THE HYALURONIDASE INHIBITOR OF HUMAN BLOOD

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The disappearance of the "spreading factor" from the blood stream after intravenous injection was demonstrated by Duran-Reynals. He suggested the presence of a substance in blood which destroys the "spreading factor" (1). Hobby et al. (2) found evidence of the inhibition of hyaluronidase prepared from Clostridium welchii and certain strains of streptococci by normal human and rabbit serum. This effect was ascribed to salt formation between albumin and hyaluronic acid.

McClean (3) reported that hyaluronidases prepared from bull, rabbit, and mouse testes were inhibited by guinea pig, rabbit, sheep, horse, mouse, and human serum. Heparin, chondroitin sulfate, and gastric mucin were found to be inhibitory, while Shiga-Kruse polysaccharide and blood group A hapten were without activity. The inhibitor in blood was not considered identical with any of these substances, since chemical properties indicated that it was pseudoglobulin in nature.

A recent series of papers by Haas (4-6) has revived interest in the nature of the hyaluronidase inhibitor in blood. On the basis of reaction rates and effects of temperature changes it was concluded that the substance in blood is an enzyme and the name "antinvasin I" was suggested. Comparison of the relative activity of blood sera from different species on different hyaluronidases led to the conclusion that there exists a complex system consisting of at least two different antinvasins and a substance accompanying hyaluronidase which was named "proinvasin." Haas suggests that this complex system is responsible for defense against bacterial invasion. This work was done with crude hyaluronidases and no account was taken of the possible effects of contaminating enzymes.

In a preliminary report (7) from this laboratory evidence was presented casting doubt on the enzyme nature of the substance in blood which interferes with hyaluronidase activity. More recently Hechter (8) has studied the effect of serum on the spreading activity of hyaluronidase and found evidence of inhibition. He quotes unpublished work by Hididian and Pirie as proving that the serum factor acts as an inhibitor rather than an enzyme.

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Leonard and Kurzrok have shown that serum also inhibits the dispersal by hyaluronidase of follicle cells surrounding the ovum (9).

The effects discussed above are all presumably due to a physiological substance in blood serum. This should be distinguished from the specific antibodies for individual hyaluronidases which have been repeatedly demonstrated (10–13).

It is the purpose of this report to present evidence showing that the behavior of the substance in blood is inconsistent with the thesis that it is an enzyme. A relatively simple method has been devised for the estimation of this substance and some success has been achieved in its purification. All studies have been concerned only with inhibition of bovine hyaluronidase by human serum.

Methods

The viscosity method of Madinaveitia and Quibell (14) was used as modified by Haas (4). Due to the tedious nature of this method it was used only for comparative purposes and was finally abandoned in favor of a simpler turbidity method. Materials used were prepared as previously described (15).

Before examining results obtained by the viscosity method it is necessary to consider the method of calculation of activity. If the time required to reach a half reduction of viscosity of a given amount of hyaluronic acid in the presence of hyaluronidase (half life time) is called \( R_0 \), the activity of hyaluronidase may be expressed as the reciprocal of the half life time \( (1/R_0) \). If the same amount of hyaluronidase is mixed with a given amount of serum, a new half life time is obtained which may be designated as \( R \). The activity of hyaluronidase remaining after the action of serum can be expressed then as \( 1/R \) and the amount of hyaluronidase that is destroyed (or inhibited) is \( 1/R_0 - 1/R \). Haas has used this method of computation in some experiments but in others he has used the expression \( (R - R_0)/R_0 \). The derivation and meaning of this expression are not given. It is not equal to the previous expression \( (1/R_0 - 1/R) \) and has no meaning when methods of hyaluronidase assay not involving the half life time are used. Using the method as described by Haas, we have been unable to find any linear relationship between activity and amount of serum. If, however, activity is expressed in terms of amount of hyaluronidase activity destroyed \( (1/R_0 - 1/R) \), linearity is obtained. Under these conditions the results obtained by the turbidity method do not differ significantly from those by the viscosity method.

The turbidity method used is essentially that described in a previous publication from this laboratory (15). A standard curve for hyaluronidase is run daily to check on reagents and methods. It has been previously pointed out that the activity of hyaluronidase varies with different prepa-
rations of hyaluronic acid. In order to obviate this difficulty we have defined our unit in terms of the activity of a given amount of a standard hyaluronidase preparation. No evidence of change has been found in 10 months storage in the dry state at -20°.

