The inhibition of testicular hyaluronidase by heavy metals

By Karl Meyer and Maurice M. Rapport

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Edward Daniels Faulkner Arthritis Clinic, Presbyterian Hospital, New York, New York)

(Received for publication, September 23, 1950)

The literature on inhibitors of hyaluronidase is both voluminous and contradictory. The contradictions are caused by the differences in methods used, by the differences in enzyme purity, and to a very high degree by the differences in the purity of the substrates used. The latter are often contaminated with protein, with glycogen, and with mucopolysaccharides other than hyaluronic acid, especially when umbilical cord is the source (1). One factor which affects enzyme activity appears to be contamination with heavy metals, especially Fe+++ and Cu++. These metals have a high affinity for some group or groups in the enzyme, causing a reversible inactivation. The most effective reversing agent when Fe+++ is the inactivating metal is pyrophosphate; with Cu++, cysteine appears to be best.

Experimental

In most experiments hyaluronidase activity was measured turbidimetrically (1, 2). Some of the data were checked by viscosimetric and reductimetric assays (1, 3).

The enzymes used in the majority of the experiments were prepared by a modification of the method of Morgan and McClean (4). All steps in the purification were carried out at about 0°. The fractionation was carried out by carefully varying the pH in the presence of excess lead acetate. The precipitates were dissolved in cold dilute acetic acid and dialyzed first against 0.05 M acetic acid, then against frequent changes of distilled water at 0°. If after 3 days of dialysis the reaction for lead was still positive, a dilute solution of Na₂S was added and the pH adjusted until the PbS flocculated and could be removed by centrifugation. The solution was again dialyzed and finally dried from the frozen state.

* Supported by a grant from the Helen Hay Whitney Foundation and the Josiah Macy, Jr., Foundation. This work was reported in part at the Thirty-second annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, March, 1948, and the Conference on the Ground Substance of the Mesenchyme and Hyaluronidase, New York Academy of Sciences, New York, December, 1948.
In a number of experiments, enzymes obtained from Dr. Joseph Seifter (Wyeth Institute) and from Dr. Monroe E. Freeman (Army Medical Department) were used.

The hyaluronate used in most of the experiments was obtained from a tumor fluid (5). Other samples were derived from human umbilical cord, cattle synovial fluid, and vitreous humor. As is customary in this laboratory, all preparations were analyzed for nitrogen, hexosamine, uronic acid, and sulfate (2). The analytical data are presented in Table I.

All reagents were dissolved in 0.1 M acetate buffer containing 0.15 M NaCl, the final pH being 5.9. Studies on inhibition were carried out at a concentration of agent not greater than $10^{-3}$ M, since we believe that non-specific effects, such as protein denaturation, may be encountered at higher concentrations.

Experiments were performed in two ways. In one, the enzyme and heavy metal were incubated at 37° for 10 minutes and then substrate and complex former (when used) were added. In the other there was no incubation period. With complex formers, the latter type of experiments are termed "prevention," the former type "reversal." In all experiments, controls of the metal or the complex former or both with the substrate were included. In the concentration used the metals and complex formers did not influence the turbidities of the substrates. At the end of this period the substrate solution was added and enzyme activity determined.

### Table I

<table>
<thead>
<tr>
<th>Source</th>
<th>Nitrogen per cent</th>
<th>Hexosamine per cent</th>
<th>Uronic acid per cent</th>
<th>Sulfate per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>2.79</td>
<td>34.9</td>
<td>39.5</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Umbilical cord</td>
<td>3.33</td>
<td>38.5</td>
<td>44.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Synovial fluid</td>
<td>3.24</td>
<td>42.5</td>
<td>41.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Vitreous humor</td>
<td>3.02</td>
<td>39.3</td>
<td>42.6</td>
<td>1.4</td>
</tr>
</tbody>
</table>

**Results**

The inhibition of hyaluronidase by increasing concentrations of ferric chloride is shown with and without incubation in Fig. 1.

It can be seen that $3 \times 10^{-4}$ M Fe+++ almost completely inhibits the enzyme and the effect of the incubation period is negligible. With the incubation period, the mean inhibition with different substrates and enzymes in seven sets of experiments with $5 \times 10^{-5}$ M Fe+++ was 67 per cent, with a minimum of 53 and a maximum of 78 per cent. When the incubation period was omitted, the mean inhibition in three different
The reaction of the enzyme with Fe⁺⁺⁺ thus appears to be very fast.

Bivalent iron as FeSO₄(NH₄)₂SO₄ or as FeSO₄ at 5 × 10⁻⁵ M inhibited the enzyme only about 30 per cent.

