FACTORS AFFECTING THE ASSAY OF HYALURONIDASE

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A number of different types of assay methods have been developed for the determination of hyaluronidase activity in vitro. These include the mucin clot test (1, 2), the viscosimetric assay (3-12), the turbidimetric assay (7, 13-17), and the reductimetric assay (18). Of these, the turbidimetric and viscosimetric assays have been used most widely.

In the work reported here a study has been made of a number of factors affecting the turbidimetric assay and a modification of the method has been developed based on this study. The effect of hyaluronate molecular weight on the viscosimetric assay has also been investigated.

The hyaluronic acid depolymerase obtained from bull testes has been used in these experiments, since this enzyme is being used therapeutically and its standardization is of considerable interest. The results obtained do not necessarily apply to bacterial or other hyaluronidases.

EXPERIMENTAL

Method of Assay

Changes in the assay procedure to test various factors affecting the activity will be covered in the descriptions of the individual experiments. The standard method of assay is the following.

Preparation of Acid Serum—Fresh, sterile, normal horse serum is lyophilized in small lots and stored in stoppered containers at 0-2°C. This material has shown no changes in turbidity-producing properties for periods up to 1 year. For use, the serum is reconstituted with water and diluted with 9 volumes of 0.5 M acetate buffer, pH 4.30. The pH is adjusted to 3.1 with 4 N hydrochloric acid and the solution allowed to age for 18 to 24 hours at room temperature. Thereafter, it is stored at 0-2°C and in this form it is stable for 3 to 4 weeks. For each day’s use, a sufficient quantity of the 1:10 serum, pH 3.1, is diluted to 1:40 with 3 volumes of 0.5 M acetate buffer, pH 4.30, and brought to room temperature.

Potassium Hyaluronate Solution—Purified potassium hyaluronate from human umbilical cord (UC hyaluronate) or bovine vitreous humor (VH hyaluronate), the preparation and properties of which have been described previously (19), is used as the substrate.

A stock aqueous solution containing 0.5 mg. per ml. of potassium hyaluronate is prepared and stored at 0-2°C. This solution is stable for several
weeks. For each day's use, a portion of this solution is diluted with an equal volume of 0.04 ionic strength sodium phosphate buffer, pH 6.3, containing 0.14 mM sodium chloride. (The pH becomes 6.4 on dilution with the aqueous hyaluronate solution.)

**Hyaluronidase Solutions**—A stock standard hyaluronidase solution is prepared by dissolving 1 mg. per ml. of the crude dry standard in cold water (see section on enzyme standard). It is stable at 0-2° for at least 1 week. For the standard curve, determined simultaneously with the unknowns, the stock standard is diluted to 3 turbidity reducing units (t.r.u.) per ml. immediately before assay with 0.02 ionic strength sodium phosphate buffer, pH 6.4, containing 0.07 mM sodium chloride. Unknown preparations are made up at 1 mg. per ml. and diluted with the same buffer at 0-2°, immediately before assay, to such a concentration that the activity can be read from the standard curve.

**Activity Determination**—All activity measurements are made in duplicate. The hyaluronate solution, in 0.50 ml. quantities, is pipetted into the bottom of 16 X 100 mm. test-tubes. Sufficient 0.02 mM sodium phosphate-0.07 mM sodium chloride buffer, pH 6.4, is then added so that the final volume after the addition of the enzyme will be 1.00 ml. At 1/2 minute intervals, varying amounts of freshly diluted enzyme solution are added to the tubes containing hyaluronate, and the tubes are immediately placed in a stirred water bath at 37° ± 0.2° and incubated for 30 ± 0.25 minutes.

The tubes are removed from the water bath at 1/2 minute intervals and 4.0 ml. of 1:40 acid serum are added immediately. The tubes are allowed to stand for 30 minutes at room temperature and the turbidity is read in an acid serum photoelectric colorimeter with a red filter. Blank determinations are run, hyaluronate being omitted. Unknown activities are then calculated from the turbidity value by reading from the standard curve. Typical standard curves are shown in Fig. 5.

