THE DETERMINATION OF FIBRINOGEN WITH PROTAMINE*

BY E. MYLON, M. C. WINTERNITZ, AND G. J. DE SÜTÖ-NAGY

(From the Laboratory of Pathology, Yale University School of Medicine, New Haven)

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This presentation deals with a method for the determination of the fibrinogen of plasma irrespective of the presence of anticoagulants like heparin. As is well known, this can be effected by salting-out methods. The older ones no longer are considered adequate for quantitative determination when fibrinogen is in small amounts or when the available plasma is limited. A more recently developed technique (1) in which sodium sulfite is employed yields results in accord with the Cullen-Van Slyke method for the quantitative determination of fibrin. Reference will be made to this later, as it prescribes a 1:25 dilution of the plasma with resultant fibrin loss.

Qualitative Determination of Fibrinogen

Addition of protamine to blood plasma causes a precipitate. When this is centrifuged and washed, it can be redissolved in 3 per cent saline. The resulting solution coagulates between 54–56° and becomes gelatinous in consistency several hours after the addition of fresh serum (thrombin). These characteristics identify this substance as fibrinogen in so far as this is possible.

Further corroboration of the nature of the protamine precipitate of plasma is available from the fact that no precipitate follows addition of protamine to dilute serum. Under this circumstance only a slight haze occasionally follows and this is not influenced by centrifugation. The native globulins of serum obviously are not precipitated by protamine. In this respect they contrast with globulins previously salted-out and redissolved. The latter, less stable, are readily precipitated with protamine. This difference in the behavior of native and redissolved globulins supports the view that the process of isolation alters characteristics of these proteins. For the purposes of the method to be presented it is sufficient that native serum proteins are not precipitated by protamine.

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DETERMINATION OF FIBRINOGEN

**Fibrin Determination**

For control of the method to be presented plasma fibrin was determined by a procedure described by a number of investigators (Foster and Whipple (4), Cullen and Van Slyke (3), Wu and Ling (12), Jones and Smith (8), Peters and Van Slyke (9)). A dilution of 1 cc. of oxalated plasma\(^1\) in 25 cc. of saline was used. This was recalcified and after 3 hours at room temperature the plasma-saline-calcium mixture was centrifuged for 20 minutes at 2500 R.P.M. A firm fibrin clot was deposited on the bottom of the tube. The supernatant was drained off, the clot and the wall of the tube washed with 30 cc. of distilled water, and the tube again centrifuged. After a second washing the clot was transferred to a Kjeldahl flask with the aid of 20 to 30 cc. of distilled water, digested with sulfuric acid and copper sulfate, and the nitrogen determined in the usual way. It will be noted that this is a slight modification of the Cullen-Van Slyke and the Wu and Ling methods. Duplicate determinations differed only between 1 and 1.5 per cent.

**Quantitative Determination of Fibrinogen**

For the quantitative determination of fibrinogen 1 cc.\(^2\) of a 1 per cent solution of protamine in saline was added to a mixture of 1 cc. of oxalated plasma (100 mg. of dry sodium oxalate to 20 cc. of blood) in 25 cc. of saline contained in a carefully cleansed 50 cc. centrifuge tube. Within a minute 3 cc. of a 1.58 per cent calcium acetate solution were added and the tube was then placed in the ice chest for 1 hour. Then it was centrifuged for 20 minutes at 2500 R.P.M., the supernatant poured off, 30 cc. of cold distilled water added, the contents again centrifuged for 10 minutes, and this wash water also discarded.

Early in the development of the method it was found that protamine remains in solution on addition of trichloroacetic acid in absolute alcohol and that after exposure to this reagent the fibrinogen disk, previously impregnated with calcium, is rendered firmer. The next step in the method, therefore, was to add 30 cc. of a 10 per cent solution of trichloroacetic acid in absolute alcohol to the centrifuge tube. Then the fibrinogen mat was loosened from the base of the tube with the sharp end of a glass rod. Small particles clinging to the wall of the tube were freed by using the mat as a wiper and after the tube was recentrifuged the alcoholic solution was poured off. The mat was transferred with the aid of 20 cc. of distilled water to a Kjeldahl flask and the last traces of fibrinogen were removed from the tube with 10 cc. of distilled water to which 3 cc. of

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\(^1\) 50 mg. of dry sodium oxalate to each 10 cc. of blood.

