STUDIES ON THE CHEMISTRY OF BLOOD COAGULATION

VII. PROTAMINES AND BLOOD CLOTTING*

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An investigation of the rôle of protamines in blood coagulation must start from the following facts. (a) Protamines act as anticoagulants both in vivo (1) and in vitro (2). (b) Protamines inhibit the anticoagulant effect of heparin both in the living organism and in plasma (3). To these observations a third must now be added. Cephalin is precipitated from its emulsions in water by the protamine salmine which forms with it an insoluble complex (4). Protamincs are of interest in an analysis of the blood clotting mechanism, as they combine with two important agents which, although not antagonists, have opposite effects on clotting; viz., with heparin which inhibits, and with cephalin which activates the coagulation of blood and plasma.

The present paper has the double purpose of supplementing a previous communication from this laboratory (3) by furnishing a brief description of the compound between heparin and salmine, and of analyzing the effects of protamines on clotting inhibitors and activators.

EXPERIMENTAL

Compound between Heparin and Salmine—In these experiments a heparin preparation from beef lungs (5) was used which had an anticoagulant potency of 530 inhibitor units per mg. (6). A neutral solution of 1.01 gm. of this material in 50 cc. of water was made slightly acid with acetic acid and filtered. On addition of 0.37 gm. of salmine sulfate in 30 cc. of water immediate precipita-

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tion took place. After addition of 1.5 cc. of glacial acetic acid the mixture was chilled for several hours. The precipitate which was removed by centrifugation formed a slightly yellow, elastic sheet. It was washed with 5 per cent acetic acid and subsequently triturated with absolute alcohol, when it disintegrated to a faintly yellow powder which after drying weighed 0.48 gm. Analysis, found, N 14.3, S (volatile) 6.7, ash 0.8, amino sugar 11.7 (calculated as glucosamine).¹

Heparin which probably is a high molecular sulfuric acid ester (8) is usually found to contain a large amount, about 35 per cent, of inorganic sulfates as ash. The fact that our product was practically free of ash and contained the total sulfur in volatile form shows that the strongly basic protamine had replaced the cations of heparin to form a salt between salmine and heparin.

As was to be expected from our previous experiments (3), a fine suspension of the heparin-salmine salt in physiological saline did not show any effect on clotting, when examined according to the method employed in this laboratory (6).

The complex between heparin and salmine here described is to a certain degree reminiscent of the compounds of clupein with various acids described by Felix and Mager (9) and of the protein complexes of chondroitinsulfuric acid prepared by Meyer, Palmer, and Smyth (10).

The protamine-heparin complex was found remarkably stable. Repeated treatment with 10 per cent CaCl₂ solution (11), for instance, did not set free any heparin. Even by extended tryptic digestion only little heparin, although of higher purity, was liberated. A suspension of 390 mg. of the salt in 15 cc. of 0.5 N ammonia-ammonium chloride buffer of pH 9.2 was incubated with 50 mg. of trypsin (Fairchild) at 38° for 48 hours. During this period the mixture did not change its appearance appreciably. The undissolved material was centrifuged off and washed with 1 N ammonia. The fraction that had gone into solution was precipitated from the combined supernatant liquids with acetone, centrifuged off, washed with acetone and ether, and dried. It weighed 53.5 mg. and formed an almost white powder. The

¹ The author wishes to thank Dr. K. Meyer of this School for the amino sugar determination which was carried out according to the method of Palmer, Smyth, and Meyer (7).
recovered heparin showed a considerably higher anticoagulant potency (2500 inhibitor units per mg.) than the original product.

In order to arrive at a better characterization of this heparin fraction it was converted into the barium salt according to the method of Charles and Scott (12). The product obtained consisted of microscopic needles, weighing 31.9 mg., but was not identical with the crystalline barium salt of heparin described by these authors. Analysis, found, C 26.6, H 5.4, N 3.1, S 8.6, ash (BaSO₄) 21.4, Ba 12.6. It can be seen that there was almost twice as much volatile S as was contained in the ash. The anticoagulant activity of this fraction was 1600 inhibitor units per mg.

A study of the influence of the pH on the precipitation of the heparin-salmine compound, as described in the preceding paper (4), showed compound formation to take place over the entire range examined between pH 2 and 11.