It has been found that, due to the high dilution of the enzyme, some activity is lost during the incubation, particularly with the more purified hyaluronidase preparations. The enzyme can be stabilized by having about 0.5 mg. per ml. of partially hydrolyzed gelatin present in the incubation solution (see the section on stability). Merthiolate (1:10,000) is

1 The tubes are not heated to destroy the enzyme after incubation as in several methods (7, 17), since, under these conditions, it has been found that there is no significant enzyme activity after the addition of the acid serum.

2 A red filter is used rather than a blue filter as described in some methods, since the red filter gives a blank of lower proportionate value. The turbidity readings reported here are proportional to optical density, 100 Klett units being equivalent to an optical density of 0.267 on a Beckman DU spectrophotometer, with a 1 cm. cell and a wave-length of 620 mμ.
used as a preservative for the gelatin solution. In the experiments reported here, gelatin was used only when noted.

Hyaluronidase Standard—The assay is based on a comparison of the unknown activity against the activity of a lot of crude lyophilized hyaluronidase, which was prepared by the initial ammonium sulfate fractionation described by Hahn (20). The standard enzyme is stored at 0–2°. We have been unable to detect any change in the activity of this preparation over a period of 2 years.

The turbidity reducing unit, as originally described by Kass and Seastone, was defined as the amount of enzyme required to reduce the turbidity produced by 0.2 mg. of hyaluronate to that given by 0.1 mg. in 30 minutes under specified conditions. This unit has been used by a number of workers but with varying conditions of pH, purity of substrate, salts, salt concentrations, etc. As shown in the following experiments, these factors may cause variations in the results obtained; therefore there is probably some difference in the value of the turbidity reducing units used in different laboratories.

The crude hyaluronidase preparation which has been used throughout as a standard had been more or less arbitrarily assigned a value of 218 t.r.u. per mg. At the conclusion of the experiments reported here, this standard was assayed according to the general method of Kass and Seastone but with the conditions of pH, salt concentration, etc., which we have adopted for the routine assay. Purified UC hyaluronate was used as the substrate and hydrolyzed gelatin as a stabilizer. The experimental value obtained was almost identical with the assigned value and therefore we have continued to use 218 t.r.u. per mg. as the activity of the standard enzyme.

Factors Affecting Hyaluronidase Activity

Effect of pH and Sodium Chloride on Activity—The effect of sodium chloride and of pH on hyaluronidase activity has been investigated by a number of workers (21, 2, 7, 10, 15, 17). To study the effects further, particularly with regard to the interrelation of salt and pH and in the presence of a relatively pure hyaluronate, the following experiments were carried out.

The same conditions were employed as described under “Method of assay” except for the changes in pH and salt concentration. Since pH and salt concentration affect turbidity, the per cent turbidity reduction was calculated from controls without enzyme and the relative activities were calculated from a standard curve.

As shown in Fig. 1, the optimal sodium chloride concentration was found to vary considerably with pH. The curves illustrate the interrela-
tionship of pH and optimal salt concentration and indicate that at low pH less salt is necessary. They also indicate that with higher salt concentrations lower pH optima will be obtained.

The optimal pH was then determined with 0.09 M sodium chloride. This salt concentration was chosen as a compromise, since it falls within the range of maximal activity at pH 4.5 and 5.2 and is only slightly above the optimal concentration at 7.1. Fig. 2 shows that, under these conditions, the optimal pH is about 6.4. The same value was also obtained for alcohol-fractionated hyaluronidase (22) containing 6000 t.r.u. per mg.