\(^2\) For very large amounts of fibrinogen (0.7 gm. and more in 100 cc. of plasma) 2 cc. of the protamine solution are recommended.
concentrated sulfuric acid were added. The slight heat generated by the addition of the acid to the water facilitated the process. This acid solution was added to the contents of the Kjeldahl flask, as was a final rinsing of the tube with a small quantity of distilled water. After addition of copper sulfate digestion and nitrogen determination were made as usual. The accuracy of the method was considered satisfactory, as parallel tests with samples of the same plasma differed only within ±2 per cent, rarely ±4 per cent. This is illustrated in Table I.

### Table I

**Fibrinogen Determination with Protamine**

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>0.02 N H₂SO₄ (neutralized with NH₄OH)</th>
<th>Average</th>
<th>Deviation per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.21</td>
<td>2.19</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>2.17</td>
<td>2.01</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1.98</td>
<td>2.50</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>2.03</td>
<td>2.50</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>2.50</td>
<td>4.51</td>
<td>0.9</td>
</tr>
<tr>
<td>6</td>
<td>2.47</td>
<td>4.72</td>
<td>0.3</td>
</tr>
<tr>
<td>7</td>
<td>4.55</td>
<td>2.33</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>4.71</td>
<td>2.33</td>
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</tr>
<tr>
<td>9</td>
<td>2.94</td>
<td>3.07</td>
<td>1.4</td>
</tr>
<tr>
<td>10</td>
<td>2.99</td>
<td>2.92</td>
<td>0.35</td>
</tr>
</tbody>
</table>

In the course of these experiments it was observed that addition of protamine to plasma leads to rapid and extensive fibrinogenolysis. The 4 or 5 per cent loss in fibrinogen of oxalated normal plasma incubated for 3 hours is increased to 35 per cent in the same period after protamine has been added. It should be recalled that under these same circumstances of time and temperature lysis of fibrin is not even initiated. This is illustrated in Fig. 1.

Difficulties in correlation persisted until it was recognized that the temperature after addition of protamine influenced lysis of fibrinogen and did not affect fibrinolysis. These facts became evident during a particularly
hot season when it seemed advisable to eliminate the influence of temperature by refrigeration of the plasma-protamine mixture at 3° during the 3 hour period believed to be desirable before centrifugation. It then became apparent that fibrinogenolysis after addition of protamine varied directly with the temperature in accordance with enzymatic action.

When fibrinogenolysis was prevented, the fibrinogen content was found to exceed by approximately 20 per cent the fibrin values obtained by the method detailed earlier in this communication. The discrepancy was less marked only when the fibrinogen content of the plasma was particularly high. Table II illustrates some of these facts. That the protamine method for the determination of fibrinogen does not yield higher values due to protamine contained in the precipitate is shown by the following experiment. 1 cc. of a fibrinogen solution prepared by repeated precipitation with sodium chloride yielded 4.025 mg. with the Kjeldahl and 3.920 mg. with the protamine method.

Similar discrepancies between fibrinogen and fibrin have been recorded by Jacques (5). Utilizing a fibrinogen solution and purified thrombin, he showed that the total fibrinogen nitrogen could not be recovered as fibrin nitrogen. He concluded that "owing to the solubility of fibrin,
however, as much as 10 per cent of the fibrin nitrogen may be present in the supernatant."

Experiments to elucidate this problem were carried out and typical examples are recorded in Table III. These two experiments show that the samples with the least dilution yielded the highest fibrin values. It is improbable that this is dependent upon impurities included in the clot. Theorell and Widström (10) used 1 part of plasma to 3 parts of diluent as

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Fibrin; 1 cc. plasma, 25 cc. saline, 3 cc. calcium acetate 0.1 N</th>
<th>Fibrinogen; protamine method, 3°</th>
<th>Fibrinogen-fibrin discrepancy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm. per 100 cc. plasma</td>
<td>gm. per 100 cc. plasma</td>
<td>per cent</td>
</tr>
<tr>
<td>1</td>
<td>0.315</td>
<td>0.383</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>0.273</td>
<td>0.352</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>0.350</td>
<td>0.437</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>0.717</td>
<td>0.804</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>0.417</td>
<td>0.530</td>
<td>21</td>
</tr>
<tr>
<td>6</td>
<td>0.357</td>
<td>0.439</td>
<td>19</td>
</tr>
</tbody>
</table>

