1765

BLOOD FIBRINOLYSIS

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STELLINGEN

I

De meeste klinische studies op het gebied van de bloedfibrinolyse behoeven herevaluatie vanwege het gebruik van onjuist gebleken werkhypothesen en inadequate meetmethoden.

II

Alvorens studies met gezuiverde komponenten over molekulaire mechanismen van kontaktaktivatie zinvol kunnen worden voortgezet, dient de verschijningsvorm van die komponenten in bloed te worden vastgesteld.

J.H. Griffin: Thrombosis and Haemostasis 38 (1977) 117.
R.J. Mandle, R.W. Colman en A.P. Kaplan: Proc. Nat. Acad. Sci. USA 73 (1976) 4179-4183.

III

Als de modellen voor humaan serum HDL, zoals voorgesteld door Verdery en Nichols juist zijn, is dat ondanks de door hun uitgevoerde berekeningen.

R.B. Verdery en A.V. Nichols: Chem. Phys. of Lipids 14 (1975) 123-134.

IV

Ten onrechte verwerpen Yu en Yu de authenticiteit van de door Downer e.a. gevonden derde grote hydrofobe subunit van cytochroom c oxidase uit runderhart.

C.A. Yu en L. Yu: Biochim. Biophys. Acta 495 (1977) 248-259.
N.W. Downer, N.C. Robinson en R.A. Capaldi: Biochemistry 15 (1976) 2930-2936.

V

Het is misleidend synthetische substraten voor proteolytische enzymen aan te duiden met de naam van een enzym.

E. Amundsen en L. Svensen. In: New Methods for the Analysis of Coagulation using chromogenic substrates (Ed. I. Witt), de Gruyter, Berlin (1977) p 211-220.

VI

De betekenis van plasma kallikreine als plasminogeenaktivator is gering.

VII

Doseringen bij infusietherapie voor patienten met hemofilie dienen gebaseerd te zijn op halfwaardetijden in plasma van de betreffende stollingsfaktor. De meeste literatuurgegevens over deze waarden zijn echter fout.

W.Th. Hermens. In: Handbook of Hemophilia (Eds. K.M. Brinkhous en H.C. Hemker), Excerpta Medica, Amsterdam (1975) p 569-589.

VIII

Het is niet waarschijnlijk dat de hoeveelheid circulerend anodaal antigeen bij Schistosoma mansoni infekties een nauwkeurige maat is voor de wormlast van de gastheer.

M.P. Bawden en T.H. Weller: Am. J. Trop. Med. Hyg. 23 (1974) 1077-1084.

IX

Voor het meten van de aktiviteit van extrinsieke plasminogeenaktivatoren in bloed verdienen de technieken met fibrine als substraat nog de voorkeur boven die met synthetische chromogene substraten.

H. Stormorken: Thrombosis and Haemostasis 36 (1976) 299-301.

X

Het rijksvaccinatieprogramma dient te worden uitgebreid met vaccinatie tegen bacteriële meningitis-verwekkers zoals Neisseria meningitidis, Haemophilus influenzae en Diplococcus pneumoniae.

XI

Het is gewenst dat exploitanten van legbatterijen eieren voor hun geld kiezen.

XII

De voortschrijdende ontkoppeling tussen lichaamsbeweging en werk betekent voor verstandige mensen een vermindering van de vrijheid vrije tijd te besteden.

XIII

Dat het bloed ons van schrik in de aderen zou kunnen stollen moet onwaarschijnlijk worden geacht.

C. Kluft 22 februari 1978

BLOOD FIBRINOLYSIS

PROACTIVATORS AND ACTIVATORS IN HUMAN PLASMA

by

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Drawings : Mr. J. J. Nieboer

to my parents
to Nini and the children

CONTENTS

Fibrin formed in the body by coagulation of fibrinogen has a temporary function or is part of a pathological process and should again disappear. This disappearance is due to the capacity of compounds present in the organism to break down the fibrin matrix into soluble components: a process called fibrinolysis.

The serine protease plasmin is an important agent in the proteolytic degradation of the network of fibrin. Plasmin is generated from a precursor in plasma, plasminogen, by a process catalyzed by activators (scheme, Fig. 1).

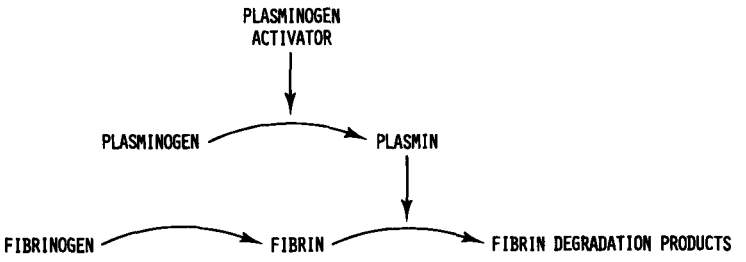

Fig. 1. Scheme for plasma fibrinolysis.

Mechanism of fibrinolysis

Initiation of fibrinolysis appears to take place preferentially at the surface of fibrin. The mechanism of plasminogen activation has been more extensively studied for only two activators: human urinary activator (urokinase) and a porcine tissue activator. Urokinase is only weakly adsorbed to fibrin, while, in contrast, the tissue activator is strongly bound to fibrin and its effect on plasminogen is greatly stimulated by the presence of fibrin (Thorsen et al., 1972; Robbins et al., 1975; Wallen, 1977).
Native plasminogen (with glutamic acid as NH_2-terminal amino acid) has a weak affinity for fibrin, whereas the modified form of plasminogen (resulting from limited proteolysis of the NH_2-terminal end and with lysine as the NH_2-terminal amino acid) has a much stronger affinity (Thorsen, 1975); (for reviews on plasminogen, see Collen and Verstraete, 1975; Collen and De Maeyer, 1975). These interactions with fibrin are related to the presence of a lysine-binding site in the plasminogen molecule (Wiman and Wallen, 1977).

The plasmin formed accumulates on fibrin (Celander and Guest, 1957). The active site of plasmin which is located in the light chain (Summaria et al., 1967) as well as the lysine-binding sites on the heavy chain (Wiman and Wallen, 1977) are probably involved in this selective accumulation on fibrin. This accumulation assures that plasmin is primarily involved in the proteolysis of fibrin. In the absence of fibrin, plasmin is rapidly inactivated by inhibitors in plasma, so that other proteolytic effects are reduced. Otherwise, plasmin has a remarkably broad specificity (reviewed by Christman et al., 1977). The α_2-antiplasmin is the most effective plasmin inhibitor, with α_2-macroglobulin as a powerful second (Collen, 1976; Aoki et al., 1977).

PLASMINOGEN ACTIVATORS AND PROACTIVATORS IN PLASMA

Plasminogen activators are widely distributed in the organism. They are presently pragmatically subdivided into groups according to their source of origin: circulating plasminogen activators (blood), tissue plasminogen activators, urinary plasminogen activators (urokinase) and tissue culture plasminogen activators. Knowledge of their molecular biology is still fragmentary and the possible structural identities of several of the activators in these arbitrary groups are still mostly undetermined.

The circulating plasminogen activators (restricted to cell-free plasma of blood) can be divided into activators of intrinsic or of extrinsic origin. Activators generated in the plasma from circulating proactivators are designated as intrinsic activators to distinguish them from the extrinsic activators released into the blood plasma from cells, presumably mainly the vascular endothelial cells. The significance of this classification is substantiated by the recent finding that it parallels the functional division of activator activity in plasma into a part which is susceptible to inhibition by C1-inactivator and a part which is resistant to this inhibitor (Kluft, 1977a, 1978).

a) *INTRINSIC* activation by a factor XII-dependent pathway

The occurrence of Hageman factor (factor XII)-dependent fibrinolysis in plasma was recognized in 1959 by Niewiarowski and Prou-Wartelle. The fibrinolytic activity produced by surface exposure was assumed to result from an interaction of surface activated Hageman factor with a hypothetical precursor of a plasminogen activator (Iatridis and Ferguson, 1962). The corresponding cascade type of mechanism is shown in figure 2. The early steps in this cascade, which concern the activation and participation of factor XII, have been

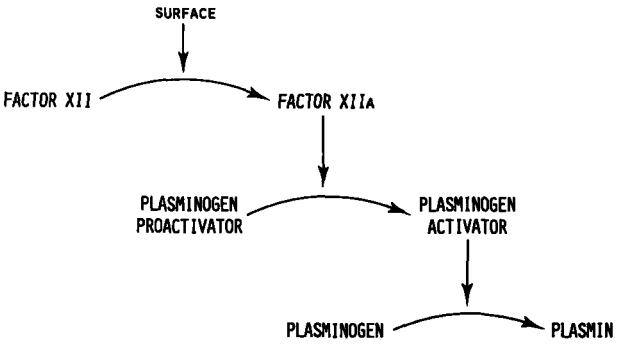

Fig. 2. Tentative scheme for the factor XII-dependent pathway of fibrinolysis.

the subject of many studies in recent years (reviews by Griffin et al., 1976 and Kaplan et al., 1976). The formation of surface-bound agglomarates of several proteins appeared to be involved. Solid surfaces like that of glass, kaolin, collagen or celite or soluble high molecular weight acid polysaccharides such as dextran sulphate serve as a rendezvous. The adsorbed factor XII and a complex of high molecular weight (HMW) kininogen with prekallikrein (Mandle et al., 1976) undergo limited proteolysis during the intimate contact on the surface which results in the production of the following activation products: factor XIIa in the form of a surface-bound and a soluble fragment (Revak and Cochrane, 1976; Revak et al., 1977), bradykinin and a soluble complex of kinin-free HMW kininogen with kallikrein (Trumpi et al., 1977). The activation is less effective in the liquid phase, where it is quenched by inhibitors.

Among the activation products generated in this early step, kallikrein is capable of activating plasminogen (Colman 1969; Bouma and Griffin, 1977). Kallikrein can therefore be included as a plasminogen activator in the scheme of factor XII-dependent fibrinolysis (Fig. 2). The major activity performed by the factor XII-dependent pathway, however, originates from a different precursor, which appears to be activated at a later stage of the cascade (Kluft, 1978).

Ogston et al. (1969) described an activating agent for which they used the term Hageman factor cofactor. The nature of this component and its position in the mechanism of activation remains unclear. While Ogston et al. (1969,1976) found it unable to induce activation of plasminogen, other investigators

(Laake and Venneröd, 1974; Kluft, 1978) have reported on the presence of plasminogen activator activity in preparations of the Hageman factor cofactor.

b) *INTRINSIC* activation by a factor XII-independent pathway

The existence of a factor XII-independent intrinsic pathway of fibrinolysis in plasma has only recently been appreciated. Originally, its possible existence was thought likely from studies on activation of factor XII-deficient plasma, in which nearly normal levels of fibrinolysis are often generated. In retrospect, this was seen as early as 1959 by Niewiarowski and Prou-Wartelle. In 1974, it was reconsidered and recognized by Astrup and Rosa and independently by Schreiber and Austen using different methods of activity generation. About 50% of the intrinsic activator potential seems to be generated by a process independent of factor XII (Kluft, 1978).
Recent experiments indicate that different activators are involved in factor XII-dependent and independent pathways (Kluft, 1978), although they might originate from a common precursor.

c) *EXTRINSIC* plasminogen activators in plasma

Several types of cells, such as endothelial cells, mesothelial cells, cells covering the synovial lining, surface epithelial cells, erythrocytes and leucocytes, are reported to contain plasminogen activator (reviewed by Astrup, 1975). Generally, the extrinsic activator(s) in plasma are assumed to originate from the vascular endothelium and to be related to the activator released by postmortem perfusion of vessels such as described by Aoki and Von Kaulla (1971). The presence and the fluctuations of the extrinsic activator in plasma have been studied mainly by nonspecific assays for fibrinolysis in blood. These studies have revealed the existence of characteristic physiological fluctuations and properties of plasminogen activators in plasma. Briefly, the following characteristics are reported:

a) There is a continuous low level of activity in normal morning plasma which increases moderately during the day, indicating a diurnal fluctuation in activity (Rosing et al., 1973).
b) The activities in plasma rise abruptly after a variety of stimuli such as venous occlusion, strenous exercise, emotional stress, surgical operations or the administration of vasoactive substances (inactive in vitro) such as catecholamines, nicotinic acid and vasopressin analogies (reviewed by Astrup, 1973,1977). The mechanism by which these rises are mediated are not clear (Åberg and Nilsson, 1975; Cash, 1975; Britton et al., 1976).
c) Elevated levels of activity in plasma decrease very rapidly, most likely

because of a clearance by the liver (half-life in the order of 15 min) (reviewed by Alkjaersig and Fletcher, 1977).

d) Increased levels of plasma activity do not produce significant fibrinogenolysis or activation of plasminogen unless fibrin is present. (Collen and Verstraete, 1976; Gurewich et al., 1974,1975).

e) The activator in plasma is strongly adsorbed to the fibrin when plasma is clotted.

Most of these characteristics (a-c,e) hold for the C1-inactivator-resistant activator present in euglobulin fractions (Kluft, 1977b,1978). Thus, the C1--inactivator-resistant activator activity is very low in morning plasma but increases tremendously by a factor of 50 to 100 after 15 min of venous stasis (Kluft, 1978).

Ogston et al. (1976) published on characteristics of an activator specifically seen in plasma after venous occlusion. This activator appeared in a high molecular weight form which was dissociable at high ionic strength.

An inventory of plasminogen activators in plasma is briefly given in figure 3.

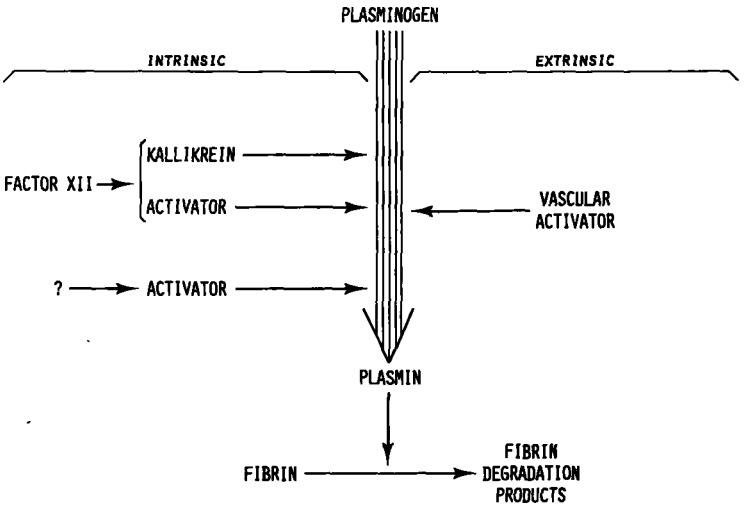

Fig. 3. Schematic inventory of plasminogen activators in plasma.

Methods of assay

The determination of levels of plasma or blood fibrinolytic activity has been usually done by methods which reduce the potent antifibrinolytic potential, which prevents the registration of activity in undisturbed whole plasma or blood (Graeff and Beller, 1971; Fearnley, 1973). One approach is by means

of dilution to reduce the effect of inhibitors (dilute blood or plasma clot lysis time methods). This method presumably provides an overall estimate of plasma fibrinolysis. Another approach is based on an isoelectric precipitation of plasma, by which most of the inhibitors remain in solution, while the fibrinolytically active components precipitate (euglobulin precipitate). The determination of the fibrinolytic activity of the redissolved euglobulin precipitates can be performed by clot lysis techniques or by evaluation of the fibrinolytic effect on preformed fibrin, such as in the fibrin plate assay. The fibrinolytic activity of the euglobulin precipitates is sometimes assumed to provide a measure of the level of extrinsic activator in plasma. However, no direct assay of different plasminogen activators or proactivators in plasma was available.

AIM AND SCOPE OF THE PRESENT INVESTIGATION

The present investigation concerns blood fibrinolysis as it is observed in euglobulin fractions of platelet-poor plasma. Several observations made in this system have attracted attention. These observations included an abnormally low diurnal increase in patients with type IV hyperlipoproteinemia, in many subjects with coronary artery disease and in a high percentage of older subjects (Rosing et al., 1973), an altered fibrinolytic response to exercise in patients with type IV hyperlipoproteinemia (Epstein et al., 1970), an abnormal plasma fibrinolysis related to increased inhibition of tissue plasminogen activator in thrombotic disease (Brakman et al., 1966; Astrup and Brakman, 1970), and a high incidence of abnormally weak fibrinolytic responses to venous occlusion in patients with recurrent venous thrombosis (Nilsson, 1975).

It was realized that the components involved in the fibrinolytic activity of the euglobulin fraction were not identified. This uncertainty seriously hampered the evaluation of the pathophysiological significance of these observations of fluctuations in degree of fibrinolytic activity by this method. The characterization of the active fibrinolytic components involved in these fluctuations was therefore pertinent to the research program on arteriosclerosis and thrombosis of the Gaubius Institute.

Two intertwined problems had to be faced, namely, the lack of specific methods of assay for known fibrinolytic factors and the deficiency in knowledge about the composition of the plasma fibrinolytic system. The study was started on the basis of a standardized euglobulin fractionation method, where the fibrinolytic activity of the fraction was assayed on fibrin plates (described in

detail by Kluft et al., 1976). In preliminary experiments, it was observed that the fibrinolytic response of these euglobulin fractions on fibrin plates did not resemble that of purified proteases (plasmin, chymotrypsin, trypsin, papain) or purified plasminogen activators (urokinase, porcine tissue activator).

Euglobulin fibrinolytic response on fibrin plates was characterized by a long time lag in the appearance of lysis in the plates (Fig. 4). Obviously, a euglobulin fraction was not simply an activator solution. This is further substantiated in the chapters of this thesis.

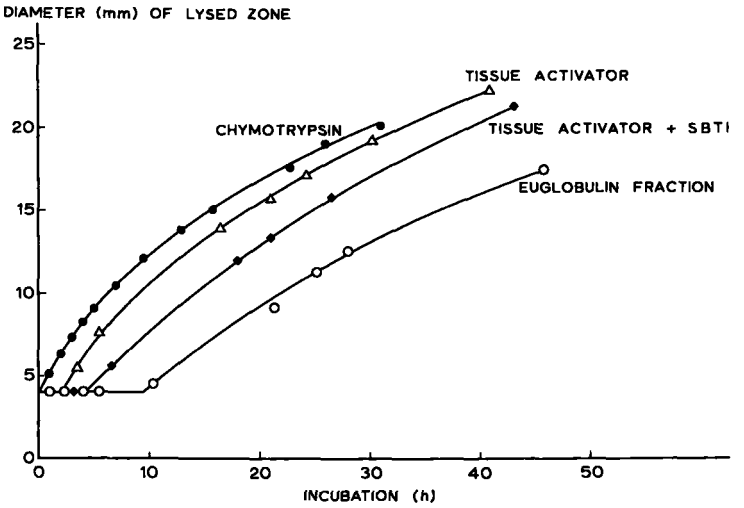

Fig. 4. Appearance of a time lag in fibrinolysis on fibrin plates. Diameters of lysed zones were read after incubation of plasminogen-rich bovine fibrin plates at 37°C for various time periods: chymotrypsin 3 µg/ml (●); tissue activator 18 U/ml (△); tissue activator (18 U/ml) mixed with 5 µg/ml soya bean trypsin inhibitor (SBTI) (◆); euglobulin fraction prepared with plasma dilution 1:10 and pH 5.9 (o).

CHAPTER I deals with the question as to which plasma protease inhibitors were precipitated in the euglobulin fractions and whether they influence euglobulin fibrinolytic activity.

CHAPTER II describes a method for eliminating inhibitory influences in euglobulin fibrinolysis. When a method was devised to avoid inhibitory effects in the assessment of euglobulin fibrinolysis, further studies on active components in the fraction could proceed.

CHAPTER III considers questions of activation of proactivators and recovery of activators and proactivators in euglobulin fractions from plasma. Quantitative assay of the total activator content is also described. A surprisingly large amount of activator appeared to be present and a start was made to identify the contributing components.

CHAPTER IV deals with the contribution of extrinsic activator in the euglobulin plasma activator potential and how this contribution can be assessed separately by means of differential inhibition.

CHAPTERS V and VI concern the intrinsic systems of fibrinolysis. A new method for assay of the total prekallikrein and kallikrein in plasma is described. On the basis of this, the controversial role of kallikrein in intrinsic fibrinolysis was reevaluated.

The knowledge obtained on the components of fibrinolysis in plasma provided the basis for presenting a new inventory of plasminogen activators and proactivators in human plasma.

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CHAPTER I

OCCURRENCE OF C1 INACTIVATOR AND OTHER PROTEINASE INHIBITORS IN EUGLOBULIN FRACTIONS AND THEIR INFLUENCE ON FIBRINOLYTIC ACTIVITY*

C. Kluft

Abstract. Considerable amounts of C1 inactivator and inter-α-trypsin inhibitor precipitate during euglobulin fractionation of human plasma. The amount precipitated depends on the ionic strength and the pH during the fractionation procedure. In contrast, α_1-antitrypsin, α_2-macroglobulin and antithrombin III are present in euglobulin fractions in trace amounts only. The fibrinolytic activity of the euglobulin fractions is inhibited by the endogenous C1 inactivator, particularly as shown by comparison of normal and hereditary angioneurotic edema (HANE) plasma.

INTRODUCTION

Euglobulin fractionation of plasma is widely used in the study of plasma fibrinolytic activity. This is based on the fact that euglobulin fractions contain considerably less inhibitors of the fibrinolytic system than does plasma, allowing the detection of fibrinolytic activity normally suppressed by the inhibitors in plasma.

The method is used for screening purposes. A number of studies showing interesting changes in the fibrinolytic activity of euglobulin fractions have been reported. In these studies, it is assumed that the measured activity represents primarily the concentration of circulating plasminogen activator. It has not been established, however, what exactly is measured in euglobulin fractions in fibrinolytic assays. The interpretation of results requires further knowledge about the fractionation procedure and the precipitating components.

A few reports have mentioned the presence of traces of inhibitory material in euglobulin fractions derived from plasma. Kowalski et al. (18) detected traces of plasmin inhibition while Lauritsen (20) reported a mean of 9% plas-

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min inhibition relative to plasma. Gallimore et al. (9) provide evidence for a low level of fibrinolysis inhibition in euglobulin fractions, depending on the pH at which the fractionation was carried out. The presence of small amounts of inhibitors of plasminogen activation is assumed by Cucuianu (6) and claimed to be dependent on the plasma level of β-lipoproteins.

These reports do not relate the detected inhibition to a specific protein. It is difficult, therefore, to interpret these results.

The present study was undertaken to determine the presence of specific inhibitors in euglobulin fractions. The inhibitors assayed were α_1-antitrypsin, α_2-macroglobulin, C1 inactivator, inter-α-trypsin inhibitor and antithrombin III. These inhibitors are known to be active against components of the fibrinolytic system (13,14,27-29) and specific antisera against them are commercially available.

A number of modifications for the preparation of euglobulin fractions are commonly used. In these procedures, dilutions of plasma of 1:20 (5,23,24), 1:16 (5,9) and 1:10 (4,15) and pH values ranging from 5.2 to 6.0 are used. Because of these variables, the influence of fractionation conditions on the precipitation of inhibitors in euglobulin fractions was determined.

The fibrinolytic activity of euglobulin fractions might be influenced by the presence of inhibitors. A natural deficiency of C1 inactivator in HANE plasma permits the contribution of this inhibitor to be studied by comparing HANE and normal human plasma.

MATERIALS AND METHODS

Reagents. Unless otherwise specified, reagents were of analytical grade and were obtained from Merck (W. Germany). Microbiological grade gelatin was purchased from Merck and Agarose for electrophoresis from British Drug Houses. Distilled water was used throughout the experiments. Rabbit anti-human antisera against α_1-antitrypsin, α_2-macroglobulin, C1 inactivator, inter-α-trypsin inhibitor and antithrombin III were purchased from Behringwerke.

Barbital/HCl buffers. Buffer containing 0.05 M sodium diethylbarbiturate, 0.093 M NaCl, 1.66 mM $CaCl_2$ and 0.69 mM $MgCl_2$ was used to prepare fibrin plates. EDTA-gelatin-barbital-buffer contained 0.05 M sodium diethylbarbiturate, 0.10 M NaCl, 0.25% (w/v) gelatin and 0.1% disodium EDTA. The buffers were adjusted to pH 7.8 with an HCl solution.

Fibrinogen solution. Plasminogen-rich fibrinogen (batch H1) was prepared from bovine plasma by ammonium sulphate precipitation according to the method

of Brakman (3). The stock solution in water contained 1.65% (w/v) fibrinogen and 0.07 M ammonium sulphate and had a clottability of 82%. Bovine thrombin (EC 3.4.21.5), 5000 NIH units (Leo Pharmaceuticals, Denmark), was dissolved in 250 ml of saline (0.15 M NaCl) containing 0.25% (w/v) gelatin. This stock solution was kept frozen at -20°C.

C1 inactivator preparation. C1 inactivator preparation Op.14470, 90-95% pure, was a gift of Dr. Heimburger, Behringwerke AG, Marburg.

Preparation of fibrin plates (12). A fibrinogen solution with a final fibrinogen concentration of 0.1% (w/v) and an ionic strength of 0.15 was prepared. 6 ml of this solution were pipetted into Petri dishes (Tradimex B.V., Vianen, Holland, 90 x 15 mm) and, after mixing with 0.2 ml of thrombin solution (20 NIH units/ml), allowed to stand for at least 30 min on a carefully leveled surface at room temperature to form the fibrin layer.

Plasma sampling. Samples of citrated blood were collected by venipuncture; 9 vol of blood were mixed with 1 vol of 0.11 M sodium citrate. All blood samples were collected from fasting subjects between 8 and 9 a.m.

Platelet-poor plasma was separated by centrifugation at 4°C for 30 min at approximately 2,000 g. Pooled platelet-poor human plasma was obtained by mixing equal amounts of plasma from men and women (10 apparently healthy donors aged between 20 and 50, not using drugs of any kind). The pooled plasma was stored in aliquots of 1 ml at -20°C. Prior to estimation, the frozen sample was thawed and kept at room temperature until clear. HANE plasma samples (patient A, woman, 39 years and patient B, man, 24 years) were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, through the courtesy of Dr. K.W. Pondman and from the 'Service central d'Immuno-Hématologie', Hôpital Lariboisière, Paris, through the courtesy of Dr. J.L. Wautier, respectively.

Preparation of euglobulin fractions. In this paper, the term euglobulin fraction refers to the fraction obtained from diluted plasma by isoelectric precipitation at acid pH values between 5 and 7. The proteins present in these fractions are called euglobulin fraction proteins. Standard euglobulin fractions (4) were prepared from 1 ml plasma diluted to 10 ml with cold distilled water. The pH of the solution was adjusted to 5.9 with 0.25% (v/v) acetic acid with constant stirring at 0°C using an automatic titrator (Radiometer). After storage at 4°C for at least 30 min, the precipitated euglobulins were separated by centrifugation at ± 4°C for 5 min at 2,000 g. The precipitate was dissolved in an amount of EDTA-gelatin-barbital buffer which was equal to the original plasma volume. In experiments to study the influence of pH and ionic

strength on euglobulin fractionation, the plasma was always diluted 1:30. Variation in ionic strength was obtained by addition of the appropriate amount of sodium chloride solutions.

Fibrinolytic activity. The fibrinolytic activity of the euglobulin fractions was measured by the fibrin plate method. From each euglobulin fraction, three drops of 30 µl each were placed onto the surface of fibrin plates (12). After incubation of the plates at 37°C for 18 h on carefully leveled shelves in a Heraeus incubator, type FB 420 (vibratory motions of the incubator being avoided), two perpendicular diameters of each lysed zone were determined with a device especially designed for this purpose (11). The mean of the diameters was taken as the diameter of the zone. Noncircular zones seldom appeared and were excluded. The diameters of the lysed zones were used to represent the fibrinolytic activity of the euglobulin fractions.

The express the measured fibrinolytic activity in units, parallel dose--response curves are required (12). The euglobulin fractions studied failed to show such a behaviour. This is probably due, amongst other things (16), to the fact that the fractions contained different amounts of the inhibitor C1 inactivator.

Inhibitor assay. The inhibitors α_1-antitrypsin, α_2-macroglobulin, inter-α--trypsin inhibitor, C1 inactivator and antithrombin III were estimated by the quantitative radial immunodiffusion assay according to Mancini et al. (22) using the corresponding monospecific antisera. The amount of each inhibitor in the euglobulin fractions was expressed relative to pooled plasma which was arbitrarily chosen as 100%.

RESULTS

Inhibitor content of the standard euglobulin fraction

The percentage of each inhibitor that precipitated in the standard euglobulin fraction was determined. The inhibitors α_1-antitrypsin, α_2-macroglobulin and antithrombin III are present in low percentages of approximately 0.5%, i.e. the detection limit of our method, and are apparently not euglobulin fraction proteins. Inter-α-trypsin inhibitor and C1 inactivator behave differently and are present in appreciable amounts (C1 inactivator: mean 21%, range 20-21%; inter-α-trypsin inhibitor: 52%, range 50-55%; mean values and ranges obtained from 5 standard euglobulin fractions derived from pooled plasma). This observation makes both inter-α-trypsin inhibitor and C1 inactivator of special interest.

Variation in ionic strength at constant pH (5.9)

The precipitation of inter-α-trypsin inhibitor and C1 inactivator in the euglobulin fractions is favoured by decreasing ionic strength (fig. 1). The fibrinolytic activity of euglobulin fractions prepared under conditions with constant pH and decreasing ionic strength does not show a consistent increase. The fibrinolytic activity reaches an optimum and decreases again within the studied range contrasting with the two inhibitors in this respect (fig. 1). The quantities of the other inhibitors α_1-antitrypsin, α_2-macroglobulin and antithrombin III remained low over the whole range, confirming that they are not euglobulin fraction proteins.

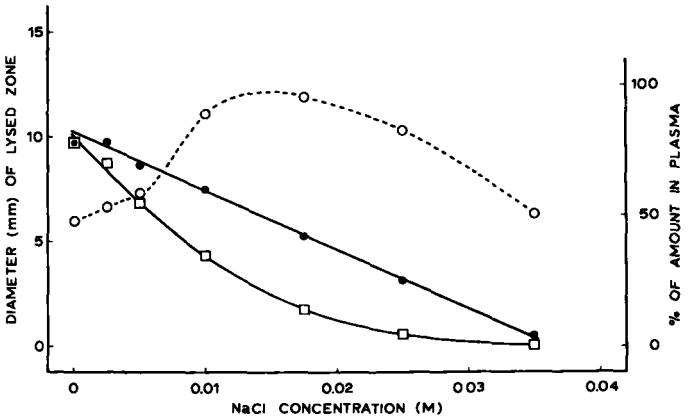

Fig. 1. Preparation of euglobulin fractions at constant pH (5.9) and variable ionic strength. Effect on the fibrinolytic activity and the inhibitor content of the fractions obtained. Each point in the figure represents a euglobulin fraction. Variation in ionic strength is recorded on the abscissas as final concentration of added NaCl in 1:30 diluted plasma. The fibrinolytic activity (o) is recorded in diameters of the lysed zones after 18 h of incubation of the fibrin plate. C1 inactivator (□) and inter-α-trypsin inhibitor (●) were determined in each fraction by the technique of Mancini et al. (22) and expressed as percentages of the amount in plasma.

Variation in pH at constant ionic strength

The variation in pH was studied at three values of ionic strength (fig. 2a-c). The precipitation of inter-α-trypsin inhibitor and C1 inactivator is optimal at low pH (5.0). In accordance with the results represented in figure 1, the precipitation is greatest at the lowest ionic strength (fig. 2c). The fibrinolytic activity shows an optimum which shifts to higher pH values as the ionic strength decreases (fig. 2a-c).

On the right hand side of the optima of fibrinolytic activity for both variables (ionic strength, fig. 1 and pH, fig. 2), all components as well as

protein (E280, not shown) exhibit the same decreasing tendency, suggesting a similar precipitation behaviour for all euglobulin fraction proteins and not for any component in particular.

The presence of appreciable amounts of C1 inactivator and inter-α-trypsin inhibitor and the low fibrinolytic activity may be related at conditions of low ionic strength and the lower pH. To study the relationship between the observed low fibrinolytic activity and C1 inactivator under these conditions, comparative studies were made in HANE plasma.

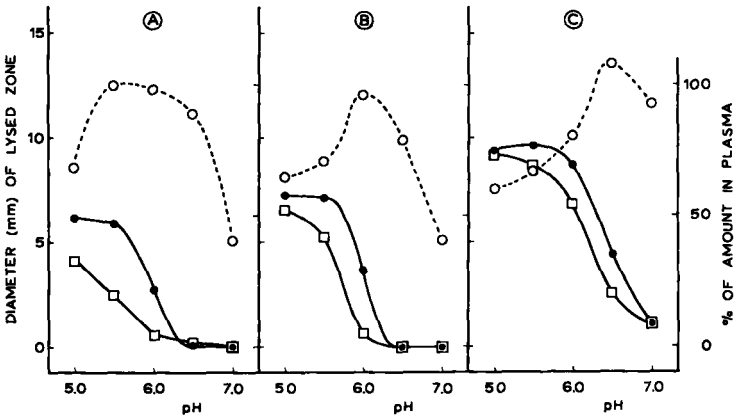

Fig. 2. Preparation of euglobulin fractions with variable pH and three fixed values of the ionic strength. Effect on the fibrinolytic activity and inhibitor content of the fractions obtained. Each point in the figure represents a euglobulin fraction prepared at the indicated pH and at a fixed value of ionic strength. The ionic strength was obtained by addition of NaCl solutions to 0.0175 M (a); 0.010 M (b), and 0.0025 M (c) final concentration in 1:30 diluted plasma. See also legends to figure 1.

Studies in HANE plasma

In plasma of patients with HANE, C1 inactivator is functionally absent (7,26); however, inter-α-trypsin inhibitor concentration appears to be normal. This provides a model for the study of the euglobulin fibrinolytic activity without the influence of C1 inactivator.

The fibrinolytic activities of euglobulin fractions of normal and HANE plasma were compared as a function of variation in ionic strength at constant pH (fig. 3) and as a function of variation in pH at constant ionic strength (fig. 4).

The fibrinolytic activity in euglobulin fractions prepared from normal plasma at low pH and low ionic strength is obviously lower than in the

corresponding fractions from HANE plasma. These experiments clearly demonstrate that the low fibrinolytic activity in these fractions is related to a high level of C1 inactivator.

