing activity in the skin, by viscosimetry and by the mucin-clot prevention test. The conclusion is drawn that all three tests measure the activity of the same agent.

I wish to express my appreciation of the keen cooperation and many constructive suggestions of Mr C. W. Hale.

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Studies on Diffusing Factors

3. A NEW BIOLOGICAL ASSAY OF DIFFUSING FACTOR IN GUINEA-PIGS

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The most accurate biological method of assaying diffusing factor hitherto published is that of Bacharach, Chance & Middleton [1940]. This method compares the activities of pairs of preparations by measurement of the mean bleb size produced in suitable rabbits, using a 'four-point' assay. Since the log dose-response curve is sigmoid these authors found that the four doses must be carefully adjusted so as to lie upon the straight portion of the curve, but if such precautions are observed they claim to be able to distinguish satisfactorily between doses

in the ratio of 2 to 1. In the course of work undertaken to establish the identity, or otherwise, of diffusing factor and hyaluronidase, it became desirable to be able to detect twofold differences in diffusing-factor activity. In view, however, of the difficulty caused by the non-linearity of the log dose-response curve [Evans & Madinaveitia, 1940], it was decided, at the suggestion of Mr C. W. Hale, to devise an accurate assay of the least dose which would produce a significant increase in bleb area in a given time, when injected intracutaneously