Salmine and Anticoagulants—The anticoagulants examined fall into two distinct groups with regard to their behavior towards salmine. The following substances were practically completely inactivated by the protamine: heparin (3), cellulose sulfuric acid, polyvinyl sulfuric acid, the lipid inhibitors of clotting recently isolated from brain and blood cells (13), and the synthetic cerebron sulfuric and kerasin sulfuric acids (14). It will be seen that all the synthetic substances are, and the natural anticoagulants may be assumed to be, strong acids which in all likelihood combine with the basic protamine to form insoluble salts. It is remarkable that the combination of the salmine with heparin can be effected during a considerable period after the injection of the latter into the blood stream or the addition of it to plasma in vitro.

The anticoagulants not affected by protamines comprise sodium oxalate and sodium citrate.

The addition of salmine, which is itself an anticoagulant, to plasma containing heparin brings about a considerable decrease in the coagulation time of the plasma. It, therefore, was thought possible to ascertain by means of the protamine reaction whether normal plasma contained an anticoagulant of the heparin type. In these experiments very small amounts of salmine (0.06 to 8.5 micrograms) were added to 0.1 cc. of unactivated chicken plasma (6). There was, however, no decrease in the clotting time, as might have been expected if small amounts of heparin had been
bound by the protamine. One may conclude that heparin, at least in an uncombined state, does not occur in chicken plasma.

Salmine and Cephalin—Cephalins, or possibly certain representatives of the cephalin group, are known to be potent activators of blood clotting (compare Figs. 1 and 2 (15)). It is assumed that the thromboplastic factor contained in most tissues is a complex between cephalin and a protein.

In the preceding paper the preparation of cephalin-salmine complexes was described (4). The addition of a suspension of this material to unactivated chicken plasma had no effect whatever on the plasma clotting time. This was in sharp contrast with the high activating potency of the uncombined cephalin. These experiments showed that under proper conditions cephalin and salmine were able to counteract each other's action: the activator cephalin reacted with the inhibitor salmine.

The antagonistic effect of salmine on the activation of plasma clotting by cephalin can also be demonstrated, although not as clearly, by the addition of small amounts of salmine to cephalin-containing chicken plasma. A typical experiment is reproduced in Table I.

### TABLE I

**Effect of Salmine on Clotting of Cephalin-Containing Chicken Plasma**

<table>
<thead>
<tr>
<th>Cephalin in 0.1 cc. chicken plasma</th>
<th>Clotting time</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>Experiment A</td>
</tr>
<tr>
<td>0.032</td>
<td>5</td>
</tr>
<tr>
<td>0.016</td>
<td>10</td>
</tr>
<tr>
<td>0.008</td>
<td>15</td>
</tr>
<tr>
<td>0.004</td>
<td>25</td>
</tr>
<tr>
<td>0.002</td>
<td>30</td>
</tr>
<tr>
<td>0</td>
<td>30</td>
</tr>
</tbody>
</table>

In Experiment A the chicken plasma contained only cephalin; in Experiment B 0.005 mg. of salmine had been added to each sample of cephalin-containing plasma.

DISCUSSION

The ability of protamines to destroy the inhibiting effect of anticoagulants may find various applications which have been
E. Chargaff

discussed in a previous publication from this laboratory (3). In the experiments cited above the protamine reaction failed to establish the presence of small amounts of heparin in normal chicken plasma. The presence of heparin or a similar substance in the blood of dogs during anaphylaxis and in peptone shock has recently been demonstrated by means of the protamine reaction (16).

In view of the results reported here a new explanation for the anticoagulant effect of protamines may be put forward. The anticoagulants can be grouped into (a) substances reacting with the calcium ion necessary for clotting (sodium citrate or oxalate), (b) substances reacting with the prothrombin or thrombin (or, perhaps, with the fibrinogen) either by blocking certain groups that are necessary for the conversion of fibrinogen into fibrin, or by offering a substitute substrate to the enzyme, thereby protecting the fibrinogen (heparin, synthetic anticoagulants, hirudin), and (c) substances reacting with the cephalin or the thromboplastic factor. No anticoagulant of the third group has as yet been satisfactorily demonstrated. From the experiments reported here it can be concluded that the action of protamines in blood clotting is at least partly due to the inhibition of the clotting activator.

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**SUMMARY**

The properties of a heparin-salmine complex are described. It is shown that the complexes between salmine and heparin or cephalin are both inactive in blood clotting. The effect of protamines on plasma containing inhibitors or activators of blood clotting is discussed. The anticoagulant effect of salmine is explained on the ground that it combines with the natural activator of coagulation.
BIBLIOGRAPHY

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