In agreement with these results, addition of purified C1 inactivator to our standard euglobulin fraction considerably inhibits the fibrinolytic activity. In a typical experiment, addition of purified C1 inactivator to the standard euglobulin fraction of normal plasma decreased the lysed zone on the fibrin plate from 11.8 to 8.1 mm. The concentration of C1 inactivator was increased from 21 to 56% relative to the plasma concentration.

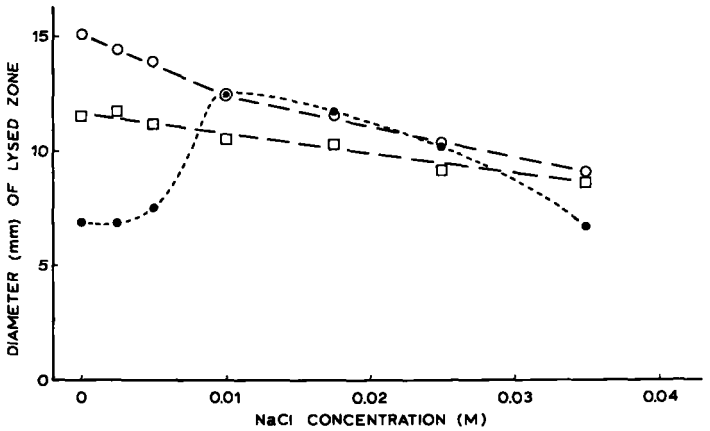

Fig. 3. Comparison between the fibrinolytic activity of euglobulin fractions from two HANE plasmas and pooled normal plasma (variation in ionic strength). Pooled normal plasma and plasma of 2 patients with HANE were used. ● = Normal plasma; o = patient A; □ = patient B. See also legend to figure 1.

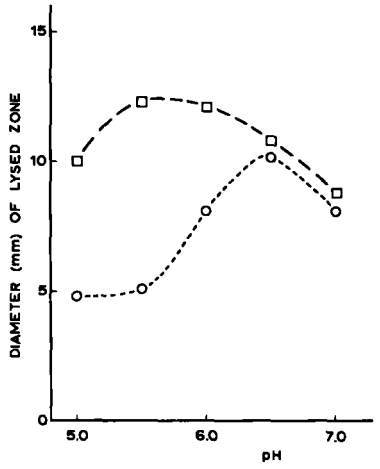

Fig. 4. Comparison between the fibrinolytic activity of euglobulin fractions from HANE plasma and pooled normal plasma (variation in pH). The fixed value of ionic strength was obtained by addition of a sodium choride solution to 0.0025 M final concentration in 1:30 diluted plasma. Pooled normal plasma (o) and plasma of patient A (□-□) was used. See also legends to figure 2b.

DISCUSSION

In this study, the precipitation of proteinase inhibitors in euglobulin fractions is clearly established. Inter-α-trypsin inhibitor and C1 inactivator are euglobulin fraction proteins in contrast to α_1-antitrypsin, α_2-macroglobulin and antithrombin III. Choosing the proper conditions in euglobulin fractionation appears to facilitate the separation of the two classes of inhibitors. Such fractionation may also be a useful step in purification procedures.

One might ask to what extent the presence of inhibitors in euglobulin fractions influences the measured activity. It is difficult to determine in a euglobulin fraction (a mixture of proteinase inhibitors and active components) to what extent the inhibitors are actually of importance. The availability of HANE plasma, however, makes it possible to study the influence of C1 inactivator. The results showed a considerable inhibition of the fibrinolytic activity of euglobulin fractions by both endogenous and exogenous C1 inactivator. Inhibition by exogenous C1 inactivator has been reported (25).

The contribution of the other four inhibitors to the inhibition of the fibrinolytic activity of euglobulin fractions has not been established experimentally. Considerable amounts of inter-α-trypsin inhibitor also precipitate in euglobulin fractions. This component is described as a weak plasmin inhibitor (29). It could therefore be of minor importance. The amounts of α_1-anti-trypsin, α_2-macroglobulin and antithrombin III are quantitatively small; the influence of them on fibrinolytic activity cannot be completely excluded at present.

In addition to the precipitation of the inhibitors inter-α-trypsin inhibitor and C1 inactivator, fractionation conditions also influence that of the substances responsible for the fibrinolytic activity. The highest fibrinolytic activity seems to be obtained in fractions prepared at low ionic strength and a pH of approximately 5.5, as strongly suggested by the results with HANE plasma (fig. 3,4). Even in this situation, it is possible with dextran sulphate, known for its so-called peptone effect (2), to obtain additional fibrinolytic activity from the supernatant which is left after removal of the euglobulin fraction (unpublished). This indicates that not all potential fibrinolytic activity is precipitated from plasma in the euglobulin fraction.

The results have consequences with regard to our standard procedure. The standard procedure we use was developed in the late fifties (1,4) and is based on studies on fractionation conditions during euglobulin fractionation. The procedure resulting in the most active euglobulin fraction as measured by the

fibrin plate assay has been chosen as a standard procedure. From a practical point of view, it is an advantage to have a highly active fraction. Among other frequently used modifications of the euglobulin fractionation method, it is the procedure resulting in a fraction with a relatively low content of C1 inactivator (16). Based on the results of this study, however, better selections of conditions can be made. For special purposes, e.g. to study the active components, fractions can be selected with a negligible content of inhibitors.

It must be emphasized that not the total amount of C1 inactivator, but rather the amount of functional C1 inactivator available to inhibit the fibrinolytic activity is important. C1 inactivator is a common factor in clotting, kinin, complement and fibrinolytic systems because of the interaction of this inhibitor with active Hageman factor (8,28). Factor XIa (8), plasma kallikrein (25), C1 esterase (21,25), plasma plasminogen activator (19) and plasmin (10, 25). Many of these enzymes can compete for C1 inactivator. Consequently, *in vivo* or *in vitro*, changes in one or more components of the systems mentioned might become manifest as a change in the fibrinolytic activity of euglobulin fractions (17). Which enzyme(s) of the fibrinolytic system of euglobulin fractions are the targets of the C1 inactivator is still to be determined.

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CHAPTER II

ELIMINATION OF INHIBITION IN EUGLOBULIN FIBRINOLYSIS BY USE OF FLUFENAMATE: INVOLVEMENT OF C1 INACTIVATOR*

C. Kluft

Abstract. The fibrinolytic activity of euglobulin fractions prepared from human morning plasma and assayed on fibrin plates is strongly inhibited by the C1 inactivator present in the fractions. Flufenamate, a potent representative of the group of synthetic thrombolytic agents, eliminates this inhibition in euglobulin fractions. This elimination is an apparently irreversible reaction dependent on concentration, time and temperature. The fibrinolytic enhancing effect of flufenamate in euglobulin fractions correlated well with a similar effect of added $C\bar{1}s$, which neutralized the C1 inactivator. The effect of flufenamate was slightly greater than that of added $C\bar{1}s$, suggesting an additional effect of the flufenamate. The activity enhancing effect of the flufenamate at the lower molarities could be separated from an activity decreasing effect at the higher molarities. A simple technique by which inhibitory effects in euglobulin fibrinolysis are selectively eliminated is described.

INTRODUCTION

The search for synthetic thrombolytic agents initiated by Von Kaulla (1,2) has yielded extensive lists of compounds with the capacity to stimulate fibrinolysis in human plasma *in vitro*. The most active compounds are members of the chemical group of aryl- or halogen-substituted salicylates and biarylcarboxylates or belonging to the pharmacological group of acidic, nonsteroidal anti-inflammatory agents - such as phenylbutazone, fenamates (among others flufenamic acid) and indomethacin.

The fibrinolysis-inducing mechanism of these compounds, as suggested by Von Kaulla (2), is believed to be a result of their properties as differential inhibitors. At low molarity, they eliminate the effect of inhibitors on the fibrinolytic system, whereas, at higher molarity, they inhibit plasmin. Each compound has its own characteristic molarity range below and above which induction of fibrinolysis does not occur.

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More generally, as emphasized by Gryglewski (3), the compounds react with various enzymatic and nonenzymatic proteins such as α_2-macroglobulin (2), antiplasmin (4), antiactivator (5), fibrin (6) and fibrin monomer (7), human serum albumin (8), prostaglandin synthethase (9), rat serum glutamic pyruvic transaminase (10) and glutamate decarboxylase (11). There are also reports of effects on collagen or casein proteolysis by trypsin, α-chymotrypsin, thrombin, plasmin, kallikrein and leukocyte collagenase (2,3,12,13). In most of the studies, however, the question of whether enzyme or substrate is affected (an aspect discussed by Baillie and Sim (13)) remains unsettled.

Most of the described effects may be accounted for by a nonspecific binding of the compounds to the various proteins, causing a change in secondary and tertiary structure which impairs activity (3).

An activity relevant for the effects of the synthetic agent on the fibrinolytic system is the inactivation of inhibitors such as demonstrated for antiplasmin (4), antiactivator (5) and α_2-macroglobulin (2). Euglobulin fractions of human plasma contain appreciable amounts of inhibitory material (14,15). We previously found that one of the most potent of the synthetic thrombolytic agents, flufenamic acid, appreciably enhances the fibrinolytic activity of euglobulin solutions, indicating indeed an elimination of inhibitors (15,16). This communication presents the results of a detailed study of the effects of flufenamate on euglobulin fibrinolysis. It is shown that it is possible to selectively eliminate inhibitory agents in the euglobulin fraction, thus enabling a more precise determination of true euglobulin fibrinolytic activity.

MATERIALS AND METHODS

Materials

Unless otherwise specified, reagents were of analytical grade and obtained from Merck, Darmstadt, West Germany. Distilled water was used throughout. Additional reagents were obtained from the following sources.

Microbiological grade gelatin and N-Acetyl-tyrosin-ethylester (ATEe) from Merck, Darmstadt, West Germany. "Agarose for electrophoresis" from BDH Chemicals Ltd., Poole, England. Flufenamic acid (N-[α.α.α.-trifluoro-m-tolyl]anthranilic acid) from Aldrich Europe, Beerse, Belgium. 3,5-dibromosalicylic acid and Dowex I from Fluka A.G., Buchs, Switzerland. Anti-human antisera raised in rabbits against C1 inactivator and C1-esterase (C1s) (laboratory preparation) from Behringwerke A.G., Marburg, West Germany. Human serum albumin from Serva, Heidelberg, West Germany. Trypsin (EC 3.4.21.4) and chymotrypsin A (EC 3.4.21.1)

from Boehringer, Mannheim, West Germany. Plasminogen-free bovine fibrinogen from Poviet, Organon-Teknika, Oss, The Netherlands. Human urokinase (EC 3.4.99.26) from Leo Pharmaceuticals, Ballerup, Denmark.

Human tissue activator prepared from uteri was kindly supplied by Drs. D. Rijken and G. Wijngaards (17) of this laboratory. Plasminogen-rich bovine fibrinogen was prepared according to Brakman (18). Platelet-poor, citrated human plasma and pooled plasma were obtained in the early morning under conditions which secure a baseline level of fibrinolysis (15). Active $C\bar{1}$-esterase ($C\bar{1}s$) was prepared from outdated plasma or serum according to Vroon et al. (19) and Haines and Lepow (20). The preparation contained 650 units/ml (21). It was contaminated with traces of other proteins but was found to be free of direct fibrinolytic or plasminogen activator activity.

Solutions

EDTA-buffer (μ = 0.15): 0.05 M sodium diethylbarbiturate, 0.10 M NaCl, 0.25% (w/v) gelatin and 2.7 mM ethylene diamine tetraacetate (EDTA) adjusted to pH 7.8 with an HCl solution.

Gelatin buffer (μ = 0.15): 0.05 M sodium diethylbarbiturate, 0.10 M NaCl, and 0.25% (w/v) gelatin adjusted to pH 7.8 with an HCl solution.

Sodium flufenamate solutions: 0.5 M flufenamic acid in equivalent concentration of NaOH (60°C), diluted to appropriate concentrations with EDTA-buffer.

Trypsin and chymotrypsin solutions: Solutions of trypsin and chymotrypsin were freshly prepared by dissolving 12 mg of the enzyme in 20 ml 0.01 N HCl. The solution was made up to 100 ml with gelatin buffer and diluted in plastic tubes to appropriate concentrations with gelatin buffer.

3,5-dibromosalicylate solutions: 0.83 M 3,5-dibromosalicylic acid dissolved in 3 volumes of ethanol was added to 6 volumes of 0.42 N NaOH, diluted with 1 volume of distilled water and adjusted with an HCl solution to pH 7.8. Changing the proportion of ethanol did not influence the results.

Methods

Preparation of fibrin plates (22): A fibrinogen solution with a final fibrinogen concentration of 0.1% (w/v) and an ionic strength of 0.15 was prepared. Six ml of this solution were pipetted into Petri Dishes (Tradimex B.V., Vianen, The Netherlands, 90 x 15 mm) and, after mixing with 0.2 ml of thrombin solution (20 NIH units/ml), allowed to stand for at least 30 min on a carefully leveled surface at room temperature to form the fibrin layer.

Preparation of euglobulin fractions: Regular euglobulin precipitates (15)

were obtained by diluting 1 ml plasma with 9 ml cold distilled water and adjusting the pH to 5.9 with a 0.25% (v/v) solution of acetic acid with constant stirring in an ice-bath for at least 30 min; the precipitates were separated by centrifugation in the cold for 10 min at 2,000 g and dissolved in an amount of EDTA-buffer equal to the original plasma volume. In experiments designed to study the influence of pH and ionic strength on euglobulin fractionation, the plasma sample was always diluted 1:30. Variations in ionic strength were obtained by addition of the appropriate amount of sodium chloride solutions.

Fibrinolytic activity: The fibrinolytic activities of the euglobulin fractions, trypsin and chymotrypsin were determined by the fibrin plate method. Three drops (each 30 µl) of each solution were applied to the fibrin plates (22). After incubation of the plates at 37°C for 18 h on carefully leveled shelves in a Heraeus incubator, type FB 420 (vibratory motions of the incubator being avoided), two perpendicular diameters of each lysed zone were determined. The mean of the diameters was taken to represent the fibrinolytic activity.

Modification with flufenamate (15): To each of three drops of the euglobulin solution placed on the fibrin plates were immediately added 5 µl of a 14 mM sodium flufenamate solution by means of a syringe. Otherwise, the procedure was as described above.

C1 esterase activity: $C\bar{1}$-s was assayed titrimetrically (Titrator TTT 2, Radiometer; Copenhagen) by its esterolytic activity towards ATEe (21).

C1 inactivator assays: a) Immunochemically. The C1 inactivator was estimated by the quantitative radial immunodiffusion assay according to Mancini et al. (23) using a monospecific antiserum. The amount of C1 inactivator was expressed in % relative to pooled plasma. b) Functionally. The inhibition was measured against the esterolytic activity of purified $C\bar{1}$s towards ATEe, according to the method of Levy and Lepow (21) and expressed in units per milliliter or alternatively in % relative to pooled plasma.

Flufenamate removal: Flufenamate can be bound to cholestyramine or Dowex I (24). To limit activation and inactivation processes in the euglobulin solutions, we used a short incubation period of 15 min at low temperature (0-4°C) and under agitation, to remove flufenamate. The resin was added as a suspension equilibrated in EDTA buffer and it was removed by cold centrifugation for 5 min at 2,000 g. With 2 g/l cholestyramine or Dowex I, more than 99% of the flufenamate in a 2 mM solution was removed by this procedure. Flufenamate was assayed by its adsorbance at 290 nm. A similarly treated euglobulin solution containing 2 mM flufenamate showed a remaining concentration of about 0.3 mM flufena-

mate with 2 g/l or more of the resin, presumably due to competition between proteins and the resin for the flufenamate. The addition of flufenamate at 0-4°C and its subsequent removal at 0-4°C produced no change in the fibrinolytic activity of the solution, indicating that the remaining low level of flufenamate did not stimulate fibrinolysis.

RESULTS

1. The effects of flufenamate on the fibrinolytic activity of euglobulin fractions

Various euglobulin fractions. Two series of euglobulin fractions were prepared. One was precipitated at varying acidities at constant ionic strength (fig. 1, left panel). The other series was precipitated at varying ionic strengths at constant acidity (fig. 1, right panel). The fibrinolytic activity of most of the resulting euglobulin fractions was increased considerably by the addition of flufenamate (2 mM). The greatest increase occurred in fractions precipitated at low ionic strength and low pH (fig. 1).

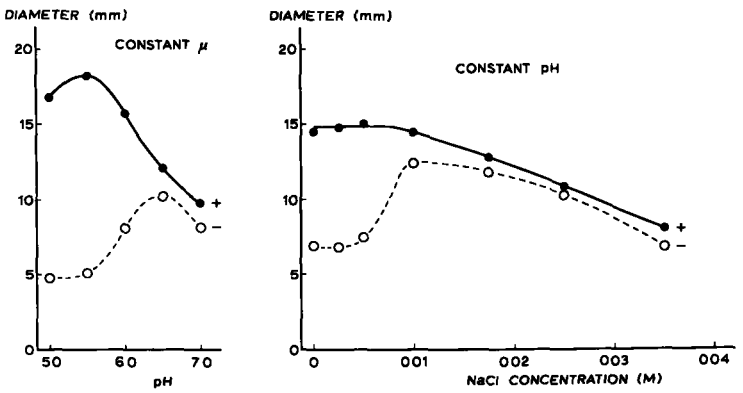

Fig. 1. Effect of flufenamate on the fibrinolytic activity of euglobulin fractions. Each point represents a euglobulin fraction prepared from pooled morning plasma at the indicated pH and ionic strength. The fibrinolytic activity (ordinate) was determined by the fibrin plate assay and recorded as diameters of lysed zones. Flufenamate to a final concentration of 2 mM was added to each fraction (+, ●–●); control without flufenamate (-, o-o). Left: effect of variation in pH with constant ionic strength (0.0025 M NaCl). Right: effect of variation in ionic strength by addition of sodium chloride solutions (final concentration in 1:30 diluted plasma recorded on the abscissae) at a constant pH of 5.9. The fraction prepared with 0.01 M NaCl, pH 5.9 corresponds with the regular euglobulin fraction.

Previous studies (14) showed that these particular fractions contain C1 inactivator, which decreases their fibrinolytic activity. Elimination of C1 inactivator by flufenamate could therefore be a mechanism by which the fibrinolytic activity of these fractions is increased.

Concentration and time dependence. The activity increasing effects of various concentrations of flufenamate were determined by using a euglobulin fraction which showed a large enhancement. As shown in figure 2, the enhancement reached a maximum at about 2-2.5 mM flufenamate. This was followed by a decrease at higher concentrations of flufenamate, indicating a detrimental effect of flufenamate at these concentrations. When the euglobulin solutions were preincubated at 37°C with various concentrations of flufenamate, results such as those shown in figure 3 were obtained. It can be seen that, at low concentrations of flufenamate (up to the optimum concentration), there is no destruction due to prolonged incubation at 37°C. At higher concentrations, the destruction progressively increases. The enhancing and decreasing effects of flufenamate seem to be separate, time and concentration dependent reactions.

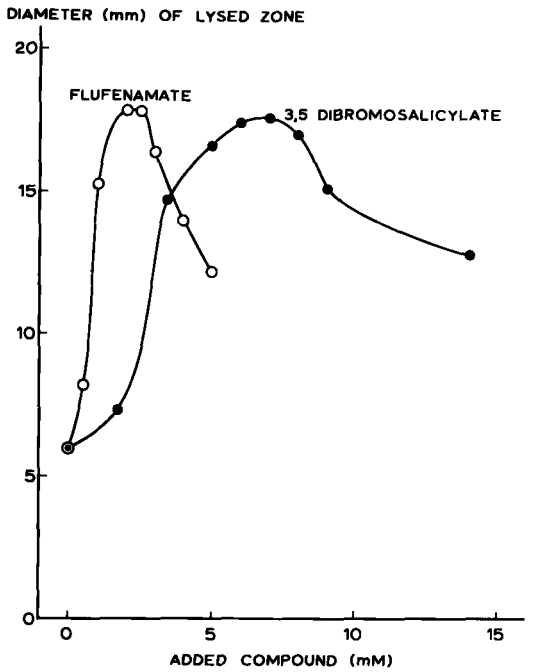

Fig. 2. Effect of various concentrations of flufenamate and 3,5-dibromosalicylate on the fibrinolytic activity of a euglobulin fraction with a high level of C1 inactivator. The fraction was prepared from pooled morning plasma at pH 5.5 and 0.0025 M NaCl. The final concentration of the added compounds in the fraction is recorded on the abscissa. See also legend to figure 1.

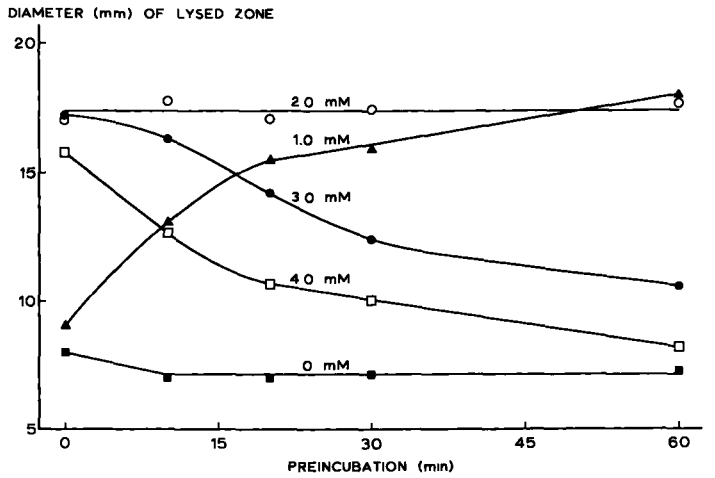

Fig. 3. Effect of preincubation with several flufenamate concentrations on the fibrinolytic activity of a euglobulin fraction (prepared at dilution 1:30, pH 5.9) with a high level of C1 inactivator. The fraction was preincubated at 37°C with flufenamate (final concentrations shown in the figure) and the fibrinolytic activity was subsequently determined by the fibrin plate assay. See also legend to figure 2.

Other drugs. Figure 2 shows that 3,5-dibromosalicylic acid has effects similar to those of flufenamate. Optimal enhancement was reached at a higher molar concentration (7 mM) than for flufenamate, which agrees with the relative potencies of these two compounds in other test systems (3). With the much less effective acetyl salicylic acid, optimal enhancement required a 3 h preincubation of the euglobulin fraction with 45 mM of the compound at 37°C.

Reversibility and influence of the incubation period during assay. In the above experiments, it is conceivable that some of the reaction between the compound and the euglobulin fraction takes place during the incubation period of the assay. The compounds may also effect the substrate of the fibrin plate, while, at the same time, diffusion on the plate progressively lowers the concentration of the compounds, raising questions about a possible reversibility of the effects.

To study the above-mentioned aspects, most of the flufenamate was removed from the euglobulin solutions as described under Methods. Curve 2 (Fig. 4) shows the activities after most of the flufenamate was removed after preincubation before assay. It is evident that the low activity of the original solution (cf t = 0, curve 2) was enhanced by the preincubation and the enhancement

was complete and practically irreversible in about 30 min. Readdition of flufenamate after removal (curve 3) produced a curve similar to curve 1, indicating that the procedure to remove flufenamate does not significantly interfere with the fibrinolytic components. The influence of the incubation period on fibrin plates can be deduced from the difference between curves 1 and 2 in figure 4. In curve 1 (in contrast to curve 2), no flufenamate is removed before the assay. The enhancing effect has occurred without preincubation, obviously due to the incubation period on the fibrin plate. In this particular experiment, the influence of the incubation of the fibrin plate is equivalent to an effective preincubation time of around 30 min (curve 2).
A possible influence of flufenamate on the fibrin substrate should have shown up as a difference between curves 2 and 3 (Fig. 4). Only a small difference is noted between the recorded activity (after 60 and 90 min preincubation) in the presence or absence of flufenamate, indicating that an effect on the fibrin substrate is small.

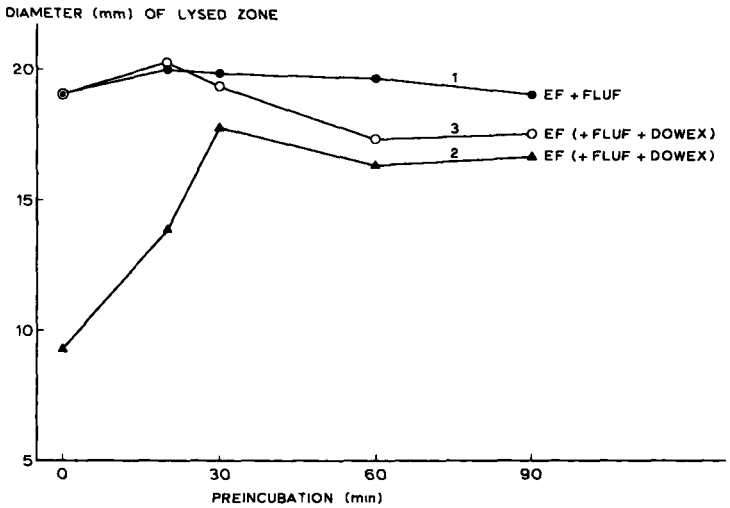

Fig. 4. Effects of the removal of flufenamate from euglobulin fractions before assay on the fibrin plate. A euglobulin fraction (EF) with a high level of C1 inactivator (prepared at dilution 1:30, pH 5.9) was preincubated for various lengths of time (abscissa) with 1.5 mM flufenamate (FLUF). After this preincubation, the fibrinolytic activity was determined on fibrin plates. A) without further treatment of the fraction, curve 1, EF + FLUF; B) after removal of the bulk of the flufenamate by DOWEX, curve 2, EF (+ FLUF + DOWEX); C) after removal of the bulk of the flufenamate by DOWEX, and subsequent readdition of flufenamate to 2 mM, curve 3, EF (+ FLUF + DOWEX) + FLUF. See also legend to figure 2.

It is concluded that the enhancing effect of flufenamate on euglobulin fibrinolysis is complete during the period of incubation in the plate assay and that a possible influence of the low concentrations of flufenamate on the assay is not significant. These circumstances can be used to simplify the determination of the flufenamate effect.

2. Effect of flufenamate on purified proteases assayed on plasminogen--free fibrin

The effects of flufenamate were also studied in a less complicated situation employing purified proteases (trypsin and chymotrypsin) and plasminogen--free fibrin. Preincubation of these proteases with two concentrations of flufenamate showed that chymotrypsin was particularly sensitive to this treatment (Fig. 5A). Incubation of this enzyme with 5 mM flufenamate resulted in a complete loss of fibrinolytic activity in 60 min. This loss was apparently irreversible, since, in an additional experiment, a 5 times dilution of the mixture to a concentration of 1 mM flufenamate (which has little inhibitory effect according to Fig. 5A) did not result in recovery of activity. Trypsin was less affected. Calculated on the basis of a dose-response curve (made in the absence

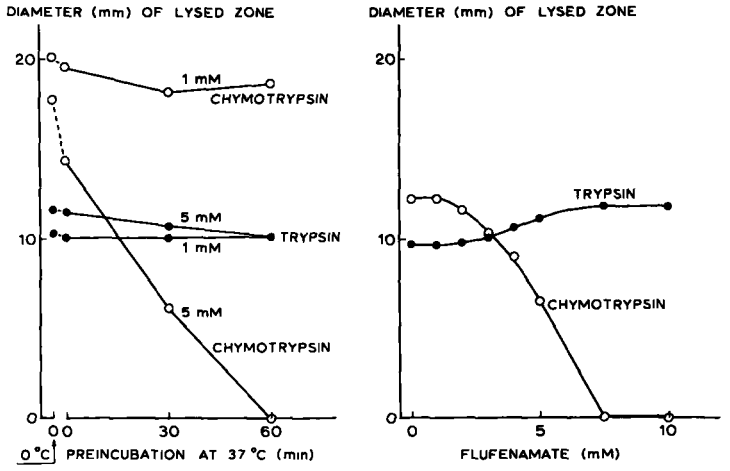

Fig. 5. Effects of flufenamate on trypsin and chymotrypsin activity on plasminogen-free fibrin plates. Fig. 5A, left: trypsin (●–●, 0.6 ppm) and chymotrypsin (o–o, 6.3 ppm) were preincubated for various lengths of time at $0^{\circ}C$ or $37^{\circ}C$ (abscissa) with 1 and 5 mM flufenamate. Fig. 5B, right: chymotrypsin (0.8 ppm) and trypsin (0.6 ppm) were mixed with various concentrations of flufenamate (abscissa) at $0^{\circ}C$ and assayed on plasminogen-free fibrin plates. The fibrinolytic activity is recorded as diameters of lysed zones after 18 h incubation (ordinates).

of flufenamate), 5 mM flufenamate caused a loss of 30% of the initial trypsin activity after 60 min at 37°C.
When the mixtures were prepared at 0°C and immediately applied to the fibrin plates, the destruction of chymotrypsin increased with the concentration of flufenamate with all activity being lost at a concentration of about 7 mM. In contrast, a slight increase in trypsin activity was seen (Fig. 5B).

The results of these experiments show marked differences between the application of cold mixtures (0°C) and the warm solutions (37°C, Fig. 5) and they stress the importance of strict temperature control when applying the technique. Furthermore, it is seen that, in the concentration range of 2-2.5 mM flufenamate which is used to eliminate inhibitor effects in euglobulin fraction, the influence on the activity of proteases on the plasminogen-free fibrin (Fig. 5B) are slight. This supports the conclusion reached earlier that, in this concentration range, flufenamate does not influence the assay.

3. Effects of flufenamate on C1 inactivator

To study the effect of flufenamate on C1 inactivator, samples of pooled human plasma were incubated for 10 min at 37°C with varying concentrations of flufenamate. This treatment resulted in the plasma losing the capacity to inhibit purified $C\bar{1}s$. The loss of inhibitory capacity was nearly complete at a concentration of 15 mM flufenamate (Fig. 6). This indicates that the major plasma inhibitor of $C\bar{1}s$, the C1 inactivator, lost its effect due to the flufenamate treatment. Mixtures of flufenamate and plasma not preincubated at 37°C showed no loss of inhibition, indicating that the presence of flufenamate did not influence the assay. When a euglobulin fraction with a high content of C1 inactivator was similarly tested, the loss of inhibitory capacity occurred at lower concentrations of flufenamate (Fig. 6) and concentrations of flufenamate above 10 mM exerted an inhibiting effect. The difference in amounts of flufenamate needed to produce loss of inhibition in plasma and the euglobulin fractions could be attributed in part to a binding to the albumin in plasma. An interaction between albumin and flufenamate was indicated by a progressive increase in the electrophoretic mobility of albumin (anodal, pH 8.3) on addition of flufenamate to plasma. Likewise, addition of purified albumin to the euglobulin fractions caused a corresponding shift of the optimum enhancement of fibrinolytic activity to higher flufenamate concentrations. Thus, with 35 mg albumin/ml the optimal enhancement was reached at 5.5 mM flufenamate. The results show that flufenamate eliminates the effect of C1 inactivator on $C\bar{1}s$, although the concentration of flufenamate producing optimal effect depended

upon the protein composition of the solution.

4. Comparison of the effects of flufenamate and $C\bar{1}s$ on the fibrinolytic activity of euglobulin fractions

The relation between the elimination of C1 inactivator and the effect of flufenamate on euglobulin fibrinolysis was further tested by addition of purified $C\bar{1}s$ to neutralize the C1 inactivator. The addition of increasing amounts of purified $C\bar{1}s$ led to a progressive increase in the fibrinolytic activity of the euglobulin fractions until an optimum was reached, confirming the inhibiting effect of C1 inactivator in these fractions. The optimum enhancement in fibrinolytic activity obtained by addition of $C\bar{1}s$ was compared with that obtained with flufenamate (Fig. 7). The effects are shown for a fraction with a low amount of C1 inactivator (prepared at dilution 1:10, pH 5.9) and also for one with a high amount of C1 inactivator (prepared at dilution 1:30, pH 5.9). A clear correlation between the effects of $C\bar{1}s$ and flufenamate can be observed. Both show a small effect on the first mentioned and a much larger effect on the last mentioned fraction. The effect of flufenamate was greater than that of $C\bar{1}s$ in both fractions, suggesting an extra effect of flufenamate.

In attempts to determine the reason(s) for the stronger effect of flufenamate on the euglobulin fractions, samples of plasma deficient in C1 inactivator (Hereditary Angioedema) were studied. The fibrinolytic activity in euglobulin fractions from this plasma prepared at dilutions of 1:10 or 1:30 at pH 5.9 (cf Fig. 7) was slightly enhanced by flufenamate, from 12.4 to 14.4 mm and from 15.1 to 18.7 mm respectively. This suggests that components other than C1 inactivator contribute to the enhancement. The extra effect of flufenamate compared to that of $C\bar{1}s$ was also found to be variable among normal individuals and randomly chosen patients.

The extra enhancement by flufenamate was maintained on several types of fibrin plates, amongst which were highly purified bovine fibrin plates (25). Solutions of purified activators such as urokinase (20.7 to 20.6 mm) and human tissue activator (14.7 to 14.0 mm) showed no enhanced activity in the presence of 2 mM flufenamate, excluding a contribution to enhancement originating from the fibrin substrate of the assay. Therefore, the major enhancing effect of flufenamate corresponds to a neutralization of the C1 inactivator. There was also a small additional enhancement (most prevalent in C1 inactivator-free (HAE) fractions) involving constituents of the euglobulin fractions. This effect is the subject of a separate study.

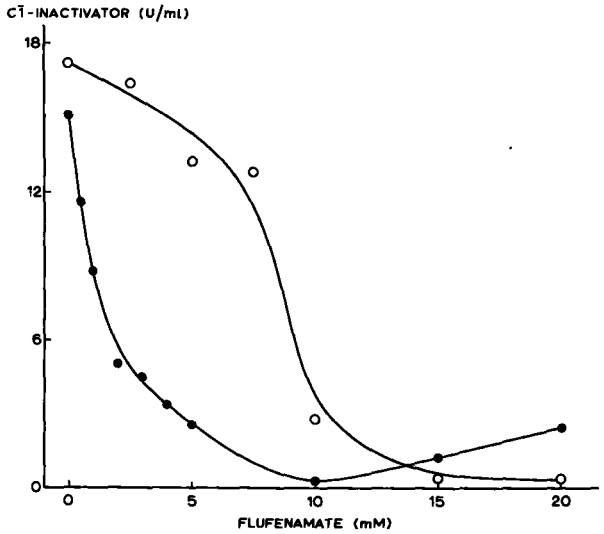

Fig. 6. Effect of flufenamate on C1 inactivator activity. Pooled morning plasma (o–o) and a euglobulin fraction (●–●) prepared at a dilution 1:30 and pH 5.9 were incubated for 10 min at 37°C with various concentrations of flufenamate (abscissa). The resulting activity of C1 inactivator assayed as inhibition of the esterolytic activity of C1̄s is recorded on the ordinate expressed in units/ml (21).