Next to Fe⁺⁺⁺ the most effective metal inhibitor is Cu⁺⁺. It can be seen from Fig. 1 that the inhibition by CuSO₄ is less than that caused by Fe⁺⁺⁺ and that the effect of incubation with this metal is marked. In other experiments (with incubation) 5 × 10⁻⁵ M CuSO₄ in four experiments with different substrates and enzymes gave a mean inhibition of 51 per cent (minimum 40, maximum 64), compared with 67 per cent for FeCl₃. At higher concentrations (with incubation), the greater inhibitory effect of Fe⁺⁺⁺ as compared to Cu⁺⁺ is even more pronounced. 5 × 10⁻⁴ M Cu⁺⁺ gave inhibitions of 62 and 66 per cent, while with 3 × 10⁻⁴ M Fe⁺⁺⁺ under identical conditions the inhibition was over 95 per cent.

Among other metal salts tested only Zn⁺⁺ (as acetate) showed a definite inhibitory action; i.e., at 5 × 10⁻⁴ M, 32 and 29 per cent inhibition. At
this concentration the following metal salts were inactive: cadmium(ous) acetate, nickel(ous) chloride, cobalt(ous) chloride, manganese(ous) chloride, lead acetate, aluminum chloride, and mercuric acetate. The ineffectiveness of Hg\(^{++}\) is in accord with the finding that \(1 \times 10^{-5}\) M p-chloromercuribenzoic acid has no inhibitory action on either testicular or bacterial (pneumococcus) hyaluronidase when studied under these conditions.

The prevention and reversal of the Fe\(^{+++}\) and Cu\(^{++}\) inhibitions \((5 \times 10^{-5}\) M\) were studied with the following metal-binding agents \((1 \times 10^{-3}\) M\): potassium cyanide, sodium pyrophosphate, cysteine hydrochloride, glutathione, oxine, sodium ethylene diamine tetraacetate ("Versene"), and dithione.

The variation in results in these experiments was considerable, depending on both the substrates and enzymes used. Table II summarizes the results.

It can be seen that with Fe\(^{+++}\) as the inhibitor pyrophosphate not only reversed the reaction, but actually appeared to activate the enzyme.
With Cu\(^{++}\) inhibition, cysteine and glutathione completely prevented and reversed the reaction. It appears noteworthy that powerful chelating agents such as oxine, versene, and dithione only incompletely prevent or reverse the metal inactivation.

**DISCUSSION**

The inhibition or inactivation of hyaluronidase by a variety of agents has been reported. Competitive inhibition was thought to be the basis of the effect of heparin (6, 1) and other sulfated mucopolysaccharides (1) and derivatives of hyaluronic acid (7). The mechanism of the most widely publicized inhibition of the enzyme, that caused by serum, is completely unknown.

The data presented in this study indicate that hyaluronidase reversibly combines with Fe\(^{+++}\), Fe\(^{++}\), Cu\(^{++}\), and Zn\(^{++}\). The affinity of the group or groups of the enzyme for the heavy metal must be quite great, since chelating agents such as oxine and versene are rather poor competitors of the enzyme for the metal. Since p-chloromercuribenzoic acid fails to inactivate the enzyme, other reports to the contrary (8, 9), SH groups are not essential for the activity and hence the group combining with the metal cannot be a sulfhydryl group.

Inactivation of hyaluronidase by Fe\(^{+++}\) and Cu\(^{++}\) may be one of the reasons for the variability in activity encountered with the enzyme. Addition of pyrophosphate and especially of cysteine at a concentration of 1 \(\times\) 10\(^{-3}\) M to the enzyme-substrate system gives enzyme activities 30 to 60 per cent higher than without the addition. This activation depends mostly on the substrates. The metal impurity of the latter appears to be Cu\(^{++}\), which has been shown to be present in tumor hyaluronate in rather high concentration by Pirie (10).

The increase in enzyme activity on addition of cysteine and other compounds raises the question of whether the addition of such compounds to the enzyme-substrate system should not be adopted for hyaluronidase determinations. Cysteine or other SH compounds would not appear to be substances of choice, since such autoxidizable reducing agents interfere in the reductimetric method. However, for any proposed standard method, there will be need for an activating and stabilizing agent.

The authors wish to express their appreciation to Miss Toby G. Bernstein and Miss Lucy F. Siegel for valuable assistance.

**SUMMARY**

Ferric, cupric, ferrous, and zinc salts were found to inhibit testicular hyaluronidase, this property decreasing in the order mentioned. Other metals including cadmium, lead, and mercury were found inactive. The
inhibition could be prevented or reversed by a number of metal complex formers. When Fe\(^{+++}\) was used as the metal inhibitor, pyrophosphate was found the most potent reversing agent, while with Cu\(^{++}\) cysteine was most active. Potent chelating agents such as oxine, ethylenediaminetetraacetic acid, and dithione were rather poor competitors with the enzyme for the metal. The group or groups in the enzyme binding the metal are not known; SH groups appear to be excluded by the failure of p-chloromercuribenzoic acid to inhibit the enzyme.

**BIBLIOGRAPHY**

THE INHIBITION OF TESTICULAR HYALURONIDASE BY HEAVY METALS
Karl Meyer and Maurice M. Rapport


Access the most updated version of this article at http://www.jbc.org/content/188/2/485.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/188/2/485.citation.full.html#ref-list-1