![Graph showing effect of sodium chloride concentration on hyaluronidase activity at various pH values.](image)

**Fig. 1.** Effect of sodium chloride concentration on hyaluronidase activity at various pH values. Acetate buffer at pH 4.5 and 5.2; phosphate buffer at pH 6.2 and 7.1. The salt concentrations and pH values are those of the incubation solution. Umbilical cord hyaluronate, 0.125 mg per ml; hyaluronidase, 218 t.r.u. per mg, 0.625 γ per ml. Mole ratio of sodium chloride to buffer 50:1.

In comparing the two experiments with crude and purified hyaluronidase it was found that the activity ratios of purified to crude hyaluronidase were relatively constant on the alkaline side of the optimal pH but were lower on the acid side. For example, at pH 5.2 the ratio was 0.45:1 as compared with 1:1 at pH 6.4. With hydrolyzed gelatin as a stabilizer the ratio at pH 5.2 was 0.83:1. Thus it would not seem desirable to run the assay at a pH much below the optimum. This is of interest, since in the various published assay methods pH values from 4.5 to 7.0 have been used.

Curves were then determined with a constant ionic strength of 0.09 at pH 6.4 with various concentrations of sodium chloride and sodium phosphate buffer. It was found that maximal activity was obtained with ionic
strengths of 0.07 sodium chloride and 0.02 sodium phosphate buffer. Therefore, these conditions have been adopted for the routine assay.

Since the optimal ionic strength of 0.09 is lower than that given in other published turbidimetric assays, it seemed desirable to determine whether the use of less salt would have a variable effect with changing enzyme concentration. Three standard turbidity reduction curves were made with 0.05, 0.09, and 0.15 M sodium chloride. The curves were then adjusted to equal activity levels on the basis of the activities at 50 per cent turbidity reduction and the points replotted. These curves were practically coincident throughout their entire length, indicating that salt concentration, over this range, had little effect on the kinetics of the reaction. Madinaveitia and Quibell (21) have shown this same result with the viscometric method but found that at very low salt concentrations there was no longer a linear relation between enzyme concentration and activity.

Stability of Hyaluronidase during Assay—It was realized that hyaluronidase is unstable in very dilute solutions, and therefore the dilutions for the assay were made as rapidly as possible with cold solutions and the incubation has been started as soon as possible after dilution. It seemed advisable, however, to determine the extent of this instability and to study the effects of stabilizing agents under the conditions of the assay.

It has been shown by McCullagh et al. (23) that partially hydrolyzed gelatin has a stabilizing effect on hyaluronidase both in solution and in the dry state. We tried various gelatin preparations and found that Difco Bacto-gelatin, autoclaved as a 5 per cent solution for 90 minutes at 15

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**Fig. 2.** Effect of pH on hyaluronidase activity. Hyaluronidase concentration, enzyme, and hyaluronate as in Fig. 1. Sodium chloride 0.09 M, buffers 0.002 M.
pounds pressure and lyophilized, gave a low viscosity material with good stabilizing properties. The optimal concentration of hydrolyzed gelatin for stabilization under the conditions of the assay was determined with a hyaluronidase having a potency of 1500 t.r.u. per mg. and was found to be about 0.33 mg. per ml.

To determine the influence of enzyme purity on stability during assay, a number of lots of hyaluronidase, prepared by different methods, were assayed at activity levels of about 0.75 t.r.u. per tube with and without hydrolyzed gelatin. The results, given in Table I, indicate an increasing loss of activity during assay with increasing purity in the absence of gelatin. The degree of stability of the different enzyme preparations in the absence of gelatin was roughly proportional to the amount of total protein present.

The differences in activity ratio indicate that, when purified hyaluronidase preparations are assayed against a crude standard, a stabilizing agent, such as hydrolyzed gelatin, should be included in the incubation solution of the standard and the unknown if accurate absolute potencies are to be obtained.

It was found that the enzyme is protected by the gelatin-buffer solution when incubated without substrate either at 37° for 30 minutes or in the cold up to 48 hours at the concentrations which are used for the assay.