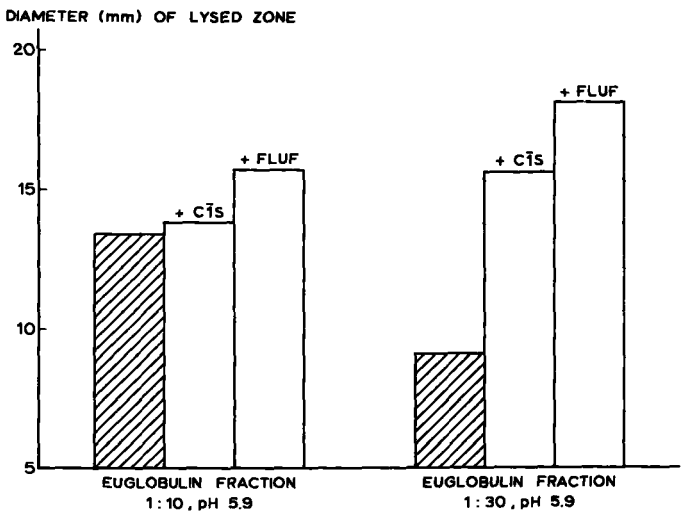

Fig. 7. Comparison of the effects of flufenamate and added C1̄s on the fibrinolytic activities of euglobulin fractions. Amounts of C1̄s (+ C1̄s) or flufenamate (+ FLUF) resulting in optimum enhancement of fibrinolytic activity were added to euglobulin fraction with either a low level of C1 inactivator (1:10, pH 5.9, left section) or a high level of C1 inactivator (1:30, pH 5.9, right section) prepared from pooled morning plasma. The fibrinolytic activity represented by the bars was measured by the fibrin plate method and expressed as diameters of lysed zones (ordinate). Activity of the fractions without additions is represented by the hatched bars.

5. Partial elimination of C1 inactivator in euglobulin fractions by intrinsic mechanisms

Up to now C1 inactivator in euglobulin fractions has been determined immunochemically (14,16). It is well-known that C1 esterase is a euglobulin (26) and we could immunochemically confirm the presence of C1s in our fractions. It is possible that, in euglobulin fractions, C1 esterase becomes activated and neutralizes part of the C1 inactivator; therefore, the C1 inactivator in euglobulin fractions was also assayed by a functional method.

In a series of euglobulin fractions prepared at various ionic strengths at constant pH, the content of C1 inactivator was determined immunochemically as well as functionally by its capacity to inhibit $C\bar{1}s$ (Fig. 8). The results show a marked discrepancy. The content of functionally active C1 inactivator is generally lower than that determined immunochemically. Fractions without functional C1 inactivator exhibited spontaneous activity on the substrate ATEe which was used to assay $C\bar{1}s$. The activity of a freshly prepared fraction on ATEe was initially low, but it gradually increased to an optimum (Fig. 9). This increase was more rapid in the presence of calcium ions than in the presence of EDTA. This finding suggested that C1 esterase is precipitated in a functionally inactive form, but is readily activated in the solution. In euglo-

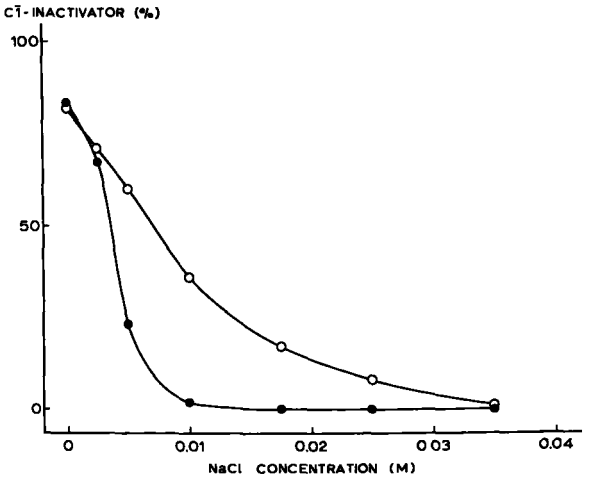

Fig. 8. Comparison of immunochemical and functional amounts of C1 inactivator in euglobulin fractions prepared from pooled morning plasma at various ionic strengths and at constant pH (5.9). The C1 inactivator concentrations were measured immunochemically (o–o) or functionally (•–•) and were expressed in % relative to the plasma. See also legend to figure 1, right. The fraction prepared at 0.01 M NaCl corresponds with the regular euglobulin fraction.

bulin fractions which have no spontaneous activity on ATEe (although they show functional C1 inactivator), the C1 esterase is possibly activated as described above, subsequently neutralizing part of the C1 inactivator. This would explain the discrepancy mentioned. In comparing the results in figures 1 and 8, it is seen that the enhancing effects of flufenamate shown in figure 2 parallels the content of functional C1 inactivator (Fig. 8). This indicates that the functional amount rather than the immunochemical amount of C1 inactivator is relevant for inhibition of euglobulin fibrinolysis.

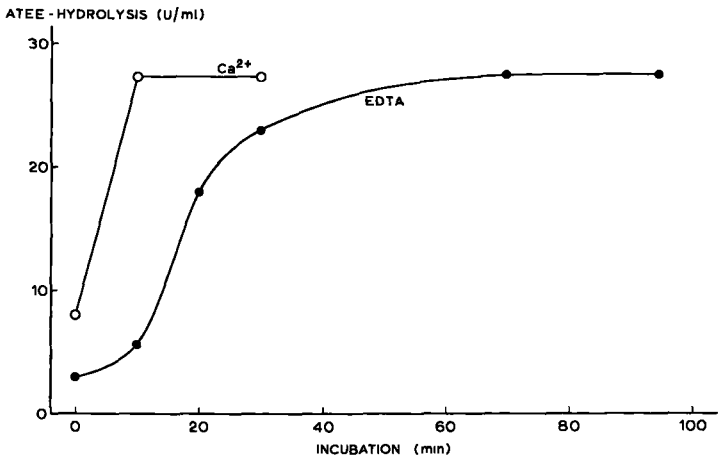

Fig. 9. Effect of calcium ions versus EDTA on the generation of ATEe hydrolysing activity in a euglobulin fraction with low level of C1 inactivator. A euglobulin fraction prepared at 0.025 M NaCl and pH 5.9 (see legends to figure 1) was incubated for various lengths of time (abscissa) at 37°C in the presence of free calcium ions or of EDTA. The activity on ATEe was assayed tritrimetrically and expressed in units/ml (ordinate) (21).

DISCUSSION

Addition of increasing amounts of flufenamate to euglobulin fractions first produces an increase in the fibrinolytic activity measured on fibrin plates. This is followed at higher concentrations by progressively decreasing activity (Figs. 2 and 3). In an appropriate test system, these two effects could be studied separately.

The fibrinolysis enhancing effect of flufenamate consists of two activities: a) the major activity is related to the elimination of C1 inactivator. This substance strongly inhibits euglobulin fibrinolysis and considerable activity can be set free by its elimination (14). This was confirmed in the present

study by the demonstration of a fibrinolysis enhancing effect of $C\bar{1}s$ on euglobulin fractions. Similarly, the elimination of C1 inactivator by addition of flufenamate to plasma or euglobulin fractions was demonstrated by the loss of capacity to inhibit $C\bar{1}s$; b) flufenamate also exerted a minor enhancing effect which could not be attributed to the elimination of C1 inactivator. Evidence is presented which indicates that this enhancement is due to an effect on constituents of the euglobulin fraction and not on constituents of the fibrin substrate in the plate assay, such as was possible according to a former study (7). It is possible that other plasma proteinase inhibitors which have been shown to be present in the euglobulin fractions (14,15) are involved; alternatively, the addition of flufenamate results in a higher degree of activation of the proactivator system, which is only partially activated in the euglobulin fractions (27).

The fibrinolysis decreasing effect is observed only at higher concentrations of flufenamate. It becomes progressively more pronounced on prolongation of the periods of preincubation at $37^{o}C$ (Fig. 3). The effect apparently involves inactivation of active fibrinolytic components in the euglobulin fractions. Since morning plasma with a baseline level of fibrinolysis was used throughout the study, these active components concern mainly the intrinsic system of fibrinolysis (28). Additional experiments to be published separately showed that the extrinsic plasminogen activator which is specifically increased in plasma after stimulation of fibrinolysis (e.g., venous occlusion, exercise) is more susceptible to flufenamate, demanding lower concentrations of this compound to be adjusted for every situation.

The common factor in the effects of flufenamate on C1 inactivator, chymotrypsin or the fibrinolytically active components in euglobulin fractions is destruction of the function of these proteins. This appeared to be a time, concentration and temperature-dependent reaction, which seemed to be irreversible under the conditions employed. The experiments show that the elimination of C1 inactivator occurs at lower flufenamate concentrations than the destruction of the fibrinolytically active components. Decreased susceptibility of the fibrinolytically active components is also seen in treatments of plasma with acid (29), acetone (30) or chloroform (31). The difference in susceptibility to flufenamate - called differential inhibition by Von Kaulla (2) - is advantageously used in euglobulin fibrinolysis. The results of the present paper demonstrate that inhibitory effects can be eliminated by a simple technique without affecting the fibrinolytically active components. A euglobulin technique modified according to this principle has been successfully used in other

studies (15,27,28). Studies of the effects of flufenamate on euglobulins from various animal species also show an enhancing effect in most cases (32). It must be emphasized that the use of the fibrin plate assay is essential. As shown, the effects of flufenamate on the preformed fibrin substrate is negligible in the molarities employed. Diffusion of components applied in drops to the fibrin plates results in a lowering of the flufenamate concentration. This is in part responsible for the clear separation between the fibrinolysis enhancing and decreasing effects of flufenamate. Preliminary experiments have shown that the effects of flufenamate are more complex and more difficult to manage in euglobulin clot lysis time methods.

Elimination of C1 inactivator may also be of importance for the effects of flufenamate on whole plasma as observed in the hanging clot method and in the plasma clot method as employed by Von Kaulla (2) or when plasma-flufenamate mixtures are assayed on fibrin plates (33). Optimum enhancing effects of flufenamate are observed in the plasma clot method at 12-13 mM (2) and on the fibrin plates at approximately 17.5 mM (33). These concentrations of flufenamate in plasma eliminate C1 inactivator (Fig. 6). This inhibitor is particularly effective against intrinsic proactivator systems of plasma *in vitro* (27,28) and its elimination may result in facilitated generation of their fibrinolytic potential. That activation of the intrinsic factor XII dependent system occurs, indeed is suggested by the finding of reduced activities in Hageman trait plasma compared to normal plasmas when plasma-flufenamate mixtures are assayed on fibrin plates (33).

The results of this study also provide insight into the factors which influence the fibrinolytic activity of euglobulin fractions (without added flufenamate) in the classical assay on the fibrin plate. This activity is partly determined by the presence of functional C1 inactivator. The available amount of this inhibitor is determined by a partial neutralization of the total amount by intrinsic mechanisms. Thus, $C\bar{1}s$ is an important eliminator of the inhibitor. Likewise, euglobulin fractions contain plasma prekallikrein, which, after activation, may neutralize part of the C1 inactivator, its major inhibitor (34). This indirect involvement of $C\bar{1}s$ and kallikrein in the fibrinolytic activity of euglobulin fractions on fibrin plates provides a link between the complement, kallikrein and fibrinolytic systems. The addition of flufenamate abolishes this link. Similarly, the influence of calcium ions or EDTA on the activation of C1 esterase introduced a calcium/EDTA effect in euglobulin fibrinolysis. The recognition of this particular effect of calcium ions may contribute to a better understanding of the reported complex effects of calcium ions on plasma and euglobulin fibrinolysis (35,36,37,38).

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CHAPTER III

STUDIES ON THE FIBRINOLYTIC SYSTEM IN HUMAN PLASMA: QUANTITATIVE DETERMINATION OF PLASMINOGEN ACTIVATORS AND PROACTIVATORS*

C. Kluft

Abstract. Effects due to plasma plasminogen activators and proactivators are usually studied in assay systems where inhibitors influence the activity and where the degree of activation of proactivators in unknown. Quantitative information on activator and proactivator levels in plasma is therefore not available. Studies on the precipitating and activating properties of dextran sulphate in euglobulin fractionation presented in this paper resulted in the preparation of a fraction in which there was optimal recovery and optimal activation of a number of plasminogen activators and proactivators from human plasma. The quantitative assay of these activators on plasminogen-rich fibrin plates required the addition of flufenamate to eliminate inhibitors. The response on the fibrin plates (lysed zones) could be converted to arbitrary blood activator units (BAU). Consequently, a new activator assay which enables one to quantitatively determine the plasma level of plasminogen activators and proactivators together is introduced. Two different contributions could be distinguished: an activity originating from extrinsic activator and one originating from intrinsic proactivators. The former could be assayed separately by means of its resistance to inhibition by C1-inactivator. Considering the relative concentrations of extrinsic and intrinsic activators, an impression of the pattern of activator content in plasma was gained. In morning plasma with baseline levels of fibrinolysis, the amount of extrinsic activator was negligible as compared to the level of potentially active intrinsic activators. Consequently, the new assay nearly exclusively determines the level of intrinsic activators in morning plasma. A pilot study gave a fairly stable level of 100 $\pm$ 15 BAU/ml (n=50). When fibrinolysis was stimulated by venous occlusion (15 min), the amount of extrinsic activator was greatly increased, reaching a total activator level of 249 $\pm$ 27 BAU/ml (n=7).

*Submitted for publication.

INTRODUCTION

The plasminogen activator activity of blood plasma is usually assayed by isoelectric precipitation of the plasma euglobulins followed by determination of the fibrinolytic activity of the redissolved precipitate. However, the fibrinolytic activity of the euglobulin precipitate varies with conditions of precipitation, such as the plasma dilution and the pH. Furthermore, the separation of blood plasminogen activators from inhibitors present in the plasma by isoelectric precipitation is incomplete; there is only a partial recovery of the total amount of potentially active plasminogen activator present in the plasma in the precipitate (1,2,3). Large amounts of additional activator activity can be recovered when the isoelectric precipitation is performed in the presence of certain polyanions such as acidic polysaccharides (1,2). Dextran sulphate of high molecular weight is particularly effective in this respect (4).

The fibrinolytic activity of the redissolved euglobulin precipitate is increased when flufenamate is added to the solution (5). This increase is mainly due to neutralization of the C1-inactivator by flufenamate (5); some of the C1-inactivator is coprecipitated in the euglobulin fractions. This paper describes how a combination of the plasminogen-activator-generating and precipitating properties of dextran sulphate with the inhibitor-eliminating property of flufenamic acid makes it possible to obtain a quantitative determination of plasminogen activator and proactivator levels in plasma. The total activity includes that contributed by the extrinsic activator derived from tissue and that caused by intrinsic activators produced by activation of precursors in plasma. These activities are determined separately, thus providing a quantitative description of the activator content of plasma.

MATERIALS AND METHODS

Materials

Unless otherwise specified, reagents were of analytical grade and obtained from Merck, West Germany. Distilled water was used throughout the experiments. Additional reagents were obtained from the following sources: Microbiological grade gelatin from Merck; "Agarose for electrophoresis" from B.D.H. Chemicals Ltd., Poole, England; Dextran sulphate, sodium salt $\overline{MW}$ 500,000, and Sepharose 4B from Pharmacia Ltd., Uppsala, Sweden; Flufenamic acid from Aldrich Europe, Beerse, Belgium; Human plasminogen, labeled 15 Casein units/mg protein, from AB Kabi, Stockholm, Sweden; Antihuman antisera raised in rabbits against C1-

-inactivator or plasminogen from Behringwerke A.G., Marburg, West Germany; Plasminogen-free bovine fibrinogen from Poviet, Organon Teknika, Oss, The Netherlands; Plasminogen-rich bovine fibrinogen was prepared according to Brakman (6). Platelet-poor, citrated human plasma and pooled plasma were prepared as previously described (7). Factor XII-deficient plasma (human male, aged 69 years) was obtained from the Department of Hematology, University Hospital, Leiden, through the courtesy of Dr. J.J. Veltkamp. Fletcher-factor-deficient and Fitzgerald-factor-deficient plasmas were purchased from George King Bio-Medical Inc., 151, Main Street, Salem, N.H. The C1-inactivator preparation (8) was from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam. Hog ovary tissue activator was kindly supplied by Drs. T. Astrup and P. Kok (9).

Solutions

EDTA buffer ($\mu = 0.15$): 0.05 M sodium diethylbarbiturate, 0.10 M NaCl, 0.25% (w/v) gelatin and 2.7 mM ethylenediamine tetraacetate (EDTA) adjusted to pH 7.8 with an HCl solution.

Dextran sulphate solutions: (A) In distilled water, 100 mg/l, stable for a few months at room temperature; (B) In EDTA buffer, 250 mg/l, fresly prepared; (C) In flufenamate solution, 175 mg/l (EDTA buffer containing 14 mM sodium flufenamate).

Sodium flufenamate solution: 0.5 M flufenamic acid in equivalent concentration of NaOH (60°C), diluted to 14 mM with EDTA buffer.

Methods

Preparation of euglobulin fractions

Schemes for the preparation from plasma of various euglobulin fractions are shown in figure 1.

Regular euglobulin fraction

Regular euglobulin fractions were prepared with a plasma dilution of 1:10 and a pH of 5.9 as described in detail elsewhere (7).

Supernatant fraction

Precipitates were prepared from the supernatant remaining after separation of the regular euglobulin fraction (cf fig. 1) by addition of 1 ml (per 1 ml plasma) dextran sulphate solution A to the supernatant. The pH was readjusted to 5.9 if necessary. The mixture was then allowed to stand in an ice bath for 30 min before centrifugation. The precipitate was redissolved in EDTA buffer in a volume equal to the original plasma volume.

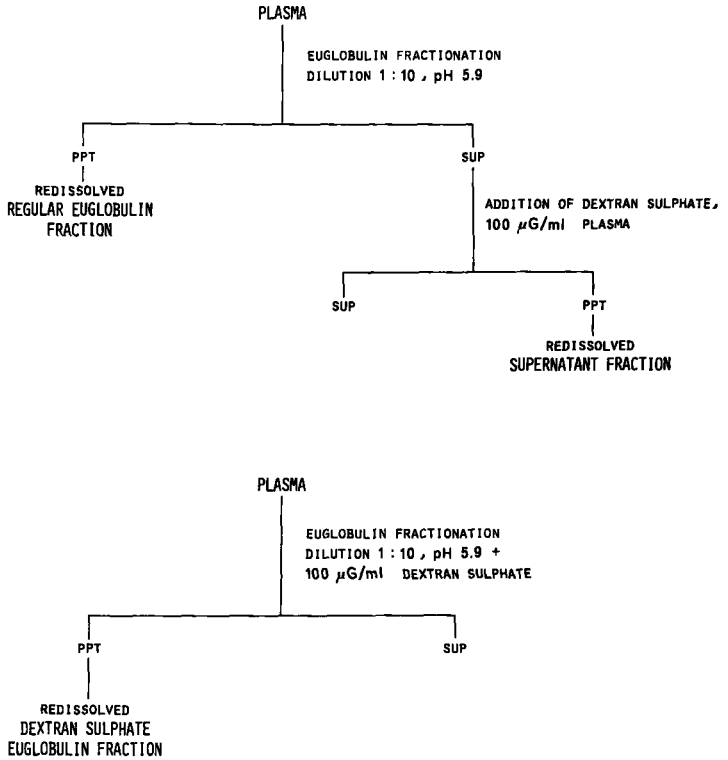

Fig. 1. Schemes of euglobulin fractionation.

Dextran sulphate euglobulin fraction

This fraction was separated from a mixture of 1 ml plasma, 8 ml distilled water and 1 ml dextran sulphate solution A. All solutions were precooled in ice water and mixed rapidly in the order mentioned. Titration with acetic acid to pH 5.9 was started immediately after mixing. After centrifugation in the cold, the precipitate was redissolved in 1 ml EDTA buffer.

Assay of fibrinolytic activity

Fibrinolytic activities were assayed by the fibrin plate method (10). Diameters of the lysed zones after 18 hrs of incubation at 37°C were used to represent the fibrinolytic activity of the solutions tested. Alternatively, the activity was expressed in arbitrary blood activator units (BAU) as defined below under "Experimental", section 3.

Regular assay

In the regular assay of fibrinolytic activity, drops of 30 µl of the euglobulin solutions were applied to the plasminogen-rich bovine fibrin plates (in triplicate).

Modified assays

In these modified assays, several reagents were added to the euglobulin fractions before assay. Immediately after application of the 30 µl drops of the euglobulin fraction to the fibrin plate, 5 µl of the appropriate solution (see below) were added to each drop with a syringe. The increase in the size of the drops from 30 to 35 µl was accounted for in controls by addition of EDTA buffer. This simple and convenient technique was found to yield results comparable to those obtained by premixing the solutions. Compounds added:

(I) *Flufenamate:* A 5 µl drop of 14 mM flufenamate in EDTA buffer was added in order to obtain a final concentration of 2 mM in the euglobulin fraction.

(II) *Dextran sulphate:* 5 µl of dextran sulphate solution B.

(III) *Dextran sulphate + flufenamate:* 5 µl of dextran sulphate solution C.

(IV) *Plasminogen:* 5 µl of an appropriate concentration of plasminogen dissolved in EDTA buffer.

(V) *C1-inactivator:* 5 µl of C1-inactivator dissolved in EDTA buffer.

The test for C1-inactivator-resistant fibrinolytic activity requires the addition of 5 µl of 700% (relative to pooled plasma) C1-inactivator. The regular euglobulin fractions usually contain about 25% of the plasma level of endogenous C1-inactivator. As a control, the amount of C1-inactivator in each euglobulin fraction was determined. If it was not between 20 and 30%, the assay with added C1-inactivator was repeated with an adjusted amount of exogenous C1-inactivator. Alternatively, a range of concentrations of exogenous C1-inactivator was used and the results obtained by interpolation at a total amount of 125% C1-inactivator.

Removal of plasminogen from the dextran sulphate euglobulin fraction for experiments presented in Table 1. Lysine was coupled to Sepharose 4B as described by Deutsch and Mertz (11). A level of 1.5% plasminogen relative to that in plasma was obtained by batch-wise treatment with the gel equilibrated in EDTA buffer (0.6 ml gel/ml fraction) and incubation for 30 min at 0°C. The fraction was diluted to about 73% of the original concentration by this procedure.

Plasminogen and C1-inactivator were assayed by the quantitative radial immunodiffusion method according to Mancini et al. (12). The pooled human

plasma served as an arbitrary reference (100%).

Venous occlusion of the arm for 15 min was performed as described by Robertson et al. (13).

EXPERIMENTAL

1. The complexity of euglobulin fibrinolysis

a) Inhibitors in the euglobulin fraction

Euglobulin fractions still contain inhibitors. The main substance inhibiting the fibrinolytic activity of the fractions in assays on fibrin plates was found to be the C1-inactivator, which coprecipitates in the fractions in varying amounts depending upon the conditions of fractionation (3). A number of synthetic thrombolytic agents owe their capacity to induce lysis of fibrin in vitro to the ability to inactivate inhibitors of fibrinolysis (14). One of the most potent of the agents, flufenamic acid, nullifies the influence of inhibitors on addition to the euglobulin fractions (5,7). Fig. 2 shows how flufenamate enhances euglobulin fibrinolytic activity.

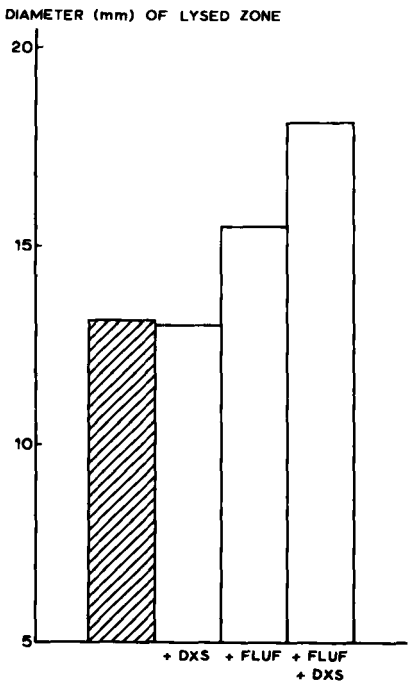

Fig. 2. Effects of dextran sulphate and flufenamate on the fibrinolytic activity of the regular euglobulin fraction. The fibrinolytic activity of the regular euglobulin fraction of pooled morning plasma is shown by the height of the shaded column and expressed as diameters of lysed zones in plasminogen-rich fibrin plates after 18 h incubation (ordinate). All open columns record activities obtained in the same regular euglobulin fraction by addition of synthetic compounds: dextran sulphate (DXS, 25 µg/ml) and/or flufenamate (FLUF, 2 mM).

b) Proactivators in the euglobulin fraction

Plasma contains a factor XII-dependent, fibrinolysis-inducing proactivator/activator system (15). This proactivator system is also present in the euglobulin fractions. When activation of proactivator is incomplete due to a failure to convert sufficient factor XII to its active form, an increase in fibrinolytic activity should follow the addition of a factor XII activator. Dextran sulphate is an excellent activator of factor XII (16). Its solubility is of particular advantage in the assay of euglobulin fractions. As shown in fig. 2, the addition of dextran sulphate alone did not enhance euglobulin fibrinolysis. In contrast, the combined addition of flufenamate and dextran sulphate produced an increase in activity greater than that obtained with flufenamate alone. Optimum activity was obtained when the euglobulin solution contained 2 mM flufenamate and 25 µg/ml dextran sulphate. The results indicate that "unconverted" proactivator is present in the euglobulin fractions as usually precipitated and assayed. Optimal activation could apparently be achieved by the dextran sulphate only when inhibitors were simultaneously inactivated by addition of flufenamate.

c) Two types of activator in the euglobulin fraction

In addition to the activator derived from the factor XII-dependent proactivator system, it is generally assumed that extrinsic activator of vascular origin is present in plasma and is separated in the euglobulin fraction. When it was found that C1-inactivator inhibits only part of the fibrinolytic activity of euglobulin fractions, the existence of two different types of activator became obvious (18,19). Experiments to be presented in detail elsewhere showed that extrinsic activators (urokinase, tissue activator, vascular activator from cadaver limb perfusion) are resistant to inhibition by the C1-inactivator. It is most likely therefore that the C1-inactivator resistant activity in the euglobulin fraction represents its content of extrinsic activator(s). To identify the part of the total activator activity which is readily inhibited by C1-inactivator, we studied samples of plasma known for their defective activation of the factor XII-dependent pathway of fibrinolysis (Hageman, Fletcher, and Fitzgerald trait). Fig. 3 shows that the measured activity of the euglobulin fraction is considerably lower in the deficient plasma samples than in the normal plasma. It is also seen that the reduction in activity primarily affects the activity which is sensitive to the C1-inactivator, while that resistant to this inactivator remains essentially unchanged. From these results, it is concluded that the factor XII-dependent activator activity belongs to that part which is decreased by the C1-inactivator. The identical magnitude of the activity which is resistant to the C1-inactivator in the norm-

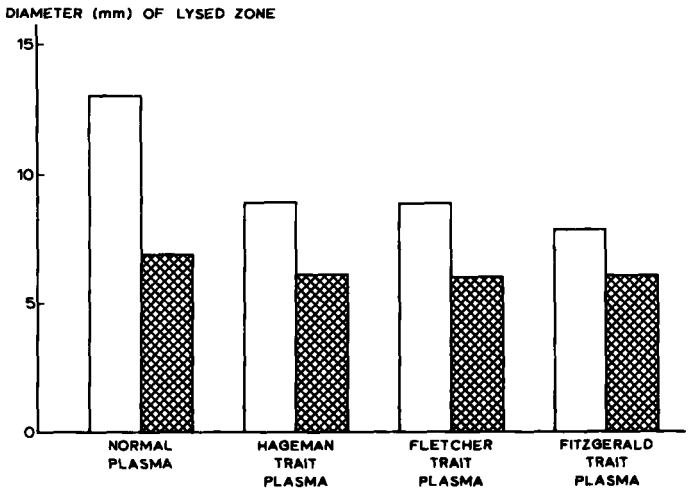

Fig. 3. Euglobulin fibrinolytic activities in normal plasma compared to plasmas deficient in essential components of the factor XII-dependent pathway. The fibrinolytic activity is expressed as diameters of lysed zones (ordinate) and determined by the regular euglobulin assay (open bars) and with added C1--inactivator (hatched bars). Morning plasmas were obtained from healthy volunteers (mean of 15 subjects), one Hageman trait patient (deficient in factor XII), one Fletcher trait patient (deficient in plasma prekallikrein) and one Fitzgerald trait patient (deficient in high molecular weight kininogen).

al and deficient plasma samples is compatible with its assumed origin from an extrinsic activator. Therefore, in the sections below, the C1-inactivator resistant part of the total activity is referred to as that representing the content of extrinsic activators, while the inhibited part represents activators derived from the intrinsic system. It will be shown that the intrinsic system includes a factor XII-dependent and possibly also a factor XII-independent pathway.

d) Incomplete recovery of activity in the regular euglobulin fractions

Only a portion of the total fibrinolytic activity in plasma is recovered in the regular euglobulin fraction. Additional activity can be precipitated with acid polysaccharides. Dextran sulphate is a particularly suitable precipitating agent (4), in addition to its capacity to activate factor XII. Fig. 4 shows that the activity exhibited by precipitates obtained by addition of dextran sulphate to the supernatant remaining after separation of the regular euglobulin fraction increases with the concentration of dextran sulphate until an optimum is reached. However, coprecipitation of the inhibitor also increases, reaching 30% C1-inactivator relative to plasma at 100 µg/ml dextran sulphate

so that it is necessary to add flufenamate to register a plateau of recovery (Fig. 4). When additional amounts of C1-inactivator were added to the precipitate from the supernatant (100 µg/ml dextran sulphate) the inhibition of fibrinolysis was complete. This indicates that only activators of the intrinsic type are present in this fraction. From these findings, it is concluded that the intrinsic activator system was only partially recovered during the preparation of regular euglobulin fractions.

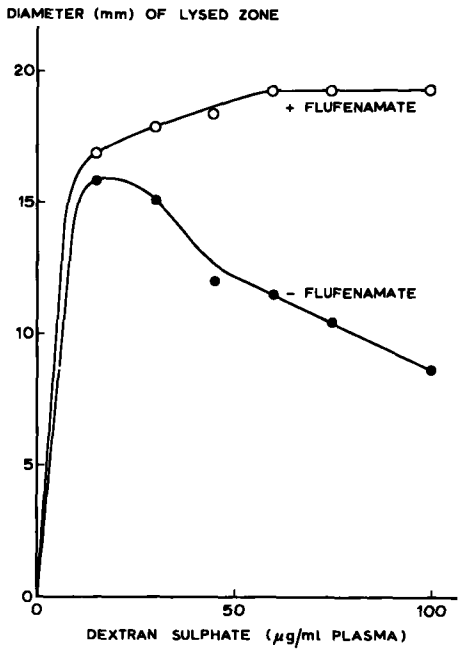

Fig. 4. Fibrinolytic activity in precipitates obtained from supernatants of euglobulin fractions by means of dextran sulphate. Each point in the figure represents a precipitate collected after addition of the indicated amount of dextran sulphate (abscissa) to the supernatant resulting from the removal of the regular euglobulin fraction. The fibrinolytic activity of the resuspended precipitates was determined on plasminogen-rich bovine fibrin plates without (●—●) and with (o—o) flufenamate (2 mM) added to the fraction and recorded as diameters of lysed zones (ordinate).

2. The optimal precipitation and activation of activators and proactivators from plasma

a) Fractionation in the presence of dextran sulphate

The above survey indicates that dextran sulphate and flufenamate are powerful tools for activating proactivators, increasing the precipitation of activators in the euglobulin fractions and eliminating the effect of inhibitors. An attempt was therefore made to use these agents to produce a euglobulin fraction from plasma in which there would be optimal activation and precipitation of

activators. The experiment presented in Fig. 5 shows that euglobulin fractions precipitated from plasma in the presence of dextran sulphate increase in activity until an optimum is reached at 100 µg/ml dextran sulphate. Addition of dextran sulphate to this fraction did not further increase the activity in the assay. Addition of flufenamate to the redissolved precipitate eliminates inhibitors, as is evident from the lower activities recorded in the absence of flufenamate. Increased coprecipitation of inhibitors occurs at higher dextran sulphate concentrations. Thus, the concentration of C1-inactivator in the fractions increases from 25% of the plasma concentration in the regular euglobulin to 65% in that precipitated with 100 µg/ml dextran sulphate. The results indicate that optimal precipitation and activation is achieved when the precipitation of a euglobulin fraction from plasma takes place in the presence of 100 µg dextran sulphate per ml plasma. Such a fraction is now referred to as "the dextran sulphate euglobulin fraction".

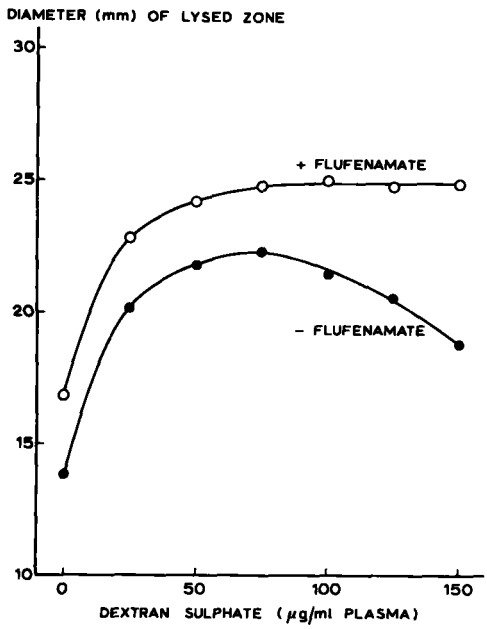

Fig. 5. Fibrinolytic activity of euglobulin fractions prepared in the presence of dextran sulphate. Each point in the figure represents a euglobulin fraction prepared from plasma in the presence of the indicated amount of dextran sulphate (abscissa). The fibrinolytic activity of the fractions was determined on plasminogen-rich bovine fibrin plates without (●–●) and with (o–o) flufenamate (2 mM) added to the fraction and recorded as diameters of lysed zones (ordinate).

