CLVII. THE NITROGEN PARTITION IN BLOOD CLOTTING

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WHILE there has been considerable controversy for many years as to the enzyme nature of thrombin, the weight of evidence, as summarized by Eagle [1935] recently, is in favour of thrombin being an enzyme. Little is known, however, regarding the nature of the reaction catalysed. Numerous workers have put forward the view that thrombin is a proteolytic enzyme similar to trypsin. This assumption was based on the fact that, under certain conditions, trypsin clots blood and plasma but the finding of Dale & Walpole [1916] that trypsin has an indirect action on the clotting system suggests other possible explanations. As a result of the supposed proteolytic action, a number of workers have assumed that fibrin is not the sole product of the reaction. Such a possibility can be tested by studying the nitrogen partition during clotting, provided that the fibrinogen is free from other protein and a sufficiently active thrombin is used. While early workers in the subject [*v*. Hsu & Wu, 1933] attempted studies of this nature, their results are vitiated by the use of impure fibrinogen. Hsu & Wu used much purer preparations and found only 6·7 % of the fibrinogen N in the serum after clotting, 93 % being recovered as fibrin. The significance of this 7 % difference has yet to be established.

MATERIALS

Fibrinogen. This was prepared by the method of Florkin [1930] which involves three precipitations of the protein with NaCl. By maintaining a *p*H of 6·0 and conducting all operations in the cold room at 2°, the method yields a chemical individual of constant and reproducible solubility, which can be kept for some weeks without change in solubility. Before use, the fibrinogen was precipitated with a half volume of $3M$ potassium phosphate buffer of *p*H 6·6 and redissolved in $M/4$ phosphate buffer containing toluene to give a concentration of 0·5 mg. N per ml.

Thrombin. We are indebted to Mr R. A. Mustard for a large amount of thrombin, prepared by the method of Mellanby [1930, 1933]. A solution was made from the dry powder and impurities precipitated by adjusting to *p*H 5·3. The solution was then brought to neutrality and toluene added. For use, it was diluted with distilled water.

METHODS

The reaction. The reaction was carried out in pyrex test tubes, $12 \times 1{\cdot}4$ cm.; 2 ml. of fibrinogen were placed in the tube, 3 ml. of water added and the tube placed in a water bath at 25°. At zero time, 1 ml. of thrombin solution was added, the tube stoppered with a paraffined cork, inverted to mix the contents and then placed in a rocker in the bath. The tube was completely immersed and was rocked gently so that the contents flowed from one end of the tube to the other about 30 times a minute. This served both to keep the solution homogeneously mixed and also to break up the fibrin clot as it formed.

The reaction was stopped by centrifuging the tube in a small angle centrifuge for 4 min. 2 min. after beginning centrifuging was arbitrarily taken as the end of the reaction time. The supernatant solution was decanted off and the fibrin washed with 2 ml. of 1 % saline, any fibrin adhering to the cork being removed with a probe and added to the saline. The tube was again centrifuged and the wash fluid added to the supernatant. N determinations were then made on the fibrin and the supernatant, the digestion of the fibrin being carried out in the same test tube as the reaction. The nitrogen was determined by the micro-Kjeldahl method using an apparatus of the improved Parnas-Wagner type. $N/140$ HCl and $N/140$ NaOH were used for absorption and titration respectively.

The nitrogen partition during clotting. As Hsu & Wu have pointed out, it is necessary to study this over the whole reaction and not just at equilibrium. This procedure was followed in all the experiments and Fig. 1 shows a curve typical of

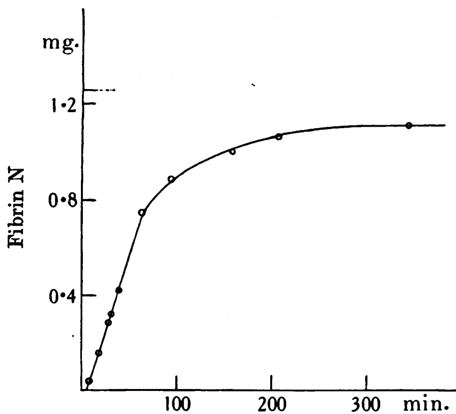

Fig. 1.

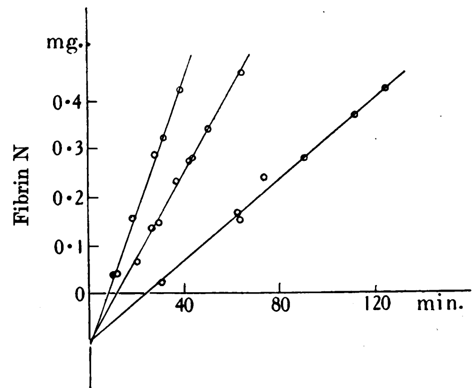

Fig. 2.

Fig. 1. Exp. 346. Fibrinogen N, 1·255 mg.; thrombin N, 0·007 mg. Phosphate $M/8$. Total volume, 6 ml.

