25. SOME FACTORS INFLUENCING THE ANTICOAGULANT ACTION OF HEPARIN

BY LOUIS BARKER JAKES AND ROBERT ALEXANDER MUSTARD

*From the Department of Physiology, University of Toronto,
Toronto, Canada*

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It has been generally assumed that the anticoagulant action of heparin is due to direct combination with prothrombin, thrombin, or kinase. The chief experimental facts on this point have been confirmed by Mustard & Jaques [1937] and may be summarized as follows. When citrated plasma and thrombin are used to test the action of heparin, the heparin acts as an antithrombin [Howell, 1925]. When kinase is added, it neutralizes the action of heparin. It is thus possible to "titrate" heparin against kinase [Fischer & Astrup, 1935]. If the prothrombin is removed from the plasma with alumina gel, the heparin is still active but the corresponding action of kinase is largely abolished [Quick, 1936]. Heparin thus acts as an antikinase indirectly. Its anticoagulant action is that of an antithrombin. It has been frequently suggested (originally by Howell) that heparin also acts as an antiprothrombin, and recently Brinkhous *et al.* [1939] have obtained direct evidence of such an antiprothrombin action. In this paper, however, only the antithrombin action of heparin will be considered. The question of the anticoagulant action of heparin is complicated by the influence of various factors outside the known clotting system. Mellanby [1934] concluded that the presence of neutral salts was necessary for the action of heparin: Howell & Holt [1918] concluded that the serum proteins, particularly the euglobulin fraction, were necessary. We have not found any evidence that salt is necessary for the action of heparin but have confirmed the work of Howell & Holt and of Quick, that the presence of serum protein is essential.

Methods

The activity of any factor in the clotting system can be measured either by direct determination of the resulting clotting time or by determination of the dilution necessary to give a fixed clotting time. In either case an arbitrary standard solution must always be used for comparison. The clotting time method has the disadvantage that the clotting time is not a simple function of the heparin concentration [Barratt, 1937]. This renders difficult the interpretation of data on the effect of other factors on the action of heparin. The dilution method with a fixed clotting time is used in the assay of heparin by Charles & Scott [1933] and this can be adapted to various purposes. Its chief disadvantage is the great number of tests which it is necessary to conduct in order to obtain quantitative results with any degree of accuracy. A third method which has definite but limited advantages is to determine the rate of clotting by measurements of turbidity, viscosity, or fibrin nitrogen [*vide* Jaques, 1938]. With any method considerable care must be taken when comparing two different anticoagulant substances since Astrup [1938] has found quite different relationships for different anticoagulants.

We have used the method which appeared to be the most suitable for the experiment planned. Constant temperature water baths were used. The experiments were carried out in 2 ml. coagulation tubes with a total volume of 1.0 ml. of the clotting mixture. The clotting time was determined at 37.5°. The dilution method was carried out at 25° with a fixed clotting time of 2 hr.

Materials

Fibrinogen and thrombin were prepared by the methods of Florkin and Mellanby respectively as previously described [Jaques, 1938]. The heparin used was supplied by the Connaught Laboratories. In the early experiments only the 5 unit per mg. material was available: later, as the 15, 60 and 100 unit materials were prepared, these were used. There was no apparent difference in these preparations as regards the experiments described here. The unit used is that of the Connaught Laboratories, Toronto, and has been found to be equivalent to the potency of 1/100 mg. of the crystalline barium salt of heparin prepared by Charles & Scott [1936].

The effect of salt on the action of heparin

Mellanby [1934] has emphasized the effect of salt concentration on the action of heparin. He found that dialysis of plasma resulted in a loss of heparin activity and that this was restored by the addition of NaCl. Hence, he concluded that the presence of neutral salt was necessary for the action of heparin. While under certain limited conditions this appears to be the case, we have found that when a more extensive study of the system is made, the effect of NaCl is to depress the activity of heparin rather than to increase it.

Fig. 1. Effect of dialysis on the antithrombin action of heparin. ○—○ Clotting time with 0.5 ml. of thrombin. ●—● Clotting time with 0.5 ml. of thrombin + 0.1 unit of heparin.