The contact activation by dextran sulphate in plasma occurs very rapidly, even at 0°C, a temperature at which prekallikrein is completely converted to kallikrein in 7 minutes at the employed dextran sulphate concentrations (16). Several time-consuming steps are involved in the preparation of the dextran sulphate euglobulin fraction. Some of these time periods may be required in order to achieve activation or, alternatively, they must be kept short to prevent inactivation or destruction of activated components. The cold technique already in routine use for euglobulin precipitation (7) was strictly adhered to in order to diminish losses of activity. Preincubation of mixtures of plasma and dextran sulphate at 0°C for 1, 4 and 10 min showed a slow, progressive loss of activity in the subsequently prepared dextran sulphate euglobulin fraction. Maximal and reproducible activity was achieved when the inactivation was delayed by dilution of the plasma with cold distilled water before the dextran sulphate was added prior to the adjustment of pH. This adjustment was made with an automatic titrator and it required approximately 3 min; faster or slower titration showed no improvement. Addition of the appropriate amount of acetic acid as one volume or addition of dextran sulphate after adjustment of pH gave inferior results as judged from the activity of the separated dextran sulphate euglobulin fraction. The titrated mixture could be left on ice for 30 min without changes in recovery of activity. This is in contrast to the kaolin activation procedures (17,20) which require incubation at 37°C at this stage. All variations in technical procedures indicated the same optimal activity; therefore, a procedure employing the optimal conditions of activation and precipitation as described here was adopted for the quantitative determination of plasminogen activators in plasma.

b) Dilution curves of the dextran sulphate euglobulin fraction

If optimal activation and recovery of activators is achieved in the dextran sulphate euglobulin fraction, dilutions of this fraction should yield a dilution curve of the activator in the fraction. The amount of dextran sulphate present in the solution of the dextran sulphate euglobulin fraction itself was found to yield an optimal activity in the presence of 2 mM flufenamate. To achieve optimal activities in every dilution of serial dilutions of this fraction, series of dextran sulphate and flufenamate concentrations were tested. Because of changes in protein concentrations, each dilution was found to require a different amount of added dextran sulphate and flufenamate for optimal activity. The optimal activities thus determined produced dilution curves as shown in Fig. 6. The curve is linear over an appreciable range in the double logarithmic plot and parallels that of dilutions of the tissue plasminogen

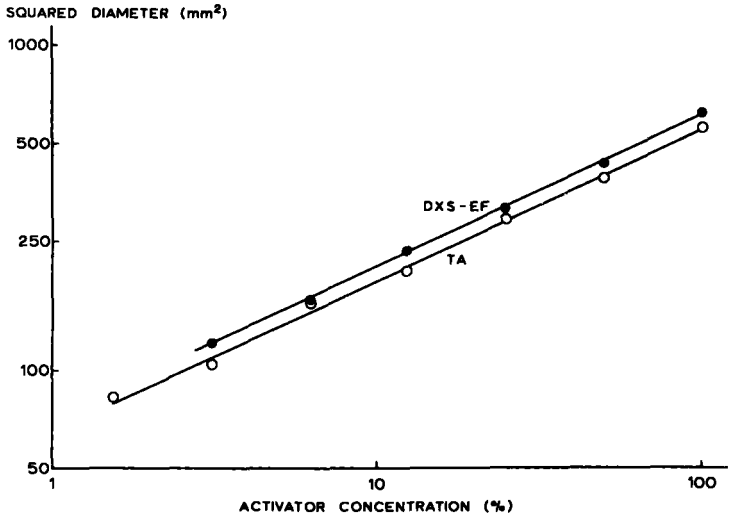

Fig. 6. Dilution curves of the dextran sulphate euglobulin fraction of pooled morning plasma and of hog ovary tissue activator. The fibrinolytic activity was determined on plasminogen-rich fibrin plates and recorded as squared diameters of lysed zones (ordinate, log-scale) after 18 hrs of incubation at 37°C. Purified hog tissue activator (TA) was diluted 1:1 with 1.0 CU/ml bovine plasminogen solution and further diluted serially in buffer containing 0.5 CU/ml plasminogen. The dextran sulphate euglobulin fraction (DXS-EF) was diluted serially in EDTA-gelatin buffer and each dilution was assayed in the presence of optimal amounts of dextran sulphate and flufenamate (see text).

activator.

c) Complementary activities of the regular euglobulin fraction and the dextran sulphate precipitate from its supernatant

To substantiate the complete recovery and activation of activators in the dextran sulphate euglobulin fraction, it is necessary to show that the maximal activity which can be elicited in the regular euglobulin fraction plus that precipitated from its supernatant by dextran sulphate (cf figures 1 and 4) equals the activity of the dextran sulphate euglobulin fraction. Pooled morning plasma was used in the study of these problems. The maximal activities in each fraction were determined in the presence of dextran sulphate and/or flufenamate as described in the previous sections. Firstly, aliquots of double concentrated solutions of the regular euglobulin fraction and of the precipitate from its supernatant with dextran sulphate were mixed. The activity of this mixture was found to closely approach the activity of the dextran sulphate euglobulin fraction (interpolation on the dilution curve gave close to 100%). Consequently, in this approach, the basis of the quantitative assay appeared valid. Secondly,

the maximal activity of the regular euglobulin fraction and the corresponding supernatant fraction (using 100 µg/ml dextran sulphate) were determined separately. The sum of these activities was calculated after interpolation on the dilution curve of the dextran sulphate euglobulin fraction. Here, the initially calculated sums were much too low. It was then found that the supernatant fraction was deficient in plasminogen (see also below). When sufficient plasminogen was added to the solution, higher activities were obtained and values close to 100% were obtained. These experiments further established the relation between the three different precipitates and showed that optimal conditions for the assay of plasminogen activators in each of the precipitates were found.

3. Dose-response curve and definition of unit

As indicated above, the linear dilution curve of the dextran sulphate euglobulin fraction can serve as a dose-response curve for the determination of plasminogen activators in the fraction. This determination was further validated when a parallel curve was obtained from assays with serial dilutions of purified tissue activator isolated from pig ovaries in the presence of added plasminogen (Fig. 6). Dilutions of regular euglobulin fractions prepared from plasma with strongly stimulated fibrinolysis (venous occlusion, nicotinic acid administration) resulting in high levels of the extrinsic activator yielded similar linear curves for the first few dilutions, with a slope of around 0.45. Therefore, a linear curve with slope 0.45, closely fitting the experimental points obtained from the pooled morning plasma (Fig. 6), was adapted as a dose-response curve for the determination of plasminogen activators in euglobulin fractions. The activator concentration in the dextran sulphate euglobulin fraction prepared from normal pooled morning plasma was arbitrarily set at 100 blood activator units (BAU)/ml. Such plasma pools were found to be stable for several months at -80°C. Three subsequently prepared pools of plasma showed approximately the same amount of activator, indicating their usefulness as a standard of reference.

4. Requirement of plasminogen

Plasminogen is essential for the assay of activators by determination of the fibrinolytic activity produced by them. The necessity to supplement the supernatant precipitates with additional plasminogen drew attention to the need to ensure that sufficient plasminogen is present during the assay of activator activity. The content of plasminogen in the regular euglobulin fraction,

in its supernatant precipitate and in the dextran sulphate euglobulin fraction from pooled plasma were found to be 85%, 7%, and 96%, respectively, relative to the concentration in the plasma. Addition of purified plasminogen to these fractions increased the fibrinolytic activity of only the supernatant fraction. When the plasminogen concentration of this fraction was increased to 70% of that in the pooled plasma, no further increase in activity was seen. Obviously, each fraction must have an amount of plasminogen which corresponds to around 70% of the amount in normal pooled plasma before optimal conditions for the registration of activator activity are established, even though so-called plasminogen-rich fibrin plates are used. An additional experiment (Table 1) in which the activities of the normal dextran sulphate euglobulin fraction and a fraction depleted of plasminogen by treatment with Sepharose-lysine (both assayed on plasminogen-rich fibrin plates) were compared verified the need to secure sufficient concentrations of plasminogen in the fraction.

Table 1. Plasminogen requirements for fibrinolytic activity assays of the dextran sulphate euglobulin fraction

	Activity of the fraction on fibrin plates	
Plasminogen content in fraction	Fibrin + plg* mm (BAU/ml)**	Fibrin - plg mm (BAU/ml)
normal (88 %)	25.0 (103)	19.6 (36)
depleted (1.5%)	19.7 (37)	10.9 (3)
readded (59 %)	23.2 (74)	18.7 (30)

* plg = plasminogen

**BAU = Blood Activator Units

Fibrinolytic activities of dextran sulphate euglobulin fractions of pooled morning plasma are assayed on plasminogen-rich and plasminogen-free fibrin plates. The activities are expressed as diameters (mm) of lysed zones after 18 hrs incubation of the plates and also as BAU/ml. Three types of dextran sulphate euglobulin fractions are distinguished according to their plasminogen status: 1) the normal fraction; 2) a fraction depleted of plasminogen by Sepharose-lysine treatment; and 3) the latter after readdition of purified plasminogen. The actual plasminogen content of each fraction relative to the plasma is given.

The possible contribution of a direct protease effect (not mediated by plasminogen) to the total activity of the dextran sulphate euglobulin fraction was determined by assays on plasminogen-free fibrin plates (Table 1). The activity of the plasminogen-depleted fraction on the plasminogen-free fibrin plates was very slight. Converted to blood activator units by interpolation on the reference curve, it corresponded to only 3% of the activator concentration in the pooled plasma. The treatment with Sepharose-lysine did not deplete the fraction of activator; its activity reappeared after addition of purified plasminogen, although this activity was lower than the original because of the dilution occurring during the Sepharose-lysine treatment (see Methods). These results show that the activity produced by the dextran sulphate euglobulin fractions on the plasminogen-rich fibrin plates is nearly exclusively due to plasminogen activators.

5. Requirement of factor XII

As shown above (section 1b), the factor XII-dependent pathway of fibrinolysis is involved in the production of activator activity in the euglobulin fractions. In Hageman trait plasma (deficient in factor XII), activation of fibrinolysis in incomplete. This offers the possibility to study the requirement for factor XII in evoking activity in the dextran sulphate euglobulin fraction. This is of importance, since activation appears to be complete when this fraction is prepared from normal plasma (see above). As shown in fig. 7, the generation of fibrinolytic activity by dextran sulphate in Hageman trait plasma is similar to the generation in pooled normal plasma, except that a considerable lower level of activity is reached. It corresponds to an activator concentration of 48 BAU/ml in this particular Hageman trait plasma, while normal plasma was found to contain 100 $\pm$ 15 BAU/ml ($\bar{x} \pm$ SD; n=50). This low level of activator activity in the deficient plasma could be due to insufficient activation of proactivator in the dextran sulphate euglobulin fraction. Therefore, factor XII was provided by the addition of normal plasma. When dextran sulphate euglobulin fractions prepared from Hageman trait plasma were mixed in various ratios with those prepared from normal plasma, a pattern indicating a correction of the deficient activation in the Hageman trait plasma evolved (Fig. 8). Eventually, practically normal levels of proactivator appeared to be present in this plasma. Complete correction in the dextran sulphate euglobulin fraction prepared from the Hageman trait plasma was obtained by addition of only 5% of a fraction prepared from normal plasma. Simple mixing of the plasmas in this ratio before the fractionation also resulted in complete

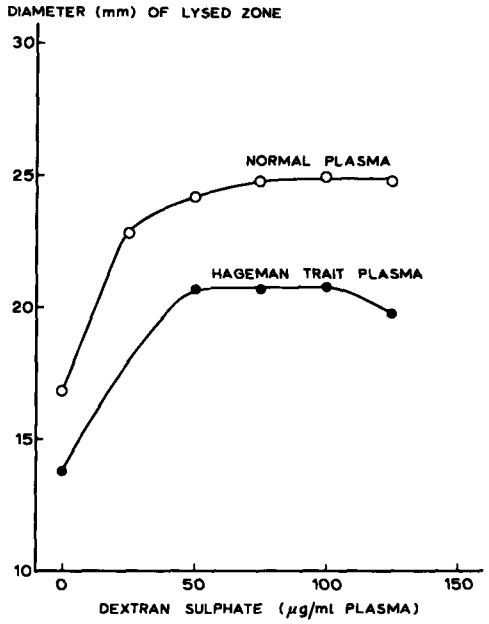

Fig. 7. Effects of dextran sulphate on euglobulin fractionation of normal and factor XII--deficient plasma. Each point in the figure represents a euglobulin fraction prepared from pooled normal (o—o) or Hageman trait plasma (●—●) in the presence of the indicated amount of dextran sulphate (abscissa). The fibrinolytic activity of the fractions was determined on plasminogen--rich bovine fibrin plates with 2 mM flufenamate added to the fraction and recorded as diameters of lysed zones (ordinate).

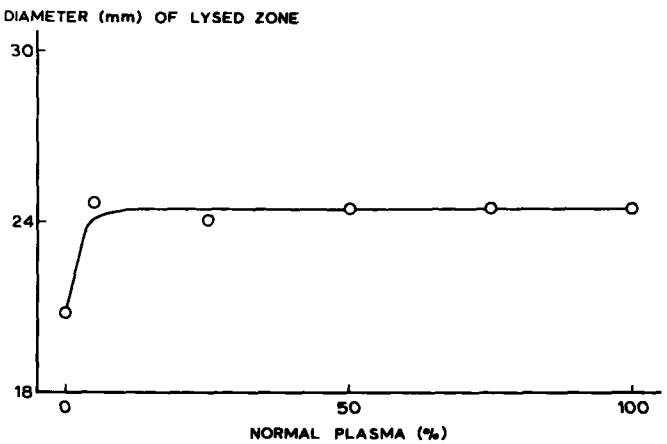

Fig. 8. Correction of factor XII deficiency with pooled normal plasma. Dextran sulphate euglobulin fractions of Hageman trait and pooled normal plasma were mixed in various ratios. The percentage contribution of normal plasma is indicated on the abscissa. The fibrinolytic activity of these mixtures was determined on plasminogen-rich bovine fibrin plates and recorded as diameters of lysed zones (ordinate) after 18 hrs of incubation at 37°C.

correction. These results lead to a number of conclusions. Firstly, all essential components for the factor XII-dependent pathway of fibrinolysis are present in the dextran sulphate euglobulin fraction. Secondly, complete activation of the factor XII-dependent proactivator(s) is achieved in the fractions prepared from normal plasma; fractions prepared from the deficient plasma require only the addition of 5% normal fraction for full restoration of activity. Sufficient factor XII therefore seems to be present in the fractions from normal plasma. Thirdly, the amount of activator activity revealed by correction in Hageman trait plasma (approximately 50% of the total) is clearly factor XII-dependent; the other half is already present in the deficient plasma and remains unspecified. It has been suggested that the latter activity represents a factor XII-independent pathway of fibrinolysis (4,21). A candidate for this is the extrinsic system of fibrinolysis. However, in the Hageman trait plasma studied, the amount of C1-inactivator resistant activity ascribed to extrinsic activator was found to be very low (a few BAU/ml; see also fig. 3). Furthermore, the activity must be classified as intrinsic, since C1-inactivator was found to completely inhibit it. The subdivision of the activator content of the dextran euglobulin fraction of morning plasma obtained from this study on Hageman trait plasma is shown in figure 9.

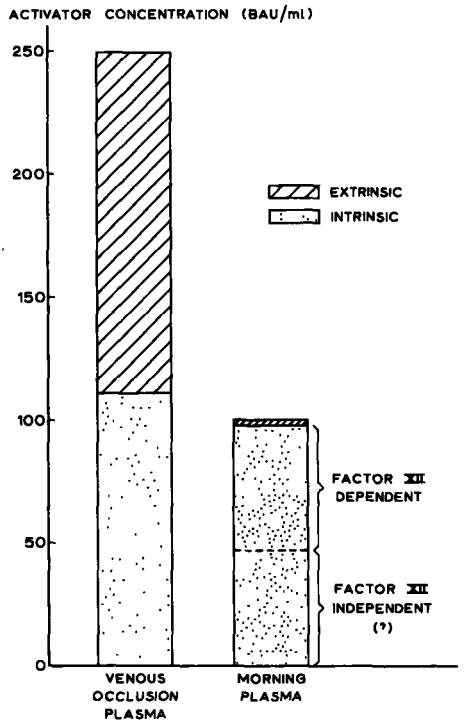

Fig. 9. Plasminogen activator composition in dextran sulphate euglobulin fractions prepared from morning and venous occlusion plasma.

6. Results with the new method and comparison with the regular euglobulin assay

A comparison of the new activator assay with the usually employed regular euglobulin assay was made by using normal morning plasma. Samples of plasma from a group of eleven apparently healthy volunteers (aged 20 to 50; not on medication) gave the results shown in figure 10.

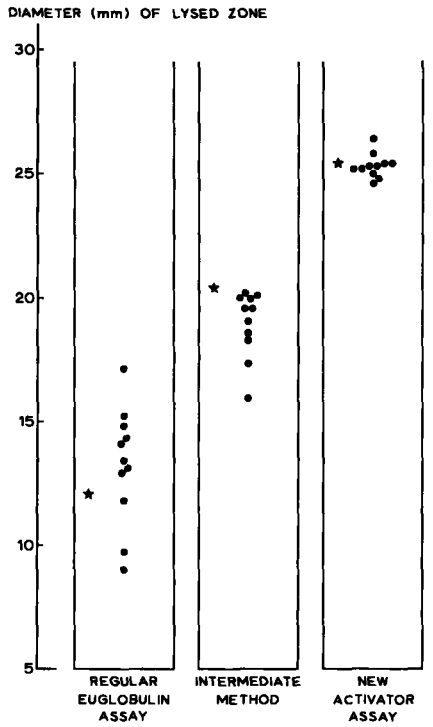

Fig. 10. Comparison of methods for the assay of fibrinolytic activity in morning plasma. The regular euglobulin method (left hand panel) and the new activator assay in the dextran sulphate euglobulin fraction (right hand panel) are compared in a pilot study on morning plasma of eleven apparently healthy volunteers (●) aged between 20 and 50 and a pooled sample (*) prepared from their plasmas. The middle panel shows an intermediate method using the fractionation step of the regular method and the assay procedures of the new method. In the intermediate method, all activator of the regular fraction is revealed by adding flufenamate (2 mM) and dextran sulphate (25 µg/ml) to the fractions before assay. The fibrinolytic activity was determined on plasminogen-rich fibrin plates and recorded as diameters of lysed zones after 18 hrs of incubation at 37°C (ordinate).

Assayed by the new method (right panel), a range of activities from 24.6-26.4 mm (corresponding to 89-117 BAU/ml) was found; the arithmetical mean of the individual concentrations (99 BAU/ml) agrees well with the activator concentration in the pooled sample (100 BAU/ml). In addition, assays of 50 normal subjects gave a fairly uniform level of total activator concentration of 100 ± 15 (SD) BAU/ml in the morning plasma. Figure 10 also shows the results of assays with the regular euglobulin method (left panel). In addition the middle panel of the figure shows results obtained when solutions of the regular euglobulins were assayed in the presence of added dextran sulphate and flufenamate. The values obtained with this intermediate method clearly show the effect of dextran sulphate on the euglobulin precipitation. With the intermediate method, activities of 15.9-20.2 mm (corresponding to 15-41 BAU/ml) are obtained. These are considerably lower than the 89-117 BAU/ml obtained with the new assay. These values demonstrate how much of the total activator concentration in the dextran sulphate euglobulin fraction is recovered in the regular euglobulin fraction. To account for individual variations, this amount was also calculated separately for each individual and was found to vary from 16-38%. This range indicates variation in fractionation.

The usual assay of the regular euglobulin fraction does not reveal all activator activity in this fraction. This is obvious when comparing the regular method (left panel) and the intermediate method (middle panel). The regular method shows a broad range of activities from 9.0-17.1 mm (corresponding to 1.2-19 BAU/ml). Calculated separately for each individual, it becomes apparent that only 4-15% of the potential activity in the fraction is revealed in the regular assay. This is obviously due to inhibition and incomplete activation of proactivators, which can be overcome by addition of flufenamate and dextran sulphate.

As shown above, samples of morning plasma with a baseline level of fibrinolysis showed a fairly uniform total activator concentration. In samples of plasma with stimulated fibrinolysis the total activator concentration was considerably higher. Such stimulation was obtained by venous occlusion of the arm for 15 min. This resulted in plasma with an estimated activator concentration of 249 ± 27 BAU/ml ($\bar{x}$ ± SD; n=7)(Fig. 9). The increase is due to the presence of extrinsic activator (of which only a few BAU/ml are present in the preocclusion plasma samples) since 138 ± 29 BAU/ml ($\bar{x}$ ± SD; n=7) was measured in the occlusion plasma after neutralization of the intrinsic activators with C1-inactivator. The amount of activator derived from the intrinsic system remains essentially unchanged by venous occlusion, showing only a slight increase due to haemo-concentration. These differences between morning and venous occlusion

plasma are clearly shown in figure 9.

DISCUSSION

Optimal recovery of a group of activators and proactivators in normal plasma in the dextran sulphate euglobulin is demonstrated. This optimal precipitation is separately demonstrated for the factor XII independent components of this group in Hageman trait (Fig. 7) and Fletcher trait plasma as well as for extrinsic activator(s) (Chapter IV of this thesis).

The activators and proactivators in the dextran sulphate euglobulin fraction are susceptible to isoelectric and dextran sulphate precipitation. Theoretically, other components not susceptible to these precipitation procedures may exist and remain in the supernatant. Fibrinolytic activity could not be demonstrated in this diluted supernatant; however, inhibitors could have prevented this. The assay of the proactivators in the dextran sulphate euglobulin fraction requires their complete conversion to active enzymes. Of the known factors participating in this conversion, sufficient quantities are present, as shown for dextran sulphate, factor XII (Fig. 7), prekallikrein (31) and high molecular weight kininogen (31). From this it is concluded that optimal conversion from proactivators to activators has been achieved. Additional evidence is obtained from unsuccessful attempts to generate more activator activity in the dextran sulphate euglobulin fraction of normal plasma by incubation, by additions of active factor XII, kallikreins, tissue activator or urokinase. Only proportional recovery of added activator activity was found.

The use of two synthetic compounds, flufenamate and dextran sulphate, as tools in the present study on euglobulin fibrinolysis was found to be essential. Flufenamate is a potent representative of the synthetic thrombolytic agents which were comprehensively studied by Von Kaulla (14) and which have, among others, the capacity to eliminate inhibitors of fibrinolysis. In an accurate assay of euglobulin fibrinolysis, it is essential to eliminate the effect of the C1-inactivator or other traces of proteinase inhibitors which may be present in these fractions. The elimination achieved by flufenamate is selective and does not affect the active components in the fraction. Flufenamate does not activate factor XII. Dextran sulphate has two equally important effects. It enhances activation of the proactivator and ensures optimal precipitation of activators. Its effect on the precipitation is most clearly demonstrated by the formation of the active supernatant precipitate, as seen in figure 4.

Precipitates prepared in the presence of dextran sulphate contain a variety of proteins including, among others, C1-inactivator, plasminogen and plasminogen proactivators, suggesting a rather nonspecific mechanism of precipitation. Most likely, at low ionic strength and low pH (5.9), dextran sulphate forms insoluble salt-like complexes which are similar to those reported for other anionic polyelectrolytes with various proteins (22). Proteins with an overall negative charge at pH 5.9 may also be involved, as shown for the C1-inactivator in our experiments (the isoelectric point of C1-inactivator is reported as 2.7-2.8 (23)). Our results show that plasma plasminogen activators are precipitated at low concentrations of dextran sulphate, while inhibitors (e.g., C1-inactivator) require higher concentrations. This difference is responsible for the shapes of the curves in figures 4 and 5, where inhibitors precipitated at high dextran sulphate concentrations result in a decrease in fibrinolytic activity. Correct interpretation of the activator precipitation curves was possible only after the elimination of inhibitors by flufenamate.

It has been suggested that polyanions naturally occurring in plasma (e.g., mucopolysaccharides) are involved in the regular preparation of euglobulin fractions (1,22,24,25). It can be imagined that variations in plasma levels of such compounds may result in variable fractionation results. Such a mechanism might be responsible for the variations (16-38%) encountered in the precipitation of plasminogen activators from eleven individuals (Fig. 10). The activating properties of peptone-like agents (polyelectrolytes to which the dextran sulphate belongs) has been a subject of speculation. Ungar and Mist (26) originally proposed that the agents split an activator-inhibitor complex present in the serum (guinea-pig). Olesen (27) found that activation and euglobulin fractionation were inseparable. He assumed, as did Macfarlane and Pilling (28), that a hypothetical enzyme-inhibitor complex was split by the euglobulin fractionation and that this splitting was reinforced by the peptone-like agents. Our experiments (Fig. 2), clearly demonstrate that dextran sulphate exerts an activating effect on the euglobulin precipitate after the fractionation has taken place. Dextran sulphate is a potent, soluble activator of factor XII (16) and it is proposed that it exerts its activating effect in this capacity. The full expression of this activating effect depends upon elimination of inhibitors in the euglobulin fraction; the full activating effect is therefore observed as a synergistic effect of flufenamate and dextran sulphate. In this context, it should be noted that the C1-inactivator which is eliminated by the flufenamate is the main inhibitor of plasma kallikrein (29) and active factor XII (30), the former being an important cofactor in the ac-

tivation of plasminogen proactivator and presumably being a plasminogen activator itself (31). As mentioned above, a portion of the fibrinolytic activity present in the dextran sulphate euglobulin fraction seemed to be independent of the presence of factor XII. The possible activating effect of dextran sulphate on this portion remains to be determined.

The new activator assay can be regarded as a contact activation test, because of the activation of the factor XII-dependent pathway of fibrinolysis initiated by dextran sulphate. Investigation of the roles of factor XII, high molecular weight kininogen and kallikrein in this process have been reported recently (31). Compared to other contact activation tests such as those described by Iatridis and Ferguson (20) and Ogston et al. (32), it is an advantage that dextran sulphate is a soluble activator of factor XII in contrast to kaolin. This makes it possible to avoid problems of adsorption and diffusion during activation, precipitation or assay. The use of flufenamate to eliminate the inhibition by C1-inactivator (33) and the complete activation and precipitation by dextran sulphate makes it possible to include all potential activators in the assay. The consequently greater effect of the dextran sulphate compared to that of kaolin has been noted earlier (4).

The present study shows that it is now possible to quantify plasminogen activators in plasma. Previous attempts to relate fibrinolytic activities of euglobulin fractions (recorded as zones of lysis on fibrin plates) with activator concentrations were unsuccessful because of the large variations in the slopes of the double logarithmic dilution curves of the euglobulin fractions (34). This failure can now be attributed to the complex composition of the regular euglobulin fraction, which contains inhibitors, "unconverted" proactivators and more than one type of activator. Euglobulin precipitation in the presence of dextran sulphate and elimination of inhibitors by addition of flufenamate yielded a dilution curve for which evidence is presented that it represents a dose-response curve for plasminogen activators in the fraction. This linear dilution curve has the same slope in the double logarithmic graph as the dilution curve obtained by the assay of a purified tissue plasminogen activator in the presence of added plasminogen. The addition of plasminogen is essential in this model. The dilution curve obtained in assays of the tissue activator without added plasminogen differed from the curve obtained when plasminogen was added to the solutions, despite the use of plasminogen-rich fibrin plates. Other studies have shown differences in the patterns of dilution curves for urokinase and tissue activator (35). Our results indicate that

plasminogen activators may show a more uniform pattern of response when they are assayed in the presence of sufficient plasminogen. In the assay of euglobulin fractions, we observed a definite requirement for plasminogen for optimal activator activity. Apart from a possibly more general validity, it is advantageous that these dilution curves yield linear curves in a double logarithmic graph and that they be parallel for a number of situations, as demonstrated in this paper. This makes it possible to interrelate the concentrations of tissue activator, extrinsic and intrinsic activators from blood and to express their concentrations on the basis of one common activator unit.

Other aspects of the study are concerned with the selection of a plasma fraction which ensures complete recovery of activators and with the question of the presence of more than one type of plasminogen activator in the euglobulin fractions. The final assay provides an inventory of plasminogen activators in plasma. It was found that the composition in plasma obtained in the morning at rest (baseline level of fibrinolysis) is relatively simple, since the quantity of extrinsic activator in this plasma is negligible. Only a few percent of the total activator concentration of 100 BAU/ml in the morning plasma can be attributed to the extrinsic or vascular activator. Consequently, assays of the total fibrinolytic activity in morning plasma determine nearly exclusively the level of intrinsic activators after complete activation (cf figure 9). Our studies reveal a reasonably constant level of total intrinsic activators among individuals, a finding compatible with the occurrence of fairly uniform levels of other precursor proteins in blood.

According to our results on Hageman trait plasma (figs. 7 and 8), and supported by results on Fletcher trait plasma (31), approximately one-half of the intrinsic activator activity seems to be generated independently of factor XII. Such a pathway of fibrinolysis has been previously suggested (4,21,36) and it can now be more specifically attributed to an intrinsic system of fibrinolysis. The other half of the intrinsic activator activity is clearly factor XII-dependent and, as previously reported, it contains an activator presumably identical to plasma kallikrein (10-15 BAU/ml) and an unidentified plasminogen proactivator (31). The plasma level of extrinsic activator fluctuates and increases due to stress. In plasma samples other than those obtained in the morning at rest, the contribution of the extrinsic activator to the activity of the dextran sulphate euglobulin fraction is therefore usually not negligible. Thus, an enormous increase in extrinsic activator concentration was demonstrated after 15 min venous occlusion. The new activator assay provides a method by which it is possible to quantify these changes in the plasma level of extrinsic activator.

The changes, however, should not be too small, because of the high background level of total intrinsic activators.

The regular euglobulin method

As demonstrated above, the regular euglobulin assay is of a complex nature. The activity determined in this assay is much smaller than the total activator potential of the plasma, as is evident from the comparison (fig. 10) of data obtained by the regular method and the new assay. The low activity found with the regular euglobulin assay results from incomplete precipitation of activators, only partial activation of activator precursors and inhibition by co-precipitated inhibitors. These aspects, and the variations among individuals, contribute to the wide, normal ranges of the usual euglobulin methods and they have limited their applicability to the consideration and study of only large deviations such as seen in pathological cases.

As a consequence of the results presented in this paper, the regular euglobulin method can now be reappraised and extended. One aspect, in particular, needs separate attention. The precipitation of intrinsic activators is incomplete in this method. The extrinsic activator, however, seems to be completely recovered. A study of extrinsic activator should therefore preferably employ the regular euglobulin fraction where interference by intrinsic activators can be easily eliminated by added C1-inactivator.

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CHAPTER IV

C1-INACTIVATOR-RESISTANT FIBRINOLYTIC ACTIVITY IN PLASMA EUGLOBULIN FRACTIONS: ITS RELATION TO VASCULAR ACTIVATOR IN BLOOD AND ITS ROLE IN EUGLOBULIN FIBRINOLYSIS*

C. Kluft

Abstract. Addition of C1-inactivator to plasma euglobulin fractions revealed that part of their fibrinolytic activity is resistant to this inhibitor, while another part is inactivated. The resistant part of the activity fluctuates in parallel with changes in blood fibrinolytic activity caused by diurnal fluctuations, stress or venous occlusion. Purified plasminogen activators of extrinsic origin (human tissue activator and vascular perfusion activator) were found to be resistant to C1-inactivator. The resistant part of the euglobulin activity therefore seems to indicate the presence of fluctuating amounts of a plasminogen activator of extrinsic origin in plasma. Optimal recovery of the resistant activity from plasma was achieved in euglobulin fractions prepared at pH 5.9 with a plasma dilution 1:10, in which resistant activity could be quantitatively assayed. Samples of morning plasma with baseline levels of fibrinolytic activity have levels of resistant activity amounting to only a few blood activator units (BAU)/ml. Samples of afternoon plasma showed a moderate increase in the resistant activity, while plasma collected after venous occlusion of the arm showed an enormous increase to 138 $\pm$ 29 (SD) BAU/ml (n=7). The C1-inactivator susceptible activity can be optimally recovered by isoelectric precipitation in the presence of dextran sulphate. It seems to originate from intrinsic proactivator systems. It was demonstrated that extrinsic activator has the capacity to enhance the generation of activator activity in these intrinsic systems in vitro.

INTRODUCTION

Increased blood fibrinolytic activity occurs in a variety of physiologic and pathologic states. Increases in physiologic fibrinolysis include stress--induced fibrinolysis (emotional stress, electric shock, exercise, etc.) and those caused by the injection of vasoactive and numerous other drugs (for a review, see ref. 1). There is also a diurnal rhythm with increased activity in

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the late afternoon (2).

The explanation for the increased activity was shown to be increased plasminogen activator activity in the blood (3,4). Release of activator activity into the blood vessels can be demonstrated by postmortem infusion of limbs with saline (5). Circumstantial evidence supports the general belief that this vascular or extrinsic activator is released from the fibrinolytically active endothelial cells (6) into the blood and is a source of the enhanced blood fibrinolytic activity (7-9). There are also, however, potent intrinsic pro-activator-activator systems in plasma (10-13). These proactivator-activator systems may also be involved in the generation of increased blood fibrinolytic activity (14,15). So far, specific methods of assay for the various plasminogen activators in plasma have not been available and the exact nature of the activator or activators responsible for the increased activity in blood remained undetermined.

Recent studies have demonstrated the presence of C1-inactivator in the euglobulin fractions of human plasma and that it considerably inhibits their fibrinolytic activity on fibrin plates (16,17). Substantial fibrinolytic activity could be generated by abolition of the effect of the C1-inactivator by the addition of either $\overline{C1s}$ preparations or flufenamate (18). It was also demonstrated that a synergistic effect of dextran sulphate and flufenamate released all potential activator activity in the euglobulin fractions (19,20). A most significant finding was that C1-inactivator inhibited only part of the fibrinolytic activity of euglobulin fractions. This partial inhibition suggested that the total activator content of the euglobulin fractions is composed of separate entities with different properties. This paper reports experiments carried out to determine the properties and origin of the C1-inactivator resistant part of the fibrinolytic activity of euglobulin fractions and its relation to extrinsic and intrinsic activator activity.