The higher values, it would seem, more nearly approximate the actual content of fibrin and dilution results in substantial loss. This is not predictable, for it is not based upon solubility alone, as is indicated by analysis of the experiments in Table III. The increase in dilution between Samples 1 and 2 is 8 cc. and between Samples 1 and 4 is 32 cc. The corresponding losses in fibrin per cc. of plasma are 0.61 mg. and 0.64 mg.
In the second group the increase in dilution between Samples 5 and 6 is 8 cc. and between Samples 5 and 7 is 17 cc. and the corresponding losses in fibrin per cc. of plasma are 0.47 and 0.56 mg.

More important is the fact that the higher fibrin values obtained with the lesser dilution differ only slightly from those secured with the protamine fibrinogen method. The difference is only ±5 per cent. Dilution decreases the fibrin value but the way this is effected is not understood. It does not act by impairing the enzyme system, thrombin, as the following experiment shows.

5 cc. of oxalated plasma were diluted with 125 cc. of saline and recalciﬁed with 15 cc. of 1.58 per cent calcium acetate; 3 hours later the well formed clot was removed and 10 cc. of fresh serum were added. This excess of thrombin did not cause reclothing and it was obvious that the low fibrin value of the dilute solution could not have resulted from lack of thrombin.

The question arises whether dilution may inﬂuence the amount of fibrin formation by interfering with the linkage of molecules essential for thread formation. That there are intermediate substances between fibrinogen and fibrin has been shown by Hammarsten (11) and more recently by Apitz (11). Hydration or greater dispersion of intermediate substances or increase of the intermolecular space may impair the final steps. Since fibrinogen precipitation with protamine is largely independent of dilution, it may aid in the solution of this problem.

It is important to note that the difﬁculty soluble compound of protamine and heparin (Chargaff and Olson (2)) does not interfere with the quantitative determination of fibrinogen. When protamine is added to a mixture of plasma and heparin, the fibrinogen is precipitated just as though no heparin were present. The quantity of heparin in the plasma may be in considerable excess of the physiological and indeed may be greater than occurs in anaphylactic shock as determined by Jacques and Waters (6). Moreover, such amounts of heparin inﬂuence the nitrogen content of the precipitate so slightly that the fibrinogen nitrogen determination is not interfered with. Parallel experiments without heparin and with 27.5 units for each cc. of blood only show differences of from 1 to 3 per cent.

Precipitation of heparin by protamine has been applied successfully by Jacques and Waters (7) for the quantitative determination of blood heparin. Small amounts of protamine shorten the coagulation time of heparinized blood, while larger amounts, as the authors point out, prolong the clotting time; and as the quantity of protamine is increased the blood may become incoagulable. This latter effect undoubtedly results from the partial precipitation of fibrinogen. The coagulation time of the blood is prolonged with less than 0.5 mg. for each cc. of plasma, owing probably to its influence on fibrinogen. With 0.75 mg., clot formation is entirely inhibited and with
1.5 mg. per cc. of plasma approximately 80 per cent of the total fibrinogen content is precipitated. As a rule 9 mg. of protamine will precipitate all of the fibrinogen but even 18 mg. will not precipitate other nitrogenous substances in the plasma.

On the basis of the above experiments the amount of protamine elected for the quantitative determination of fibrinogen was 10 mg. per cc. of plasma except when the fibrinogen is in great excess, when 20 mg. per cc. are recommended.

SUMMARY

1. A method is described for the quantitative determination of fibrinogen in plasma with the aid of protamine.

2. Heparin does not interfere with this determination when it is present even in the higher amounts shown to occur in anaphylactic shock (25 units per cc. of blood).

3. Fibrinogen values secured by this technique are about 20 per cent higher than fibrin values obtained by the methods of Cullen and Van Slyke and others that require high plasma dilution.

4. These differences are reduced to about 5 per cent if the comparative fibrin determinations are made with smaller plasma dilutions.

5. The discrepancies are not explained by the assumption of a soluble form of fibrin.

6. After the addition of protamine to plasma the enzymatic fibrinogenolysis is markedly increased. About 35 per cent of the fibrinogen is split off after 3 hours incubation of the fibrinogen-protamine precipitate.

BIBLIOGRAPHY

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