Citrated horse plasma was dialysed against distilled water and 5 ml. samples were removed from time to time and filtered. 0.5 ml. of a standard thrombin solution was added to 0.5 ml. of the sample and the clotting time determined. The clotting time was also determined with a second 0.5 ml. of the sample to which was added the standard thrombin + 0.1 unit of heparin. The results are shown in Fig. 1. The clotting time of the plasma with thrombin alone decreased

to a minimum, due to removal of salts, and then gradually increased. The effect on the action of heparin was different. In the early stages of the dialysis the clotting time with heparin decreased until it was the same as the control, as observed by Mellanby. With prolonged dialysis, however, the clotting time with heparin increased over that of the control, i.e. there appeared to be a point during dialysis where the heparin had least activity. In further experiments plasma was dialysed for 18 hr. and then 20 % NaCl was added to samples of it to give a series of plasmas with known salt concentrations. Varying amounts of heparin were then added to 0.5 ml. of the samples and the clotting times with a constant amount of thrombin determined. The results are shown in Fig. 2. It can be observed (Fig. 2 (1)) that for each heparin concentration, as the salt

Fig. 2. Effect of sodium chloride concentration on heparin activity.

concentration increased, the clotting time decreased to a minimum and then increased. The value of the salt concentration for this minimum for the various curves was approximately the same. The average figure of a number of experiments was 0.5 %. To make it possible to examine the data more fully, they have been replotted in Fig. 2 (2). Clotting time is plotted against heparin concentration and a series of curves are obtained, the slope of any one of which gives a measure of the heparin activity for that particular salt concentration. It can be seen that on dialysis the heparin activity has been increased. With 0.4 unit of heparin, for example, the clotting time has been increased from 30 to 80 sec. and this appears to be a direct effect on heparin as the clotting time with thrombin alone has not changed. When NaCl was added to the dialysed plasma, however, the action of heparin was almost completely neutralized. This is in contrast to the action of NaCl on thrombin alone (Fig. 2 (1)). NaCl added to dialysed plasma first increases the activity of thrombin (lowers the clotting time) and then decreases it, i.e. traces of NaCl have a coagulant action whilst larger amounts have an anticoagulant action. At all salt concentrations, however, the NaCl tends to neutralize the action of heparin. Only in the case of 1.5 % NaCl does the heparin

show any activity and this may be wholly due to the fact that the thrombin activity has by then been greatly reduced (clotting time for zero heparin has been doubled). None of the heparin curves with added salt show any resemblance to those obtained with either the original plasma or the dialysed plasma. It should be observed that all the above results were obtained with one sample of dialysed plasma. The experiment has been repeated a number of times with the same results, using plasmas from different horses. It appears from these results that NaCl decreases the activity of heparin. This can only be observed at very low concentrations of heparin and salt (i.e. highly dialysed plasma). At higher concentrations the clotting time increases so rapidly with an increase in the concentration of either that it is not possible to detect the much smaller effect on the activity of the heparin due to the presence of the salt. Thus, with undialysed plasma or plasma which has been dialysed only a short time, the addition of more NaCl to that already present causes a marked increase in clotting time and disguises the direct effect of the salt on the heparin.

The effect of plasma proteins on the action of heparin

Howell concluded that heparin did not act as a simple antithrombin since it only acted as an antithrombin in plasma or blood but was inactive with fibrinogen solutions. This was confirmed in an experiment to test the purity of the fibrinogen. A fibrinogen solution and a sample of the original horse plasma were used, and increasing amounts of heparin were titrated against increasing amounts of thrombin, a fixed clotting time of 2 hr. at 25° being the end-point. Analyses for fibrin N, chloride and phosphate and pH determinations by the glass electrode showed the fibrinogen solution and the plasma to be identical in regard to these factors. It was observed, however, that while heparin is highly potent when plasma is used for testing its potency, it is almost completely inactive when a solution of fibrinogen is substituted for the plasma. Addition of dialysed plasma to the fibrinogen solution restores the activity of heparin.