To determine inhibitor in serum the following method is used. Standard enzyme is diluted in 0.1 M borate buffer at pH 7.4 so as to contain 24 units per ml. 0.5 ml. of this solution is mixed with 0.5 ml. of diluted serum (serum is diluted with 0.15 M NaCl to appropriate concentration, depending on activity) and the mixture is incubated for 10 minutes at 24°. At the end of this time the mixture is brought to 38° and 1 ml. of hyaluronic acid made up in 0.3 M phosphate buffer at pH 5.5 is added. This mixture is incubated at 38° for 45 minutes, after which time the turbidity is de-

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Viscosity</th>
<th>Turbidity, units per ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\frac{1}{R_0} - \frac{1}{R}$ per ml</td>
<td>$\frac{R - R_0}{R_0}$ per ml</td>
</tr>
<tr>
<td>1</td>
<td>86</td>
<td>46</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>51</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>32</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>92</td>
<td>64</td>
</tr>
</tbody>
</table>

Turbidity developed due to the addition of the serum is corrected for by a blank containing all reagents except hyaluronidase. The activity of serum is determined in terms of amount of enzyme which has been inhibited. 1 unit of inhibitor is that amount which will inhibit 1 unit of hyaluronidase. Results are expressed in terms of units of inhibitor per ml. of serum. Table I shows the results of assay of six sera by both the viscosity and turbidity methods. With the methods as outlined above the results obtained are comparable to those obtained by the viscosity method, provided calculations are made by the formula $\frac{1}{R_0} - \frac{1}{R}$. If the formula $(R - R_0)/R_0$ is used, this is not true. It will be noted that not only do the absolute values differ but the relative values on different sera do not agree.

Since good agreement between the turbidity and viscosity methods was obtained, the turbidity method was used in the remaining studies.

EXPERIMENTAL

Relationship of Activity to Concentration of Serum—It has previously been demonstrated by the viscosity method that the activity of serum varies
linearly with serum concentration (4). In this case activity was expressed as a function of hyaluronidase destroyed. Similar experiments by the turbidity method have confirmed this finding. The results of such an experiment are shown in Fig. 1. The curve shown is the calculated regression line by the method of Fisher (16) from the observed points up to and including 0.05 ml. of serum. It is obvious that above this point the curve shows flattening. For this reason, values were accepted as valid only if destruction did not exceed 4 units (in the presence of 6 units) of hyaluronidase. If the amount of enzyme inhibited falls below 2 units, the intrinsic error of the turbidity method becomes very large. This is illustrated by

The following example. If 6 units of hyaluronidase are added and 1 unit is inhibited by a given amount of serum, 5 units will remain. In a previous publication it has been shown that at this level the coefficient of variation in the turbidity determination is about 5 per cent; thus the value of 5 units may be considered as 5 ± 0.25. Since the inhibitor value is obtained by difference from 6, its value will be 1.0 ± 0.25 unit; thus the error in the inhibitor becomes ±25 per cent, although the actual experimental error in the determination is only ±5 per cent. By this same type of calculation it can be shown that with between 2 and 4 units of inhibitor the coefficient of variation should vary between 5 and 20 per cent. In a series of 300 determinations done under these conditions the coefficient of variation was found to be empirically ±14 per cent.

\[ Y = \bar{Y} + b(X - \bar{X}) \]
\[ b = 0.93 \]
\[ s_b = 0.068 \]
Effect of Hyaluronidase Concentration on Activity of Serum—It has previously been claimed that the activity of serum is directly proportional to hyaluronidase concentration (4). This is to be expected whether the substance in serum is an inhibitor or an enzyme, provided the amount of hyaluronidase is low. If the concentration of hyaluronidase is in excess of that required for maximum activity, the activity of the serum should become independent of hyaluronidase concentration. That this is so is shown by the data in Table II.

**Table II**

<table>
<thead>
<tr>
<th>Hyaluronidase present, units</th>
<th>6.0</th>
<th>8.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot; inactivated, units</td>
<td>3.1</td>
<td>3.2</td>
<td>3.1</td>
</tr>
</tbody>
</table>

**Fig. 2.** Rate of reaction between serum and hyaluronidase

Effect of Time on Reaction between Hyaluronidase and Serum—The next group of experiments was performed in order to determine the effect of time on the activity of blood serum. This is one of the criteria upon which Haas has concluded that the substance in question is an enzyme. The results of such an experiment are shown in Fig. 2. It will be noted that the
reaction is apparently complete in 5 minutes, despite the fact that only 50 per cent of the hyaluronidase present has been inhibited. When Haas' data (4) are recalculated by the formula \(1/R_0 - 1/R\), his results are found to agree well with those in Fig. 2.

The findings in this experiment are inconsistent with the assumption that the substance in question is an enzyme.

**Effect of Temperature on Activity of Serum**—The next experiments were performed to study the effect of temperature on the rate of reaction between serum and bovine testicular hyaluronidase. Table III illustrates the results of a group of such experiments. The serum and enzyme were mixed and kept at the indicated temperature. After 10 minutes hyaluronic acid dissolved in 0.3 M phosphate buffer was added and the mixture brought to 38°C for the determination of the remaining hyaluronidase. It will be noted that higher activity was consistently obtained at lower temperatures.

**Table III**

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>7°C</th>
<th>17°C</th>
<th>27°C</th>
<th>38°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units per ml. serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>79</td>
<td>76</td>
<td>66</td>
<td>47</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>100</td>
<td>71</td>
<td>51</td>
</tr>
<tr>
<td>3</td>
<td>94</td>
<td>92</td>
<td>79</td>
<td>48</td>
</tr>
</tbody>
</table>

With some purified preparations (prepared according to the method of Hahn (17)) no difference between different temperatures was obtained.