**Effect of Various Factors on Turbidity**

Meyer and Palmer (24) showed that in the presence of acetic acid various proteins produce a precipitate with hyaluronic acid. They suggested that the reaction was due to the combination of the positively charged protein with the negatively charged hyaluronate. Seastone (25) showed that a turbidity could be produced by hyaluronate and acid serum which
could be used as a measure of the hyaluronate concentration and noted that the amount of turbidity was decreased by an excess of either protein or hyaluronate. Kass and Seastone (13) modified this procedure for their hyaluronidase assay. We have investigated a number of conditions which affect this reaction such as salt, hyaluronate and serum concentration, pH, temperature, and time. The effects of most of these variables on turbidity appear to be due to variations of the charges on the hyaluronate, the protein, or the complex.

While uniform turbidity production requires careful control of a number of factors, variation in turbidity-producing properties from one serum lot to another (17) has not been observed when the method of acid serum preparation outlined under "Method of assay" is employed. An essential part of the preparation is the acidification to pH 3.1 after the 1:10 dilution with buffer. Failure to acidify (14, 16) results in a lower turbidity and in a standard curve having poor proportionality to enzyme concentration. The pH of 4.3 for the buffer used to dilute the serum to 1:40 is a critical factor, since slight variations will result in variable turbidities. This pH is, however, in the region of maximal turbidity and minimal variation.

The effect of variation in serum concentration was studied both with intact and enzymatically degraded hyaluronate. When no enzyme was used, turbidity was maximal at 1:80 to 1:40 serum, with an appreciable decrease at 1:20. However, when sufficient enzyme was used to reduce the turbidity almost completely by the normal assay procedure (1:40 serum), considerable turbidity was obtained at lower serum concentrations. Thus there is no single serum concentration which will give maximal turbidity over the entire range of enzymatic degradation products and the concentration of 1:40 appears to be a suitable compromise.

The concentration of salt in the 1 ml. of incubation solution affects the amount of turbidity obtained. There is a slight increase in turbidity from 0 to 0.1 ionic strength, a plateau of maximal turbidity from 0.1 to 0.25, then a rather rapid diminution to zero at 0.6.

The length of time of standing affects the amount of turbidity as does the temperature. After 30 minutes standing the rate of increase in turbidity is small, however, and the readings are customarily made at 30 ± 2 minutes. The effect of temperature on turbidity is appreciable, varying in a typical experiment from 98 Klett units at 17° to 102 at 25° and 110 at 37°. Thus it is desirable to keep the tubes at relatively uniform temperatures.

In trials for the standard curves with highly viscous, umbilical cord hyaluronates, it was found that, at the initial enzyme concentrations, the curve did not fall immediately but showed a flat or rising section before
falling. To examine this phenomenon further a curve was obtained with very low enzyme concentrations, a potassium hyaluronate giving a specific viscosity of 5.4 as a 0.1 per cent solution in 0.05 M phosphate buffer, pH 7.0, containing 0.05 M sodium chloride being used. Enzyme dilutions were made up at 0.015 t.r.u. per ml. and at intervals up to 0.15 t.r.u. per ml. with an additional concentration of 0.30 t.r.u. per ml., the latter two being approximately the first concentrations on the usual standard curve. The results, shown in Fig. 3, indicate that there is a distinct rise in turbidity with small quantities of enzyme. We have interpreted this result to mean that the highly polymerized hyaluronate molecule can be broken down into 2 or more molecules, each of which is still large enough to produce turbidity with acidified serum.

These results might also be considered as additional evidence that the hyaluronidase produces a random splitting of the polymer, rather than end-group splitting (18).

**Comparison of Umbilical Cord and Vitreous Humor Hyaluronates As Substrates**

A comparison was made of the assay results obtainable with human umbilical cord and bovine vitreous humor hyaluronates prepared as described previously (19). Fig. 4 shows a comparison of turbidity production with the two hyaluronates at various concentrations. Although the curves are slightly divergent at the higher levels, they are in good agree-
ment, considering the differences in source and viscosity between the two preparations. The concentration of 0.125 mg. per ml. was chosen for the routine assay, since this amount gives a practical working turbidity with the purified hyaluronates and falls in the more linear portions of the turbidity curves.

