TWO NEW FACTORS IN BLOOD COAGULATION—HEPARIN AND PRO-ANTITHROMBIN

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A survey of the results of recent work indicates that at least six different substances are concerned in one way or another in the process of blood coagulation; namely, fibrinogen, thrombin, prothrombin, calcium, antithrombin and the so-called zymoplastic or thromboplastic substances furnished by the body cells in general, including the blood corpuscles. With regard to the last mentioned factor satisfactory evidence has been obtained to show that the active material in tissue extracts is a phosphatid (1) (cephalin). Work that has been going on in this laboratory during the past two or three years shows that we must add two other substances to this list of fibrin factors. So far as we know, neither of these substances has been recognized by other workers.

The two new factors are: First, a phosphatid, not previously described, which exists in various tissues but is found in greatest abundance in the liver. This phosphatid is designated as heparin to indicate its origin from liver. It inhibits coagulation, partially or completely according to the concentration. Second, a substance present in blood plasma and blood serum which is converted into antithrombin by a reaction with heparin. Just as in plasma and serum there is a mother substance from which thrombin is formed, so there is an antecedent substance from which antithrombin is formed. By analogy this substance is designated as pro-antithrombin. Prothrombin is activated to thrombin by calcium, the pro-antithrombin is activated to antithrombin by heparin.

Preparation of heparin. Attention was first called to this substance during some work done in this laboratory by Jay McLean (2). In the

1 In a previous publication—Harvey Lectures, 1916–17, Series xii—this substance was described under the name of Antiprothrombin.
course of his work McLean prepared cuorin from heart muscle by the
method of Erlandsen (3) and attempted to prepare heparphosphatid
from the liver by the method of Baskoff (4). The cuorin obtained corre-
sponded in all of its properties and in its N to P ratio with the mate-
rial described by Erlandsen. When freed from contamination with
cephalin the cuorin in solution in sodium chloride, 0.9 per cent, was
found to have an inhibiting action on clotting. The phosphatid
obtained from the liver did not correspond in its properties or in its
N to P ratio with the heparphosphatid described by Baskoff. When
freed from mixture with cephalin, a result most difficult to accomplish,
this substance had a very marked inhibiting effect upon coagulation
of blood, its action in this respect being much stronger than that of
cuorin. We have varied the method of preparation in many different
ways in the hope of devising a rapid and economical process for the
isolation of this inhibiting phosphatid. The method finally selected,
while yielding a reliable preparation of heparin, is time-consuming and
expensive in material. The main obstacle lies in the fact that the
solubilities of heparin and cephalin are so similar that there is great
difficulty in separating them. But until the cephalin or cephalin-like
material is removed the properties of the heparin, so far as coagulation
is concerned, are masked or antagonized. The method of preparation
finally adopted is as follows:

Fresh livers, preferably from dogs, are perfused, when possible, through the
portal vein with a solution of sodium chloride, 0.9 per cent. They are then
ground to a pulp, spread thin on a glass plate and dried in a current of hot air.
The dried material is pulverized and extracted with ether in the proportion of
300 cc. of ether to each 100 grams of dried liver. The extraction may be made in
a few hours with a shaking machine or by allowing the mixture to stand for 24 to
48 hours with an occasional shaking. The extract is then filtered and refiltered
in a closed space until the filtrate is free from turbidity. The dark red filtrate
is evaporated to dryness before an electric fan, and the sticky residue is treated
with water-free ether (Squibb's) in as small quantities as suffice to dissolve it.
There is always in this extract an insoluble residue which must be centrifugalized
off. The clear red extract is then precipitated by acetone, the extract being
added slowly with stirring to four times its volume of acetone. The precipita-
tion is done in centrifugal tubes and the mixture is then centrifugalized. The
supernatant liquid is drained off and discarded. The residue is dried in a cur-
rent of air to drive off the adherent acetone. The residue is again dissolved in
water-free ether, using as little ether as possible, and the solution is centrifuga-
lized for a few minutes and poured off from any residue that may be obtained.
The ether solution is then added slowly with stirring to four times its volume of
absolute alcohol at 50°C. The mixture is kept in the warm bath for some time
until the precipitate has settled. The supernatant liquid is decanted as far as
possible and the balance is poured into centrifugal tubes and centrifugalized while warm. The supernatant liquid is poured off and the residue is dried in a current of warm air until the excess of alcohol is driven off. The residue is again dissolved in water-free ether, centrifugalized, precipitated at 50°C. by four volumes of absolute alcohol and so on as described above. This procedure is repeated until the precipitate formed by the absolute alcohol begins to lose its solubility in water-free ether. This change in solubility of the alcohol precipitate takes place quite suddenly sometimes after twelve to twenty precipitations, but sometimes more gradually. When, after at least two hours treatment with ether, the residue on centrifugalization is suddenly increased in amount, the looked-for change is taking place. Thereafter each such residue is preserved. It is first washed with fresh ether and is then dissolved in water-free chloroform (Squibb's). The chloroform solution of the material is given two final precipitations. First, it is precipitated by two volumes of absolute alcohol at 50°C. This precipitate is separated by centrifugalization and is again treated with chloroform. After the material is completely dissolved the solution is precipitated, this time at room temperature, by the addition of an equal volume of absolute alcohol. The precipitate is centrifugalized off and dried in a current of warm air. The dark residue is ground in a mortar to a chocolate colored powder and kept until needed in a desiccator. This material should be completely soluble in water giving, according to concentration, a clear brownish or red solution, with no indication of opalescence. Material kept now for two years shows no deterioration in its property of preventing coagulation.