The activities at these temperatures are at variance with those previously reported (4) and are again inconsistent with the assumption that the active substance in blood is an enzyme.

The decrease in activity at higher temperatures might be due to the presence of contaminating enzymes in the hyaluronidase preparations, which destroy the inhibitory substance in serum. That this is true is suggested by the fact that no change with temperature was obtained with some purified preparations.

Such contaminating enzymes may be responsible for the effects described by Haas as due to proinvasins. It seems unnecessary to postulate a complex scheme for the explanation of these effects.

**Effect of pH and Salt Concentration on Activity of Serum**—Studies on the effect of pH and salt concentration on the reaction between serum and hyaluronidase were made difficult by the high buffer capacity of serum. Marked inhibition of activity was found at an ionic strength above 0.2, while if the ionic strength was below 0.1, difficulty was encountered in
maintaining a constant pH with different amounts of serum. If a 0.1 M borate buffer at pH 7.4 was used, this difficulty was circumvented.

Table IV shows the variation in serum activity with variation of pH at constant ionic strength (μ = 0.12). In general these results confirm those previously reported.

The inhibition of the reaction by phosphate and sulfate ions has been confirmed. The effect of sulfate is of practical importance, since the purification of hyaluronidase usually involves ammonium sulfate purification. Care must be taken to remove sulfates completely prior to use of hyaluronidase for determination of inhibition by serum.

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>pH 5.8</th>
<th>pH 6.6</th>
<th>pH 7.6</th>
<th>pH 8.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>53</td>
<td>58</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>34</td>
<td>46</td>
<td>40</td>
</tr>
</tbody>
</table>

**Table V**

Variation of Serum Hyaluronidase Inhibitor with Age and Sex

The results are expressed in units per ml.

<table>
<thead>
<tr>
<th>Age, yrs.</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-15</td>
<td>126.7</td>
<td>112.4</td>
</tr>
<tr>
<td>16-30</td>
<td>85.1</td>
<td>117.6</td>
</tr>
<tr>
<td>31-45</td>
<td>89.1</td>
<td></td>
</tr>
<tr>
<td>&gt;45</td>
<td>149.4</td>
<td></td>
</tr>
</tbody>
</table>

**Chemical Properties**

*Stability*—As previously pointed out, the substance in blood which inhibits hyaluronidase is quite unstable. At 50° the activity is completely destroyed in 10 minutes at pH 7.4. Even at 4° at this pH there is about 25 per cent destruction in 48 hours. Some variation has been found in different sera, thus making it difficult to draw final conclusions as to the stability at lower temperatures.

All sera tested were stable at -20°, some having been kept for as long as 6 months with no detectable loss of activity.

*Purification*—The inhibitor is apparently of large molecular weight, since it does not dialyze. It can be separated from serum by Method 6 of Cohn et al. (18) and the activity can be quantitatively recovered in Fractions II and III.
All of the activity is found in the plasma. No difference between the activity of serum and plasma has been found.

**Distribution in Normal Human Serum**

Nothing is known regarding the distribution of this substance in normal or pathological states. Table V gives the results from a number of normal individuals divided by age and sex. By Fisher's t test it is found that males of reproductive age (15 to 45) have a significantly lower range than any other group studied. An insufficient number of female children or females over 45 years have so far been studied from which to draw definite conclusions regarding these groups. The wide variations within the normal group remain to be explained. Preliminary evidence indicates that the inhibitor level in the serum of males is inversely related to the hyaluronidase concentration of semen.

**DISCUSSION**

There seems to be little question that there exists in the blood of a number of species of animals an inhibitor of the enzyme hyaluronidase. This substance is apparently quite distinct from specific antibodies to particular hyaluronidases. The question of specificity of the inhibitor for particular hyaluronidases remains for future work, since a final answer can only be obtained when pure hyaluronidases are available. The work reported here has been concerned only with the reaction between bovine testicular hyaluronidase and human serum.

The evidence presented in this paper lends no support to the thesis that the substance in blood is an enzyme. The findings are consistent with the idea that this substance is an inhibitor. McClean (3) has shown that a number of polysaccharides act as inhibitors of hyaluronidase, and the suggestion has been made that this substance in blood is a competitive inhibitor (8). Since present evidence indicates that the inhibitor in blood is protein in nature, it is unlikely that any of the carbohydrates used by McClean are responsible for the inhibition in serum. It is possible, however, that it may have a polysaccharide prosthetic group which permits it to act as a competitive inhibitor.

The significance of variations in distribution of this substance remains to be determined. The relationships to sex and age together with the known rôle of hyaluronidase in fertilization suggest that this enzyme system is probably under endocrine control. Elucidation of these mechanisms may serve to further our understanding of the mechanism of action of certain hormones.
SUMMARY

1. A simple and rapid method has been devised for the estimation of hyaluronidase inhibitor in human blood.

2. Kinetic studies on the inhibition of hyaluronidase of bovine testes indicate that this substance is an inhibitor rather than an enzyme, as previously claimed.

3. Chemical studies suggest that this substance is protein in nature.

4. Studies of blood levels in normal individuals show a lower level in males of reproductive age than in other groups studied.

BIBLIOGRAPHY

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