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Hyaluronidase: Correlation between Biological Assay and other Methods of Assay

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(Received 25 June 1952)

Different workers have at various times sought to correlate the *in vivo* and *in vitro* activities of hyaluronidase preparations. Chain & Duthie (1940), drawing attention to the association between diffusion in the tissues and the mucolytic activity of testicular extracts, pointed out that the viscosity-reducing and diffusing activities of various enzyme preparations were in good agreement. McClean (1943) compared enzymes from different sources by their diffusing activity with Humphrey's (1943) technique, by their viscosity-reducing activity at pH 7.0 and by the mucin-clot prevention test; he found that the results of the viscosimetric assay could be very closely correlated to those of the skin-diffusing activity. Madinaveitia, Todd, Bacharach & Chance (1940), on the other hand, were unable to correlate satisfactorily the viscosity-reducing activity of hyaluronidase preparation with their skin-diffusing activity and stated that diffusing factors ought not to be assayed in terms of their activity in the physicochemical test. In his review on the biological role of hyaluronic acid and hyaluronidase, Meyer (1947) pointed out that the spreading reaction could not be considered as an accurate assay of hyaluronidase and that the correlation between spreading reaction and physicochemical methods of hyaluronidase assay was poor. In certain instances the comparison was vitiated by the use of different conditions of pH and salt concentration in the different tests, but even when this was avoided the error of the biological assays was too great to permit any conclusion that *in vitro* tests are a true measure of *in vivo* activity. The question is of some importance because of the need to standardize hyaluronidase in view of its increasing use in clinical medicine. Having devised a biological method of hyaluronidase assay which gives reproducible results (Jaques, 1953) we undertook to

assay seven different enzyme preparations by the more convenient viscosimetric and turbidimetric methods and to correlate the results with those of the bioassay. All tests were performed under approximately physiological conditions of pH and salt concentration in order to imitate more closely the conditions of the skin test (McClean, 1941, 1943).

METHODS

Substrates. Two different hyaluronate preparations were used. (a) A commercial potassium hyaluronate (Allen and Hanbury) containing approximately 60% hyaluronate (measured as glucosamine) dissolved in 0.9% (w/v) NaCl containing potassium phosphate buffer, pH 7.0, *I* = 0.1 with respect to phosphate, and 1:100 000 thiomersalate. This preparation will be referred to below as 'commercial hyaluronate'; it was used at 0.2 and 0.35% (w/v) in viscosimetric and turbidimetric assays respectively. (b) Purified potassium hyaluronate prepared by Dr H. J. Rogers from umbilical cords by the method of Hadidian & Pirie (1948). This preparation contained no N other than glucosamine N. It will be referred to as 'purified hyaluronate'. It was used, in the same solvent, at 0.1 and 0.2% (w/v) in viscosimetric and turbidimetric assays respectively.

The two batches of potassium hyaluronate were employed for both the viscosimetric and turbidimetric assays of all seven enzyme preparations. In the light of the observations of Meyer (1951) that various metals, notably Fe, are potent inhibitors of hyaluronidase, many of the assays with gelatin as stabilizer and commercial hyaluronate as substrate were repeated in the presence of 0.2% (w/v) sodium pyrophosphate; this substance was added in all assays with the purified substrate, although with our reagents it did not appear to have any appreciable effect.

Enzyme solutions. These were made in 0.9% (w/v) NaCl containing phosphate buffer, pH 7.02, *I* = 0.1 with respect to phosphate, containing 0.2% (w/v) gelatin and 1:100 000 thiomersalate. Stock solutions containing 1–10 mg. enzyme/ml. lost no appreciable activity during 3 weeks' storage at 4°.

Biological assay. The procedure was that described by Jaques (1953).

Viscosimetric method. The method of McClean & Hale (1941) was used.

Viscosimeters. The viscosimeters employed were of an Ostwald type calibrated in absolute viscosity units over a range of viscosities studied. (Supplied by Dr L. A. Steiner, 76 Cavendish Road, London, S.W. 12.) Flow time with water at 30° was of the order of 65 sec. The water bath in which the viscosimeters were immersed was kept at 30 ± 1°. The general conduct of the tests followed the lines suggested by McClean & Hale (1941) who defined a viscosity-reducing unit as the amount of enzyme which will reduce the viscosity of the substrate in 20 min. to a level half-way between its original figure and that of the solvent employed. Our results were calculated on this basis.

Turbidimetric method. The method used was based upon that of Tolsdorf, McCready, McCullagh & Schwenk (1949) in which the unit of activity is taken as the quantity of enzyme required to reduce the turbidity given by 0.2 mg. of purified hyaluronate to that given by 0.1 mg. when incubated together for 30 min. at 37° in 0.1 M-acetate buffer, pH 6.0, containing 0.15 M-NaCl. The turbidity is developed, after arresting the enzyme action by heating for 10 min. at 60°, by addition of a 1:10 dilution of serum or plasma previously heated at pH 3.1, and an excess of acetate buffer, pH 4.2.