Curves of turbidity reduction with two other lots of hyaluronate are shown in Fig. 5. The specific viscosities of the UC and VH hyaluronates were 4.2 and 0.60, respectively, as 0.1 per cent solutions in 0.05 M phosphate buffer, pH 7.0, containing 0.05 M sodium chloride. The sulfur content was less than 0.1 per cent (26).

![Fig. 4. Comparison of turbidity production with umbilical cord and vitreous humor hyaluronate. O, UC hyaluronate; ●, VH hyaluronate. Standard assay conditions.](http://www.jbc.org/)

The UC hyaluronate curve shows the initial break previously discussed and the curve is nearly linear throughout most of its length, but flattens out in the lower turbidity range. The VH hyaluronate curve is slightly lower and does not show the initial break but has a slope in the linear portion almost identical with that of the UC hyaluronate curve. The difference between the two curves, which occurs with all of our preparations of UC and VH hyaluronate, is due, at least in part, to the lower molecular weight of the VH hyaluronate. In comparison with UC hyaluronate, the VH hyaluronate is less polymerized and therefore less splitting is required to reduce the turbidity to the same level.

However, the amounts of hyaluronidase required to reduce the turbidities to the same degree are quite similar. The results illustrate that the turbidimetric assay gives relatively consistent values, even with hy-
aluronates from different sources and with considerable variation in degree of polymerization.

With regard to the effect of non-inhibiting impurities in the substrate, it was found that, when the amount of hyaluronate is varied 25 per cent, the turbidity reduction curves remain parallel in the linear portions.

**Fig. 5**  
Standard turbidity reduction curves. O, UC hyaluronate; O, VH hyaluronate. Conditions as described under “Method of assay.” Enzyme potency 218 t.r.u. per mg. Assayed with hydrolyzed gelatin, 0.3 mg. per ml.

**Fig. 6**  
Relationship of specific viscosity of hyaluronates to concentration and to weight average molecular weight times concentration. O, UC hyaluronate versus C; O, UC hyaluronate versus $M_w \times C$; O, VH hyaluronate versus C and $M_w \times C$ (two curves identical). Conditions as for the viscosimetric assay.

**Viscosimetric Assay**

Influence of Hyaluronate Molecular Weight on Viscosimetric Assay

Viscosimetric units for the standardization of hyaluronidase have been established on the basis of the amount of enzyme required to reduce the viscosity of a hyaluronate solution to one-half the original viscosity under specific conditions (3, 4, 7, 9). Other modifications have also been developed (8, 12) which depend upon a hyaluronate standard. However, Hadidian and Pirie (10) and Meyer (27) have pointed out that half viscosity reduction times may vary as much as 10-fold with hyaluronates of

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*A preliminary report on this section was given at the Miniature meeting of the Philadelphia section of the American Chemical Society, Philadelphia, January 18, 1951.*
different initial viscosities. The former attributed the variation to differences in molecular size and in some instances to the presence of inhibitor. The latter suggests that the opening of labile linkages in the more viscous preparations contributes to the more rapid viscosity reduction.

Our experience with purified UC and VH hyaluronates has been similar. These hyaluronates, which show almost identical behavior in the turbidimetric assay, have shown up to 25-fold differences in rate of viscosity reduction.

The presence of inhibitor in hyaluronate is evidenced by variation in the rate of turbidity reduction between hyaluronates or by differences in the slopes of the linear portions of the turbidity reduction curves. The hyaluronates used here show no such differences and behave quite similarly to those shown in Fig. 5. The differences which we have found in viscosity reduction may be accounted for by the difference in the molecular weights of the hyaluronates.