Properties of heparin. As stated above the material is soluble in water without opalescence. The occurrence of opalescence in the solutions is an indication of admixture with cephalin and if much of the latter is present the inhibiting effect on coagulation is lessened or absent altogether in freshly made preparations. When such preparations are kept for some weeks or months it will be found that the inhibiting action of their solutions is greatly improved. The explanation of this change is found in the fact that cephalin exposed to air loses gradually its favoring action upon coagulation (5).

The mode of preparation of heparin indicates that it belongs to the group of phosphatids—it gives reactions for nitrogen and phosphorus. Through the kindness of Doctor Levene of the Rockefeller Institute analyses for N and P were made in two samples. As these samples were made a year apart the mode of preparation was slightly different in the two cases but both preparations gave clear, non-opalescent solutions which exhibited a very marked inhibiting effect upon coagulation and when added to plasma or serum caused the formation of antithrombin. The results of these analyses were as follows:
Nitrogen ................................................ 5.01 per cent
Phosphorus ............................................. 4.75 per cent
Ratio of nitrogen to phosphorus ....................... 2.4 to 1

Preparation II—March, 1918

Nitrogen ................................................ 5.92 per cent
Phosphorus ............................................. 4.93 per cent
Ratio of nitrogen to phosphorus ....................... 2.6 to 1

The preparations had therefore a fairly constant composition in nitrogen and phosphorus, the ratio of one to the other being probably 2.5 to 1.

As compared with the phosphatids that have been obtained from the liver, heparin resembles most some of the preparations of jecorin that have been described. For his preparations of jecorin, Baskoff gives a mean value of the N to P ratio of 2.38 to 1, but in the preparations of other workers this ratio has varied from 1.8 to 1 (Drechsel) to 6.08 to 1 (Siegfried and Mark). For his preparations of heparphosphatid, Baskoff gives the N to P ratio as approximately 1 to 1.5. The two characteristic reactions of heparin that are of special physiological interest are first, that it retards or prevents the coagulation of blood; and second, when added to plasma or serum it causes the formation of a notable amount of antithrombin. Both reactions have been studied as carefully as possible.

The inhibiting action on coagulation. This effect may be demonstrated on the shed blood or upon the circulating blood by intravascular injections. The solutions of heparin used were made up in 0.9 per cent sodium chloride to prevent hemolysis of the blood corpuscles. In shed blood it was found that in mixtures containing 0.1 per cent of heparin the coagulation was prevented entirely. In more dilute solutions the coagulation was delayed in proportion to the concentration. When injected intravenously kymographic records showed that there was no disturbance of heart rate, blood pressure or respiratory rhythm and no variation in rectal temperature. If the injection of heparin is made in the proportion of one decigram per kilogram of body weight, blood drawn within a few minutes after the injection shows no spontaneous coagulation. Later the effects of the injected heparin wear off gradually so that in three or four hours the coagulation time of the blood has returned to normal. The gradual wearing off of the effect of the injected heparin was shown also in the variations of the prothrombin
time (6)—that is to say, the time of coagulation of the oxalated plasma when recalcified with the optimum amount of calcium chloride. Thus in one of the earlier experiments the following figures were obtained:

<table>
<thead>
<tr>
<th>Time after Injection</th>
<th>Prothrombin Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 minutes</td>
<td>5 minutes</td>
</tr>
<tr>
<td>10 minutes</td>
<td>No clot in 24 hours</td>
</tr>
<tr>
<td>1 hour</td>
<td>38 minutes</td>
</tr>
<tr>
<td>2 hours</td>
<td>14 minutes</td>
</tr>
</tbody>
</table>

Experiments made to determine how the heparin acts in preventing coagulation gave decisive proof that it inhibits the activation of prothrombin to thrombin. Its action certainly is entirely different from that of hirudin. This latter substance is a true antithrombin—that is to say, when added in minute amounts to mixtures of thrombin and fibrinogen it prevents clotting. Heparin has no such effect. Added in relatively large amounts to solutions of thrombin it has no retarding effect upon the action of the thrombin on fibrinogen. Since the heparin has no action upon fully formed thrombin and since it prevents the formation of thrombin when oxalated plasma is recalcified, it follows that the heparin must prevent the activation of prothrombin to thrombin, probably either by a reaction with the prothrombin or by a reaction with calcium. There are no facts to support the latter view. Heparin gives no precipitate with calcium salts and no indication of combining with the calcium in any way. Blood that has been rendered incoagulable with heparin cannot be made to clot by adding calcium chloride in any amounts. For example: in one experiment blood was caught as it flowed from an artery into a solution of heparin so that the concentration of the mixture was 0.15 per cent in heparin. This mixture was centrifugalized and the clear plasma was removed. This plasma remained unclotted as long as it was observed (24 hours). Mixtures were made with a solution of calcium chloride 0.5 per cent in the proportions of 5 drops of plasma to 1, 2, 3, 4 and 8 drops of the calcium chloride. None of these mixtures, or of similar mixtures in which the calcium chloride was replaced by water, showed any signs of clotting within 24 hours. The probability therefore is that the heparin reacts in some way with the prothrombin to prevent its activation by calcium. This conclusion is supported by direct experiments with solutions of prothrombin. Repeated experiments of this character were made with prothrombin prepared from the oxalated plasma of cat's blood (7). For example:
BLOOD COAGULATION: HEPARIN AND PRO-ANTITHROMBIN

Prothrombin solution ............................................. 5 drops
Heparin (0.2 per cent) ........................................... 2 drops
Calcium chloride (0.5 per cent) ............................... 2 drops
Fibrinogen .......................................................... 8 drops

This mixture gave no clot in 24 hours—whereas a control mixture in which the heparin was replaced by an equal amount of water gave a solid clot within 5 minutes.

Heparin is a stable compound. As stated above, preparations kept in desiccators have retained their properties unimpaired for at least two years. Its solutions are not injured by heating to the boiling point. Specific experiments of this kind have been made with solutions containing heparin in concentrations of 0.5, 0.25 and 0.12 per cent. The test tubes containing these solutions were immersed for five minutes in boiling water. Subsequent examination showed that the activity of these solutions in preventing clotting and in activating pro-antithrombin had not been impaired.

The activation of pro-antithrombin. Early in this work it was observed that while heparin itself does not act as an antithrombin nevertheless it causes a marked increase in the antithrombin of plasma or serum. The following experiment may be used to illustrate this reaction. Clear oxalated and centrifugalized plasma from cat's blood was heated to 54°C. in a water bath to remove its fibrinogen. The precipitated fibrinogen was removed by filtration and the plasma was then tested for antithrombin by the method used in this laboratory (8) with and without the addition of a solution of heparin. One drop of each solution was added to 2, 3, 4 and 5 drops of thrombin and after an incubation of 15 minutes 10 drops of fibrinogen were added to each specimen and the time of coagulation was noted.