The nature of the plasma protein factor involved was studied by a fractional precipitation of the plasma with $(\text{NH}_4)_2\text{SO}_4$. 25 ml. portions of plasma were precipitated with $(\text{NH}_4)_2\text{SO}_4$, centrifuged and the precipitates dissolved in 25 ml. of physiological saline. Their potency was then tested by determining the amount it was necessary to add to 1 unit of heparin in order to keep a clotting mixture fluid for 2 hr.—0.5 ml. of fibrinogen solution + 0.02 ml. of thrombin + 1.0 unit of heparin + saline to 1 ml. were taken for the test. Without plasma this mixture clotted in 10 min. The results are shown in Table 1. The protein

Table 1

Fraction	ml. required for heparin end-point	Potency as % of plasma	Antithrombin potency— clotting time min.
Plasma	0.005	—	25
Precipitated by $\frac{1}{2}$ sat. $(\text{NH}_4)_2\text{SO}_4$	> 0.50	0	10
Precipitated by $\frac{1}{4}$ sat. $(\text{NH}_4)_2\text{SO}_4$	0.06	7	10
Full sat. $(\text{NH}_4)_2\text{SO}_4$	0.005	100	25
Crystalline serum albumin	> 0.5	0	10

The serum albumin was crystallized from 250 ml. horse plasma and dissolved in 25 ml. of saline. The solution contained 3.82 mg. protein N per ml.

factor necessary for heparin activity is found in the albumin fraction, but is not associated with crystalline albumin. Quick [1938] has identified the protein factor with the normal antithrombin of plasma. The antithrombin potencies of

the various fractions are also shown in Table 1. The thrombin + 0.1 ml. of the protein solution were mixed and allowed to stand in the ice-box overnight. 0.5 ml. of fibrinogen was then added and the time taken to clot was noted. It can be observed that the antithrombin potency is also found in the albumin fraction but not in the solution of crystalline serum albumin.

DISCUSSION

The results reported here indicate that the presence of NaCl decreases the anticoagulant action of heparin. It is evident, however, that usually this action is overshadowed by the direct anticoagulant action of sodium chloride as observed by Mellanby. It is also shown that heparin only acts as an antithrombin in the presence of plasma protein. There are two possible explanations for this fact. Either heparin is the anticoagulant but is only active when combined with protein, or the protein is the anticoagulant and heparin acts by combining with it and enhancing its activity. The latter hypothesis has been advanced by Quick. Quick suggests that heparin acts by combining with the normal antithrombin of serum. This so increases the affinity of the normal antithrombin for thrombin that it competes with fibrinogen for the thrombin, thus becoming an anticoagulant. The results reported here are in agreement with those of Quick although the low activity of the plasma antithrombin renders difficult its assay and an adequate testing of the hypothesis. Quick has suggested the term "heparin antithrombin" for the combined anticoagulant. Further evidence in favour of Quick's hypothesis is the work of Fischer on the union of heparin with proteins. He found that, while heparin combines with a large number of proteins under suitable conditions (on the acid side of the isoelectric point of the protein), the resulting heparin-protein compound is inactive provided that it is stable at the pH of the assay. We have obtained results similar to those of Fischer.

Howell, in his original paper on the subject, showed that the protein was destroyed by heating to 70°. Howell thought it was a eugloblin, Quick thinks that it is the serum albumin. From the results reported, it is seen that the substance is found in the albumin fraction of plasma but is not crystalline albumin. Provided that the reaction of heparin with this protein is similar to that of heparin with other proteins, as described by Fischer, it is probable that its isoelectric point is more alkaline than the pH of plasma.

Little is known of the resulting heparin antithrombin. Howell concluded that heparin antithrombin is different from serum antithrombin as the latter is destroyed by heat and as no metathrombin develops in heparinized serum. Schmitz & Kühl [1935], however, observed destruction of heparin antithrombin in serum at 56°. We have found that it is precipitated by saturated Na_2SO_4 and $(\text{NH}_4)_2\text{SO}_4$. The formation of heparin antithrombin is not instantaneous but involves a considerable period of time after the addition of the heparin to the blood. This was observed by Quick who found that the apparent potency of heparin in blood *in vitro* increased on standing. The same phenomenon can be observed *in vivo*.

SUMMARY

1. Prolonged dialysis of plasma has little effect on the activity of heparin. Addition of sodium chloride to this plasma decreases the activity of the heparin.
2. The results of Howell which indicated that heparin is only active in the presence of a protein in plasma are confirmed. The protein is found in the albumin fraction but is not crystalline serum albumin.
3. These results are in agreement with Quick's view that heparin acts by combining with and enhancing the activity of the normal plasma antithrombin.

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