The relative molecular weights were determined by the intrinsic viscosity method which has been applied to hyaluronate solutions by Blix and Snellman (28) and recently by Jeanloz and Forchielli (29). Fig. 6 shows the specific viscosity ($\eta_s$) of VH and UC hyaluronates as a function of hyaluronate concentration ($C$). Tangents were drawn to the curves at zero concentration and the ratio of the slopes, 3.74:1, was taken to be the ratio of the weight average molecular weights ($\bar{M}_w$) (30). When the data were then plotted with $\eta_s$ as a function of relative $\bar{M}_w \times C$, also shown in Fig. 6, the curves were almost coincident at the lower viscosity levels, but were somewhat divergent at the higher levels. The close proximity at the lower concentrations indicates that, under the assay conditions in which $\eta_s$ is about 1, approximately the same relationship holds between $\eta_s$ and $\bar{M}_w \times C$ for the two preparations.

It has been shown by Kraemer and Lansing (31) that for linear polymers $\eta_s$ is more proportional to weight average molecular weight than to number average molecular weight ($\bar{M}_n$), as used by Staudinger (32). However, Florey (33) has shown for linear condensation polymers that the ratio $\bar{M}_w:\bar{M}_n$ is relatively constant if the molecular size is above 10 monomer units per molecule.

Therefore, if $N$ is the number of molecules present at any given viscosity and $K$ is the ratio $\bar{M}_w:\bar{M}_n$

$$N = K \frac{C}{\bar{M}_w}$$

If this relationship is assumed to hold for hyaluronate, then the relative

4 Alburn, H. E., and Whitley, R. W., unpublished work.
number of molecules present with any two hyaluronates is

\[
\frac{N_1}{N_2} = \frac{C_1\overline{M}_w}{C_2\overline{M}_w}
\]  

(2)

With two hyaluronates, at equal viscosities, at which \(\eta_{\text{sp.}} = f(\overline{M}_w \times C)\), the ratio \(N_1:N_2\) is constant.

To facilitate interpretation of the viscosity data with regard to enzyme action, the hyaluronates were used at equal initial viscosities and therefore at unequal concentrations. Data obtained turbidimetrically indicate that the difference in hyaluronate concentration should have but little effect on the enzyme reaction rate for the conditions used. The reaction rate is considered to be nearly independent of hyaluronate concentration and directly proportional to enzyme concentration.

Viscosity reduction curves were obtained with the UC and VH hyaluronates, whose relative molecular weights were determined previously, at initial specific viscosities of 1.4, the concentrations being 0.515 and 2.0 mg. per ml., respectively. The viscosity determinations were made at 25° in an Ostwald-Fenske pipette (water flow time 48 seconds) with 4.0 ml. of hyaluronate solution and 0.2 ml. of a solution of hyaluronidase assaying 1500 t.r.u. per mg. The hyaluronate and enzyme were dissolved in 0.05 M phosphate buffer, pH 6.3, containing 0.05 M sodium chloride. These conditions are those employed by Hadidian and Pirie and are quite similar to those which we have found to be optimal for the turbidimetric assay. The amounts of enzyme required for half viscosity reduction in 30 minutes were 0.94 \(\gamma\) for the UC hyaluronate and 14.8 \(\gamma\) for the VH hyaluronate or a ratio of apparent activity of 15.7:1.

If it is assumed that the initial distribution of molecular sizes is nearly equal (i.e., the ratio \(\overline{M}_n:\overline{M}_w\) is a constant), then for moderate changes in viscosity or molecular weight the viscosity reduction rates for the two hyaluronates should be constant, assuming random splitting within the molecule. The similarity in configuration between the viscosity reduction curves of the high and low viscosity preparations indicates that these assumptions are probably valid. This similarity is expressed quantitatively by the ratios of the times required to reach various viscosity levels, which are quite constant as shown in Table II.