Specimen I. Plasma plus an equal volume of 0.1 per cent heparin

<table>
<thead>
<tr>
<th>Thrombin</th>
<th>Specimen</th>
<th>Time of Clotting</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 drops</td>
<td>No clot</td>
<td>24 hours</td>
</tr>
<tr>
<td>3 drops</td>
<td></td>
<td>95 minutes</td>
</tr>
<tr>
<td>4 drops</td>
<td></td>
<td>42 minutes</td>
</tr>
<tr>
<td>5 drops</td>
<td></td>
<td>17 minutes</td>
</tr>
</tbody>
</table>

Specimen II. Control—Plasma plus an equal volume of water

<table>
<thead>
<tr>
<th>Thrombin</th>
<th>Specimen</th>
<th>Time of Clotting</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 drops</td>
<td>Clot</td>
<td>17 minutes</td>
</tr>
<tr>
<td>3 drops</td>
<td>Clot</td>
<td>5 minutes</td>
</tr>
<tr>
<td>4 drops</td>
<td>Clot</td>
<td>4 minutes</td>
</tr>
<tr>
<td>5 drops</td>
<td>Clot</td>
<td>3 minutes</td>
</tr>
</tbody>
</table>
As will be seen, the specimen containing heparin showed a greatly increased amount of antithrombin. This experiment was repeated a great many times with specimens of serum as well as of plasma and always with similar results. In attempting to explain this reaction it was at first supposed that the heparin reacted with some of the known substances in serum to produce an antithrombin, for example, with prothrombin or antithrombin itself, or that in some way it sensitized or intensified the action of the antithrombin already present in the plasma or serum (9). Experiments made to test these hypotheses have given negative results. Prothrombin can be isolated in an impure but effective form by the acetone method. Solutions of this kind have no antithrombic action and addition of heparin does not alter the reaction in this respect. On the other hand it was found that the antithrombin in cat’s plasma can be removed or destroyed by heating to 60°C. for five to ten minutes. Nevertheless on adding heparin to such a plasma there is an immediate production of a considerable amount of antithrombin. Hence the heparin does not act by intensifying the activity of antithrombin already present but produces new antithrombin by a reaction with an unknown constituent of the blood. This conclusion is strengthened further by the fact that this antecedent or mother substance can be precipitated from plasma by one-third or one-half saturation with ammonium sulphate. The precipitation by one-third saturation is less complete that with one-half saturation but in neither case is the active antithrombin thrown down, or if it is precipitated it is destroyed or denatured in the process. For example: a specimen of cat’s plasma, oxalated and centrifuged, was divided into two portions; to one portion ammonium sulphate was added to one-third saturation and to another to one-half saturation. The precipitates were collected by centrifugalizing and were then dissolved by the addition of water. Each solution was tested for antithrombin after adding an equal volume of heparin (0.2 per cent) and, as a control, after adding an equal volume of water, with the following result:

I. The precipitate obtained by one-third saturation with ammonium sulphate

With heparin

| Thrombin 2 drops | No clot in 1 hour |
| Thrombin 3 drops | No clot in 1 hour |
| Thrombin 4 drops | Clot in 53 minutes |
| Thrombin 5 drops | Clot in 15 minutes |
| Thrombin 2 drops | No clot in 1 hour |
With water (control)
Thrombin 2 drops......................... Clot in 4.5 minutes
Thrombin 3 drops......................... Clot in 3.5 minutes
Thrombin 4 drops......................... Clot in 2.5 minutes
Thrombin 5 drops......................... Clot in 2.5 minutes

II. The precipitate obtained by one-half saturation with ammonium sulphate

With heparin
Thrombin 2 drops......................... No clot within an hour
Thrombin 3 drops......................... No clot within an hour
Thrombin 4 drops......................... No clot within an hour
Thrombin 5 drops......................... No clot within an hour

With water (control)
Thrombin 2 drops......................... Clot in 4 minutes
Thrombin 3 drops......................... Clot in 3 minutes
Thrombin 4 drops......................... Clot in 2.5 minutes
Thrombin 5 drops......................... Clot in 2 minutes

These results can scarcely be interpreted otherwise than upon the assumption that there is a material in plasma and serum which by a reaction with heparin is converted to or gives rise to antithrombin. In the nomenclature of the day we may speak of this substance as a pro-antithrombin which is activated to antithrombin by heparin, just as prothrombin is activated to thrombin by calcium.

