ANTITHROMBOPLASTIN: THE DEGENERATION OF INTRINSIC THROMBOPLASTIN IN NORMAL SERUM

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It is well known that a powerful thromboplastin is generated during blood coagulation, and a high titre of thromboplastin can be demonstrated in specimens of fresh serum. Its generation may be observed by means of the thromboplastin generation test (Biggs and Douglas, 1953) when a mixture of antithaemophilic globulin, Christmas factor, platelets, factor V, and factor VII is recalcified. If this test is prolonged beyond the usual six-minute incubation period, it will be noticed that the thromboplastin titre rapidly falls; a mixture, which after five minutes' incubation coagulates the substrate plasma in 10 seconds, may take well over 40 seconds to clot the same plasma after another 10 minutes' incubation. Although this degeneration of thromboplastin must have been noticed by all who have performed the test, there appears to have been little research into the mechanism of its removal. There would seem to be two possibilities: either thromboplastin deteriorates by virtue of its own physical instability, or it is destroyed by a specific antithromboplastin which may be present in plasma or serum.

Tocantins has postulated the existence of an antithromboplastin, by which he means an inhibitor of thromboplastin formation (1954). Similarly, circulating anticoagulants, described by Lawrence and Johnson (1942), Munro (1946), Fantl and Nance (1946), Biggs and Macfarlane (1953), Hardisty (1954), Hougie and Fearnley (1954), and many other workers, have no action on formed thromboplastin, and are more correctly described as antithromboplatinogens.

During the course of work at this laboratory, the ability of serum to destroy brain thromboplastin was observed, and it has been postulated that there is present in serum an antithromboplastin, the function of which is to remove tissue products and extrinsic thromboplastin from the circulating blood (Berry, 1957). Following these experiments a simple test was devised to follow the rate of degeneration of naturally-produced intrinsic thromboplastin, liberated during coagulation in specimens of fresh serum. This test was used in an endeavour to confirm the existence of an antithromboplastin in normal serum. The technique of the test and the results obtained are described in the present paper.

Reagents

The specimens of blood examined were obtained by venipuncture from members of the laboratory staff, and from surgical patients shortly after their admission to hospital and at intervals after operation. Blood obtained after a difficult venipuncture (whenever there is excessive tissue trauma, or whenever there is possible platelet destruction during a slow collection) is unsuitable for the test.

Physiological calcium-fibrinogen-saline was prepared for use as a diluent. This consisted of normal saline containing 10 mg. of calcium chloride and 200 mg. of fibrinogen per 100 ml.

Fibrinogen was supplied by the Lister Institute. Each ampoule was made up with 10 ml. of distilled water, and subsequently diluted to give a concentration of 200 mg. %.

To avoid further dilution of serum specimens, a set of glass tubes was prepared, each of which contained the dehydrated equivalent of 0.2 ml. of M/40 calcium chloride.

Technique of the Test

Blood, 15 ml., is collected, 10 ml. of which is citrated (0.6 ml. citrate/5 ml. blood) and received into siliconed glass tubes. The remaining 5 ml. is left uncitrated in another siliconed tube. All specimens are immediately placed in a beaker of melting ice which is taken to the patient's bedside.

As soon as possible after venipuncture all specimens are centrifuged at 1,000 r.p.m. for five minutes. The uncitrated specimen of plasma is then transferred (using a siliconed pipette) to another siliconed tube and returned to the beaker of melting ice. It will remain unclotted long enough for the completion of the other preparations for the test.
The platelet-rich supernatant plasma from the citrated blood is centrifuged for a further 15 minutes at 3,000 r.p.m. The resulting platelet-free supernatant is used as the substrate plasma during the test. This specimen is returned to the beaker of melting ice, and it will remain at a temperature of 0° C. during all subsequent procedures.

In a rack in the 37° water-bath are placed a number of tubes containing dehydrated calcium chloride. When these preparations have been completed, 20%, 10%, and 1% dilutions of the uncitrated specimen of plasma are prepared in non-siliconed tubes, using the calcium-fibrinogen-saline as the diluent. The remaining undiluted plasma and the three dilutions are then transferred to the water-bath, each one being thoroughly agitated using a wooden applicator stick, where they will coagulate. The clotting times are observed, and also the time when each one clotted. Ten minutes after coagulation the fibrin is removed from each tube, the contained serum being expressed. The more dilute specimens may show a continued deposition of fibrin, and this is removed as it forms.

At intervals of 15, 30, 45, 60, 90, 120, and 180 minutes after coagulation, specimens from each dilution are examined for ability to promote coagulation of the substrate plasma as follows: 0.2 ml. of the serum is transferred to one of the tubes containing calcium chloride, and the tube is agitated to ensure complete solution of the calcium. Then 0.2 ml. of substrate plasma is added to the contents of this tube, and the clotting time of the mixture is noted. The serum is added to the calcium chloride immediately before each clotting time is to be performed.

In order to determine the effect of residual thrombin which may be present in the serum specimens, a parallel series of clotting times was performed, the procedure being the same as that described above, but using 0.2 ml. of fibrinogen solution in place of the substrate plasma.

By using saline instead of serum, the calcium clotting time of the substrate plasma was determined in every case.