Since we wished to make comparison with other assays performed at physiological pH and salt concentrations, and to guard against inactivation of hyaluronidase which occurs spontaneously in high dilutions in the absence of other

colloids, the method was modified. The buffered saline described above was used throughout, and gelatin or, in some cases, gum acacia was added to give concentrations of 0.1 or 0.25% respectively in the enzyme-substrate mixtures. The period of incubation at 37° was shortened to 10 min., since evidence was obtained that the rate of enzyme action diminished quite markedly with further incubation. Furthermore, the reaction was stopped by chilling at 0°, followed by addition of dilute acidified horse serum, rather than by heating at 60° which permits considerable further enzyme action to take place.

RESULTS

Table 1 compares the results of the three different methods of assay, the potency of the standard enzyme, which was always assayed simultaneously, being taken as unity. At least two assays, and often many more, were performed with each enzyme by each method. The potencies of the various enzymes as determined with the viscosimetric method are in reasonable agreement with those of the bioassay. In the viscosimetric assay the nature of the substrate apparently did not affect the estimation of potency of the majority of enzymes tested, the only marked difference occurring with the streptococcal enzyme (*G*) which was less active upon the purified substrate. With the pure hyaluronate the value for *G* corresponds much better with

Table 1. *Comparison of the activity of several hyaluronidase preparations using various assay methods*

Enzymes	Bioassay activity in terms of standard (b)	Viscosimetric method activity in terms of standard		Turbidimetric method activity in terms of standard		Ratios			
		Commercial hyaluronate (v ₁)	Purified hyaluronate (v ₂)	Commercial hyaluronate (t ₁)	Purified hyaluronate (t ₂)	b/v ₁	b/v ₂	b/t ₁	b/t ₂
A, standard; testis	1	1	1	1	1	1	1	1	1
B, testis	5.1	4.5	5.4	2.1	2.2	1.1	0.9	2.4	2.3
C, testis	5.4	6.6	6.9	2.6	2.3	0.8	0.8	2.1	2.3
D, testis	6.8	7.6	7.2	3.8	2.6	0.9	0.95	1.8	2.6
E, testis	0.59	1.0	0.61	0.5	0.38	0.6	1.0	1.2	1.5
F, <i>Staphylococcus</i>	2.3	2.6	1.7	0.8	0.54	0.9	1.4	2.9	4.3
G, <i>Streptococcus</i>	0.53	1.4	0.65	0.2	0.16	0.4	0.8	2.7	3.4

Table 2. *The potency of several hyaluronidase preparations*

Enzymes	Concentration of enzyme which reduced the viscosity of the substrate by half in 20 min. at 30° (μg./ml.)		Concentration of enzyme which reduced the turbidity value of the substrate by half in 10 min. at 37° (μg./ml.)	
	Substrate commercial potassium hyaluronate (0.2 mg./ml.)	Substrate purified potassium hyaluronate (0.1 mg./ml.)	Substrate commercial potassium hyaluronate (0.35 mg./ml.)	Substrate purified potassium hyaluronate (0.2 mg./ml.)
A, standard	5.5	7.03	71	91
B	1.19	1.3	34.5	41.6
C	0.83	1.01	27	40
D	0.73	0.98	19.2	35
E	5.4	11.5	133	240
F	2.04	4.13	91	167
G	4.05	10.9	370	570

that obtained in the bioassay. The overall agreement of biological and viscosimetric assay is brought out in the column wherein are listed the ratios of both assays with each substrate. On the other hand, the correlation between the turbidimetric results and this bioassay is less good. Thus, in the biological assay the preparations *B* and *C* were both about five times as potent as the standard, whereas turbidimetrically their activities in terms of the standard were respectively 2.1 and 2.6. With the bacterial enzymes *F* and *G* the discrepancy between the results of both methods was even more pronounced. In Table 2 some experimental values are given, expressed as reciprocal dilutions (w/v) of the various enzyme preparations required to reduce the viscosity due to the substrate by half in 20 min., or the turbidity by half in 10 min., under the conditions outlined above.

When 0.2% (w/v) gelatin was used as stabilizer in the diluting fluid, the shape of the curves of turbidity (absorptiometer reading), after the set period of incubation, against quantity of enzyme was closely similar for all the enzyme preparations, and the relative potencies calculated in terms of any arbitrary turbidity end point were likewise similar. In earlier experiments 0.5% (w/v) gum acacia was used instead of gelatin, and it was regularly observed that preparations *A*, *B* and *D* gave curves which had a steeper slope than those given by preparations *C* and *G*. The relative potencies in the gum acacia experiments were therefore dependent upon the turbidity end point chosen, and consequently also upon the time taken to reach this end point.

Table 3. *Relative activities of enzyme preparations per mg. compared with standard preparation by the turbidimetric method, using a single preparation of the substrate*

(Method (a): incubated 10 min. in presence of 0.2% (w/v) gelatin. Method (b): incubated 30 min. in presence of 0.5% (w/v) gum acacia.)