The two sets of compounds show an interesting difference in their reactions to acetone. The precipitate obtained by adding an equal volume of acetone to blood plasma yields on appropriate treatment an active prothrombin but no pro-antithrombin. So also from solutions in which it is contained thrombin may be precipitated uninjured by acetone while antithrombin is destroyed or denatured. On the other hand ammonium sulphate to one-half saturation precipitates uninjured prothrombin, thrombin and pro-antithrombin, while antithrombin is either not precipitated or else is destroyed or denatured. It was observed also that in cat's oxalated plasma, after heating to 54°C, to remove the fibrinogen, both prothrombin and pro-antithrombin may be precipitated in part by the addition of acetic acid to distinct acid reaction. The precipitate may be collected by centrifuging and then dissolved in a solution of sodium chloride, 1 per cent, made slightly alkaline with sodium carbonate. This solution furnishes thrombin on the addition of calcium chloride and antithrombin on the addition of heparin.

The pro-antithrombin or antithrombogen, as it might also be called, is thermolabile but less so than antithrombin. As stated above, this
difference is especially well shown in cat’s plasma. In this plasma all the ready formed antithrombin may be destroyed by heating to 60° for 5 minutes while the pro-antithrombin is apparently not affected. Heating this plasma to 70°C. however will destroy all the pro-antithrombin very promptly, and the same result is obtained by prolonged heating at lower temperatures. The following results were obtained from the plasma of cat’s blood, oxalated and centrifugalized, subsequently heated to 60° for 5 minutes and 15 minutes and to 70° for 5 minutes and then examined for antithrombin with and without the addition of heparin.

I. Specimen heated to 60°C. for 5 minutes

<table>
<thead>
<tr>
<th></th>
<th>Heparin</th>
<th>No clot in 24 hours</th>
<th>No clot in 24 hours</th>
<th>No clot in 24 hours</th>
<th>No clot in 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin 2 drops</td>
<td>With</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>water (control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin 2 drops</td>
<td>With</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>water (control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin 3 drops</td>
<td>With</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>water (control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin 4 drops</td>
<td>With</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>water (control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin 5 drops</td>
<td>With</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>water (control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

II. Specimen heated to 60°C. for 15 minutes

<table>
<thead>
<tr>
<th></th>
<th>Heparin</th>
<th>Clot in 32 minutes</th>
<th>Clot in 10 minutes</th>
<th>Clot in 5 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin 2 drops</td>
<td>With</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>water (control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin 3 drops</td>
<td>With</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>water (control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin 4 drops</td>
<td>With</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>water (control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin 5 drops</td>
<td>With</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>water (control)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

III. Specimen heated to 70°C. for 5 minutes

<table>
<thead>
<tr>
<th></th>
<th>Heparin</th>
<th>Clot in 4.5 minutes</th>
<th>Clot in 3.5 minutes</th>
<th>Clot in 2.5 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin 2 drops</td>
<td>With</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>water (control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin 3 drops</td>
<td>With</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>water (control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin 4 drops</td>
<td>With</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>water (control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin 5 drops</td>
<td>With</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>water (control)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It will be seen that the reaction with heparin was much weakened by heating to 60°C. for 15 minutes, and completely destroyed by heating to 70° for 5 minutes.

The source of pro-antithrombin. An effort was made to determine whether or not the pro-antithrombin is furnished to the plasma by the blood corpuscles. The experiments were not completed but they were carried far enough to show that neither the blood plates nor the leucocytes give origin to this substance. Platelets obtained from oxalated
pig's blood by differential centrifugalization and subsequent washing with a 0.9 per cent solution of sodium chloride give a solution when extracted with water which contains prothrombin but no pro-antithrombin—that is to say, addition of calcium chloride causes the formation of thrombin, but addition of heparin does not produce antithrombin. A similar negative result, so far as pro-antithrombin is concerned, was obtained by extracting lymphocytes furnished by the thymus gland and blood leucocytes obtained from the buffy coat of centrifugalized oxalated blood.