Results

The clotting times of the uncitrated plasma, undiluted, after transferring from melting ice to the water-bath, varied from 200 to 38 seconds in 38 cases investigated. Dilution of the plasma with calcium fibrinogen saline did not result in a very great increase in the clotting time; the difference between clotting times of the 100% and 1% specimens was never greater than five minutes. Omission of calcium chloride from the mixture leads to considerable prolongation, however, some specimens taking as long as one hour before deposition of fibrin was observed.

The calcium clotting times of substrate plasma from these 38 cases varied from 150 to 344 seconds.

The clotting times of the substrate plasma when added to 15-minute-old serum varied from 25 to 62 seconds, the average time being 40 seconds. With increasing age of the serum, the plasma clotting time also increased, until eventually the calcium clotting time figure was reached. Specimens of serum over two hours old were able to bring about little or no shortening of the substrate plasma clotting time. These results are shown in Fig. 1.

Dilution of the uncitrated specimens before coagulation did not greatly affect the ability of fresh (15 minute) serum to reduce the clotting time of the substrate plasma. The difference between the "clotting activity" of undiluted specimens and the 1% dilutions, represented in terms of clotting time, was never more than 50 seconds. Whereas, however, the undiluted serum rapidly lost its ability to shorten the clotting time of substrate plasma. This deterioration was far slower in the 20% dilution. The 10% and 1% specimens showed little loss of potency even after several hours' incubation (Fig. 2).

Repeating the investigations, with fibrinogen solution in place of the substrate plasma, gave an indication of the part played by thrombin in producing these results. It was observed that, even though fibrinogen clotting times were always greater than the corresponding substrate plasma
This residual clotting times, serum specimens still contained a significant amount of thrombin up to 45 minutes after coagulation. Older specimens of serum contained little if any thrombin, however, and the fibrinogen clotting times of serum specimens older than 45 minutes were almost invariably greater than 10 minutes.

**Interpretation of Results and Discussion**

A fresh specimen of serum may contain unconverted prothrombin, thrombin, and factor VII, in addition to the thromboplastin generated during coagulation. Each of these may be concerned in the coagulation of the substrate plasma. In the absence of platelets in the substrate, it is believed that Christmas factor in the serum will not be involved, and can be ignored when interpreting the results of the test.

In a normal specimen, having a normal rate of prothrombin conversion, it is believed that the amount of prothrombin remaining in a 15-minute-old specimen of serum will be insignificant compared with the amount in the substrate plasma. This residual prothrombin will have little effect in determining the plasma clotting time.

Thrombin is rapidly removed from serum after coagulation, by taking part in the reaction converting fibrinogen to fibrin, by adsorption on to the fibrin clot, and by the action of antithrombin. It has been demonstrated that following the recalcification of a citrated specimen of plasma, most of the thrombin has disappeared from the resulting serum four minutes after clotting (Biggs and Macfarlane, 1953; Berry, 1956). It was assumed for the purpose of the present investigations that thrombin disappeared with equal rapidity after the coagulation of whole blood (or uncitratated plasma). To encourage this, the fibrin clot was allowed to remain undisturbed for 10 minutes before the serum was expressed from it and the clot removed, in order that as much thrombin as possible might be adsorbed.

It was observed, however, when parallel fibrinogen clotting times were performed, that significant amounts of thrombin could be demonstrated in the serum as long as 45 minutes after coagulation. The thrombin persisted for the longest time in the diluted specimens, presumably a result of delayed prothrombin conversion, and of dilution of antithrombin. When interpreting the results, therefore, it was considered that the earlier readings (represented in section A of Fig. 2) were produced by the combined effect of thrombin and thromboplastin, and only those readings from older serum specimens (section B, Fig. 2) could be expected to reflect thromboplastin activity alone.

In the absence of platelets there can be no liberation of intrinsic thromboplastin in the substrate plasma itself. It is thought that even the most carefully collected specimen of blood must contain traces of tissue fluid; for this reason specimens were collected and stored in melting ice to discourage the production of extrinsic thromboplastin in the substrate plasma.

The results of these experiments have shown that fresh serum (up to three hours after coagulation) has coagulant properties, and that with increasing age the serum loses this ability to clot the substrate plasma. The shortening of the plasma clotting times is greater than can be explained by the presence of prothrombin or thrombin in the serum, and must depend largely on the thromboplastin content of the serum. A study of the results as shown in Fig. 2, and especially those results in section B of the graph, suggests that this thromboplastin rapidly degenerates, and that the rate of degeneration varies with the dilution of the serum, being most rapid in the undiluted specimens. In fact the most dilute specimens showed little degeneration over a period of three hours.

If thromboplastin is a labile substance, and disappears from serum because of its lability, one would expect the rate of degeneration to be constant in all dilutions of serum. The varying rates
of degeneration shown by these experiments are in favour of the existence of an antithromboplastin in normal serum.

Summary

Investigations into the rate of degeneration of intrinsic thromboplastin in fresh serum are described.

It has been shown that the rate of thromboplastin degeneration varies with serum dilution, being more rapid in the more concentrated specimens.

It is suggested that these results can only be explained by postulating the existence of a specific antithromboplastin in normal serum.

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References