INHIBITION OF HYALURONIDASE ACTION BY DERIVATIVES OF HESPERIDIN

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In a previous communication (1) it was reported that certain vitamin P compounds exerted an inhibitory action on the breakdown of hyaluronic acid by testis hyaluronidase, as determined turbidimetrically. This action was manifested for the most part only when the compounds in question were combined with ascorbic acid, and only when their concentration was relatively high (0.1 mg. per cc.). Since the original object of the investigation was its application to the field of capillary fragility, it was considered to be of importance to attempt an activation of the hyaluronidase-inhibiting properties manifested by vitamin P compounds, without destroying their vitamin P specificity.

Heparin has been found to cause a marked inhibition of hyaluronidase activity at very low concentrations (2). This action depends on the sulfate groups present in the molecule, for it has been shown that when heparin is desulfonated it no longer functions as an inhibitor of hyaluronidase (2). Heparin has no vitamin P specificity, but these findings suggested that a potentiation of the action of vitamin P compounds in inhibiting the breakdown of hyaluronic acid by hyaluronidase might be accomplished by the formation of suitable derivatives.

Hesperidin was chosen as a representative vitamin P compound. The demonstrated importance of the sulfate groups in heparin suggested the synthesis of a sulfonated hesperidin. It has been shown that phosphate groups may potentiate the action of inhibitors on enzyme systems (3); accordingly a phosphorylated hesperidin was synthesized. Finally, hesperidin was acetylated to form a representative organic derivative.

The methods employed for the synthesis of these compounds are presented in detail below. In each case the starting material was hesperidin which had been purified by treatment with formamide.

EXPERIMENTAL

Acetylated Hesperidin—35 gm. of hesperidin were suspended in 350 cc. of glacial acetic acid. A few cc. of concentrated H₂SO₄ were added slowly with stirring. The mixture was placed on the steam bath and heated with occasional stirring for about 20 minutes. At the end of this time a dark red solution formed. This was allowed to cool to room temperature and
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treated with 5 volumes of water. The precipitate which formed was filtered off after standing for several hours in the ice box and washed with water.

A yield of 20 gm. of a dark yellow material was obtained. The substance was insoluble in water, and freely soluble in alcohol. It was purified by solution in 100 cc. of alcohol, and reprecipitation by the addition of 10 volumes of water. A few cc. of acetic acid were added to aid in the formation of a precipitate. The precipitate was filtered off and washed with water. Yield, 11 gm.; m.p., 120°.

Analysis showed the material to have an acetyl content of 13.2 per cent. The calculated acetyl content for the diacetate of hesperidin is 12.1 per cent; for the triacetate, 17.1 per cent. The material is thus a mixture, with the diacetate predominating.

Sulfonated Hesperidin (Na Salt)—To 40 gm. of hesperidin were added 100 cc. of H₂SO₄ slowly and with cooling. A dark red solution formed, which was poured slowly and with cooling into 500 cc. of alcohol. The solution was neutralized with saturated NaOH, and the precipitated Na₂SO₄ was filtered off. The precipitate was washed on the filter with alcohol and the washings added to the filtrate. The combined solutions were made alkaline with NaOH and poured into 500 cc. of alcohol. The orange-colored precipitate which formed was filtered off, washed with alcohol, and dried.

Purification was accomplished by solution in a minimum volume of water and reprecipitation with 5 volumes of alcohol. Yield, 27 gm. The melting point was indeterminate, the compound decomposing gradually over a wide temperature range.

Analysis showed a sulfur content of 6.3 per cent. The calculated content for the monosulfonated hesperidin Na is 4.4 per cent; for the disulfonated, 7.8 per cent. The material is thus a mixture of the mono- and disulfonates.

Phosphorylated Hesperidin—37 gm. of hesperidin were suspended in 400 cc. of anhydrous pyridine. 100 cc. of POCl₃ were added slowly, with stirring. Heat was evolved, and a dark brown solution formed. This was allowed to cool to room temperature, and a bulky precipitate settled out. Water was added cautiously with cooling until there was no further reaction and the precipitate was dissolved. The solution was filtered to remove any insoluble particles and then poured into 5 volumes of alcohol. The precipitate which formed was filtered off and washed with alcohol. Purification was accomplished by solution in a minimum volume of water and reprecipitation with 5 volumes of alcohol. Yield, 33 gm. The melting point was indeterminate, the compound decomposing gradually over a wide temperature range.

Analysis showed a phosphorous content of 14.5 per cent. The calculated
content for the pentaphosphate is 15.7 per cent; for the tetraphosphate, 13.7 per cent. The material is thus a mixture of the penta- and tetraphosphates of hesperidin.