Fig. 2. *a*, Fibrinogen No. 43, thrombin No. 46 (0·007 mg. N). $b+c$, Fibrinogen No. 55, thrombin No. 70. In *b* thrombin N was 0·0028 mg.; in *c*, 0·0014 mg. Total fibrinogen N was 1·09 mg. Equations of the lines were found to be: (*a*) F(fibrin nitrogen) $=0{\cdot}01344t-0{\cdot}1078$; (*b*) $=0{\cdot}00876t-0{\cdot}1038$; (*c*) $=0{\cdot}00416t-0{\cdot}0953$. Average value of $a=0{\cdot}1021$ mg. N $=0{\cdot}0170$ mg. N/ml.

those obtained. Two points are to be observed with regard to this curve—(1) not all the fibrinogen N is recovered as fibrin N, $1{\cdot}255-1{\cdot}125=0{\cdot}130$ mg. N remaining in the supernatant, and (2) a small lag phase is present. Before assuming that the N in the serum represents another product of the reaction it is necessary to investigate the possibility that it is due to the solubility of fibrin, and the lag phase provides a means of testing this hypothesis. If fibrin has a measurable solubility, a measurable time will elapse at the beginning of the reaction before the solution is saturated with fibrin and the fibrin begins to precipitate. Therefore, by continuing the initial linear portion of the curve below the time axis, it should cut the fibrin axis at the value representing the dissolved fibrin. This has been done in Fig. 2 for various concentrations of thrombin and it can be seen that the intercept on the fibrin axis is 0·10 mg. in each case. The close agreement observed suggests that the lag phase is simply due to the solubility of fibrin. Hence, it is probable that the difference between the fibrinogen and fibrin nitrogen is also due to the solubility of the fibrin, so that,

if any N is split off in the conversion, it is less than 0·5 % of the original N. The value reported represents a solubility of 0·0170 mg. N per ml. or 0·102 g. protein per litre. Hsu & Wu's values when converted to the same basis show close agreement, 0·017 ± 0·006 mg. per ml.[1]

It can be observed that when the progress curve is drawn to allow for the solubility of the fibrin, the curve is that typical of many enzyme reactions. This is in contrast to the results of Klinke & Elias [1931] and Kugelmass [1925] who found the reaction to be autocatalytic. The difference may be due to the different physical conditions used.

Fibrinolytic action of purified thrombin. A number of reports have appeared in the literature suggesting that thrombin has a fibrinolytic action (see Hirose). The most conclusive is that of Hirose [1934]. She reported that using sterile thrombin and fibrinogen, fibrinolysis occurred in 3 to 6 days. To our surprise, we found that our purified thrombin had a very high fibrinolytic action under the conditions of our experiments, measurable fibrinolysis occurring 4 hr. after the addition of thrombin. Fig. 3 shows an experiment of this nature. The fibrin formed reached a maximum in 3 hr. and then rapidly decreased. Increasing the thrombin concentration decreased the total amount of fibrin formed and increased the rate of fibrinolysis. Controls showed that it was not due to the shaking, and that removal of the supernatant containing most of the thrombin at the end of clotting resulted in the fibrinolysis being decreased proportionately. That such a marked action of thrombin has not been observed before may be due to the use of less purified reagents. As found by Hirose, the product of the reaction appears to be Hammarsten's fibrinoglobulin, no non-protein nitrogen appearing during the reaction.

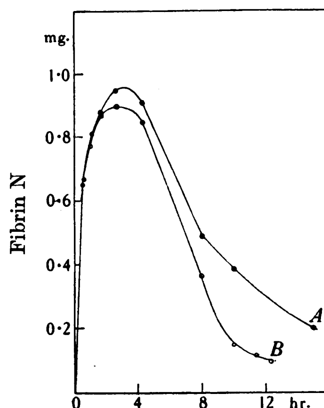

Fig. 3. Fibrinogen N = 1·09 mg. Thrombin N in *A*, 0·064 mg.; in *B*, 0·128 mg.

It is difficult to prove that the fibrinolytic enzyme is actually thrombin and not another enzyme which has accompanied it through the purification process. As Mellanby's preparation of thrombin involves the preparation of prothrombin followed by its spontaneous activation and the removal of impurities which do not undergo the same change in properties, it seems unlikely that the fibrinolysis is due to an accompanying enzyme.

This work was originally undertaken with a view to applying the method (determination of the fibrin nitrogen by micro-Kjeldahl analysis) as a quantitative basis for the study of various problems in blood clotting. This has not proved feasible chiefly because of the difficulty in stopping the reaction to take samples. Any other method than centrifuging results either in the precipitation of the fibrinogen or the re-solution of the fibrin.

[1] A further indirect proof of the solubility hypothesis is the solubility of fibrinogen in salts used as precipitants. Campbell & Hanna [1937] have recently shown that fibrinogen is precipitated with 12·5% sodium sulphite so as to yield values which check with the Van Slyke & Cullen method of estimating fibrin. In a series of experiments the solubility of fibrinogen under the conditions of their method was found to be 0·043 mg. N per ml., the equivalent of 0·014 mg. fibrin N per ml. in the Van Slyke & Cullen method.

Summary

When thrombin acts on a solution of fibrinogen, free from other proteins, all the fibrinogen N appears as fibrin nitrogen. Owing to the solubility of fibrin, however, as much as 10% of the fibrin nitrogen may be present in the supernatant. Purified thrombin preparations have a marked fibrinolytic action.

Note added 5 July, 1938. Since this paper was sent to press the results of a similar study by Presnell (*Amer. J. Physiol.* **122**, 596, 1938) have been published. When his results are analysed, they indicate an interpretation similar to that given above, namely that the difference in the fibrinogen nitrogen is due to the solubility of fibrin rather than to a "proteolytic" action of thrombin.

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