INHIBITORY ACTION OF VITAMIN P COMPOUNDS ON HYALURONIDASE

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In 1936 Szent-Gyorgyi and coworkers (1) described the isolation of a material which was capable of increasing capillary resistance in man, and which they called citrin or vitamin P. Subsequent work has shown that many substances have this property, and vitamin P activity has been attributed variously to flavones, flavanones, and flavonols.

Higby (2) found that crude hesperidin possessed vitamin P activity, but that upon purification it became inactive. He attributed the activity of the crude preparations to the presence of hesperidin in soluble form, as the chalcone. Majovski et al. (3) postulated that pure hesperidin was inactive because of its relative insolubility. Scarborough (4), however, found that hesperidin, as well as its aglycone hesperetin, was active in pure form. Rutin has been reported to increase capillary resistance in man (5), as have esculin and its aglycone esculentin (6).

The mechanism by which these and the other known vitamin P substances act to increase capillary resistance is unknown, although Lavollay (7) has postulated that they function as inhibitors of adrenalin oxidation, and that adrenalin itself or its first oxidation product is the true vitamin P.

It has been suggested that hyaluronidase plays a rôle in the maintenance of capillary strength. Duran-Reynals (8) reported that the permeability of the vascular system could be increased by preparations rich in hyaluronidase. More recently hyaluronidase has been considered as a factor "in accentuating capillary fragility rather than in inducing direct changes in capillary permeability" (9). The possibility is thus raised that the effect of vitamin P substances in increasing capillary resistance is due to an inhibition of the action of hyaluronidase. It appeared that an investigation of this point might bear fruitful results.

EXPERIMENTAL

Hyaluronidase was prepared from bull testes by the method of Kass and Seastone (10). The hyaluronic acid used was prepared from bovine vitreous humor by the method of Seastone (11). The estimation of hyaluronidase activity was performed turbidimetrically (11).

For each assay two tubes were set up. Into one were pipetted 1.5 cc. of a solution of hyaluronic acid in 0.1 M acetate buffer, pH 6.0, plus 0.5 cc.
of a water solution of the substance whose effect was being tested. The other tube contained 2.0 cc. of a solution of the hyaluronidase in buffer. The two tubes were allowed to stand for 5 minutes at 37°, and their contents were then mixed; the mixture was incubated for 1/2 hour at 37°. At the end of this time 1 cc. of the contents of the tube was pipetted into a mixture of 3 cc. of 0.5 M acetate buffer, pH 4.2, and 1 cc. of acidified horse serum (11). Turbidity was allowed to develop for 1/2 hour and was then determined in the turbidimeter. The readings were converted to concentration of hyaluronic acid by reference to a standard curve. The concentration of hyaluronic acid in the reacting tubes was 0.2 mg. per cc.; the concen-

**Table I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Per cent inhibition</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0.1 mg. per cc.</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>25</td>
</tr>
<tr>
<td>Dicoumarol</td>
<td>0</td>
</tr>
<tr>
<td>Hesperidin (purified)</td>
<td>0</td>
</tr>
<tr>
<td>&quot; (purified) methyl chalcone</td>
<td>0</td>
</tr>
<tr>
<td>Rutin</td>
<td>0</td>
</tr>
<tr>
<td>Esculin</td>
<td>0</td>
</tr>
<tr>
<td>Esculetin</td>
<td>0</td>
</tr>
<tr>
<td>4-Methyl esculetin</td>
<td>0</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>0</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>0</td>
</tr>
<tr>
<td>Salicyluric acid</td>
<td>0</td>
</tr>
</tbody>
</table>

tration of hyaluronidase was adjusted so as to bring the final reading to the flattest part of the standard curve.

Controls were run with and without hyaluronidase and with and without the substance whose effect was being tested. Results were obtained in a typical experiment by a comparison of four tubes, whose contents were as follows: (1) hyaluronic acid, (2) hyaluronic acid + hyaluronidase, (3) hyaluronic acid + hyaluronidase + test substance, (4) hyaluronic acid + test substance.

When combinations of substances were tested, additional controls were run.