The reaction between heparin and pro-antithrombin. We have no knowledge regarding the chemical nature of pro-antithrombin, or the change that it undergoes in being converted to active antithrombin, but it seems evident that the reaction is not enzymatic or catalytic. The amount of antithrombin formed increases in proportion to the amount of heparin added. If one takes several specimens of oxalated plasma of 0.5 cc. each and adds one, five and ten drops of a 0.2 per cent solution of heparin it will be found that the amount of antithrombin formed increases with the heparin added. Unfortunately the mode of demonstrating antithrombin does not lend itself readily to a quantitative determination, except within narrow limits, so that it is not possible to express the relationship between the amounts of heparin and the amounts of antithrombin in a definite form. But the fact that the reaction between the heparin and the pro-antithrombin is of the nature of a definite chemical reaction is borne out also by the following observation. A specimen of clear cat's plasma, oxalated, was heated to 60°C. for ten minutes, thereby precipitating its fibrinogen and destroying all the antithrombin present. After filtration, a 0.2 per cent solution of heparin was added in equal volume to a part of this plasma. Examination showed now the presence of a considerable amount of antithrombin, due to the action of the heparin on the pro-antithrombin. This mixture was then heated again to 60°C. for ten minutes to destroy the newly formed antithrombin, and that this really occurred was determined by experiment. To the mixture there was again added an equal volume of heparin, 0.2 per cent, with the result that there was again a formation of a considerable amount of antithrombin. The experiment serves not only to indicate that the reaction between the heparin and pro-antithrombin takes place in definite proportions after the manner of an ordinary chemical reaction, but it serves to show also that the reserve supply of pro-antithrombin in blood is considerable. The reaction between antithrombin and thrombin, as has been pointed
out previously (10), is a colloidal reaction the extent of which varies with the time, so that in any effort to estimate the amount of antithrombin in a quantitative way in different solutions it is necessary to give a definite time for the interaction between the two substances. The longer they are in contact the larger will be the amount of thrombin inactivated by the antithrombin. The relations are quite different in the reaction between heparin and pro-antithrombin. When a mixture is made the maximal reaction is completed as soon as an examination can be made, and a longer exposure leads to no further increase in antithrombin.

**DISCUSSION**

One, at least, of these new factors in coagulation, the pro-antithrombin, is present normally in blood. The other, the heparin, has not been shown directly to be a normal constituent of blood but the indirect evidence speaks strongly in favor of this view. The direct rôle of the pro-antithrombin is to give rise to antithrombin and it may be assumed that the amount of the latter constantly present in normal blood is dependent upon the extent to which the pro-antithrombin is activated, and this in turn is dependent upon the amount of heparin since so far as we know the pro-antithrombin cannot be changed to antithrombin otherwise than by a reaction with heparin. The fact that there is a supply, and a considerable supply, of pro-antithrombin in circulating blood indicates that the antithrombin probably takes an important part in regulating the process of coagulation. What is the rôle of antithrombin? Two views have been suggested: Nolf (11) and afterwards Howell (12) have stated their belief that the antithrombin is directly concerned in maintaining the normal fluidity of the blood. The latter in his theory of coagulation has assumed that some inhibiting substance, presumably the antithrombin, functions by preventing the activation of prothrombin by calcium and that in this way intravascular coagulation is avoided. When blood is shed or when in any other way thromboplastic substance is added to the blood, the inhibiting influence of the antithrombin is neutralized, the prothrombin is converted to thrombin and coagulation ensues. At the time this theory was proposed antithrombin was the only inhibiting substance known in the blood, but the work described in this paper makes it probable that the blood contains a second inhibiting substance, heparin, which so far as its action is concerned, may be designated as an anti-prothrombin since it inhibits clotting by preventing
the activation of prothrombin. It would be more logical perhaps to assume that heparin rather than antithrombin is the substance in blood which prevents spontaneous clotting and which is neutralized in shed blood by the thromboplastic material, the cephalin. Laboratory experiments show readily that the inhibiting effect of heparin can be neutralized by the addition of cephalin, but in order to give this modification of Howell's original theory a sound experimental basis it is necessary to show that heparin is a normal constituent of blood. This demonstration has not yet been furnished. But admitting the probability of this view the question arises as to the function fulfilled by the antithrombin which we know does exist in normal blood. In several pieces of work done in this laboratory (Weymouth, 13), (Gasser, 14), (Rich, 15), evidence has been furnished which tends to show that metathrombin found in the serum of clotted blood is a combination of thrombin and antithrombin. The antithrombin inactivates thrombin by forming the substance designated as metathrombin by Mora-witz. In accordance with this view Gasser has suggested that this may be at least one function of the antithrombin. It is very probable that thrombin in small amounts is formed constantly in circulating blood and the presence of antithrombin may be a device to neutralize this thrombin. On the other hand, when blood is shed thrombin is formed suddenly in relatively large amounts and the antithrombin under such conditions is inadequate for protection, especially as its rate of action upon thrombin is greatly reduced at temperatures below that of the body (16). Provisionally we are inclined to adopt the view that the value of the antithrombin and therefore of the pro-antithrombin is to safeguard the blood from the action of any free thrombin that may be liberated in the circulation.