MATERIALS AND METHODS

Materials

Unless otherwise specified, reagents were of analytical grade and obtained from Merck, Darmstadt, West Germany. Distilled water was used throughout. Further reagents were obtained from the following sources.
Microbiological grade gelatin from Merck. 'Agarose for electrophoresis' from BDH Chemicals Ltd., Poole, England. Flufenamic acid from Aldrich Europe, Beerse,

Belgium. Antisera raised in rabbits against human C1-inactivator from Behringwerke A.G., Marburg, West Germany. Dextran sulphate, sodium salt, $\overline{MW}$ 500,000, from Pharmacia Ltd., Uppsala, Sweden. Plasminogen-rich bovine fibrinogen was prepared according to Brakman (21). Plasminogen-free bovine fibrinogen from Poviet, Organon-Teknika, Oss, The Netherlands. Plasminogen-containing human fibrinogen (grade L) from KABI, Stockholm, Sweden. Samples of Fitzgerald trait plasma and Fletcher trait plasma (case 3 as described in (22)) were obtained from George King Biomedical Inc., 151, Main Street, Salem, N.H., USA. Factor XII-deficient plasma was obtained from the Department of Hematology, University Hospital, Leiden, through the courtesy of Dr. J.J. Veltkamp. Perfusion activator preparations obtained by perfusion of human cadaver limbs, as described by Aoki and Von Kaulla (5), were kindly supplied by Dr. V. Noordhoek Hegt of this laboratory. Human tissue activator was prepared from uteri and kindly supplied by Drs. D. Rijken and G. Wijngaards of this laboratory (23). Platelet-poor citrated human plasma and pooled plasma were prepared as previously described (24). Samples of morning plasma were collected under conditions designed to obtain undisturbed plasma with baseline levels of fibrinolytic activity (24). The donors had fasted from 10:00 p.m. the day before and had to be at rest at the time of venipuncture. Blood was drawn from the arm between 8:00 and 9:00 a.m. with minimum stasis. Samples of afternoon plasma were collected around 5:00 p.m. Venous occlusion plasma was collected after venous occlusion of the arm for 15 min as described by Robertson et al. (25). Partially purified C1-inactivator preparation (26) from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, was used throughout, yielding results identical to those obtained with a highly purified C1-inactivator preparation (Op. 14470, 90-95% pure), kindly supplied by Dr. N. Heimburger, Behringwerke, Marburg, West Germany.

Solutions

EDTA buffer (μ = 0.15): 0.05 M sodium diethylbarbiturate, 0.10 M NaCl, 0.25% (w/v) gelatin and 2.7 mM ethylene diamine tetraacetate (EDTA) adjusted to pH 7.8 with an HCl solution.

Sodium flufenamate solutions: 0.5 M flufenamic acid in equivalent concentration of NaOH (60^{o}C), diluted to appropriate concentrations with EDTA buffer.

Dextran sulphate solution: Appropriate amounts of the sodium salt of the dextran sulphate preparation were dissolved in distilled water.

Methods

Preparation of euglobulin fractions

Regular euglobulin fractions were prepared with a plasma dilution of 1:10 and pH 5.9 as described in detail elsewhere (24).

Supernatant fraction. To the supernatant resulting from the centrifugation of the regular euglobulin fraction, 1 ml of dextran sulphate solution (100 µg/ml) per 1 ml plasma was added and the mixture was left for 30 min in an ice--bath before centrifugation. The separated precipitate was redissolved in a volume of EDTA buffer equal to the original plasma volume.

Dextran sulphate euglobulin fraction. This fraction was prepared from a mixture of 1 ml plasma, 8 ml distilled water and 1 ml dextran sulphate (100 µg/ml) solution. All solutions were precooled in ice water and mixed rapidly in the order mentioned. Titration with acetic acid to pH 5.9 was started immediately after mixing. After centrifugation in the cold, the precipitate was redissolved in 1 ml EDTA buffer.

In experiments designed to study the influence of pH and ionic strength on euglobulin fractionation, the plasma samples were always diluted 1:30. Variations in ionic strength were obtained by addition of the appropriate amount of sodium chloride solutions.

Assay of fibrinolytic activity

Fibrinolytic activities were assayed by the fibrin plate method (27). Diameters of lysed zones after 18 hrs of incubation on fibrin plates at 37°C were taken as a measure for the fibrinolytic activity of the solutions tested. Alternatively, the activity was expressed in arbitrary blood activator units (BAU) as defined earlier (20). Normal pooled morning plasma served as a reference standard set at 100 BAU/ml.

Regular assay. In the regular assay of fibrinolytic activity of euglobulin fractions, drops of 30 µl of the fraction were placed on plasminogen-rich bovine fibrin (in triplicate).

Assay with flufenamate (24). Immediately after application of the 30 µl drops of the euglobulin fraction to the fibrin plate, 5 µl of 14 mM flufenamate in EDTA buffer was added. This assay was regularly applied to the dextran sulphate euglobulin fraction and the supernatant fraction.

Assay of C1-inactivator resistant fibrinolytic activity. Immediately after application of the 30 µl drops of the regular euglobulin fraction to the fibrin plate, 5 µl of 700% (relative to pooled plasma) C1-inactivator in EDTA

buffer was added.

C1-inactivator assay

The C1-inactivator was estimated by the quantitative radial immunodiffusion assay according to Mancini et al. (28) using a monospecific antiserum. The amount of C1-inactivator was expressed in % relative to the pooled plasma.

RESULTS

Occurrence of a C1-inactivator-resistant fibrinolytic activity in euglobulin fractions

Description

Addition of purified C1-inactivator to solutions of the regular euglobulin fraction of pooled morning plasma considerably reduces the fibrinolytic activity determined on plasminogen-rich fibrin plates (fig. 1).

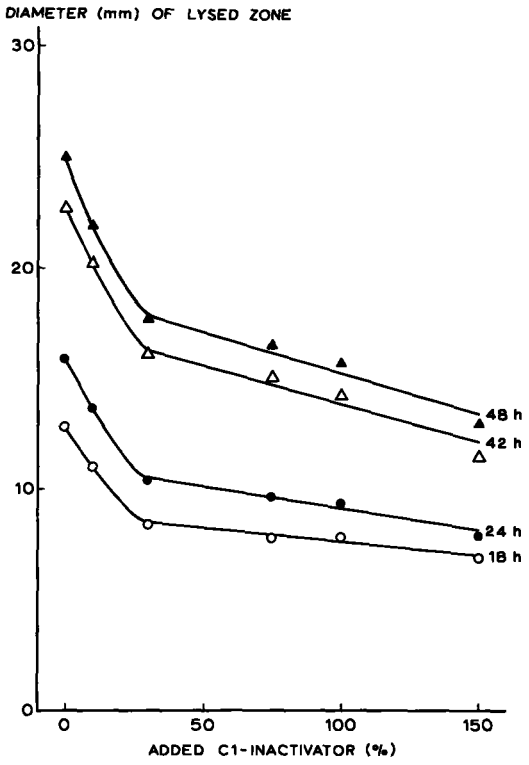

Fig. 1. Effects of added C1-inactivator on fibrinolytic activity of the regular euglobulin fraction.
Various amounts of C1-inactivator are added to a regular euglobulin fraction of pooled morning plasma. The amounts of added C1-inactivator are recorded on the abscissa relative to pooled normal plasma (100%). Fibrinolytic activities were recorded on plasminogen-rich bovine fibrin plates and expressed as diameters of lysed zones (ordinate) after 18, 24, 42 and 48 hrs incubation of the plates at 37°C.

Even the presence, however, of large amounts of C1-inactivator does not completely inhibit the fibrinolytic activity. The inhibition pattern shown in figure 1 suggests the existence of a two-stage inhibition in which low concentrations of the inhibitor react with a fibrinolytic component highly susceptible to C1-inactivator, while the second less susceptible component remains active. In the remainder of this paper, the term 'resistant activity' refers to the latter component. Figure 1 shows the results obtained after various periods of incubation of the fibrin plates. The lysed zones at high concentrations of added C1-inactivator are seen to increase progressively in size with the incubation period, indicating the presence of a genuine fibrinolytically active component. Essentially the same inhibition pattern was obtained on fibrin plates prepared from other batches of bovine plasminogen-rich fibrinogen, human plasminogen-containing fibrinogen and plasminogen-free bovine fibrinogen.

Occurrence in various types of plasma

Figure 2 shows patterns of inhibition for pooled morning plasma, pooled afternoon plasma and two plasma samples obtained after venous occlusion. It can be seen that a specific increase in C1-inactivator-resistant activity occurs, which parallels the increase in activity in the regular euglobulin fraction without added inhibitor. Results for individual plasma samples are shown in table I, where resistant activity is determined with a fixed amount of added C1-inactivator.

Table 1. Resistant activities in various types of plasma

Type of plasma	Resistant activity (mm)	Range (mm)	N
Morning	6.9	5.5 - 9.2	20
Afternoon	9.6	7.3 - 12.5	11
Preocclusion	9.1	6.7 - 14.8	7
After occlusion	26.1	23.5 - 28.7	7

C1-inactivator-resistant activity was assayed by adding 100% C1-inactivator (final concentration) to the regular euglobulin fraction of various types of plasma. The activity is expressed as diameter (mm) of lysed zones on fibrin plates. The mean, range and number of apparently healthy individuals is given.

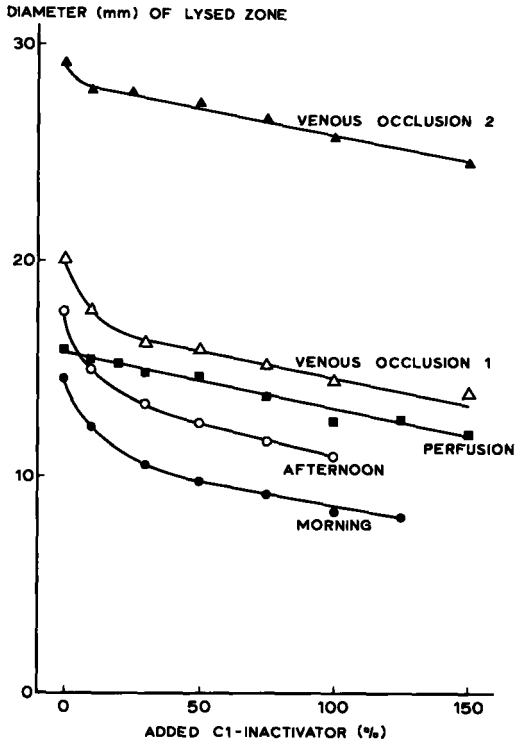

Fig. 2. Effects of C1-inactivator on fibrinolytic activities of perfusion activator and euglobulin fractions from plasmas with enhanced fibrinolysis. Various amounts of C1-inactivator are added to a preparation of vascular, perfusion activator and regular euglobulin fractions of morning plasma, afternoon (collected at 5:00 p.m.) plasma or plasma obtained after 15 min venous occlusion of the arm (no. 1 from a subject with low, no. 2 with high response). See also legend to figure 1.

The level is lowest in the early morning. In plasma obtained in the late afternoon (5:00 p.m.), the mean level of resistant activity was slightly higher (table I), with considerable overlap with the morning plasmas as evident from the range. Compared to the morning samples of each individual in this group, an increase was, however, always observed. Increased levels were also found in several samples of preocclusion morning plasma from a group of seven volunteers for the venous occlusion test. Possibly, the stress of the planned venous occlusion test had caused an increase. Samples obtained after 15 min venous occlusion showed enormously increased levels of resistant activity (table I).

Plasma from individuals with a deficiency in the factor XII dependent intrinsic pathway of fibrinolysis gave regular euglobulin fractions with low spontaneous activity compared to normal plasma (fig. 3). The levels of resistant activity, however, remained within the normal range and appeared to constitute the main activity in the euglobulin fractions of these deficient plasmas.

From these results, it can be concluded that the resistant part of the ac-

tivity fluctuates in parallel with changes in blood fibrinolytic activity believed to originate from fluctuations in extrinsic activator in plasma such as in diurnal fluctuations, stress or venous occlusion. A low level appeared to be always present in undisturbed morning plasma. The resistant activity is not influenced significantly by defects in the factor XII-dependent intrinsic pathway of fibrinolysis.

Recovery in euglobulin fractions

As shown previously (16,17), the fibrinolytic activity recovered in euglobulin fractions depends upon the conditions of fractionation, such as dilution of plasma, pH and the presence of polyanions (dextran sulphate). It was investigated to what extent the recovery of the C1-inactivator resistant part of the activity depended upon these parameters of fractionation.

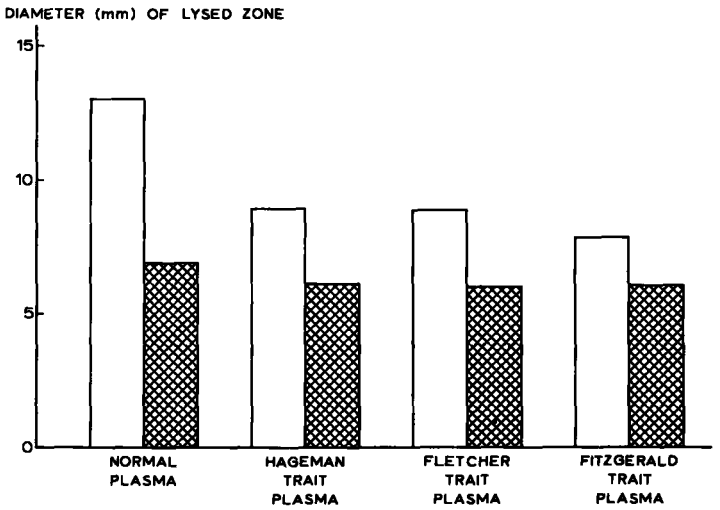

Fig. 3. Euglobulin fibrinolytic activities in normal plasma compared to plasmas deficient in essential components of the factor XII-dependent pathway. The fibrinolytic activity is expressed as diameters of lysed zones (ordinate) and determined by the regular euglobulin assay (open bars) and with added C1-inactivator (hatched bars). Morning plasmas were obtained from healthy volunteers (mean of 15 subjects), one Hageman trait patient (deficient in factor XII), one Fletcher trait patient (deficient in plasma prekallikrein) and one Fitzgerald trait patient (deficient in high molecular weight kininogen).

Influence of pH and ionic strength on recovery of the resistant activity

Samples of morning plasma with a low level of resistant activity and samples of highly active plasma obtained after venous occlusion of the arm (15 min) were used to prepare euglobulin fractions. One series of euglobulin fractions was prepared at constant ionic strength by varying the pH of fractionation (fig. 4, left panel). The other series was prepared at constant pH (5.9) by varying the ionic strength during fractionation (fig. 4, right panel). Recovery of resistant activity did not strongly depend on pH and ionic strength; however, a pH distinctly below 6.0 and high ionic strength appeared unfavourable. The highest levels of resistant activity were obtained at a pH close to 6.0 and at low ionic strength.

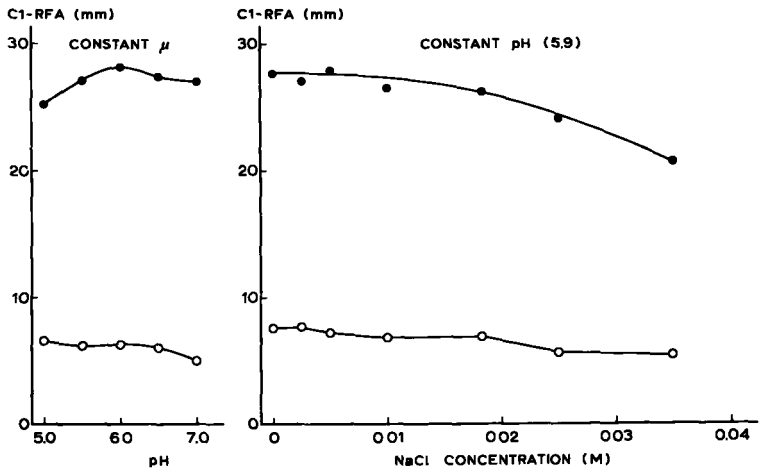

Fig. 4. Effects of fractionation conditions on the amounts of C1-inactivator resistant fibrinolytic activity (C1RFA) in euglobulin fractions.
Each point in the figure represents a euglobulin fraction prepared from pooled morning plasma (open circles) or a plasma obtained after 15 min venous occlusion (closed circles) with the indicated pH and ionic strength (μ). Plasma was always diluted 1:30 and the ionic strength varied by additions of sodium chloride solutions. The constant ionic strength (left panel) was achieved with the addition of a final concentration of 0.0025 M NaCl. C1RFA was determined on plasminogen-rich bovine fibrin plates with a constant total amount of 125% C1-inactivator (final concentration) in the fractions. The response is expressed as diameter (mm) of lysed zones (ordinate).

Recovery of resistant activity in the regular and dextran sulphate euglobulin fractions

Not all fibrinolytic activity potentially present in plasma is recovered in the regular euglobulin fraction. Additional activity can be precipitated from the supernatant by addition of dextran sulphate (see fractionation scheme in fig. 5). Optimal recovery can be achieved in one step when the isoelectric precipitation is performed in the presence of appropriate amounts of dextran sulphate (the dextran sulphate euglobulin fraction, fig. 5). Assays in the presence of C1-inactivator (fig. 6) show that the regular euglobulin fraction and the dextran sulphate euglobulin fraction contain similar amounts of resistant activity.

In contrast, the supernatant fraction was free of resistant activity. A similar supernatant fraction prepared from venous occlusion plasma with a high level of resistant activity was also found to be free of resistant activity (not shown). It is concluded that, under the conditions employed (plasma dilution 1:10, pH 5.9), the dextran sulphate precipitable activity concerns only activity susceptible to inhibition by C1-inactivator.

From the experiments presented above, it cannot be decided whether or not resistant activity is susceptible to dextran sulphate precipitation, since there could have been no resistant activity in the supernatant after complete recovery in the regular euglobulin fraction (see scheme fig. 5). To determine the precipitability of resistant activity, dextran sulphate was added to a supernatant from a euglobulin fraction prepared from venous occlusion plasma at pH 5.9, a plasma dilution of 1:30 and addition of 0.035 M NaCl (cf fig. 4). This supernatant contained resistant activity because of a less complete recovery in the removed euglobulin fraction compared to the regular euglobulin fraction (cf fig. 4). Addition of dextran sulphate produced a precipitate which contained a distinct amount of resistant activity in this case, thus demonstrating the susceptibility of resistant activity to dextran sulphate precipitation under these conditions. It seems legitimate to conclude therefore that the above demonstrated absence of resistant activity in the supernatant fraction (fig. 6) of the regular euglobulin fraction indicates optimal recovery of resistant activity in the regular euglobulin fraction. This also applies to the dextran sulphate euglobulin fraction.

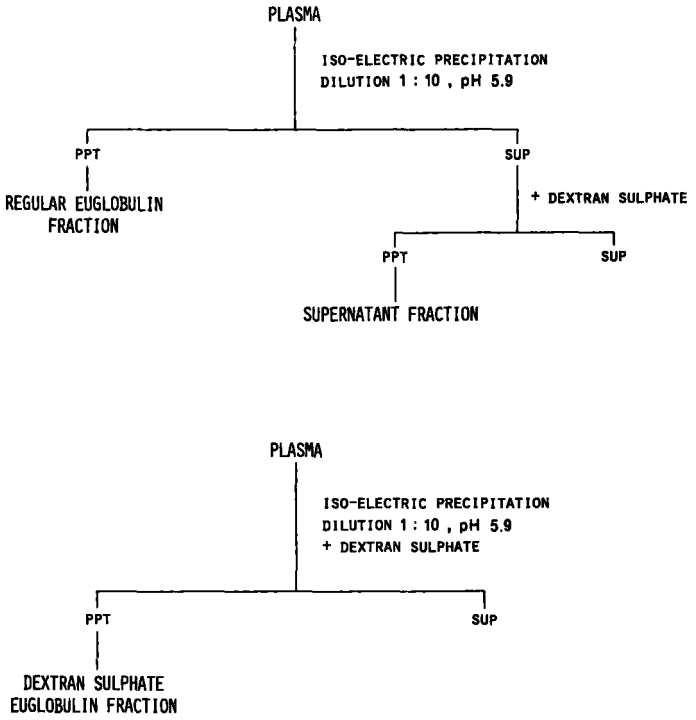

Fig. 5. Scheme for euglobulin fractionation.

Influence of C1-inactivator on the fibrinolytic activity of purified extrinsic activators and their use for quantification of resistant activity in euglobulin fractions

Purified extrinsic activators

The previous experiments suggested that the resistant activity in euglobulin fractions was related to extrinsic activator in plasma. We determined therefore how purified preparations of extrinsic activators were influenced by C1-inactivator.

Figure 7 shows that the purified human tissue plasminogen activator was only slightly inhibited by C1-inactivator, resembling the endogenous resistant activity of plasma in figures 1 and 2 in this respect. The activator obtained by perfusion of cadaver limbs also belongs to a group of C1-inactivator resistant activators (fig. 2). Mixtures of the human tissue activator with a regular euglobulin fraction retained the susceptible activity, while the resistant activity increased with the concentration of the human tissue activator (fig. 7).

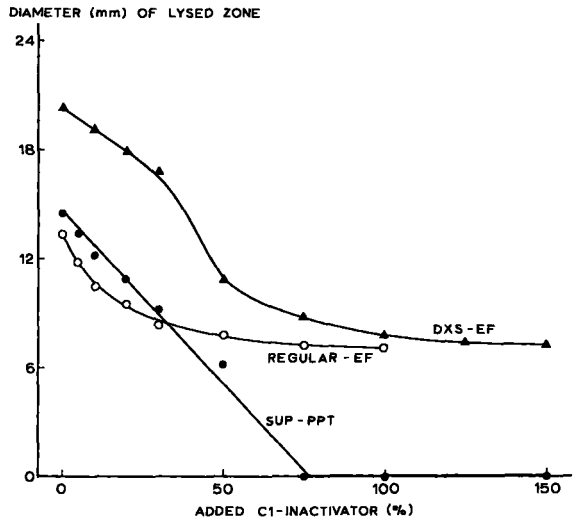

Fig. 6. Occurrence of resistant activity in various plasma fractions. Increasing amounts of purified C1-inactivator (abscissa, in % relative to pooled plasma) were added to three different fractions of pooled plasma (cf fig. 5). DXS-EF = dextran sulphate euglobulin fraction prepared with 50 µg dextran sulphate/ml plasma; regular EF = regular euglobulin fraction; sup-ppt = supernatant precipitate prepared with 45 µg dextran sulphate/ml plasma. The fibrinolytic response on plasminogen-rich bovine fibrin plates is recorded on the ordinate as diameters (mm) of lysed zones after 18 hrs incubation of the plates.

In experiments where the human tissue activator was preincubated with C1--inactivator, it was observed that the degree of inhibition did not change. This indicated that the activator was not bound and itself inactivated by the inhibitor but that the minor inhibition observed at increasing concentrations of C1-inactivator must be due to inhibition in the assay (29).

It is concluded that the purified extrinsic activators mentioned are C1--inactivator resistant. Figures 2 and 7 show a striking similarity of resistance in the patterns of inhibition by C1-inactivator produced by activator obtained by perfusion, from tissues or activity induced in blood by venous occlusion or as a result of the diurnal increase. The small amount of resistant activity in the regular euglobulin fractions prepared from undisturbed morning plasma also resembles this pattern. It is most likely therefore that resistant activity in plasma euglobulin fractions is due to extrinsic activators.

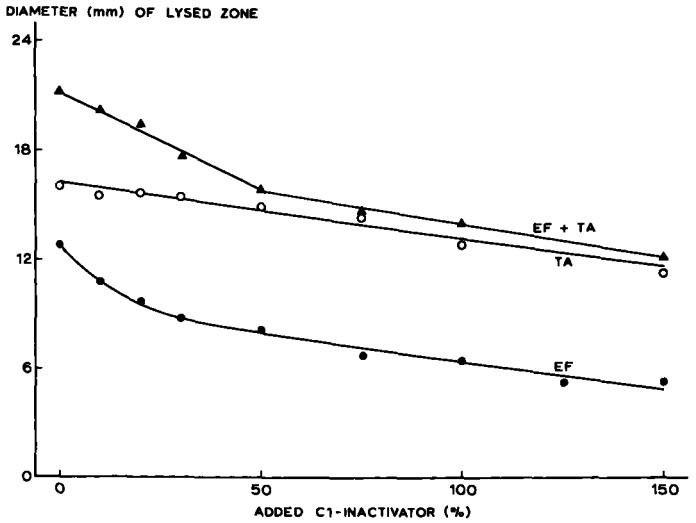

Fig. 7. Effects of C1-inactivator on fibrinolytic activities of human tissue activator alone and when mixed with a regular euglobulin fraction. Various amounts of C1-inactivator were added to a regular euglobulin fraction of pooled morning plasma (EF), to a purified human tissue activator (TA), and to a 1:1 mixture of double concentrated EF and TA. See also legends to figure 1.

Quantification of resistant activity in the regular euglobulin fraction

The preceeding experiments indicated that optimal recovery of the resistant activity from plasma is achieved in the regular and dextran sulphate euglobulin fractions. Determination of the resistant activity in these fractions therefore yields a quantification of the resistant activity in plasma which is most likely caused by extrinsic activator. Specific determination of the resistant activity can be made by addition of an excess of C1-inactivator (cf figs. 1, 2 and 6). This inhibitor, however, slightly influences the activity of the so-called resistant activity. To account for this effect, the recovery of added components responsible for the resistant activity can be determined. Referring to the experiments described above (fig. 7) showing great similarities between purified human tissue activator and endogenous resistant activity in euglobulin fractions, this purified activator was used to represent such components. The tissue activator was added to a regular euglobulin fraction prepared from pooled morning plasma, because this plasma contains the lowest level of resistant, endogenous activity. Activities recorded as lysed zones were converted to activator units as recently described (20). Recovery of the

tissue activator was calculated and plotted (fig. 8) on the assumption that the level of resistant, endogenous activity was negligible. Assayed in the presence of a fixed amount of added C1-inactivator (50% of the concentration present in the pooled plasma), the activities closely followed the calculated recovery. When the concentration of added C1-inactivator was raised to 100% (of the concentration in the pooled, normal plasma), the recovery did not show a linear increase at the lower concentrations of tissue activator and the levels were generally below those calculated. The close approximation of the calculated and the measured recovery at a level of 50% added C1-inactivator indicates that the assumption of a negligible level of resistant, endogenous activator was valid. The amount of resistant endogenous activity is low relative to the concentrations of added tissue activator and it can be estimated to amount to only a few BAU/ml.

Quantification of the resistant activity present in the regular euglobulin fraction can thus be tentatively achieved by determination of the fibrinolytic activity in the presence of 100% added C1-inactivator (an excess to be sure of complete inhibition of susceptible activity) and determination of the corresponding level of extrinsic activator by interpolation on the recovery curve of figure 8.

Application of this method to plasma with stimulated fibrinolysis obtained after 15 min venous occlusion showed a tremendously increased amount of resistant activity of 138 $\pm$ 29 (SD) BAU/ml (n=7). Obviously, the amount of resistant activity can fluctuate considerably from a few to distinctly above 100 BAU/ml.

Relation of the resistant activity of euglobulin fractions to the spontaneous activity of these fractions

Situation in the regular euglobulin fraction

a) Influence of increased levels of added tissue activator on the spontaneous activity of the regular euglobulin fraction

When tissue activator was added to the regular euglobulin fraction of pooled morning plasma with a level of spontaneous activity corresponding to 5 BAU/ml, increase of activator activity was not linear when assayed without or with addition of flufenamate (fig. 8). Extra activator activity which belongs to the C1-inactivator susceptible activity is revealed, since addition of sufficient C1-inactivator prevents generation of this extra activity (fig. 8). The amount of extra activator activity reaches an optimum. The optimal amount strikingly resembles the amount of activator activity which can be induced by

the combined effect of dextran sulphate and flufenamate (fig. 8) as described previously (20). It seems that the addition of extrinsic activator can result in the generation of a distinct amount of C1-inactivator susceptible activator activity.

b) Influence of increased levels of endogenous resistant activity on the spontaneous activity of the regular euglobulin fraction

It was explored whether the endogenous resistant activity had similar effects on the spontaneous activity of euglobulin fractions as did the added tissue activator discussed above. A wide range of resistant activities was obtained

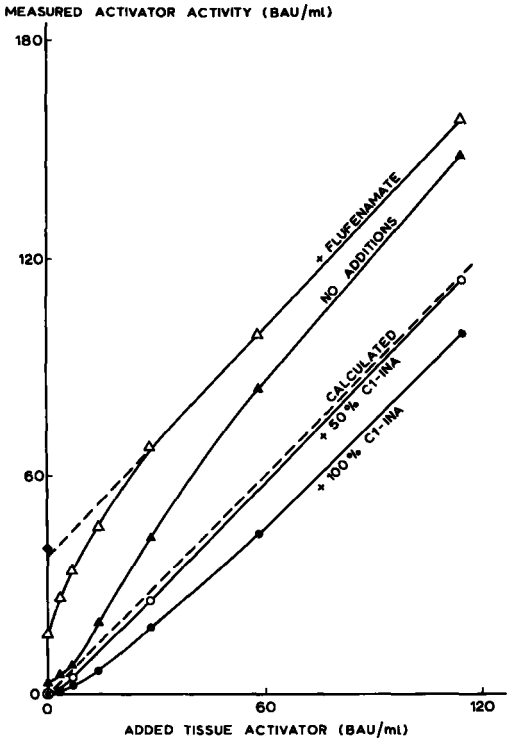

Fig. 8. Recovery of activator activity from purified tissue activator added to the regular euglobulin fraction. Various amounts of purified tissue activator were added to the regular euglobulin fraction (abscissa). The activity of the mixtures was assayed on fibrin plates with added flufenamate (Δ, 2 mM), without additions (▲), with 50% added C1-inactivator (o) and with 100% added C1-inactivator (●). The activator activity is expressed as blood activator units (BAU)/ml. The activity evoked in the regular euglobulin fraction by addition of dextran sulphate (25 µg/ml) plus flufenamate (2 mM) is indicated on the ordinate (◆). The extrapolation of the '+ flufenamate curve' intersects the ordinate at 38 BAU/ml. (----): represents the optimal recovery of added tissue activator assuming no contribution from the regular euglobulin fraction.

by studying morning plasma, afternoon plasma and venous occlusion plasma from a number of healthy individuals and patients.

As shown in fig. 9, the relation between the resistant activity and the spontaneous activity of the regular euglobulin fractions followed a pattern similar to that found in the experiment with added tissue activator. The endogenous C1-inactivator resistant activity therefore also appeared to be involved in the generation of extra C1-inactivator susceptible activity, contributing to an increased spontaneous activity. Two features of this effect are of importance. Firstly, at the higher concentrations of resistant activity, an optimal, distinct amount of susceptible activity is generated. This is also evident from the finding that, in

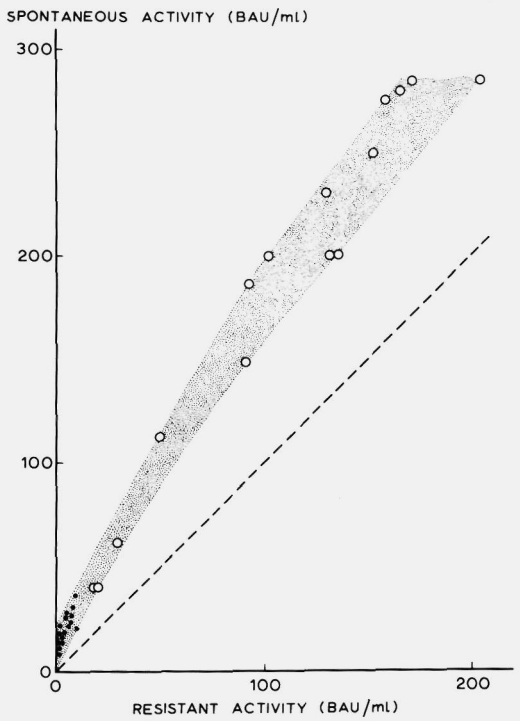

Fig. 9. Correlation between spontaneous and C1-inactivator resistant activity in regular euglobulin fractions of various types of plasma. The spontaneous activity of the regular euglobulin fraction was assayed on fibrin plates and expressed in blood activator units (BAU)/ml (ordinate). The C1-inactivator-resistant fibrinolytic activity of the regular fraction was assayed with 100% added C1-inactivator and expressed in BAU/ml. (o) represents plasma samples after 15 min venous occlusion obtained from healthy volunteers and a few patients with poor response. (•) represents plasma samples obtained in the morning or afternoon from healthy volunteers.

the regular euglobulin fraction of plasma with a significant increased activity due to venous occlusion, no extra activity is generated by the addition of dextran sulphate and/or flufenamate. Secondly, at the lower levels of resistant activity, only a part of this amount is revealed in a dose-dependent fashion. These situations are schematically presented for morning, afternoon and venous occlusion plasma in figure 10.

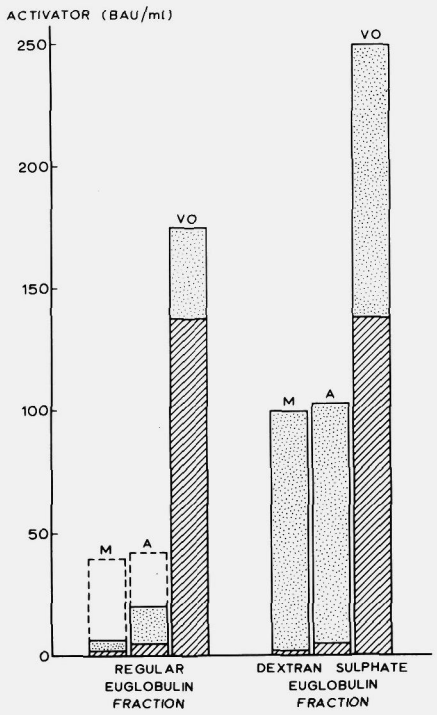

Fig. 10. Schematic summary of recorded and potential activator amounts in various types of plasma assayed in the regular or dextran sulphate euglobulin fraction. Activator amounts are recorded on the ordinate in BAU/ml. Three types of plasma are presented: M = morning plasma with baseline level of fibrinolysis, A = afternoon plasma showing diurnal increase in fibrinolysis, VO = venous occlusion plasma after 15 min occlusion of the arm. The amounts of resistant activity are indicated by the hatched parts of the bars; the remaining parts of the bars represent susceptible or intrinsic activity. In the regular assay of the regular euglobulin fraction, the intrinsic portion is sometimes (M,A) only partially seen (filled with dots) and a part remains latent (open part of the bars).