The group of vitamin P substances tested included rutin, hesperidin, a sample of hesperidin purified by treatment with formamide, hesperidin methyl chalcone, esculin, esculetin, and 4-methyl esculetin. Other sub-
stances tested were ascorbic acid, salicylic acid, salicyluric acid, sulfanilamide, and dicoumarol. These compounds were tested at concentrations of 0.1 and 1.0 mg. per cc. Several of the vitamin P compounds, notably hesperidin and esculetin, were not completely soluble at the higher concentration and precipitated during the incubation, settling to the bottom of the tubes. In these cases the clear supernatant was pipetted into the diluted serum.

Results

Of the vitamin P substances tested, only rutin showed an inhibitory action on hyaluronidase, and this only at high concentrations. The only compounds possessing activity at the lower concentration were ascorbic acid and dicoumarol. However, a combination of ascorbic acid and the vitamin P compounds showed a marked potentiation of inhibitory action, especially in the case of hesperidin methyl chalcone. The results are presented in Table I.

There was no synergistic effect noted when hesperidin methyl chalcone was tested in combination with dicoumarol.

Discussion

Of the vitamin P substances tested, only rutin showed any effect on hyaluronidase, and that only at a relatively high concentration. It is problematic whether this reaction can be considered to be specific; it may well be due to some impurity present in the rutin.

In the presence of ascorbic acid, however, the compounds having vitamin P activity manifest a well marked inhibitory action on hyaluronidase. It appears to be possible that a hyaluronidase-ascorbic acid combination is susceptible to inhibition by these compounds, while hyaluronidase itself is not. The inhibitory action of ascorbic acid is not entirely unexpected; this compound has long been known as a specific remedy against hemorrhagic lesions (12). It seems possible that its action is due to an inhibition of hyaluronidase, and that it is two-fold: first, a direct inhibition of hyaluronidase, and second a potentiation of the action of vitamin P. Because they seem to be closely associated in natural products, it is probable that a deficiency in ascorbic acid, one of whose symptoms is a marked increase in capillary fragility, will also be accompanied by a deficiency in vitamin P; there may thus be two factors to be considered here.

The fact that crude hesperidin showed no activity in the presence of ascorbic acid, while the sample of purified hesperidin did, is believed to be due merely to a concentration of the hesperidin in purification. The crude hesperidin contained a very considerable quantity of impurities, as is evidenced by the losses in the purification process; the purified hesperidin
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thus contained more actual hesperidin per unit weight than did the crude form.

Heparin is known to be a specific inhibitor of hyaluronidase (13). In view of this it becomes of interest that dicoumarol, which also functions as an anticoagulant in the body, manifests this property. It is a question for speculation whether the anticoagulating properties of these compounds are related to their effect on hyaluronidase. It should be noted that heparin is a much more specific inhibitor than dicoumarol, or, indeed, than any of the compounds tested; it has been found to give greater inhibition at very much lower concentrations (14). Dicoumarol showed no synergism when it was tested with hesperidin methyl chalcone, indicating that this property is peculiar to ascorbic acid.

Salicylate has been reported to inhibit the spreading effect of India ink in rabbits injected with hyaluronidase (15). No inhibition was noted with this substance in vitro. Further, salicyluric acid, a metabolite of salicylic acid, was found to be without effect. No synergism was noted between salicylic acid and ascorbic acid. This suggests that this synergism is limited to compounds having vitamin P activity, although, of course, extensive further work is necessary to prove this point.

SUMMARY

Ascorbic acid and dicoumarol were found to inhibit the action of hyaluronidase at concentrations of 0.1 mg. per cc. Of the other compounds tested, only rutin showed an inhibitory action, at a concentration of 1.0 mg. per cc.

Combining ascorbic acid with the vitamin P substances tested caused a marked potentiation of the inhibitory action. This was not true of a combination of dicoumarol and hesperidin methyl chalcone.

Neither salicylic acid nor salicyluric acid had an inhibitory effect. There was no synergism between salicylic acid and ascorbic acid.

Sulfanilamide was without effect.

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