Determinations of the effect of the various compounds on hyaluronidase activity were made turbidimetrically by the method previously reported (1). The hyaluronidase used was prepared from bull testes by the method of Kass and Seastone (4). Hyaluronic acid was prepared from bovine vitreous humor by the method of Seastone (5).

In addition to the hesperidin derivatives, determinations were made on suramin (germanin, Bayer 205) and on salmine sulfate. Combinations of salmine sulfate and both sulfonated and phosphorylated hesperidin and ascorbic acid and acetylated and sulfonated hesperidin were tested.

**Table I**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Inhibition</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1000 γ per cc.</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>0</td>
</tr>
<tr>
<td>Acetylated hesperidin</td>
<td>20</td>
</tr>
<tr>
<td>Sulfonated &quot;</td>
<td>85</td>
</tr>
<tr>
<td>Phosphorylated hesperidin</td>
<td>80</td>
</tr>
<tr>
<td>Suramin</td>
<td>85</td>
</tr>
<tr>
<td>Salmine sulfate</td>
<td>0</td>
</tr>
</tbody>
</table>

**Results**

The sulfonated and phosphorylated hesperidins proved to be extremely potent inhibitors of hyaluronidase. Suramin was found to be even more effective, while salmine sulfate was without activity. These results are presented in Table I. Results previously obtained with hesperidin are included to provide a standard of comparison.

Salmine sulfate, while without effect on hyaluronidase, was found completely to neutralize the inhibition caused by both sulfonated and phosphorylated hesperidin. As may be seen from Table I, sulfonated hesperidin at a concentration of 100 γ per cc. caused an inhibition of 85 per cent, phosphorylated hesperidin one of 80 per cent. When either of these substances was combined with salmine sulfate, however, at a concentration of 100 γ per cc., no inhibition at all was found.

It had previously been reported (1) that, with both at concentrations of 100 γ per cc., hesperidin caused a potentiation of 100 per cent in the inhibitory action of ascorbic acid on hyaluronidase. This was found to be the
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case also with acetylated hesperidin. Because of the large inhibition caused by sulfonated and phosphorylated hesperidin at 100 \textmu{}g per cc., these could not be tested at this concentration in combination with ascorbic acid. Sulfonated hesperidin was tested at 10 \textmu{}g per cc. with ascorbic acid. At this concentration no potentiation of the inhibitory effect of the ascorbic acid was found.

DISCUSSION

The results obtained show that the formation of derivatives of hesperidin can result in a great potentiation of its ability to inhibit the action of hyaluronidase on hyaluronic acid. Hesperidin was originally chosen for the investigation as a representative vitamin P compound, and it seems probable that the formation of similar derivatives of any member of the vitamin P group would result in the same potentiation.

It is true that inhibition of hyaluronidase action has been demonstrated by many compounds which show no vitamin P activity in vivo. The action of such substances on hyaluronidase in vitro, however, differs in one respect from that of the vitamin P compounds: they cause no potentiation of the inhibitory effect on hyaluronidase of ascorbic acid. It can therefore be postulated (on the basis of necessarily incomplete studies) that the property of potentiating the action of ascorbic acid on hyaluronidase in vitro is a measure of vitamin P specificity.

If, as has been suggested (6), hyaluronidase plays a rôle "in accentuating capillary fragility" the importance of an effective inhibitor of hyaluronidase in the clinical treatment of this condition is manifest. It would appear obvious, however, that such a substance must have a vitamin P specificity; heparin, for example, although an extremely effective hyaluronidase inhibitor, would be useless in this connection because of the other physiological effects which it is known to produce.

The importance of the derivatives of hesperidin which have been synthesized and tested, then, lies in the fact that they exert a very much more powerful inhibitory effect on hyaluronidase than do the vitamin P compounds themselves, while retaining their vitamin P specificity, as evidenced in the case of the acetylated hesperidin. A more specific evaluation of the utility of these compounds must of course await testing in vivo.

SUMMARY

Three derivatives of hesperidin have been synthesized. These include acetylated hesperidin, sulfonated hesperidin, and phosphorylated hesperidin.

All three have been shown to exert a greater inhibitory effect on hyaluronidase action than does hesperidin itself. In the case of the acetylated hes-
peridin, the same potentiation of the action of ascorbic acid as was evidenced by hesperidin has been demonstrated.

The inhibitory effect of the phosphorylated hesperidin and the sulfonated hesperidin has been shown to be neutralized by salmine sulfate.

Suramin has been shown to be an effective inhibitor of hyaluronidase action.

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