The amount of splitting required to reduce the viscosities to the same level, on the basis of these assumptions, should be proportional to the number of molecules present, \(N_1:N_2\), the value of which may be calculated from Equation 2.

For the experiments reported here this value is

\[
\frac{N_1}{N_2} = \frac{3.74 \times 2.00}{0.515 \times 1.00} = 14.5
\]
Since the relative number of cleavages is considered to be nearly equal to the relative enzyme concentrations, the value 14.5 should be the ratio of the amounts of enzyme required for the two hyaluronates. The theoretical value is therefore in fairly good agreement with the experimentally determined value of 15.7.

An alternative but less direct procedure for approximating the relative amounts of enzyme required to give the same degree of viscosity reduction with two hyaluronates of different molecular size would be to determine the concentrations of the hyaluronates which give equal viscosities. At

<table>
<thead>
<tr>
<th>Viscosity reduction (per cent)</th>
<th>VH hyaluronate*</th>
<th>UC hyaluronate†</th>
<th>Time ratios, UC:VH</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>184</td>
<td>225</td>
<td>1.22</td>
</tr>
<tr>
<td>25</td>
<td>373</td>
<td>408</td>
<td>1.25</td>
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<td>35</td>
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<td>45</td>
<td>1068</td>
<td>1335</td>
<td>1.25</td>
</tr>
<tr>
<td>55</td>
<td>1684</td>
<td>2110</td>
<td>1.25</td>
</tr>
</tbody>
</table>

* With 20 y per ml. of hyaluronidase.
† With 1 y per ml. of hyaluronidase.

low and equal viscosities, since

\[
\frac{N_1}{N_2} = \frac{C_1 \bar{M}_w}{C_2 \bar{M}_w} = \frac{C_1}{C_2}
\]

then

\[
\frac{N_1}{N_2} = \frac{C_1^2}{C_2^2}
\]

or the relative amounts of enzyme required should be proportional to the squares of hyaluronate concentrations. Similarly, it can be shown that the amounts of enzyme required will also be inversely proportional to the square of the relative hyaluronate molecular weights. For the experiments reported here, \(C_1^2:C_2^2 = 15.1\) and \(\bar{M}_w:C_1^2 = 14.0\). It can be seen from Fig. 6 that, if the experiments were run at lower viscosities at which the two \(\eta_{sp} = f(\bar{M}_w \times C)\) curves are more coincident, these values and that for \(\bar{M}_w\) would be identical, and that the experimentally determined value for \(\bar{M}_w\) would be in closer agreement with the theoretical values.

Hadidian and Pirie (34) have tabulated the viscosities of a number of
hyaluronate preparations isolated from various natural sources by several methods. The wide variations in the viscosities of these preparations may be partly due to impurities but are probably principally due to differences in degree of polymerization. Thus it is evident that a viscosimetric unit cannot be established with hyaluronate as the standard unless the molecular weight of the hyaluronate is rigidly defined. Until such a standard is defined, no definite relationship between the viscosimetric and turbidimetric units can be established.

SUMMARY

Factors affecting the turbidimetric assay of hyaluronidase have been studied and the following observations made. (1) With increasing hydrogen ion concentration, less sodium chloride is required for maximal enzyme activity. (2) An increasing inhibitory effect of sodium chloride is apparent above pH 4.5. (3) Owing to the instability of the enzyme in dilute solution, activity is lost during the assay, but this loss can be prevented by including hydrolyzed gelatin in the incubation solution. (4) The use of substrates of widely different molecular weights has little effect on the assay. (5) Uniform turbidity production is dependent upon careful control of a number of factors such as pH, salt concentration, method of serum preparation, etc.

It was found to be impracticable to correlate the turbidimetric unit with the viscosimetric unit until a standard hyaluronate is defined. At equal viscosities, the magnitude of the viscosimetric unit appears to vary inversely with the square of the hyaluronate molecular weight.

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