Situation in the dextran sulphate euglobulin fraction

In the dextran sulphate euglobulin fraction, the C1-inactivator susceptible activity is optimally generated by the combined effect of dextran sulphate and flufenamate. The recovery of this activity in the fraction is also optimal (20). In morning plasma with baseline levels of fibrinolysis, an average level of 100 ± 15 (SD) BAU/ml was found in 50 subjects (20). According to results described above, only a few BAU/ml of this amount are due to the presence of resistant activity. A group of 7 volunteers showed a total level of 249 ± 27 BAU/ml after 15 min of venous occlusion. The level of resistant activity assayed in the regular euglobulin fraction as described above was 138 ± 29 BAU/ml.

The difference of 111 BAU/ml represents the level of C1-inactivator susceptible activator activity. This situation is schematically represented in figure 10. It is concluded that, in contrast to the resistant activity, the level of susceptible activity is not changed significantly in occlusion plasma. The small increase could be due to changes in the haematocrit during the occlusion.

DISCUSSION

A part of the fibrinolytic activity of plasma euglobulin fractions shows a considerable resistance to inhibition by C1-inactivator, while the other part of the activity is inactivated. Evidence is provided that these two parts of activity can be related to two different sources of fibrinolytic activity.

The resistant activity most likely originates from extrinsic activator which is released into the blood (presumably from endothelial cells), as is supported by the following arguments:

a) Purified extrinsic activators are resistant to C1-inactivator. This is shown for purified human tissue activator and a crude preparation of vascular perfusion activator (Figs. 2,7).
b) The resistant activity follows fluctuations in fibrinolytic activity due to physiologic stimuli, as shown for the diurnal increase, stress and venous occlusion (Fig. 2, table I).
c) No extra resistant activity is generated in vitro with dextran sulphate or contact activation and by addition of purified tissue activator (Figs. 6-8). This is also clearly evident from the absence of resistant activity in the supernatant precipitates (Fig. 6).

The following characteristics of the susceptible activity make it likely

that this activity originates from plasminogen proactivators from the intrinsic fibrinolytic systems in plasma.

a) The spontaneous, susceptible activity in the regular euglobulin fraction is only part of the total amount present, indicating the occurrence of unconverted proactivators. All activity could be revealed by the combined effect of dextran sulphate and flufenamate or by increased levels of tissue activator or endogenous resistant activity (Figs. 8,9, ref. 20).
b) The total amount of susceptible activity remains fairly constant under conditions of stimulated fibrinolysis (Figs. 8,9, section III). This is compatible with an origin from fairly constant levels of precursor proteins.
c) In plasma samples deficient in a factor of the intrinsic fibrinolytic systems, the susceptible activity was specifically reduced (Fig. 3).
d) Inhibition of known factors from intrinsic fibrinolytic systems such as factor XIIa and kallikrein by C1-inactivator has been demonstrated (30,31).

The above arguments strongly support the view that the C1-inactivator resistant and susceptible activities in euglobulin fractions originate from separate entities which have been related to extrinsic and intrinsic systems of blood fibrinolysis, respectively. Additional support for the separate origin of both activities is provided by the fractionation experiments where part of the susceptible activity could be separated from the resistant activity (Fig. 6). Definite conclusions, however, can only be made after purification and characterization of the plasminogen activators and their proactivators in plasma. Further, we use as a working hypothesis that the C1-inactivator resistant activity can be ascribed to one or more extrinsic type of activators while the susceptible activity is from intrinsic systems of fibrinolysis.

The studies on euglobulin fractionation provided further details on the composition of the regular and dextran sulphate euglobulin fractions. The results are schematically represented in figure 10. The resistant or extrinsic activity is optimally recovered in both fractions. The intrinsic activity is, however, only optimally recovered and optimally activated in the dextran sulphate euglobulin fraction (20). In the regular euglobulin fraction, only partial recovery and moreover only partial activation of the present amount occurs.

Concerning the degree of activation of intrinsic systems in the regular euglobulin fraction, the unexpected observation was made that it depended upon the level of extrinsic or resistant activity. At low levels of extrinsic activator, the response in the intrinsic systems is dependent on the dose of extrinsic activator (Figs. 7 and 8). Thus, in euglobulin fractions from morning

plasma with baseline level of fibrinolysis and little resistant activity, only a slight activation of the intrinsic systems occurred. The slightly higher level of resistant activity in euglobulin fractions from the afternoon plasma increased the intrinsic activation as shown in figure 10. The very high levels of extrinsic activator in euglobulin fractions from healthy volunteers after venous occlusion led to complete activation of intrinsic proactivators (cf Figs. 7,8,9).

From this outline, it is clear that increases in blood fibrinolysis due to release of extrinsic activator are not unambiguously revealed by the regular euglobulin method. The increases observed by this method are built up of two contributions: one consists of an increase in the amount of extrinsic activator; the other is an increase in the activity of activators originating from intrinsic proactivators. Responses to venous occlusion, exercise or increases due to the diurnal fluctuations can consequently be altered by changes in both of these contributions. The addition of excess of C1-inactivator as described in this paper enables one to separately study the activity due to the extrinsic activator.

The plasma fractionation studies also provided evidence for a complete recovery of all resistant activity of plasma in the regular as well as in the dextran sulphate euglobulin fractions. Consequently, the quantitative determination of resistant activity in these fractions that was developed gives a measure of its plasma level. The plasma level of resistant activity is very low in morning plasma with baseline levels of fibrinolysis, amounting maximally to a few BAU/ml. This amount of activator comprises only a few percent of the total plasma level of plasminogen activator activity recovered in the dextran sulphate euglobulin fraction. This situation in morning plasma is visualized in figure 10. Stimulation of fibrinolysis by venous occlusion produces a tremendous increase in the plasma level of extrinsic activator activity, by a factor of 50 to 100. These tremendous fluctuations in the level of resistant activity raise questions about the significance of studies of the very low levels seen in morning plasma with baseline levels or small fluctuations such as seen in the diurnal fluctuations. As shown in table I, an increased level was even found in preocclusion morning plasma samples from volunteers for venous occlusion. One can wonder whether the range observed in the other groups of volunteers (table I) reflects merely different individual responses to normal venipuncture.

Compared to the intrinsic activator potential, the plasma level of resistant

activity ascribed to extrinsic activator during the day remains rather low (a few percent). This ratio of activities in plasma, however, should not be used as an indication for their relative importance in fibrinolysis in the circulation. Extrinsic activator can be released locally and may reach high concentrations locally. The plasma level provides only a distorted picture of such events; furthermore, elevated levels of extrinsic activator activity rapidly decline in blood, presumably due to clearance by the liver (32). In addition, in comparing the resistant activity with the susceptible or intrinsic activator amount, it must be realized that the former is caused by a directly active activator while the intrinsic activators occur as precursors which require activation. The resistant activity also has the capacity, at least in fibrinolysis in vitro, to initiate activation of the intrinsic system and its importance therefore lies possibly not only in its effect and concentration as a direct plasminogen activator. It would be interesting to know whether the extrinsic activator is also able, just as in euglobulin fractions, to recruit the intrinsic activators in whole blood and in vivo. Obviously, such a mechanism could have great implications for current concepts of thrombosis.

ACKNOWLEDGEMENT

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CHAPTER V

DETERMINATION OF PREKALLIKREIN IN HUMAN PLASMA: OPTIMAL CONDITIONS FOR ACTIVATING PREKALLIKREIN*

C. Kluft

Abstract. A method for the assay of human plasma prekallikrein in which a chromogenic synthetic tripeptide, PPAN, is used as a substrate for kallikrein is described. The conversion of prekallikrein to kallikrein is achieved by cold activation ($0^{o}C$) with water-soluble dextran sulphate. Conditions for obtaining optimal amounts of free kallikrein with respect to concentration of dextran sulphate, activation time, inhibitors (C1 inactivator), and requirement of factor XII have been determined. The activation procedure is compared to other known procedures. The assay system was worked out for pooled normal plasma and is applicable to any plasma sample not liable to unwanted preactivation or incomplete activation, as revealed by control experiments. A survey in 15 apparently healthy individuals showed a mean activity of 476 $\pm$ 58 (S.D.) mU/ml with a range of 385 to 586 mU/ml.

Abbreviations: α-N-benzoyl-L-proline-L-phenylalanine-L-arginine-p-nitroanalide (PPAN), high molecular weight (HMW), sodium dodecyl sulphate (SDS), hereditary angioneurotic edema (HANE).

INTRODUCTION

Plasma prekallikrein is the precursor of kallikrein, a proteolytic enzyme that releases the nonapeptide bradykinin from the plasma α_2-globulin HMW kininogen. Functional assays for prekallikrein have been based on its conversion to kallikrein and the action of this enzyme on synthetic substrates (TAME, BAEE) or kininogen.

One complicating factor in the assays is the required activation of prekallikrein. Factor XII, present in native plasma as a proenzyme, can be activated by both enzymatic activators (trypsin, kallikrein, plasmin) and non-enzymatic ones (glass, kaolin, ellagic acid). The activated factor XII activates prekallikrein for conversion to kallikrein by proteolytic cleavage. The

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kallikrein formed is able to activate factor XII enzymatically in a reciprocal activation mechanism, thus amplifying the activation of factor XII and its own generation (for a review, see ref. 1). Another factor which is important for the activation system is HWM kininogen. In plasma deficient in this factor (known as Fitzgerald trait (2,3), Williams trait (4) or Flaujeac trait (5)), all factor XII-dependent activations are defective. HMW kininogen is bound to prekallikrein and serves as a cofactor in the activation and activity of factor XII (6).

Another complicating factor in the assays is the inactivation of kallikrein in the plasma milieu which is achieved by a number of plasma proteinase inhibitors. C1 inactivator and α_2-macroglobulin are the main contributors, but α_1-antitrypsin and antithrombin III are also reported to inhibit plasma kallikrein (7,8,9). Antithrombin III inhibits much more potently in the presence of heparin (8,9). The presence of heparin in plasma may also be of importance for the functioning of C1 inactivator (10).

This present paper describes an assay system for prekallikrein where it is converted to free kallikrein under optimum conditions. Three important characteristics of the system are the following:

1. A cold activation procedure is chosen to diminish the influence of inhibitors, which has been shown to be considerably decreased at $0^{\circ}C$ (11).

2. Compounds such as cellulose sulphate (12,13) and chondroitin sulphate (12,14) have been shown to have the capacity to activate the kallikrein system. The commercially available synthetic compound dextran sulphate is introduced as a favourable agent to initiate the cold activation of the kallikrein system. As a water-soluble agent, it forms homogeneous mixtures with plasma and does not interfere with the spectrophotometric assay, which is in contrast to the light-scattering properties of the insoluble kaolin.

3. The activity of the kallikrein is measured in a sensitive assay with the substrate PPAN (2). Paranitroanalide which is released from the substrate by kallikrein can be determined spectrophotometrically.

The assay was worked out for pooled normal plasma, but conditions are described and examples given for application to other plasma samples.

MATERIALS AND METHODS

Materials

Unless otherwise specified, reagents were of analytical grade and purchased from E. Merck, Darmstadt, West Germany. Distilled water was used throughout

the experiments. Additional reagents and materials were obtained from the following sources: "Agarose for electrophoresis" and kaolin, light, from BDH Chemicals Ltd., Poole, England; dextran sulphate, sodium salt, $\overline{MW}$ 500,000, from Pharmacia Ltd., Uppsala, Sweden, or Sigma Chemical Co., St. Louis, Mo.; flufenamic acid from Aldrich Europe, Beerse, Belgium; ellagic acid, batch No. 39175, from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England; chromozym PK or PPAN from Pentapharm AG, Basle, Switzerland; human plasmin (EC 3.4.21.7) labeled 15 casein units/mg protein from AB Kabi, Stockholm, Sweden; antihuman antiserum raised in rabbits against plasminogen from Behringwerke AG, Marburg, West Germany; Fletcher factor-deficient plasma (case 3 as described by Hattersley and Hayse (15)); 53-year-old man, from George King Bio-Medical Inc., Salem, N.H., lot 06 GK-1701. Platelet-poor human plasma samples and pooled samples were prepared as previously described (16) and stored at -80°C. Factor XII preparation, partly purified essentially according to the method of Revak et al. (17), was kindly supplied by Dr. R.L. Jenks of this laboratory. C1 inactivator preparation, kindly supplied by Dr. M.M. Trumpi-Kalshoven of this laboratory, showed one band on SDS polyacrylamide electrophoresis and had a concentration of 25 units/ml (18). Active C1s was prepared from outdated plasma or serum according to the method of Vroon et al. (19) and Haines and Lepow (20). The preparation contained 650 units/ml (18) and was contaminated with traces of other proteins but found to be free of direct fibrinolytic and plasminogen activator activity.

Solutions

Tris-Imidazole buffer (μ = 0.15; pH 7.9). This was prepared according to the directions of Pentapharm (21).

Dextran sulphate solution. The sodium salt of the dextran sulphate preparation was dissolved in distilled water.

Ellagic acid solution. Ellagic acid (30 mg) was dissolved in 50 ml of 0.02 M NaOH under slight warming. The warm solution was adjusted to pH 7.8 with 0.1 M HCl and diluted to a final volume of 100 ml. The 10^{-3} M sodium ellagate was prepared immediately before each experiment and used after rapid cooling of an aliquot to 0°C.

Kaolin suspension. Kaolin (10 mg) was suspended in 1 ml of phosphate-saline buffer according to the method of Colman et al. (22).

PPAN solution. The powder was dissolved in distilled water to 10^{-3} M.

Assay of prekallikrein

Activation procedure with dextran sulphate. Plasma was freshly thawed until clear. A 0.1 ml sample was transferred to a plastic tube and carefully cooled to 0°C in ice water; 0.1 ml from a precooled solution of 25 mg/l dextran sulphate in distilled water was added and mixed thoroughly. The mixture was incubated in ice water for 7 min for optimal activation. Study of unknown plasma samples routinely included activations of a 1:1 mixture of the plasma with pooled plasma in order to check for incomplete activation (see Results).

Activations with kaolin and ellagic acid were done similarly with 0.1 ml of kaolin suspension or sodium ellagate 10^{-3} M. For activation with acetone, pooled plasma was incubated with 20% (v/v) acetone for 3 hr at 25°C. The acetone was removed by evaporation in vacuo at room temperature.

Measurement of activity. In a semimicro quartz cuvet (1 cm), a mixture of 1.0 ml of prewarmed Tris-imidazole buffer and 0.2 ml of PPAN solution was equilibrated at 37°C in a thermostat-controlled cuvet holder. The absorbance at 405 nm was determined in a spectrophotometer (Unicam SP 1700; double beam with recorder AR 25). After addition and mixing of 15 µl of the activated sample, changes in absorbance at 405 nm were followed continuously for 4 min. The initial slope was used to give ΔOD/min (except for the 20 sec 25°C kaolin-activated sample as described in Results, where a lag phase appeared). In experiments with kaolin-activated samples, absorbance at 405 nm was read against buffer containing appropriate amounts of kaolin as a blank. These suspensions were of limited stability in the cuvet but permitted 2 min assays. Results were expressed in milliunits per milliliter of plasma, with a molar extinction for p-nitroanilide for 10 cm^2/µMol at 405 nm. One enzyme unit is defined as the amount of enzyme that converts 1 µMol of substrate per minute under optimal or defined conditions (1 U = 1,000 mU). To check for preactivation of a plasma sample, spontaneous activity was assayed on freshly thawed plasma samples cooled to 0°C, 30 µl of which were applied to the cuvet.

RESULTS

Activation of prekallikrein by means of dextran sulphate

Time and concentration dependence. Incubation of pooled plasma with dextran sulphate at 0°C generated activity on the synthetic substrate PPAN. Fig. 1 shows the amount of activity produced during incubation at 0°C in the presence of three different amounts of dextran sulphate. In each instance, the activity reached a peak and then decreased. This can be explained as being the result

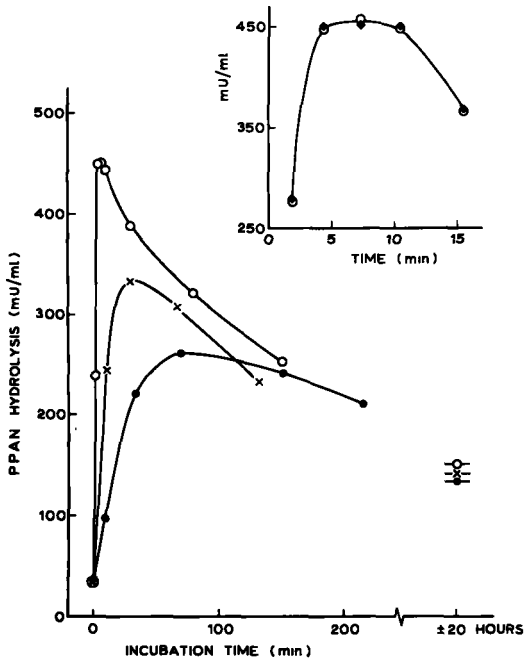

Fig. 1. Influence of incubation time on cold activation of pooled plasma with dextran sulphate. Pooled human plasma was mixed 1:1 with three different dextran sulphate solutions: 25 µg/ml (o), 3.1 µg/ml (×) and 1.5 µg/ml (•). After various times (recorded in min on the abscissa) at 0°C, activity on PPAN was measured (mU/ml plasma on the ordinate). In the inset, incubation times from 0 to 15 min are given in more detail for 25 µg/ml (o) and 100 µg/ml (◆) dextran sulphate.

of a rapid production of active kallikrein from its precursor, followed by a slower inactivation of the enzyme. Obviously, the rate of production of active kallikrein is dependent upon the amount of dextran sulphate; however, as shown in the inset of Fig. 1, addition of 100 µg of dextran sulphate was not more effective than adding 25 µg of the compound. This is shown in more detail in Fig. 2, where the amount of activity produced in a fixed incubation time (10 min) is given for a wide range of dextran sulphate concentrations. Apparently, the rate of prekallikrein activation was maximally stimulated by addition of 12.5 µg or more of dextran sulphate. The inset of Fig. 1 also demonstrates that the amount of kallikrein activity obtained after incubation with 25 or 100 µg of dextran sulphate was constant during a period of around 5 min before enzyme inactivation was observed. This suggests that under these conditions,

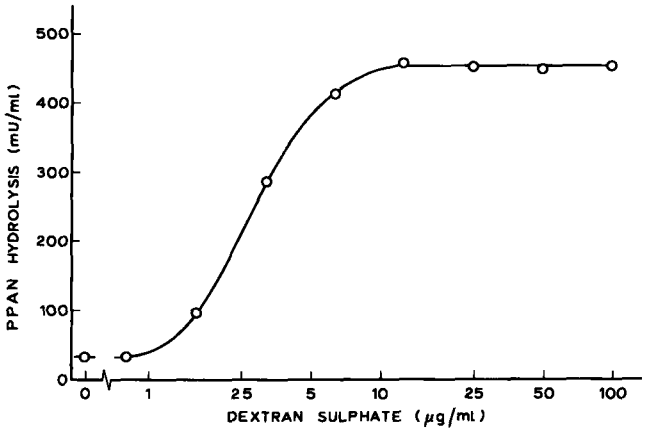

Fig. 2. Effect of cold activation (10 min) with various concentrations of dextran sulphate on PPAN hydrolysis of pooled plasma. Pooled human plasma was mixed 1:1 with dextran sulphate solutions (concentrations on logarithmic scale of the abscissa) and incubated for 10 min at 0°C. Activity on PPAN (mU/ml plasma on the ordinate) was recorded spectrophotometrically as described in Methods.

all prekallikrein has been converted to active kallikrein during this period.

Because of these findings, an incubation time of 7 min in the presence of 25 µg of dextran sulphate was chosen for the further experiments described below.

Prolonged incubation of plasma after activation with dextran sulphate led to inactivation of the free formed kallikrein. After one night, the remaining activity on the PPAN was about the same for the three concentrations of dextran sulphate tested (Fig. 1). This activity is probably due to the formation of a complex between kallikrein and α_2-macroglobulin which is known to be able to hydrolyze smaller synthetic substrates of kallikrein (23).

Influence of factor XII. The role of factor XII in the activation of prekallikrein was studied by varying the factor XII concentration in the test plasma. Such variation was achieved by mixing pooled normal plasma and factor XII-deficient plasma in different proportions. Fig. 3 shows that the production of kallikrein was dependent upon the factor XII concentration. With the factor XII content in the mixture being provided by 50% or more pooled plasma, 96% of the calculated maximum amount of kallikrein activity was obtained after 7 min of cold activation with dextran sulphate. With less than 50% pooled plasma, the production of kallikrein was too slow to prevent considerable loss

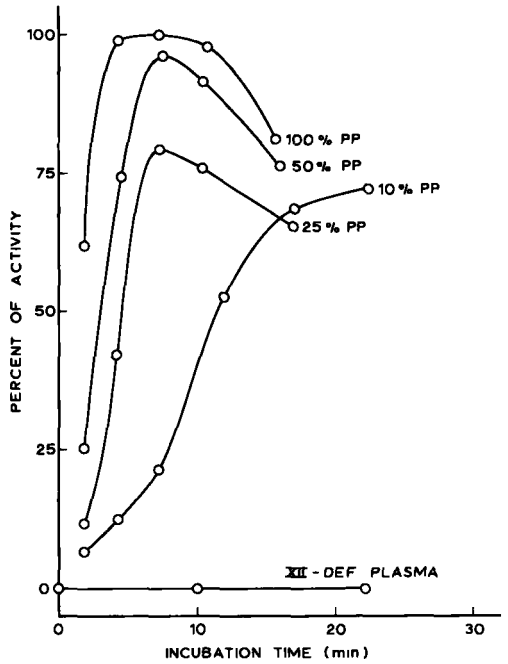

Fig. 3. Influence of decreased factor XII levels on cold activation with dextran sulphate. Cold activation with a solution of 25 µg/ml of dextran sulphate was studied in the following mixtures (v/v) of pooled plasma (PP) with factor XII-deficient plasma: 100:0, 50:50, 25:75, 10:90, 0:100. The prekallikrein content of pooled plasma and factor XII-deficient plasma (with the use of purified factor XII for correction) was determined to be 466 and 425 mU/ml, respectively. For each mixture, the theoretical optimal activity was calculated from these data. For various times of incubation (abscissa), the activity on PPAN was recorded and expressed as a percentage of the calculated optimal activity for each mixture (ordinate). Factor XII-deficient plasma (man, 69 years of age) was obtained from the Department of Haematology, University Hospital, Leiden, The Netherlands.

of activity.

No activity was produced in the deficient plasma alone after 22 min of incubation with dextran sulphate.

Influence of C1-inactivator. The influence of increased C1-inactivator was tested by enriching pooled normal plasma with purified C1-inactivator. The resulting levels of activity were about 95% of the activity obtained with the pooled plasma alone (Fig. 4), indicating a minor influence of increased C1--inactivator levels (up to 240% of the normal plasma level) on the prekallikrein activation in normal plasma in our test system.

Activation of prekallikrein by other activation procedures

Kaolin at 0°C. With kaolin, the rate of activation at 0°C was higher than with dextran sulphate (25 µg/ml), requiring an incubation time of only 2 min to reach optimum kallikrein activity. After the optimum was reached, the destruction of active kallikrein was comparable for both procedures. The requirement of factor XII for cold activation with kaolin was tested in a way similar to that described for dextran sulphate activation and showed essentially the same results. Kaolin differed from dextran sulphate in that activation of prekallikrein could also proceed in the diluted system in the cuvet. Addition of a mixture of cold plasma and kaolin (not preincubated) to the cuvet resulted in increasing consumption of substrate to a maximal rate of around 80% of the optimal activity obtained in the same plasma with dextran sulphate. Although the activation mechanisms of kaolin and dextran sulphate seem to differ in some aspects, essentially the same optimal activity on PPAN was found when either was used as activation agent, as shown in Fig. 5 (columns A and C).

Kaolin at 25°C. Activation with kaolin (final concentration 5 mg/ml) at 25°C was performed as described by Colman et al. (22). Activation was very rapid, resulting in an optimal preincubation time of 20 sec, in contrast to an optimal preincubation of 1 min reported by Colman et al. (22). The inactivation of the kallikrein formed was also very rapid, resulting in a loss of 30% of activity at 60 sec of preincubation. After 20 sec of preincubation, the activation was not completed in the tube, but further activation proceeded in the cuvet. This was evident from an increasing rate of liberation of p-nitroanilide from the PPAN. The optimal activity finally achieved in this manner has not been produced by further preincubation. This activity is presented in Fig. 5 (column B).

Sodium ellagate at 0°C. Results with freshly prepared solutions of sodium ellagate were not consistent. In only two out of four instances was a potent activation observed with a 10^{-3}M solution, which required 10 min of incubation for optimal activity (Fig. 5, column D). Dilution to 10^{-4} abolished the activ-

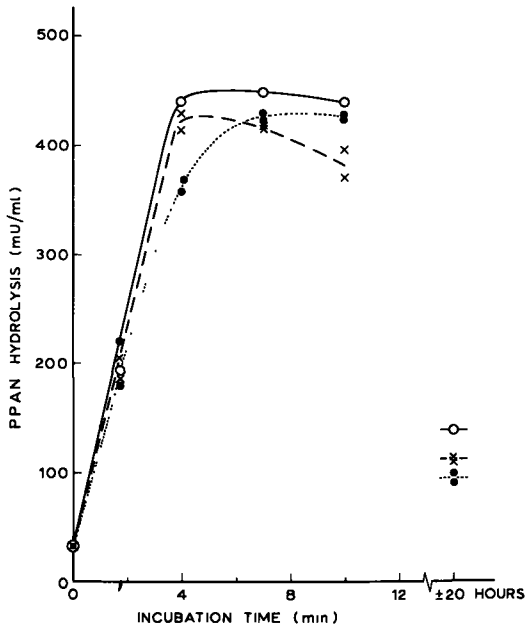

Fig. 4. Influence of elevated C1-inactivator on the cold activation of prekallikrein. Highly purified C1-inactivator (25 U/ml) was mixed with pooled normal plasma (18 U of C1-inactivator/ml) to study the influence of increased C1-inactivator on the cold activation by a solution of 25 µg/ml of dextran sulphate. Activity on PPAN (ordinate) was recorded after various preincubation times (abscissa) at $0^{o}C$ in the following mixtures; pooled plasma: :buffer:purified C1-inactivator:dextran sulphate = 2:2:0:4 (o); 2:1:1:4 (×); 2:0:2:4 (•).

ating properties of the solution in these cases.

Acetone activation at 25°C. The acetone procedure resulted in lower activities (Fig. 5, column E). Attempts to further activate with dextran sulphate were unsuccessful.

Fig. 5 shows the optimal activities obtained with the various procedures in pooled plasma. With four activation procedures (Fig. 5, columns A to D), essentially the same activity could be generated, supporting the view that optimal activation of prekallikrein was achieved.

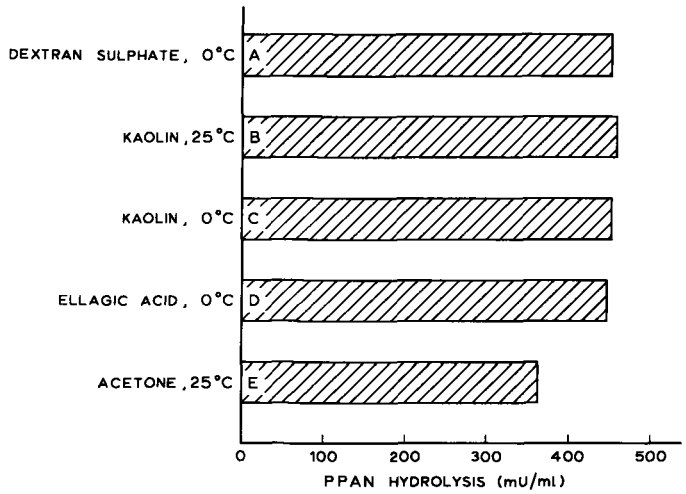

Fig. 5. Optimal activities obtained with dextran sulphate as compared with other activation methods. Optimal activities obtained in human plasma after activation with A, dextran sulphate (25 µg/ml) at 0°C for 7 min (n=4); B, kaolin (final concentration 5 mg/ml) at 25°C for 20 sec (n=2); C, kaolin (final concentration 5 mg/ml at 0°C for 2 min (n=2); D, sodium ellagate at 0°C for 10 min (n=2); and E, acetone at 25°C (n=2). See also Methods. The activity of PPAN is recorded as mU/ml of plasma.

Specificity of the new assay.

Purified human factor XII, $C\bar{1}s$, and plasmin were tested for possible activity on PPAN. Purified factor XII before and after incubation with dextran sulphate did not hydrolyze PPAN at concentrations of this factor in the cuvet twice as high as when added together with pooled plasma in the kallikrein assay. Neither did enrichment of pooled plasma with purified factor XII lead to increase in activity produced by incubation with dextran sulphate. Activated purified $C\bar{1}s$ added in amounts of up to 6 U/cuvet also failed to show activity on PPAN. In contrast, human plasmin had an appreciable activity of 120 mU/casein unit. Therefore it was investigated whether plasminogen activation could have occurred during the cold activation procedure with dextran sulphate. After 7 and 30 min of cold activation, the position and size of the immunoprecipitate in crossed immunoelectrophoresis with monospecific antiserum used against plasminogen appeared to be essentially the same and was in the region of the β-globulins, as is known for native plasminogen (24). The small differences compared with the control sample are attributed to the presence of

dextran sulphate in the activated samples interfering with the electrophoresis in agarose. It is concluded that no significant activation of plasminogen is evoked during cold activation.

Fletcher factor (plasma prekallikrein)-deficient plasma showed no activity on the substrate PPAN, even if activation was attempted after twofold enrichment with purified, partly activated factor XII. Furthermore, a 1:1 mixture of pooled normal plasma and Fletcher factor-deficient plasma (enriched twofold in factor XII) showed close to 50% of the activity of the pooled plasma, further establishing the specificity of the method for plasma kallikrein.

Examples and result obtained with the new assay for plasma prekallikrein

The assay for prekallikrein which was worked out with pooled normal plasma is applicable to any plasma sample on the following two conditions:

1. The spontaneous activity of the plasma sample must be recorded and should be within a normal range, providing a check on the quality of the plasma sample. High spontaneous activity (83 and 115 mU/ml), indicating uncontrolled conversion of prekallikrein before use of the plasma sample, was found in two out of 70 samples.

2. A check for adequate activation of prekallikrein in the test plasma must be included. For this purpose, after activation of prekallikrein, the activity of the test plasma alone is compared with its activity in a 1:1 mixture with pooled normal plasma. These activities are in agreement when adequate activation occurs in the test plasma. Incomplete activation of prekallikrein in the test plasma shows up as a discrepancy between these activities, since the pooled normal plasma in the mixture provides a normal activation process which can correct deficiencies in the test plasma, as demonstrated above for factor XII-deficient plasma (Fig. 3). This control experiment is essential for the discrimination between variations in activation of prekallikrein and variations in prekallikrein levels.

Table I gives examples of results obtained in this way with plasma from apparently healthy volunteers and a few random patients. These serve only for demonstration purposes and are not necessarily representative for the diseases involved. An example of an elevated level of prekallikrein is found in plasma 8 (osteoarthritis) and of decreased levels in plasmas 7 (liver cirrhosis) and 10 (hyperlipoproteinemia), as also previously reported (22,25,26). Incomplete activation of prekallikrein is seen for plasmas 6 (liver cirrhosis) and 9 (cystic fibrosis,(27)). In a series with 15 apparently healthy volunteers, a plasma level of prekallikrein of 476 $\pm$ 58 (S.D.) mU/ml (range 385 to 586) was

demonstrated. A pooled plasma was assayed on 10 different days in a 3-month period, yielding values ranging between 450 and 480 mU/ml (mean, 463; S.D., 11). Assay of this pooled plasma at a lower substrate concentration yielded an activity of 327 mU/ml, which agrees well with a reported value of 322 mU/ml for such a pooled plasma (2).

Table 1. Results of the new prekallikrein assay

No.	Plasma*	Control experiments: Spontaneous plasma activity (mU/ml)	Control experiments: Contribution in mixture (mU/ml)	Assay: activated sample (mU/ml)
1	Control	23	419	408
2	Control	23	413	428
3	Control	24	549	505
4	Control	12	460	465
5	Control	14	479	450
6	Severe liver cirrhosis patient†	0	162	42
7	Severe liver cirrhosis patient†	7	174	139
8	Osteoarthritis patient‡‡	7	595	604
9	Cystic fibrosis patient§	13	302	26
10	Hyperlipoproteinemia IIb patient‖	20	148	139

*Plasma of five apparently healthy individuals and of five patients was studied. Spontaneous activities of the plasma on PPAN are recorded in freshly thawed and cooled plasma. Activity after 7 min of cold activation with dextran sulphate (activated sample) and activity in a likewise activated 1:1 mixture with pooled normal plasma are assayed. The contribution of the plasma sample to the total activity in the mixture is calculated by subtraction of the known kallikrein activity of the pooled plasma.

†Obtained from the Department of Internal Medicine, Wilhelmina Gasthuis, Amsterdam.

‡‡Obtained from the Institute for Rheumatism Research, State University of Leiden.

§Obtained from the Department of Internal Medicine, Zuiderziekenhuis, Rotterdam.

‖Obtained from the Xanthoma Group, Leiden, classified according to the W.H.O. (45).

DISCUSSION

The assay system described was designed to measure the level of prekallikrein in plasma. The plasma prekallikrein can be assayed functionally via the active enzyme kallikrein which is an unstable intermediate in two sequenced reactions, namely, the formation of kallikrein from its precursor and the subsequent inactivation of kallikrein by inhibitors. The amount of free kallikrein will therefore depend upon the relative rates of these two reactions. In the cold procedure described in this paper, both reactions are slower; however, the inactivation of kallikrein is affected much more. An optimal activity of kallikrein, which is assumed to reflect a practically complete conversion of prekallikrein to kallikrein, is found. This assumption is based on the results showing (1) a large difference between the activation and inactivation rates of kallikrein, with a period of nearly constant kallikrein activity (Fig. 1); and (2) the failure to produce more kallikrein activity even if the activation at 0°C is reinforced by the addition of purified, partly activated factor XII, by the use of kaolin or if higher temperatures are employed (kaolin, 25°C).