Enzyme preparation	Method	
	(a)	(b)
<i>B</i>	2.1	1.26
<i>C</i>	2.6	2.5
<i>D</i>	3.8	1.4
<i>G</i>	0.2	0.27

The importance of this effect is shown in Table 3, where the apparent activities of four preparations upon the same commercial hyaluronate substrate compared with the activity of the standard are shown (a) measured in presence of 0.2% (w/v) gelatin with 10 min. incubation and (b) measured in presence of 0.5% (w/v) gum acacia with 30 min. incubation. Whatever the cause of the discrepancies in the presence of gum acacia, it appears to be unsuitable for use as a stabilizing agent in this test.

DISCUSSION

The possession of a reasonably accurate biological assay method for hyaluronidase has made it possible to test the validity of the correlation between activity *in vivo* and *in vitro*, measured by viscosimetric and turbidimetric methods. We have shown that, provided suitable precautions are taken, among them the use of a reference standard enzyme preparation in each test, the correlation for the viscosimetric method is good and nearly as good for the turbidimetric method. If the bacterial enzymes are excluded, which is probably justified since the reference standard was a testicular preparation, it may be deduced that the viscosimetric assay gave an accurate measure of the biological activity, especially when the purified substrate was used. The turbidimetric assays all appear to have given relatively low values compared with the bioassay. Inspection of the figures in Table 1, however, suggested that if one of the purified testicular enzymes had been used as the reference standard, the results of the assays by all three methods would have shown ratios close to unity, at least in so far as other purified testicular preparations were concerned. The figures were therefore recalculated on the basis of unit potency for preparation *C*, and in Table 4 are shown the ratios obtained. The agreement between the results of the biological and the other assays is very good for the purified testicular preparations, and it seems likely that for use as a practical biological standard, valid for all three methods, a partly purified testicular preparation would be preferable to crude seminal fluid, although the latter was originally chosen with the idea that it would contain any enzyme or enzymes to be found in the purer preparations.

The fact that the correlation is less good with the two bacterial enzymes is not surprising, since there is no evidence that they are single enzymes or that their action is identical with that of testicular preparations.

The influence of the nature of the substrate upon the results of *in vitro* methods of hyaluronidase assay has been mentioned by many workers. We began this work with the expectation that if a reference preparation of enzyme were used in all assays no influence of the substrate would be apparent, since all like enzyme preparations, at equivalent concentrations of active material, should be similarly affected by variations in degree of polymerization, content of sulphated polysaccharide or presence of enzyme inhibitors. This expectation was not fulfilled, and the apparent variations in activity of the enzymes with the pure and the cruder substrate were outside the range of experimental error. Furthermore, the effect of using gum acacia as stabilizer instead of gelatin was

Table 4. *Ratio of activities by different assay methods, when activity of preparation C is taken as 1*

Enzyme source	Biological assay Viscosimetric assay		Biological assay Turbidimetric assay	
	Commercial substrate	Pure substrate	Commercial substrate	Pure substrate
A, seminal fluid	1.26	1.26	0.5	0.45
B, testis	1.38	1.22	1.17	1.0
C, testis	1	1	1	1
D, testis	1.1	1.2	0.87	1.1
E, testis	0.75	1.2	0.58	0.69
F, <i>Staphylococcus</i>	0.91	0.58	0.72	0.54
G, <i>Streptococcus</i>	2.1	0.9	0.8	0.7

markedly different upon different enzyme preparations. These observations imply some degree of heterogeneity of the enzyme preparations compared. It is of interest, from a practical point of view, that the closest agreement between *in vitro* and *in vivo* activity was obtained with the purest substrate.

SUMMARY

1. Five preparations of testicular hyaluronidase, one of streptococcal and one of staphylococcal origin were compared by an accurate skin-diffusion

assay and by viscosimetric and turbidimetric methods.

2. With testicular preparations the results of the three methods agree well, provided that the potency is measured in terms of a reference preparation of enzyme, and that the pH and ionic strength of the solvents are approximately physiological. It is desirable to use highly purified substrate, and to stabilize the enzymes with gelatin rather than gum acacia.

3. The activities of the two bacterial enzymes showed a close but not complete correlation between the three tests.

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The Differentiation of True and Pseudo Cholinesterase by Organo-phosphorus Compounds

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(Received 1 July 1952)

True cholinesterase is the enzyme present in the brain and erythrocytes of many species which hydrolyses acetylcholine at a higher rate than butyrylcholine and is inhibited by excess substrate. Pseudo cholinesterase is present in the serum of many species and hydrolyses butyrylcholine at a higher rate than acetylcholine and is not inhibited by excess substrate. Mendel, Mundell & Rudney (1943) introduced the substrates acetyl β -methylcholine and benzoylcholine for true and pseudo cholin-

esterases respectively. Differentiation of true and pseudo cholinesterases has also been achieved by the use of selective inhibitors. Diisopropyl fluorophosphonate (DFP) inhibits pseudo cholinesterase at a lower concentration than the true (Mazur & Bodansky, 1946; Mendel & Hawkins, 1947; Adams & Thompson, 1948). However, in some species the sensitivities of the two enzymes are not very different (Ord & Thompson, 1950). A series of organo-phosphorus compounds have been ex-