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Research Article

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Diagnostic and Genetic Studies on Fibrin-Stabilizing Factor with a New Assay Based on Amine Incorporation

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ABSTRACT Fibrinoligase, the fibrin cross-linking enzyme, transiently appearing during the course of coagulation in normal blood, was shown to catalyze the incorporation of a fluorescent amine, monodansylcadaverine [or *N*-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide] into casein. The reaction provided the basis of a sensitive fluorimetric method for measuring the activity of the enzyme (and also of similar other transpeptidases, such as transglutaminase).

In tests involving plasma, certain difficulties had to be overcome which were mainly due to the fact that the enzyme itself does not occur in citrated plasma. Only its precursor (fibrin-stabilizing factor or factor XIII) is present, still requiring limited proteolytic activation by thrombin. Thus, in order to measure amine incorporation with plasma as a source of the factor, thrombin must be added. This necessitated a differential desensitization of the intrinsic fibrinogen so that the latter could not clot and could not thereby interfere with amine incorporation. Also, the thrombin-inactivating capacity of plasma had to be saturated to enable full conversion of the factor to the transpeptidase. Concentrations of casein, monodansylcadaverine, calcium, and hydrogen ions were chosen to permit almost maximal velocity of amine incorporation. A linear relationship with regard to plasma concentration could be obtained only under such conditions. No similar assay is presently available for quantitatively evaluating fibrin-stabilizing factor levels in plasma.

The amine incorporation test was applied to a clinical case of hereditary total fibrin-stabilizing factor deficiency. The effect of transfusion therapy was studied, and some of the patient's relatives were examined. Whereas a paternal aunt and uncle gave values well within the normal range, a brother and the mother proved to be partially deficient and could be considered as heterozygous carriers. The father appeared to have a reduced level of fibrin-stabilizing factor, though not quite as low as the other two relatives.

Two infusions (1 liter each) of fresh normal plasma, administered about 26 hr apart, brought levels in the patient's plasma close to those found in the mother and brother. The corrective power of the transfusions, however, rapidly declined within 5–6 days. Futility of the last transfusion could be ascribed to the appearance of a neutralizing antibody directed against the precursor stabilizing factor, a serious complication.

General diagnostic versatility and potential of the quantitative amine incorporation assay with plasma is discussed.

INTRODUCTION

Covalent cross-linking of fibrin by transpeptidation constitutes the last step in normal blood coagulation (1). It involves the selective formation of γ-glutamyl-ϵ-lysine bridges (approximately 1 mole/100,000 g of protein) between fibrin units (2–4) and results in the production of more stable structures which can no longer be dispersed in 1% monochloroacetic acid (5) or in 5 M urea (6). Some important aspects of this fibrin-stabilizing system have already been uncovered. Normal plasma contains a precursor protein (7) (called fibrin-stabilizing

An account of this work was given as a lecture at the Federation Meetings, Atlantic City, 1968. (Urayama, T., and L. Lorand. 1968. *Fed. Proc.* **27**: 693; Abstract No. 2682.)

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factor or factor XIII [8]) which is converted to the active transpeptidase (fibrinoligase) by the action of thrombin. In this limited proteolytic process, the electrophoretic mobility of the factor (bovine) is changed, but sedimentation characteristics (3) are not altered appreciably. Fibrinoligase functions as a transpeptidating (i.e. transamidating) enzyme catalyzing a reaction between (donor) ϵ-amino-lysine (9) and (acceptor) γ-glutamyl (10) carbonyl groups of adjacent fibrin molecules.

Cross-linking of fibrin was shown to be selectively inhibited by certain amines (2, 9, 10–12) which are themselves substrates for the enzyme. As such, these compounds become incorporated into fibrin and block its acceptor groups for normal cross-linking. The amine-incorporating activity of the enzyme forms the basis of a quantitative analysis. In specific terms, the incorporations of hydroxylamine, hydrazine, glycine ethyl ester, monodansylcadaverine,[1] and histamine have been studied (Table I). The first two served as chemical labels, forming fibrin hydroxamate and hydrazide, respectively. Glycine ethyl ester and histamine were used in the form of radioisotopes. As a fluorescent compound, monodansylcadaverine proved to be a very sensitive tracer and was chosen as a donor substrate for the investigations reported in the present paper.

Hitherto, assay methods (13) for the stabilizing system depended on the ability of the activated factor to convert simple fibrin gels into the acid- or urea-insoluble form. The extent of "insoluble" clot formation was taken as an index of cross-linking. Dilution of the factor to a threshold level of activity provided the basis for empirical quantitation. In its most recently developed version (14), a multistage assay consisted of (*a*) the activation of fibrin-stabilizing factor by thrombin, followed (*b*) by the quenching of thrombin (unless water-insoluble enzyme was used (3), which could be removed by filtration); (*c*) the activated factor was then mixed with isolated fibrin and (*d*) cross-linking was allowed to proceed. Finally, (*e*) 1% monochloroacetic acid was added to test for the extent of cross-linking either by visual inspection or by quantitatively analyzing the acid-insoluble residue. Though this and related methods could be applied usefully for studying purified factor preparations, assays based on clot solubility are particularly difficult to interpret quantitatively when direct testing of the fibrin-stabilizing factor content of plasma is required. Since the thrombin-activated factor is sensitive to sulfhydryl-blocking reagents, efforts have been directed towards a method based on "iodoacetate tolerance" in relation to the formation of urea- or acid-insoluble clots (15). Also, attempts were made to estimate the corrective power (16) of a given plasma (normal or partially deficient in the factor) when mixed with one which is totally deficient in fibrin-stabilizing factor.