Reduction of the influence of inhibitors

The reduction of the influence of inhibitors in the whole assay system is achieved by the cold procedure and also by dilution.

During the cold procedure, the inhibitors remain active but only slowly inhibiting kallikrein, as can be deduced from the decrease in activity following the optimum in Fig. 1 for 25 µg of dextran sulphate. Increased levels of C1-inactivator in pooled plasma had a minor effect (Fig. 4), showing that the cold procedure effectively reduced the influence of this inhibitor. The need for a cold procedure is confirmed by the effect of inhibitors observed in the experiments with kaolin at 25°C. As early as 60 sec of incubation, a 30% loss of activity was found. It was peculiar in this activation that optimal activity could not be achieved by preincubations alone; only a combination of preincubation (20 sec, 25°C) and activation in the diluted system in the cuvet (37°C) gave optimal activity.

The use of dilution to minimize the influence of inhibitors is intrinsic in spectrophotometric assays. In this sensitive assay with the subtrate PPAN (the concentration in the cuvet was 1.7 times higher than used by others, (2,21)), only a small plasma sample needs to be added to the cuvet and the final dilution of plasma is 1:162. Under these conditions a good indication of initial

activity is obtained. However, at longer recording times the p-nitroanilide release progressively slows down, which effect is caused at least partly by the kallikrein inhibitors in plasma, since it is much less pronounced if activity is assayed in acetone-activated plasma (no inhibitors interfering) or with purified kallikrein or plasmin.

Other procedures studied for reduction of the influence of the inhibitors were less successful. Acetone treatment of plasma both activates the kallikrein system and inactivates inhibitors, but the procedure studied showed low activity (cf. Fig. 5, column E). It can be suggested that the temperatures used for this and other modifications of the procedures, namely, 20 to 25°C (28,29), are responsible for the low activity, since the inactivation of inhibitors needs to be extremely rapid to avoid loss of kallikrein at these temperatures.

Treatment with sodium flufenamate to eliminate C1-inactivator (30) and α_2-macroglobulin (31) was unsuccessful, since it formed precipitates with the substrate (PPAN).

Interference by plasminogen/plasmin

Plasmin was also found to be quite active on the substrate PPAN. At pH 7.9 with our kallikrein test system, an activity of 120 mU/casein unit was detected, essentially in accordance with 133 mU/casein unit at pH 7.4 reported by Pentapharm (21). Calculation indicates that if all plasminogen in human plasma is activated, an activity of around 500 mU/ml of plasma can be found. This calculation is based on reported values of 20.3 $\pm$ 2.6 mg of plasminogen/100 ml of plasma (32), 1 casein unit equals 0.6 nMol plasmin (24), plasminogen being around 90,000 daltons. During the cold activation procedure of the prekallikrein assay and after prolonged cold activation, we found, however, no significant activation of plasminogen. It can be concluded that the specificity of the test system for prekallikrein depended not only on the specificity of the substrate but also on the selectivity of the activation procedure, as also pointed out by Colman et al. (33).

The absence of interference by plasmin and the specificity of the method for plasma kallikrein are further evident from the results showing absence of activity in Fletcher trait plasma (with a normal plasminogen level), whereas in a 1:1 mixture of this plasma with pooled normal plasma, close to the expected 50% of the pooled plasma activity is found.

Application of the new assay to individual plasma samples

In two situations, the aim of the new method to assay for the plasma prekallikrein level cannot be achieved. These situations are (1) incomplete activation of prekallikrein and (2) partial or complete activation of prekallikrein prior to the use of the plasma sample.

In a plasma sample with incomplete activation of prekallikrein, the activity detected with the new assay in the plasma sample alone will be lower than the activity detected in the mixture with pooled plasma, where the intact activation system of the pooled plasma contributes. Examples were shown in Table 1 where incomplete activation apparently occurred in plasma samples 6 (liver cirrhosis) and 9 (cystic fibrosis). The incomplete activation of prekallikrein observed in the patient with liver cirrhosis (plasma 6) is possible related to a reported discrepancy between an immunological and functional assay for kallikrein in such a patient (25). In plasma samples with incomplete activation of prekallikrein, further study of the activation of this precursor is required before the prekallikrein level can be ultimately determined. Incomplete activation of prekallikrein can be due to abnormalities in factor XII, HMW kininogen, prekallikrein, or inhibitors interfering with prekallikrein activation. This study demonstrates that, for absence of only factor XII, correction of the incomplete activation of prekallikrein is achieved in the 1:1 mixture with pooled normal plasma (Fig. 3). A combination of unfavourable circumstances may also occur, and separate assays for the mentioned factors in instances of decreased activation are useful. In the 1:1 mixture of Fletcher factor-deficient plasma with pooled normal plasma, only a slow activation of prekallikrein (originating from the pooled plasma) resulting in an optimum activity of 37% was found. By enriching the system with increasing amounts of purified, partly activated factor XII, the expected 50% activity of the pooled plasma was eventually obtained. A combination of a deficiency in prekallikrein and high C1-inactivator (142% relative to pooled plasma) possibly explains this. Alternatively, an abnormal prekallikrein molecule in the deficient plasma interfering with the activation may occur.

The second situation concerns the activation of prekallikrein before use of the plasma sample. This can be traced, because the occurrence of free kallikrein or a kallikrein-α_2-macroglobulin complex activity can be detected with PPAN. Therefore the spontaneous activity of plasma samples on PPAN was studied. With the defined procedures for plasma sampling and handling, only two samples in 70 showed a high spontaneous activity (83 and 155 mU/ml), whereas in a control group of 15 healthy volunteers, low spontaneous activity of 12 to 28

mU/ml was found. One of the samples with high spontaneous activity was from an apparently healthy man, the other from a patient deficient in C1-inactivator (HANE plasma). The absence of C1-inactivator possibly predisposes for easier activation of the kallikrein system, for spontaneous esterase activity in plasma samples of HANE patients has been reported by others (12,34).

Mechanism of spontaneous cold activation

Activation of prekallikrein prior to use can be due to cooling of plasma and blood. This effect is closely related to cold activation of factor VII in the coagulation system, a phenomenon which is well documented by Gjonnaess (35-39). It is usually a slow process with lag periods of 2 to 13 hr (35,40) and can usually be prevented by rapidly freezing the plasma sample after collection to temperatures distinctly below -20°C. This phenomenon is found only occasionally in plasma of normal men (12%) and women (17%) but more frequently in plasmas from women using estrogen-containing contraceptives (65%) and pregnant women near term (93%) (35,41).

The mechanism of the cold activation is only partly understood. Apparently, factor XII is activated, and the absence of C1-inactivator and elevated prekallikrein levels (42) facilitate this activation and further effects. The decrease in the effectiveness of inhibitors at 0°C presumably explains the requirement for cooling to observe the phenomenon (in HANE plasma in the absence of C1-inactivator, possibly activation also proceeds at higher temperatures). It is not yet clear what triggers the activation of factor XII. Activation of the kallikrein system on cooling was originally reported by Armstrong and Mills (43) and was suggested to involve a dissociation of an enzyme-inhibitor complex at 0°C. Our observation that soluble dextran sulphate triggers the activation of factor XII at 0°C makes it possible that naturally occurring soluble activators of factor XII occasionally appear in plasma, pushing the factor XII activation. Plasma mucopolysaccharides possibly serve as sources of such natural activators of factor XII activation. In this respect, it must be noted that 1.5 µg/ml dextran sulphate (within the normal range of plasma mucopolysaccharides (44)) achieved complete activation of the kallikrein system in pooled plasma at 0°C and of factor VII (unpublished) after a night of incubation.

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CHAPTER VI

FACTOR XII-DEPENDENT FIBRINOLYSIS: A DOUBLE FUNCTION OF PLASMA KALLIKREIN AND THE OCCURRENCE OF A PREVIOUSLY UNDESCRIBED FACTOR XII- AND KALLIKREIN-DEPENDENT PLASMINOGEN PROACTIVATOR*

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Abstract. Fibrinolytic studies in euglobulin fractions of Fletcher trait plasma (deficient in prekallikrein) revealed reduced activities as compared to normal plasma. A quantitative assay for total plasminogen activator plus proactivator in plasma showed that the amount in Fletcher trait patients is about half of normal (normal = ± 100 blood activator units (BAU)/ml). Plasma kallikrein partially purified in a high molecular weight form and in a subunit form exerted plasminogen activator activity amounting to 10-15 BAU/ml plasma. So, the absence of kallikrein in the deficient plasma cannot fully account for the reduction in activator activity. Additions of kallikrein preparations or normal plasma fractions resulted in additional activator activity in Fletcher trait plasma which was assessed at 30-40 BAU/ml. This activity was assumed to originate from a previously undescribed plasminogen proactivator whose activation is kallikrein- and factor XII-dependent.
Fractionation experiments demonstrated the presence of two major activator activities and a minor activity caused by kallikrein in normal plasma. It is concluded that plasma kallikrein has two functions in the generation of factor XII-dependent fibrinolytic activity: one as a direct plasminogen activator and another as a factor in the activation of a major factor XII-dependent plasminogen proactivator.

INTRODUCTION

The occurrence of Hageman factor (coagulation factor XII) dependent fibrinolysis in plasma was recognized in 1959 by Niewiarowski and Prou-Wartelle (1). The fibrinolytic activity produced by surface exposure was assumed to result from an interaction of surface activated Hageman factor with a precursor of a plasma plasminogen activator (2).

*Submitted for publication.

One of the factor XIIa substrates in plasma is prekallikrein. Purified kallikrein preparations obtained from plasma activated by surface contact were found to possess plasminogen activator activity (3,4). Kaplan and Austen (5) initially reported the separation of plasminogen activator activity from kallikrein activity. This separation has, however, been a matter of dispute (6,7,8,9). Very recently, Kaplan et al. (10) presented new data which showed kallikrein to possess activator activity, while their formerly described separate plasminogen proactivator was suggested to be a degradation product of prekallikrein.

The presently available information suggests the following scheme for factor XII-dependent fibrinolysis (Fig. 1)

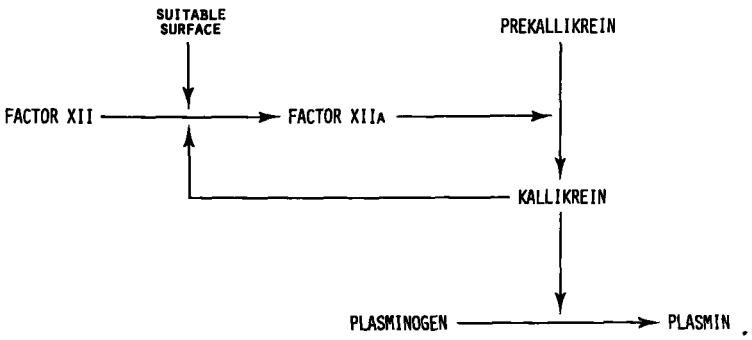

Fig. 1. Factor XII-dependent pathway of fibrinolysis in plasma.

The role of kallikrein in the factor XII-dependent pathways is complex, as indicated by the arrow pointing back to the factor XII activation in the scheme in figure 1. Prekallikrein occurs in plasma as a protein complex with high molecular weight (HMW) kininogen (11,12). Rapid contact activation of factor XII in plasma is achieved only by the participation of this complex, while kallikrein causes limited proteolysis of the surface-bound factor XII (13-15).

Accordingly, in the absence of prekallikrein, such as in Fletcher trait plasma, only a slow contact activation of coagulation is observed (16-18). Similarly, the contact activation of fibrinolysis was found to be slow in Fletcher trait plasma (17,18). That Fletcher trait plasma is able to generate plasmin at all by contact activation, however, implies the formation of an

activator different from kallikrein. The precursor of this activator has not yet been identified.

A recently developed method using euglobulin fractionation procedures allowed the quantitative assessment of the total activity of plasminogen activators and proactivators in plasma (19,20). Two sources of activity were distinguished: an activity originating from an extrinsic activator and one originating from intrinsic proactivators. In samples of morning plasma obtained at rest, the amount of extrinsic activator was negligible in comparison with the level of potentially active, intrinsic proactivators. A fairly stable level of 100 ± 15 blood activator units (BAU)/ml was found in a survey on 50 apparently healthy individuals (20). In plasma deficient in factor XII (Hageman trait plasma), the total activator concentration reached only 52 BAU/ml (3 patients) but the values increased to normal when factor XII was added to the assay (19). These observations suggested a subdivision of the intrinsic activator activity into two roughly equal contributions by a factor XII-dependent and a factor XII-independent pathway (19,20,21).

The present study was undertaken to determine the contribution of kallikrein to the intrinsic plasminogen activator potential. For this purpose, samples of plasma deficient in prekallikrein (Fletcher trait plasma) were used. The activator activity of partially purified kallikrein preparations was also assessed. Furthermore, the intrinsic activators present in euglobulin fractions of normal plasma prepared in the presence of dextran sulphate were submitted to gel filtration and DEAE-cellulose chromatography. All results led to the conclusion that there is a major plasminogen proactivator in the factor XII--dependent pathway which is different from kallikrein.

MATERIALS AND METHODS

Materials

Unless otherwise specified, reagents were of analytical grade and purchased from E. Merck, Darmstadt, West Germany. Distilled water was used throughout. Additional reagents and materials were obtained from the following sources. 'Agarose for electrophoresis' from BDH Chemicals Ltd., Poole, England; dextran sulphate, sodium salt, $\overline{MW}$ 500,000 from Pharmacia Ltd., Uppsala, Sweden; flufenamic acid from Aldrich Europe, Beerse, Belgium; chromozym PK or α-N-benzoyl -L-proline-L-phenylalanine-L-arginine-p-nitroanilide HCl (PPAN) from Pentapharm A.G., Basle, Switzerland; Carbowax 20,000 from Fluka A.G., Buchs, Swit-

zerland; ammonium sulphate and catalase from Serva, Heidelberg, West Germany; ultrogel ACA 34 from LKB, Bromma, Sweden; antisera raised in rabbits against human plasminogen, α_2-macroglobulin, and plasma prekallikrein (a laboratory preparation) from Behringwerke A.G., Marburg, West Germany; diethylaminoethyl (DEAE) cellulose DE 52 from Whatman Ltd., Kent, England; plasminogen-free bovine fibrinogen from Poviet, Organon Teknika, Oss, The Netherlands. Plasminogen-rich bovine fibrinogen (batch PF_2) was prepared according to Brakman (22); platelet-poor, citrated human morning plasma and pooled plasma were prepared as previously described (23); Fletcher trait plasma A (case 3 as described in (24)) was obtained from George King Biomedical Inc., Salem, N.H. USA; Fletcher trait plasma B was obtained through the courtesy of Drs. J. and C. Soria, Hôpital Hotel-Dieu and Laroboisière, Paris, France; outdated human plasma was obtained from the Blood Bank, University Hospital Leiden, The Netherlands. the presence of a normal level of prekallikrein was confirmed before use (25).

Solutions

EDTA buffer (μ = 0.15): 0.05 M sodium diethylbarbiturate, 0.10 M NaCl, 0.25% (w/v) gelatin and 2.7 mM ethylene diamine tetraacetate (EDTA) adjusted to pH 7.8 with an HCl solution.

Sodium flufenamate solutions: 0.5 M flufenamic acid in equivalent concentration of NaOH (60°C), diluted to appropriate concentrations with EDTA buffer.

Dextran sulphate solution: The sodium salt of the dextran sulphate preparation was dissolved in distilled water.

TRIS-imidazole buffer (μ = 0.15; pH 7.9) as described in (25).

Methods

Preparation of euglobulin fractions

Regular euglobulin fractions were prepared with a plasma dilution of 1:10 and pH 5.9 as described in detail elsewhere (23).

Supernatant fraction. 1 ml (per ml plasma) of dextran sulphate (100 µg/ml) in distilled water was added to the supernatant resulting from the removal of the regular euglobulin fraction. The mixture was allowed to stand for 30 min in an ice-bath before centrifugation. The precipitate was redissolved in EDTA buffer in a volume equal to the original plasma volume.

Dextran sulphate euglobulin fraction. This fraction was prepared from a

mixture of 1 ml plasma, 8 ml distilled water and 1 ml dextran sulphate (100 µg/ml) solution. All solutions were precooled in ice-water and mixed rapidly in the order mentioned. Titration with acetic acid to pH 5.9 was started immediately after mixing. After centrifugation in the cold, the precipitate was dissolved in 1 ml EDTA buffer.

Assay of fibrinolytic activity

Fibrinolytic activities were determined by the fibrin plate method (26). Diameters of lysed zones after 18 hrs of incubation on fibrin plates at 37°C were used to represent the fibrinolytic activity of the solutions tested. Alternatively, the activity was expressed in blood activator units (BAU) as defined earlier (C. Kluft. Submitted for publication). Pooled, normal morning plasma served as a standard arbitrarily set at 100 BAU/ml.

Regular assay. In the regular assay of the fibrinolytic activity of euglobulin fractions, drops of 30 µl of the fraction were placed on the plasminogen-rich bovine fibrin (in triplicate).

Assay with flufenamate (23). In this assay, immediately after application of the 30 µl drops of the euglobulin fraction to the fibrin plate, 5 µl of 14 mM flufenamate in EDTA buffer was added to each drop. This assay was routinely used with the dextran sulphate euglobulin fraction and the supernatant fraction.

Assay of C1-inactivator-resistant fibrinolytic activity. Immediately after application of the 30 µl drops of the regular euglobulin fraction to the fibrin plate, 5 µl of a 700% solution (relative to the pooled plasma) of C1-inactivator in EDTA buffer was added.

Plasminogen and prekallikrein related antigen were assayed by the quantitative radial immunodiffusion method according to Mancini et al. (27) or the rocket immunoelectrophoresis method according to Laurell (28).

Amidolytic activity of kallikrein: 3 µl of the test solutions were added to a prewarmed (37°C) mixture of 0.2 ml Tris-imidazole buffer (µ = 0.15; pH 7.9) with 0.1% Carbowax and 40 µl PPAN solution (1 mM in distilled water). After rapid transfer to a micro quartz cuvet (type 178, Hellma Benelux, The Hague, The Netherlands) in a thermostat controlled cuvet holder, the increase in optical density at 405 nm was determined continuously for about 4 min with a Unicam SP 1700 spectrophotometer. The initial slope read as ΔOD/min was converted to mU/ml (25). Plasma prekallikrein was assayed after conversion to kallikrein by incubation of 0.1 ml plasma and 0.1 ml dextran sulphate solution

(25 µg/ml) for 7 min at 0°C as previously described (25).

Partial purification of kallikrein

A euglobulin fraction precipitated at plasma dilution 1:7.5 and pH 5.9 was prepared from 200 ml defibrinated outdated plasma at 0-4°C. From the remaining supernatant, a precipitate was obtained by addition of 30 µg dextran sulphate/ml plasma and further addition of water to a plasma dilution of 1:10. After centrifugation, the precipitate was dissolved in 100 ml 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.5. Addition of ammonium sulphate to 30% saturation produced a precipitate which was removed by centrifugation. The supernatant was then brought to 50% with ammonium sulphate and the precipitate formed was collected by centrifugation and dissolved in 4 ml 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.5. This preparation was fractionated on a column of Ultrogel ACA 34 (2x100 cm) in the same buffer. Kallikrein and plasminogen activator activity were assayed in the fractions. Fractions with a high ratio of kallikrein/activator activity were pooled, dialysed against 0.005 M ammonium bicarbonate and lyophilized. After gel filtration on Ultrogel ACA 34 in phosphate buffer containing 1.0 M NaCl, the kallikrein peak fraction usually showed a specific activity of about 250x that of normal plasma and an optimal ratio of kallikrein over activator activity. Such fractions served as partially purified kallikrein complex preparations. Separation from the contaminating plasminogen activator activity type IIB (see Results section) was almost complete. The kallikrein subunit was prepared from the pooled kallikrein peak obtained in the gel filtration on Ultrogel ACA 34 run in 0.01 M phosphate buffer, 0.15 M NaCl. After dialysis and lyophilization, the powder was dissolved in 0.02 M phosphate buffer, pH 8.0, and chromatographed on DEAE-cellulose in the same buffer. The kallikrein subunit formed during this procedure passed through the column and was collected.

RESULTS

1. Fibrinolytic activities of Fletcher trait and normal plasma

The contribution of plasma prekallikrein to the fibrinolytic activity of plasma was assessed by the assay of euglobulin fractions prepared from normal and prekallikrein deficient plasma (Fig. 2). In two of these assays, the fibrinolytic activity was recorded as diameters of the lysed zones produced on the fibrin plates. The regular euglobulin fractions show that samples of

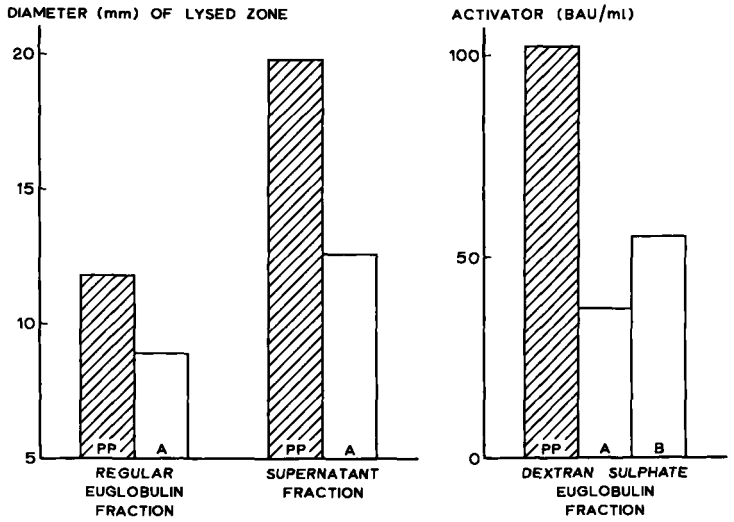

Fig. 2. Comparison of fibrinolytic activities in euglobulin fractions prepared from Fletcher trait plasma and normal, pooled plasma. The fibrinolytic activities were determined in a regular euglobulin fraction (left), a supernatant fraction precipitated by dextran sulphate (middle) and a dextran sulphate euglobulin fraction (right) (see Methods). The activities were recorded as diameters of lysed zones (left and middle) or as arbitrary blood activator units (BAU) (right). Hatched bars: pooled, normal plasma (PP); open bars: two Fletcher trait plasma samples (A and B).

Fletcher trait plasma exhibit lower activity than does the pooled plasma, although it is only slightly below the range of individual samples from the ten healthy individuals making up this pool (9.0-17.1 mm).

The redissolved precipitate obtained after addition of dextran sulphate to the supernatant remaining after separation of the regular euglobulin fraction displayed considerable fibrinolytic activity (Fig. 2), which is in agreement with previous findings (29). Individually, the ten plasma samples making up the pool gave a range of 17.7 to 19.2 mm. The corresponding fraction prepared from Fletcher trait plasma showed considerably lower activity (Fig. 2).

The third method of assay permits the quantitative determination of plasminogen activators and proactivators together (20). The pooled plasma yielded 102 arbitrary blood activator units (BAU)/ml. A range of 89-117 BAU/ml was previously found in a group of eleven healthy volunteers (20). In contrast, samples of Fletcher trait plasma A yielded only 38 $\pm$ 3 BAU/ml, while a single assay of a sample of Fletcher trait plasma B yielded 55 BAU/ml.

It can be seen that all three methods of assay indicate a reduced generation of fibrinolytic activity in Fletcher trait plasma. This is suggestive of a distinct role for kallikrein in plasma fibrinolysis. The quantitative assay indicates that Fletcher trait plasma retains about half of the activator concentration present in normal plasma (38 and 55 BAU/ml).

2. Attempts to correct reduced fibrinolysis in Fletcher trait plasma

a) *with kallikrein preparations*

The previous experiments indicated that about half of the total activator activity in normal plasma (± 50 BAU/ml) is missing in Fletcher trait plasma. As will be shown in detail in section 4 below, partially purified preparations of kallikrein high molecular weight complex and kallikrein subunit has plasminogen activator activity which seemed to reside in the kallikrein molecule. To determine whether this activator activity could account for the missing activity in the deficient plasma, the kallikrein preparations were added to the dextran sulphate euglobulin fractions of normal and Fletcher trait plasma (Fig. 3). When added to normal plasma, there was a proportional increase in

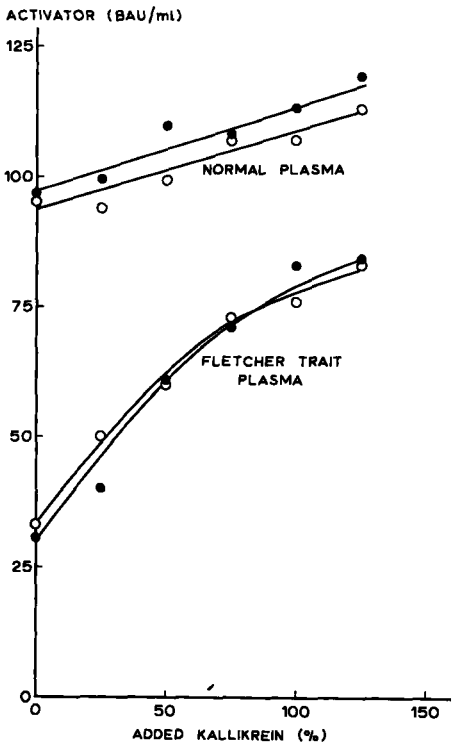

Fig. 3. Effect of preparations of kallikrein on the activator activity of dextran sulphate euglobulin fractions of normal, pooled plasma and Fletcher trait plasma. Increasing amounts of partially purified high molecular weight (●—●) and low molecular weight (○—○) kallikrein are added to the redissolved dextran sulphate euglobulin precipitates. Optimal conversion of prekallikrein to kallikrein in normal plasma yielded 476 ± 58 mU/ml (n=15) (30). This amount of kallikrein was arbitrarily taken as 100%.

activator activity, with both kallikrein preparations contributing around 10-15 BAU/ml at a concentration corresponding to normal plasma. This level obviously does not fully account for the activator activity missing in Fletcher trait plasma.

Addition of the kallikrein preparations to the dextran sulphate euglobulin fractions of Fletcher trait plasma initially produced a steep increase in activator activity which decreased in increment at higher concentrations of kallikrein and then approached the slope of the additions to normal plasma. It appeared that, besides an increase in activator activity resulting from the activator activity of the added kallikrein preparations, a considerable extra activator activity was generated in the Fletcher trait plasma.

b) *with normal plasma fractions*

The above experiments indicate a double function for plasma kallikrein in the generation of fibrinolytic activity in the dextran sulphate euglobulin fraction. Besides behaving as a plasminogen activator, it seems to participate in the generation of extra activator activity which remains inactive in Fletcher trait plasma.

This second effect of kallikrein should show up in mixtures of normal and Fletcher trait plasma, because the normal plasma should provide the kallikrein necessary for the generation of this extra activator activity in the Fletcher trait plasma.

When the supernatant fraction (precipitated by dextran sulphate) from normal plasma was diluted either with buffer or with a solution of the corresponding supernatant fraction prepared from Fletcher trait plasma, additional plasminogen activator was seen to be generated in the latter case (Fig. 4). Figure 5 shows a similar experiment involving the dextran sulphate euglobulin fractions. The measured activator activities in this case are converted to units allowing a calculation of the apparent contribution of the Fletcher trait plasma to the total activity of the mixture (Fig. 5). This calculation shows that the apparent activator activity in the dextran sulphate euglobulin fraction from the Fletcher trait plasma increases from 35 to about 80 BAU/ml and that it seems optimal in the 1:1 mixture with normal plasma fractions. The amount of extra activator activity induced in the Fletcher trait plasma is assessed at 30-40 BAU/ml. It must be noted that the calculated activator activity in Fletcher trait plasma does not include the contribution of kallikrein. Surprisingly, no generation of extra activator activity was observed when normal plasma and Fletcher trait plasma were mixed before the preparation of dextran sulphate euglobulin fractions. This could not be ascribed to an abnormal fractionation behaviour of activators or proactivators, since extra

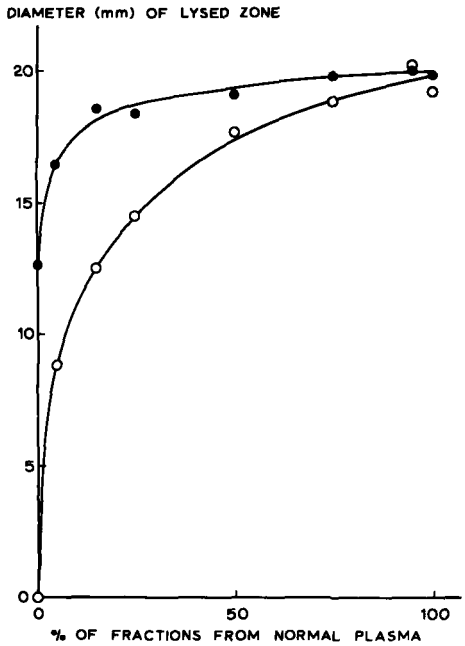

Fig. 4. Plasminogen activator activities determined in mixtures of supernatant fractions prepared from normal plasma and Fletcher trait plasma.
Supernatant fractions (precipitated with dextran sulphate) prepared from normal and Fletcher trait plasma A were mixed in various ratios (●–●). A supernatant fraction from normal plasma was diluted with EDTA buffer (o–o). The abscissa records the percentage of the normal plasma fraction in the mixtures. The ordinate records the fibrinolytic activities of the mixtures expressed in diameters of lysed zones on plasminogen-rich bovine fibrin plates with 5 µl of 14 mM flufenamate added to each drop.

activator activity could be again generated in hybrid fractions by adding dextran sulphate euglobulin fractions from normal plasma.

From these experiments, it is concluded that the reduction in activator activity in the Fletcher trait plasma is mainly due to a failure to activate a plasminogen proactivator, which is not prekallikrein in this plasma.

3. Gel filtration of the dextran sulphate euglobulin fraction

Previous studies on Hageman trait plasma (19-21) and the above-mentioned studies on Fletcher trait plasma suggest that the normal dextran sulphate euglobulin fraction contains the precursors of three different activators of plasminogen. One type of activator is present in both deficient plasmas. It amounts to about half of the total activator activity in normal plasma and seems to be activated independently of factor XII or prekallikrein. There are two factor XII-dependent contributions to the plasminogen activator activity of normal plasma: one from prekallikrein (10-15 BAU/ml) and another from an unidentified plasminogen proactivator (30-40 BAU/ml). In an attempt to separate these three components, the dextran sulphate euglobulin fraction from normal plasma was exposed to gel filtration.

To remove C1-inactivator, the fraction was first fractionated with ammonium

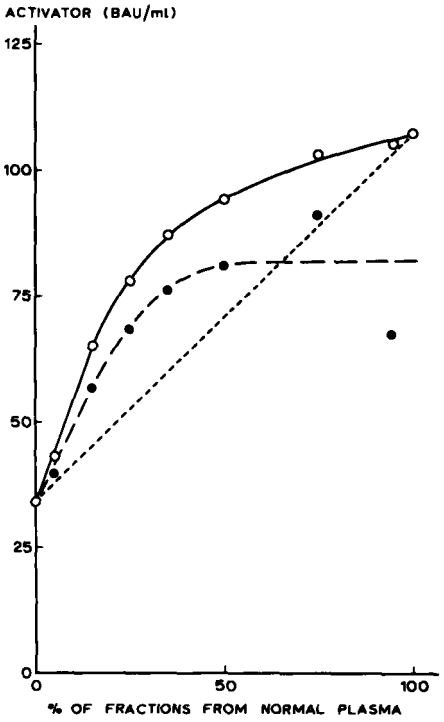

Fig. 5. Plasminogen activator activity determined in mixtures of dextran sulphate euglobulin fractions prepared from normal plasma and Fletcher trait plasma (o–o). The activator activity was determined on plasminogen-rich bovine fibrin plates in the presence of flufenamate and expressed in arbitrary blood activator units (BAU) /ml. The abscissa records the percentage of the normal plasma fraction in the mixtures. The dotted line indicates the calculated activator activities which would be obtained by a simple mixing of the two solutions. The broken line (•) gives the calculated difference in activator activity expressed as the activator activity in the dextran sulphate euglobulin fraction of Fletcher trait plasma which could explain the experimental results in the mixing experiment, assuming that the contribution of the normal plasma is invariable.

sulphate and the precipitate obtained between 30 and 50% saturation was collected, redissolved in a small volume and applied to a column of Ultrogel ACA 34 (Fig. 6). Two major peaks with activator activity (I and II) and one peak with kallikrein activity can be distinguished. No activity was observed on plasminogen-free fibrin plates. Corresponding peaks occur in dextran sulphate euglobulin fractions without prior ammonium sulphate fractionation or in the precipitate prepared from the euglobulin supernatant by addition of dextran sulphate. Activator peak I appears at a position corresponding to a very high molecular weight (above 10^6 daltons). Activator peak II is at a position of about 250,000 daltons. The relative quantities of activator in the two main peaks varies from experiment to experiment. A minor third activator peak, seen in figure 6, was not present in all preparations. This small peak shows up in the overlapping parts of activator peak II and the plasminogen peak, suggesting an increased response of activator-containing fractions on the fibrin plate because of the extra plasminogen. An additional small activator peak was occasionally seen at low molecular weight outside the fractionation range of

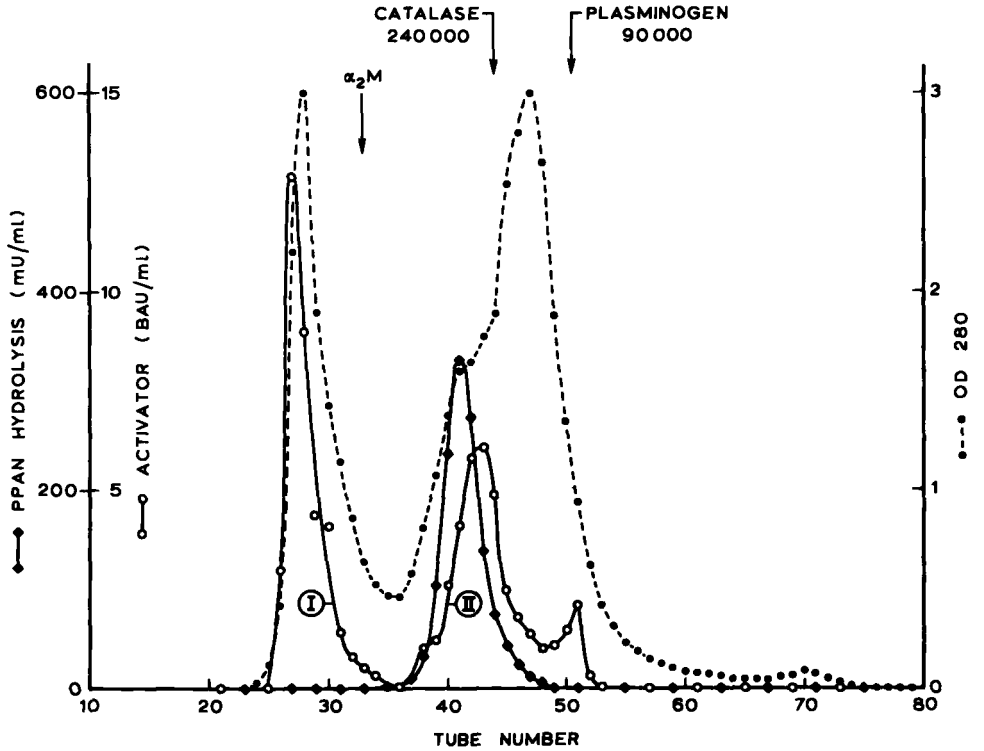

Fig. 6. Gel filtration of the dextran sulphate euglobulin fraction from normal plasma after ammonium sulphate fractionation.
The 30-50% ammonium sulphate fraction of the dextran sulphate euglobulin fraction prepared from 50 ml outdated human citrated plasma was dissolved in 4 ml phosphate buffer, 0.01 M, pH 7.5, with 0.15 M NaCl and applied to a 100 x 2 cm Ultrogel ACA 34 column. Elution by the same buffer gave the recorded profile. Protein (OD 280, •-•); activator concentration determined on plasminogen-rich fibrin plates with added flufenamate expressed in blood activator units (BAU /ml, o-o); kallikrein determined on PPAN (mU/ml, ◆-◆). The position of the plasminogen peak is indicated by an arrow. In separate runs, the positions of human α_2-macroglobulin in plasma (800,000 d) and purified catalase (240,000 d) were determined. They are indicated by an arrow.

