PREPARATIONS OF PLASMINOGEN-DEFICIENT FIBRINOGEN AND THROMBIN

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In view of developing clinical applications of plasmin, plasminogen, and activators of the latter, there is a critical need for reproducible methods yielding fibrinogen and thrombin suitable for accurate measurements of fibrinolytic materials. The presence of contaminating plasminogen in clots used for fibrinolytic assays has also been responsible for frequent misinterpretations of biochemical experiments in this field. We have found that modifications of the charcoal treatment of fibrinogen mentioned by Lüscher and Käser-Glanzmann (1961), together with proper selection and processing of fractions from the chromatography of thrombin on Amberlite IRC-50, enable the formation of fibrin clots which are highly resistant to the action of urokinase and streptokinase.

FIBRINOGEN. Fibrinogen was isolated from frozen bovine or human plasma as described by Ware, et al. (1947) up to the final cold saline washing. The protein was then dissolved to a concentration of 15 mg./ml. in 0.22 M tris(hydroxymethyl)aminomethane.HCl containing 0.9% NaCl, pH 7.4, and Darco G-60 was added at the level of 5 gm./100 ml. After 1 hour of gentle stirring at 25°C, the charcoal was removed by centrifugation and filtration. There was usually a loss of about 25% of the protein in this treatment. The fibrinogen may be stored frozen, or lyophilized. The dried material is sometimes difficultly soluble, but apparently suffers no change in pertinent characteristics. The protein component was not less than 90% clottable by thrombin.

Higher concentrations of protein, or lower levels of charcoal were found to be generally unsuccessful in achieving fibrinogen of the desired quality; occasionally it was necessary to repeat the procedure, especially in the case of human fibrinogen. Commercially available human Cohn Fraction I, or human fibrinogen isolated by ammonium sulfate fractionation of plasma, have thus far proved less suitable as starting materials.

THROMBIN. This purification was based on the observations of Rasmussen (1955), with modification for larger scale processing and adequate recovery of the thrombin in a convenient form for use. All glassware was silicone-coated to prevent losses by adsorption. Sixty 5,000-unit vials of Thrombin Topical (Parke, Davis), for example, were applied to an 8.5 cm. x 21 cm. column of Amberlite CG-50, type 2 (Fisher Chemical Co.) in 0.05 M sodium phosphate, pH 7.0. Development was with 0.3M phosphate, pH 8.0; about 200 fractions of 30 ml. were collected at a flow rate of about 150 ml. per hour. Fractions with specific activities greater than 500 units/mg., assayed by the method of Ware and Seegers (1949), were desalted on Sephadex G-50 columns and lyophilized. There may be overall losses in this procedure amounting to about 50% of the original thrombin unitage, but the yield is practical for analytical purposes.

Electrophoretic studies of the above preparations, using the Arronson-Grönwal (1958) buffer and cellulose acetate membranes, indicated complete removal of one of two (bovine) or three (human) components observable in the original fibrinogen, and substantial decrease of 3 of 4 components of Thrombin Topical. Further investigation of the electrophoretic fractions is in progress.

FIBRINOLYTIC STUDIES. All materials were dissolved in the above tris(hydroxymethyl)aminomethane- NaCl buffer. The clot mixture consisted of 0.3% fibrinogen with addition of various levels of thrombin and urokinase or streptokinase in a total volume of 0.5 ml. Lysis time at 37° C was determined by free flow of the tube contents on tilting. The human urokinase used had an activity of 5,300 units/mg. (Ploug and Kjeldaard, 1957) and was supplied by Leo Pharmaceutical Products, Copenhagen. Typical results with a bovine fibrinogen preparation are shown in Table 1; similar results may be obtained with human fibrinogen. Optimum streptokinase (Varidase, Lederle) levels for lysis of human unpurified fibrin clots in a few minutes had no effect on clots made from the treated material in more than 48 hours, nor did any other level tested over a range of 2 to 1000 units/ml.

DISCUSSION. It is apparent that the procedure as described does not completely remove potential lytic activity from the thrombin, but the results are more than adequate for assay purposes. Thrombin fractions of greater purity may be selected, if desired, with further sacrifice of yield. Susceptibility of the clots to lysis by chloroform-activated bovine plasmin was not significantly modified by the charcoal treatment.

TABLE I.

THROMBIN TYPE	THROMBIN LEVEL Units/ml.	FIBRINOGEN TYPE	UROKINASE LEVEL Units/ml.	LYSIS TIME
Topical	5	Original	50	9 Min.
Topical	5	C-Treated	500	42 Min.
Topical	50	C-Treated	500	15 Min.
Chromatographed	5	Original	50	9 Min.
Chromatographed	5	C-Treated	500	>19 Hr.
Chromatographed	50	C-Treated	500	ca. 5 Hr.
Chromatographed	5	C-Treated	1000	ca. 3 Hr.

There is, of course, the possibility that alterations of the proteins other than decrease of plasminogen contamination could be involved. It is of interest that the initial protein effluent from the Amberlite columns is rich in plasminogen. It can also be shown that charcoal removes plasminogen quantitatively from solutions of bovine euglobulin. Finally, plasminogen added to purified fibrinogen could be removed by a subsequent charcoal treatment.

In our experience, these preparations have been the most useful and reproducible in the development of unequivocal fibrinolytic assays of plasminogen and mixtures of urokinase and plasmin, and they should facilitate more clearly interpretable investigations of the activation of plasminogen. It would also appear that plasminogen-low human fibrinogen may be of value in the treatment of patients with hypofibrinogenemia due to an excessive level of circulating plasminogen activators.

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