The function of the heparin in coagulation is implied in its two characteristic properties described above. As previously stated, the presence of this substance in the blood has not been demonstrated but it seems very probable that it is one of the normal constituents of this liquid. The fact that it has the specific reaction of changing pro-antithrombin to antithrombin supports this view. If we can make this assumption it would follow that heparin constitutes an inhibiting material which restrains clotting by a double action; by its power to prevent the activation of prothrombin to thrombin and by its property of activating pro-antithrombin to antithrombin. Through this material the body is able to modify coagulability of the blood either locally or in general. An instance of local modification is furnished by the
work of Schickele (17) who has shown that an inhibiting substance to blood coagulation is produced in the uterine mucous membrane. A special study of this material in this laboratory by Dr. Jessie King has shown that it is undoubtedly heparin or a substance with properties similar to those of heparin, since it inhibits clotting by preventing the activation of prothrombin and is also effective in causing an increase in antithrombin when added to plasma or serum. It is quite possible indeed that some of the normal and abnormal variations in the coagulability of blood may be traced to variations in the amount of heparin. The properties of hemophiliic blood, for example, as described by one of us (18), are in fact identical with those exhibited by blood to which heparin has been added in concentrations sufficient to retard coagulation. If subsequent work is successful in devising a method for the quantitative estimation of heparin in blood, a great amount of light may be thrown upon some of the difficult problems in blood coagulation.

SUMMARY

1. Two new substances are described which are connected with the process of coagulation of blood:
   a. A phosphatid designated as heparin since it is obtained most readily from the liver (dog). It has an N to P ratio of 2.5 to 1. It is easily soluble in water giving a clear, non-opalescent solution of a yellowish or reddish color according to concentration. It possesses two characteristic reactions: First, it retards or prevents the coagulation of blood, both in the body and when the blood is shed. Second, it causes a marked increase in antithrombin when added to blood or serum.
   b. An antecedent or mother substance for antithrombin, present in plasma and serum in considerable amounts. It is converted promptly to antithrombin by heparin and is designated as a pro-antithrombin.

2. Heparin may be obtained from lymph glands and it has been detected by its reactions in the uterine mucous membrane. It is present in largest amounts in the liver, especially the dog’s liver.

3. Heparin inhibits clotting mainly by preventing the activation of prothrombin to thrombin. It acts as an antiprothrombin rather than as an antithrombin.

4. The action of heparin in preventing the activation of prothrombin and in causing the activation of pro-antithrombin is not destroyed at a temperature of 100°C.
5. Pro-antithrombin in blood plasma is destroyed promptly by heating to 70°C. and more gradually by prolonged heating at lower temperatures (60°C.).

6. Pro-antithrombin may be precipitated uninjured from blood plasma by acetic acid or by ammonium sulphate added to one-half saturation.

7. On the theoretical side it is suggested that heparin and pro-antithrombin are normal constituents of the circulating blood (not demonstrated for the heparin) and together fulfill the function of safeguarding the fluidity of the blood, that is to say, of preventing intravascular clotting. The pro-antithrombin by its conversion to antithrombin provides a protection against any small quantities of thrombin that may arise in the circulating blood (metathrombin formation). The heparin in addition to functioning as a specific activator to pro-antithrombin exerts an inhibiting influence upon the conversion of prothrombin to active thrombin. When blood is shed or when in other ways thromboplastic substance (cephalin) is added to the blood the protection afforded by the heparin is overcome and thrombin is formed in amounts sufficient to cause clotting. It is suggested that variations in the amount of heparin in the blood may suffice to explain some of the known abnormalities in coagulation, hemophilia, for example.

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