Ultrogel ACA 34.

Kallikrein activity measured on the synthetic tripeptide Bz-Pro-Phe-Arg--pNA.HCl (PPAN, Chromozym PK) shows a peak at about 300,000 d (Fig. 6), indicating that it existed as a protein complex similar to the one described for the precursor molecule (11,12,31). The peak fractions reacted with an antiserum against kallikrein.

From the kallikrein and plasminogen activator peaks in figure 6, it is

evident that kallikrein can only be a minor contributor to the activator activity in peak II. The occurrence of two major activator peaks different from kallikrein is in general agreement with the subdivision of activator activities in the dextran sulphate euglobulin fractions proposed from the above-mentioned studies on deficient plasmas.

4. Plasminogen activator activity of kallikrein preparations

On gel filtration, kallikrein activity (PPAN-hydrolysis) partly coincides with the plasminogen activator peak II (Fig. 6). To distinguish between a possible activator activity originating from the kallikrein and that from the plasminogen activator in peak II, further separation studies were made.

Gel filtration at high ionic strength

To obtain further separation between kallikrein and activator activity from peak II (Fig. 6), a pool of fractions corresponding to the left half of the kallikrein peak showing low coinciding activator activity was again subjected to gel filtration on Ultrogel ACA 34. The gel filtration was carried out in 1.0 M NaCl, since this might result in a change in the molecular weight of plasminogen activator such as described by Ogston et al. (32). The presence of a small amount of high molecular weight material, presumably denaturated protein, and some low molecular weight material was observed (Fig. 7). The kallikrein activity and the activator activity, however, remained at their position. This indicated that the kallikrein protein complex was not split and, consequently, not held together by weak electrostatic forces. Obviously, the plasminogen activator activity studied in these fractions differed from that reported by Ogston et al. (32). The kallikrein peak fraction was used for further studies.

DEAE-cellulose chromatography

The kallikrein protein complex can be split by ion-exchange chromatography (11,31) which results in an active kallikrein molecule of about 100,000 d. It was investigated how much activator activity accompanied the kallikrein activity when it was subjected to DEAE-cellulose chromatography and subsequent gel filtration on Ultrogel ACA 34.

For this experiment, the kallikrein peak fraction of figure 7 showing low contaminating activator activity was used. In accordance with other reports (11,31), the kallikrein activity nearly completely passed through the column in 0.02 M phosphate buffer, pH 8.0 (Fig. 8A). The plasminogen activator acti-

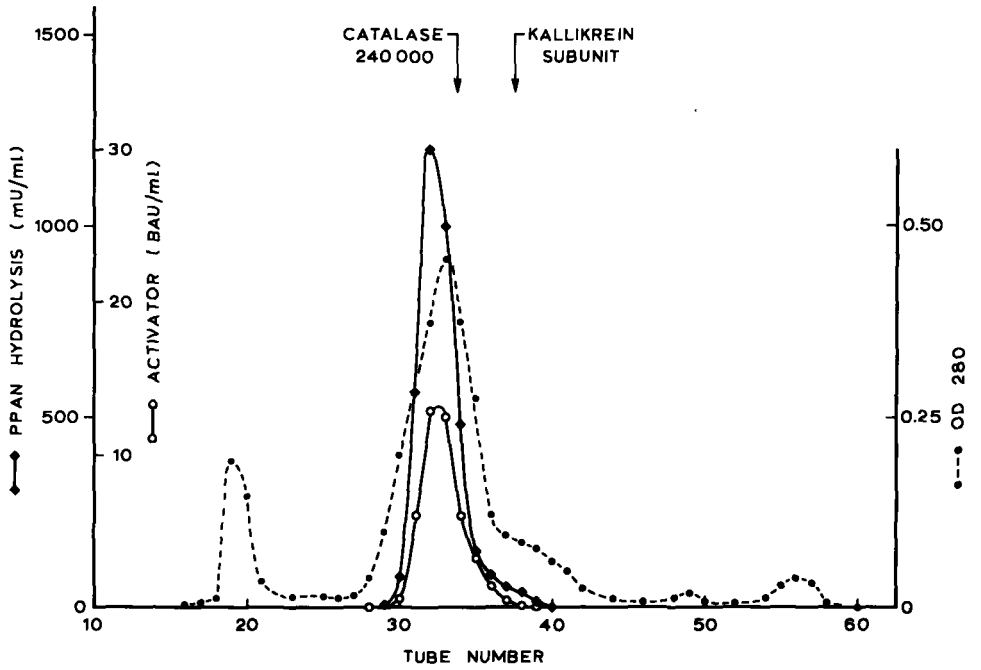

Fig. 7. Gel filtration of the kallikrein high molecular weight complex in 1.0 M NaCl.
The fractions of the left half of the kallikrein peak in figure 6 are pooled, dialysed, lyophilized, redissolved in 1.0 M NaCl, 0.01 M phosphate buffer, pH 7.5 and applied to an Ultrogel ACA 34 column 100 x 2 cm. Eluted protein (OD 280, ●-●); activator activity (BAU/ml, o-o); kallikrein activity on PPAN (mU/ml, ◆-◆). The position of catalase (240,000 d) and the kallikrein subunit were determined under the same conditions in separate runs and are indicated by arrows.

vity also passed through the column. No kallikrein and only a trace of plasminogen activator activity could be eluted from the DEAE-cellulose with 0.3 or 1.0 M NaCl (Fig. 8A). The kallikrein/activator material which had passed through the DEAE-cellulose column was applied to an Ultrogel ACA 34 column and both activities remained closely associated at a position around 100,000 d (Fig. 9).

It was concluded that kallikrein as well as some plasminogen activator activity originally resided in a protein complex of around 300,000 d and that both could be split to give a subunit of around 100,000 d by ion-exchange chromatography.

In further fractionation experiments on the subunit using SP-Sephadex,

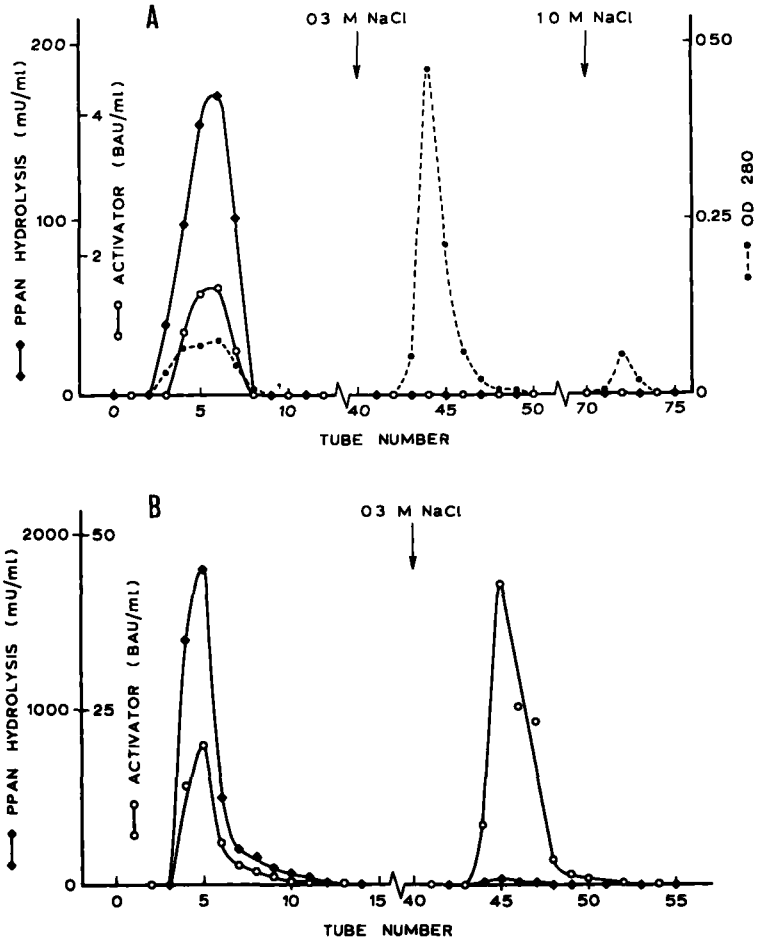

Fig. 8. DEAE-cellulose chromatography of the kallikrein high molecular weight complex and plasminogen activator peak II.
A: kallikrein peak fraction 33 of figure 7 was dialysed against 0.02 M phosphate buffer, pH 8.0, and applied to DEAE-cellulose equilibrated in the same buffer. The column was stepwise eluted with 0.3 and 1.0 M NaCl in the phosphate buffer.
B: eluted fractions from kallikrein + activator peak II (cf Fig. 6) were pooled, dialysed, lyophilized and applied to DEAE-cellulose in 0.02 M phosphate buffer, pH 8.0. The column was stepwise eluted with 0.3 M NaCl.
Eluted protein (OD 280, dotted line); plasminogen activator (BAU/ml, o–o); kallikrein (mU/ml, ◆–◆).

Agmatine-Sepharose and Concanavalin A Sepharose, the kallikrein and activator activity remained closely associated. These studies also showed great losses of activity, which was particularly true for the kallikrein subunit in contrast to the complex.

It has, not actually been demonstrated yet, however, that the activator activity which accompanied the kallikrein activity is separate from the other activator activity in peak II. To clarify this aspect, the DEAE-cellulose chromatography experiment using the kallikrein preparation (Fig. 8A) was compared to a similar experiment using a pool of eluted fractions containing both kallikrein activity and plasminogen activator activity of peak II (cf Fig. 6). In figure 8B, it can be seen that all kallikrein activity and some activator activity passed through the column in a similar manner as in the experiment with the kallikrein preparation (Fig. 8A). A difference between the two experiments lies in the considerable amount of activator activity (IIB) bound to the column and eluted with 0.3 M NaCl. This activator activity obviously represents the main activator activity of peak II and behaves in a clearly different manner than does the activator activity (IIA) associated with the kallikrein activity.

DISCUSSION

Several experiments in this study showed different activator activities to be present in dextran sulphate euglobulin fractions of plasma. This resulted in attempts to fractionate them. Gel filtration experiments showed the existence of two groups of plasminogen activators of different molecular weights (peak I and peak II in figure 6). The activity in peak II could be further divided into a fraction (IIA) closely following the plasma kallikrein activity (Figs. 7 and 8A) and another fraction (IIB) distinct from plasma kallikrein (Fig. 8B). Both activator and kallikrein activities in fraction IIA are present in a high molecular weight complex which can be split by ion-exchange chromatography. Most likely, as reported for prekallikrein (11), this complex contains high molecular weight (HMW) kininogen in precursor form and, after contact activation, the kinin-free kininogen (12). The complex remains intact under conditions of high ionic strength (1.0 M NaCl), excluding weak electrostatic interaction as the only glue, but suggesting hydrophobic or strong electrostatic forces to be involved in the subunit interaction. That both the plasminogen activator activity in this fraction (IIA) and the kallikrein activity reside in the same molecule is strongly suggested by the fractionation

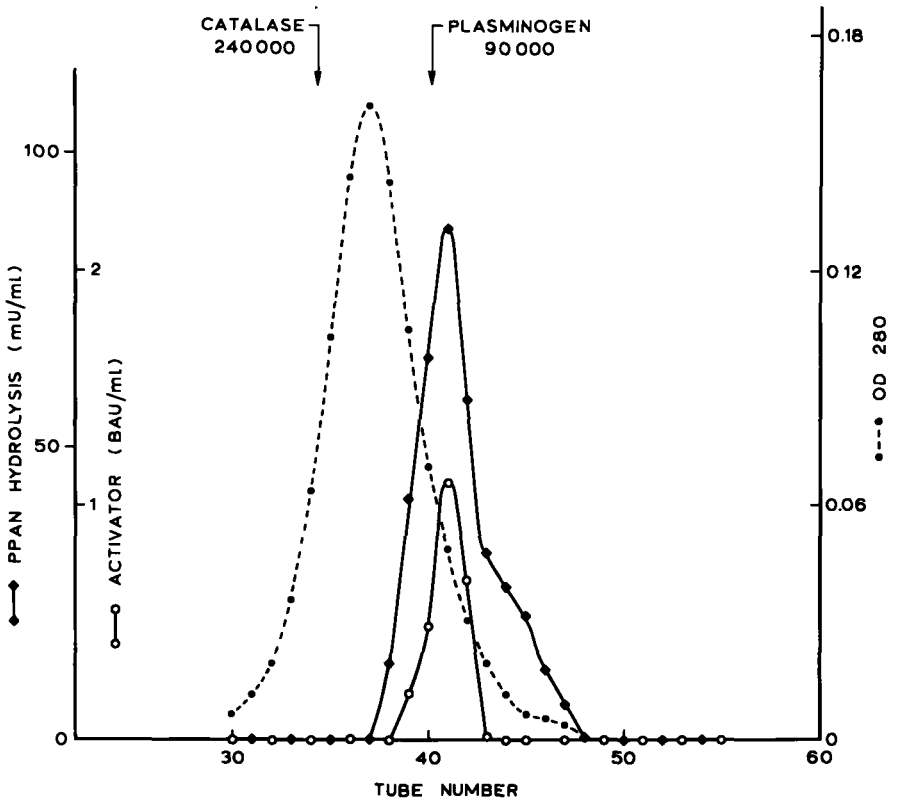

Fig. 9. Gel filtration of kallikrein after DEAE-cellulose chromatography. The kallikrein containing fractions obtained after DEAE-chromatography as shown in figure 8A, were pooled, dialysed, lyophilized and gel filtered in 0.01 M phosphate buffer, pH 7.5, with 0.15 M NaCl on Ultrogel ACA 34 column. Eluted protein (OD 280, ●-●); activator (BAU/ml, o–o); kallikrein (mU/ml, ◆–◆). In separate runs, the positions of purified catalase and plasminogen in plasma were determined. They are indicated by arrows.

results. This is also in accordance with conclusions reached from other studies (see Introduction).

The contribution of plasma kallikrein in its capacity of a plasminogen activator (IIA) to the total activator content of the dextran sulphate euglobulin fraction is assessed at 10-15% (Fig. 3). This relatively small contribution is in accord with the fractionation experiments, which showed two main activator peaks (I and II) and a small contribution of kallikrein as part of peak II (Figs. 6, 7 and 8B). Likewise, a considerable amount of activator is

generated in the dextran sulphate euglobulin fraction prepared from Fletcher trait plasma leaving only a small contribution to the kallikrein (Figs. 3 and 5).

Another important role of the prekallikrein/kallikrein system in fibrinolysis in plasma is revealed by the correction experiments on Fletcher trait plasma (Figs. 3-5). Extra activator activity is produced when kallikrein is supplied to the deficient plasma either by mixing with fractions from normal plasma or by addition of partially purified preparations of kallikrein. No difference was observed between the effects of the kallikrein protein complex and the kallikrein subunit, which is in agreement with the fact that the subunit prekallikrein/kallikrein is the missing factor in the deficient plasma (16-18).

Kallikrein is needed for the effective and rapid activation of factor XII. Therefore, the above-mentioned need for kallikrein could indicate the participation of factor XII in the activation of the plasminogen proactivator.

In any event, factor XII becomes involved because of its role in kallikrein activation. Therefore, the activator activity appearing after correction of Fletcher trait plasma by addition of kallikrein is ascribed to a factor XII--dependent plasminogen proactivator.

These results suggest the scheme for factor XII-dependent fibrinolysis in plasma presented in figure 10. It has not yet been determined which component of the factor XII/prekallikrein activation process actually activates the new plasminogen proactivator. Two enzymes produced by factor XII-dependent pathways have been reported to exert plasminogen activator activity, viz, factor XIIa (33) and factor XIa (10). These factors seem not to explain the activator activity observed after correction of Fletcher factor deficiency. Variation in amounts of factor XII ranging from 5 to 100% of normal plasma in Hageman trait plasma produced no significant increase in the activator activity generated in the dextran sulphate euglobulin fraction, indicating a very small contribution of factor XIIa itself (20).

Preliminary unpublished observations have indicated a small contribution of factor XI, since, in the dextran sulphate euglobulin fraction of plasma deficient in this factor, a normal activator content (109 $\pm$ 22 (SD) BAU/ml; 3 patients) is determined. Similarly, Iatridis and Ferguson (34) showed normal contact activation of fibrinolysis in factor XI-depleted plasma.

Ogston et al. (35) previously described the participation of a component called Hageman factor cofactor (HFCOF) in factor XII-dependent fibrinolysis. It has been suggested that this cofactor may be kallikrein (6,36). A prepara-

tion has been shown to contain plasminogen activator activity (6); however, this capacity of the HFCOF is considered negligible by Ogston's group (32,35) in comparison with its capacity to stimulate contact mediated fibrinolysis in glass-adsorbed plasma.

Preliminary results on a lyophilized preparation of purified Hageman factor cofactor (37), kindly provided by Dr. D. Ogston, showed plasminogen activator activity on plasminogen-rich bovine fibrin plates, confirming its ability to activate plasminogen. No detectable activity on the kallikrein substrate PPAN and no reaction with an antiserum directed against the kallikrein subunit was observed. When Hageman factor cofactor was added to Fletcher factor deficient and factor XII-deficient plasma, no detectable activity on PPAN could be generated by activation procedures which generate kallikrein in plasma (25). These results exclude the occurrence of kallikrein, prekallikrein and factor XII in the preparation. Consequently, HFCOF preparations seem to contain a separate fibrinolytic component. Further studies on our unidentified factor XII-dependent activator and the Hageman factor cofactor are needed to determine their mutual relation.

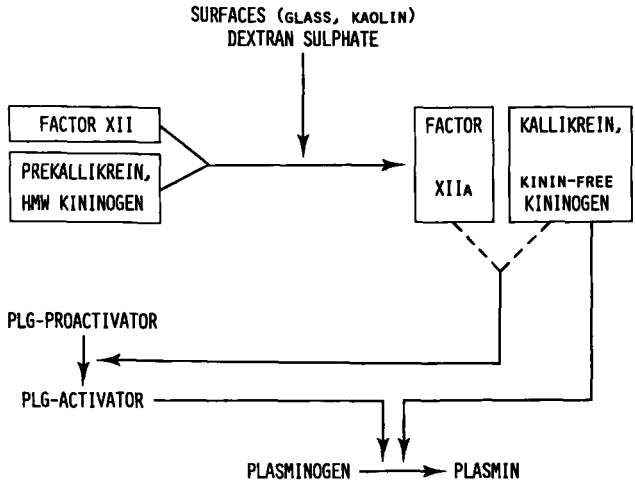

Fig. 10. Scheme of factor XII-dependent fibrinolysis in plasma. Contact activation by surfaces or by dextran sulphate in plasma results in the formation of active factor XII and the kallikrein complex containing kinin-free HMW kininogen (12). Two products of the active factor XII can be distinguished (not shown): one remains bound to surfaces; another is fragmented into a soluble product (Hageman factor fragments) containing the active centre (13).

The correction experiments on Fletcher trait plasma led to a surprising observation. Extra activator activity was generated only when kallikrein preparations were added or when euglobulin fractions from normal plasma were used. Mixing with normal plasma before the euglobulin fractionation was without effect. The effective corrections have the presence of partially or completely activated prekallikrein in common. In the dextran sulphate euglobulin fractions prepared from normal plasma, partial activation of prekallikrein occurs, as was demonstrated by the splitting of PPAN. In whole plasma, the dextran sulphate concentrations employed completely activated prekallikrein in 7 min at $0^{o}C$ (30). A possible explanation for the missing effect in mixtures of normal and Fletcher trait plasma might be an inhibitory influence of the Fletcher trait plasma on the activation of prekallikrein or plasminogen proactivator. Such an inhibition might originate from the presence of an abnormal prekallikrein, an excess of free HMW kininogen or of antibodies. It is of interest in this context to note a previous observation of a delayed activation of prekallikrein in a 1:1 mixture of the same Fletcher trait plasma A with normal plasma compared to the dilution of the normal plasma with buffer (25). Rapid activation was obtained by the addition of a partially activated factor XII preparation.

Evidence was previously presented for a subdivision of the total activator content in the dextran sulphate euglobulin fraction prepared from normal morning plasma into two nearly equal portions of a factor XII-dependent and an independent plasminogen activator activity, while an additional amount of around 1-2% is contributed by extrinsic or vascular activator (19-20).

The data presented in this paper fit into this scheme. The two samples of Fletcher trait plasmas yielded 38 and 55 BAU/ml, respectively, of a plasminogen activator attributable to a factor XII-independent pathway.

Two factor XII-dependent activators could be related to the presence of kallikrein (yielding 10-15 BAU/ml) and an unidentified proactivator yielding 30-40 BAU/ml. A scheme for this tentative inventory of plasminogen activator activity in the dextran sulphate euglobulin fraction prepared from normal plasma is presented in figure 11. The proposed occurrence of two major, distinct plasminogen activator activities agrees well with the two activator peaks (I and II) in the gel filtration profile of the dextran sulphate euglobulin fraction. Much remains to be done in order to characterize these two entities and their mode of formation.

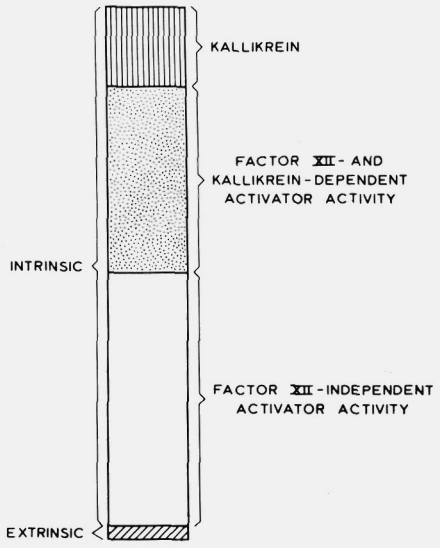

Fig. 11. Quantitative inventory of plasminogen activator activities of plasma as present in the dextran sulphate euglobulin fraction of plasma. The total column represents 100 BAU/ml of normal morning plasma.

The participation of plasma kallikrein in a double function as a source of a plasminogen activator and as a factor in the activation of a plasminogen proactivator, in total amounting to about half of the latent activator activity in plasma, implies that fluctuations in plasma prekallikrein levels or of prekallikrein activation may influence current methods used to assess plasma fibrinolytic activity. This is indeed observed in the euglobulin method of assay in which Fletcher trait plasma shows a reduced activity.

Interpretation and evaluation of data on euglobulin fibrinolysis should therefore include consideration of the status of the plasma kallikrein system.

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SUMMARY

Fibrinolysis takes care of the removal of fibrin which, after its formation from fibrinogen, serves a temporary function or is part of a pathological process.

This thesis is concerned with blood fibrinolysis as observed in euglobulin fractions of platelet-poor plasma. Decreased euglobulin fibrinolysis had been reported in relation to arteriosclerosis and thrombosis. It was realized, and clear experimental evidence was initially obtained, that most of the components involved in the fibrinolytic activity of euglobulin fractions were not identified. This uncertainty seriously hampered the evaluation of the pathophysiological significance of the observations of decreased euglobulin fibrinolysis. Especially, the knowledge concerning plasminogen activators and their precursors was only fragmentary.

Studies were carried out to characterize and assay the components involved in euglobulin fibrinolysis. A standardized euglobulin fractionation procedure, where the fibrinolytic activity of the fraction was measured on fibrin plates, was selected.

CHAPTER I concerns the presence of plasma protease inhibitors in euglobulin fractions. Considerable amounts of C1-inactivator were recovered in euglobulin fractions and were demonstrated to inhibit the fibrinolytic activity.

This inhibition could be overcome by the addition of flufenamate to euglobulin fractions. CHAPTER II describes experiments which demonstrate that flufenamate can be used to selectively eliminate C1-inactivator and possibly additional minor inhibitory influences in euglobulin fractions. A simple technique where flufenamate is used for elimination of inhibitory effects formed the basis for studies on the fibrinolytically active components in euglobulin fractions.

In CHAPTER III, it is demonstrated that, in the regular euglobulin fraction, only partial conversion of proactivators to activators occurs, and recovery of proactivators from plasma is also only partial. With the use of dextran sulphate, optimum recovery and optimum conversion of proactivators to activators was achieved and a quantitative assay for activators in euglobulin fractions was worked out. Two different contributions to the total activator activity could be distinguished: an activity attributed to extrinsic activator (mainly originating from endothelial cells) and one mediated by intrinsic proactivators present in blood. Evidence was provided that approximately half of the intrinsic proactivator potential was contributed by a factor XII-dependent and the

rest by a factor XII-independent pathway of activator generation.

CHAPTER IV describes in detail how the extrinsic activator activity could be assessed separately by means of its resistance to inhibition by C1-inactivator, while, in contrast, the intrinsic systems were completely blocked by this inhibitor. In morning plasma with baseline levels of fibrinolysis, the amount of extrinsic activator activity was negligible as compared to the fairly stable level of activity that could be obtained from intrinsic proactivators. When blood fibrinolysis was stimulated by venous occlusion (15 min), the amount of extrinsic activator activity was greatly increased, reaching levels of 50-100 times that in morning plasma. Some experiments indicated that extrinsic activator was involved in the induction of proactivator conversion in intrinsic systems in vitro.

CHAPTERS V and VI are concerned with further studies on the intrinsic proactivator systems. In Chapter V, determination of prekallikrein in human plasma using the chromogenic tripeptide Bz-Pro-Phe-Arg-p.NA as a substrate for kallikrein after optimal generation of this enzyme from its precursor is described.

In Chapter VI, the controversial role of kallikrein in factor XII-dependent intrinsic plasma fibrinolysis is reevaluated using Fletcher factor (prekallikrein)-deficient plasma and partially purified preparations of kallikrein. A double function for kallikrein is established: one as a direct plasminogen activator and another as an essential factor in the activation of a major previously undescribed plasminogen proactivator in the factor XII-dependent pathway. The occurrence of an intrinsic factor XII-dependent pathway of fibrinolysis is also further substantiated.

Finally, an inventory of plasminogen activator activities in morning plasma is made with the following contributions: a small percentage from extrinsic activator; three contributions from substances of intrinsic origin, namely, one from a factor XII-independent pathway amounting to 40-50% and two from a factor XII-dependent pathway amounting to 10-15% of prekallikrein and 30-40% of a plasminogen proactivator.

SAMENVATTING

Fibrinolyse is het oplossen van fibrine dat, na door stolling uit fibrinogeen te zijn ontstaan, normaal een tijdelijke funktie heeft of deel is van een ziekteproces. Dit proefschrift behandelt een aantal aspekten van fibrinolyse in bloed. De fibrinolytische aktiviteit is voornamelijk bepaald in de zogeeuglobulinefrakties uit bloedplasma. Sommige auteurs hebben verlaagde fibrinolytische aktiviteit in deze euglobulinefrakties in verband gebracht met arteriosclerose en trombose. De komponenten die deel uitmaken van de fibrinolytische werking van euglobulinefrakties waren echter vrijwel niet bekend. Dit maakt de beoordeling van het belang van de gevonden afwijkingen erg moeilijk. Met name was de kennis over plasminogeenaktivatoren en hun precursors niet of slechts fragmentarisch aanwezig. Het onderzoek werd daarom gericht op de karakterisering en meting van die komponenten die in euglobulinefrakties de fibrinolytische aktiviteit bepalen. Er werd uitgegaan van een gestandaardiseerde bereidingsmethode voor euglobulinefrakties en meting van hun fibrinolytische aktiviteit met behulp van de fibrineplaatmethode.

HOOFDSTUK I beschrijft een onderzoek naar het voorkomen van remmers voor eiwitsplitsende enzymen in euglobulinefrakties. Van C1 inaktivator, een remmer bekend uit het complement systeem, bleek een aanzienlijke hoeveelheid neer te slaan met de euglobulinefraktie. Bovendien bleek deze remmer de fibrinolytische aktiviteit negatief te beïnvloeden.

Deze remming kon worden opgeheven door toevoeging van natrium flufenamaat, bekend om zijn fibrinolytische werking in vitro.

HOOFDSTUK II beschrijft hoe deze verbinding bij goed gekozen concentratie in staat is C1 inaktivator en mogelijk nog meer remmende invloeden uit te schakelen. Deze bevindingen leverde een eenvoudige methode op om remmende invloeden in euglobulinefrakties te elimineren, wat essentieel is voor het onderzoek naar de fibrinolytisch aktieve bestanddelen in de euglobulinefrakties.

HOOFDSTUK III laat zien, dat in de standaard euglobulinefraktie slechts een gedeeltelijke omzetting van proaktivatoren naar aktivatoren optreedt. Bovendien bevat de fraktie slechts een deel van de proaktivatoren uit plasma. Dextran sulfaat bleek hier bruikbaar. Toepassing ervan verhoogde niet alleen de opbrengst in de euglobulinefraktie maar gaf ook totale omzetting van proaktivatoren naar aktivatoren. Dit resulteerde in een kwantitatieve bepaling van aktivatoren in euglobulinefrakties met optimale opbrengst van die aktivatoren uit plasma. In de aldus gemeten aktiviteit kon worden onderscheiden: aktiviteit, veroorzaakt door extrinsieke aktivatoren (voornamelijk uit de cellen van

de vaatwand) en die door intrinsieke aktivatoren/proaktivatoren (in bloed aanwezig). Aannemelijk kon worden gemaakt, dat de aktivatie van ongeveer de helft van het intrinsieke proaktivatortotaal afhankelijk was van faktor XII en die van de rest onafhankelijk van deze faktor.

HOOFDSTUK IV beschrijft gedetailleerd de afzonderlijke meting van extrinsieke aktivator-aktiviteit na blokkeren van het intrinsieke systeem met C1 inaktivator. In ochtendplasma (met basale waarden voor de fibrinolyse) bleek de extrinsieke aktivator-aktiviteit gering te zijn in vergelijking met de totale aktiviteit. Sterk verhoogde bloed fibrinolyse optredend na veneuze stuwing in de arm gedurende 15 minuten, doet de extrinsieke aktivator-aktiviteit sterk stijgen tot waarden die 50 tot 100 maal hoger liggen dan die in ochtendplasma. Sommige waarnemingen duiden op een mogelijke rol, althans in vitro, van de extrinsieke aktivator in de omzetting van proaktivatoren tot aktivatoren uit het intrinsieke systeem.

HOOFDSTUK V en VI behandelen een aantal aspekten van de intrinsieke proaktivator systemen.

In HOOFDSTUK V wordt een methode beschreven om prekallikreine (plasminogeen proaktivator) in plasma te bepalen. In deze methode wordt prekallikreine volledig omgezet in kallikreine, waarvan de aktiviteit bepaald wordt met behulp van een recentelijk geïntroduceerd tripeptide substraat (Bz-L-Pro-L-Phe-L-Arg-pNA.HCl).

In HOOFDSTUK VI wordt vervolgens de omstreden rol van prekallikreine/kallikreine in de faktor XII-afhankelijke fibrinolyse nader bestudeerd. Daartoe is gebruik gemaakt van Fletcher faktor (prekallikreine) deficient plasma en van gezuiverde kallikreinepreparaten. Voor kallikreine werd een tweeledige funktie vastgesteld. Enerzijds werkt kallikreine als een direkte plasminogeenaktivator, anderzijds is het een onmisbare faktor in de omzetting van proaktivator in aktivator in de faktor XII-afhankelijke fibrinolyse. De laatste komponent is als zodanig nog niet eerder beschreven. Verder is de aanwezigheid van een intrinsiek, faktor XII-onafhankelijk aktiveringsmechanisme van de fibrinolyse zeer aannemelijk gemaakt.

Uit de verkregen gegevens kon een voorlopige onderverdeling worden gemaakt van het totale plasminogeen aktivator/proaktivator bestand in euglobulinefrakties van plasma.

In ochtendplasma blijkt extrinsieke aktivator-aktiviteit slechts voor enkele procenten bij te dragen, de rest is van intrinsieke origine. Bij de intrinsieke aktivator-aktiviteit kunnen ten minste drie bijdragen onderscheiden worden, namelijk één van een faktor XII-onafhankelijk aktivatieproces (40-50%) en twee

van een faktor XII-afhankelijk aktivatieproces. Beide laatsten komen voor rekening van prekallikreine (10-15%) en van een plasminogeen proaktivator (30-40%).

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