Meaningful quantitative tests for fibrin-stabilizing factor in plasma would, of course, be of great clinical value. Using amine incorporation as a tool, we have now developed a suitable assay and applied it for studying the inheritance pattern of the fibrin-stabilizing factor deficiency disease (17) and for evaluating transfusion therapy in a totally deficient individual with a severe bleeding disorder. Though studies at Northwestern University now encompass six totally deficient unrelated individuals and their families, the present paper will deal only with the first such case which was initially investigated at Columbia University. The other studies will be reported within the framework of a more extensive genetic survey in due course.

METHODS

The sulfate salt of monodansylcadaverine or *N*-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide was prepared by Dr. N. G. Rule as described previously (2). Approximately 30 mg of this compound was dissolved in 3 ml of 0.05 M tris (hydroxymethyl) amino methane (Trizma base, Sigma Chemical Co., St. Louis, Mo.) adjusted to pH 7.5 with hydrochloric acid. Concentration of the stock solution is determined by measuring the absorbancy (at 326 mμ) of a suitable (10 μl) aliquot in (2.0 ml) 20% (v/v) dioxane-water. We assume that the molar extinction is identical (4.64×10^6 cm^2 mole^{-1}) with that of dansylamide (see reference 18).

Bovine thrombin was purified from Parke Davis & Co. Thrombin Tropical by a modification (Lorand and Urayama, unpublished data) of the Rasmussen procedure (19). Activity of the enzyme was matched against a National Institutes of Health (NIH) standard (lot No. B-3).

Acid citrate dextrose (ACD) human plasma for the isolation of fibrin-stabilizing factor (3, 20) was obtained through the courtesy of Dr. J. N. Shanberge of Evanston Hospital. Factor activity was expressed in terms of fibrin cross-linking units, based on the visual disappearance of clots in monochloroacetic acid as previously described (13). Solutions of the factor were kept at 0°C in 0.05 M Tris–0.1 M NaCl, 1 mM ethylenediaminetetraacetate at pH 7.5.

Casein (Hammarsten), lot no. T-1371, was purchased from Mann Research Laboratories, Inc., New York, reduced glutathione from Cyclo Chem. Corp., Los Angeles, Calif., and sodium dodecylsulfate from K. and K. Laboratories, Jamaica, N. Y. Other compounds were reagent grade. All solutions must be clear and, if necessary, filtered.

Fluorescence intensities were obtained with an Aminco-Bowman spectrophotofluorimeter (excitation at approximately 355 mμ; emission at approximately 525 mμ). In order to provide a suitable solvent system for redissolving the trichloroacetic acid-precipitated proteins, all fluorescence measurements were carried out in a mixture containing 8 M urea, and 0.5% sodium dodecyl sulfate in 0.05 M Tris buffer at pH 8. Monodansylcadaverine at a concentration of 1 μmole/liter in the same solvent system served as reference.

Test plasmas from the patient and her relatives were obtained by centrifuging the blood samples (collected into

[1] The trivial name "monodansylcadaverine" will be used for the compound "*N*-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide."

plastic syringes;[2] 9 ml of blood mixed with 1 ml of 4% trisodium citrate) at 2500 rpm for 20 min at 4°C. The supernatant plasmas were stored in plastic tubes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) at −70°C. Plasma for infusion had been separated from blood collected into ACD and stored in plastic containers at −20°C.

The antibody against chromatographically purified fibrin-stabilizing factor (3) appearing in the patient's plasma at the end of December (Results) was demonstrated on Ouchterlony double immunodiffusion plates (Hyland Laboratories, Los Angeles, Calif., Immuno-plate, pattern A). Diffusion was allowed to take place for 3 days at 24°C and the plate, after 3 days of washing in 0.05 M Tris, pH 7.5, was stained with 1% Amidoblack in 5% acetic acid for 10 min and washed in the acetic acid subsequently.

RESULTS

I. Detailed methodology for measuring fibrin-stabilizing factor in a purified system

In order to study the complex properties of the fibrin-stabilizing factor system, the analytical procedure was first worked out with the use of a purified factor preparation. This made it easier to overcome the added difficulties encountered in studies with plasma.

A. Selection of Substrates

1. *The amine donor.* As already mentioned in the introduction, monodansylcadaverine (2, 9) seemed to be the substrate of choice among the compounds studied so far. Of course, the over-all aspects of methodology of amine incorporation would have to be quite similar with any of the other analogous compounds, e.g., those in Table I. Selection of monodansylcadaverine, however, may be readily justified, for example, by a comparison with glycine-1-^{14}C ethyl ester which we have earlier introduced (12, 13) as a similar substrate. The former compound has a $K_{m,app}$ for the transpeptidase which is about tenfold more favorable (2). Fluorescence renders it as sensitive a tracer as would be obtainable with practical levels of radioactivity. More important, monodansylcadaverine is far more stable under our experimental conditions than glycine ethyl ester. The latter rapidly decomposes into diketopiperazine even in the absence of enzymes. Esterases in the plasma could also conceivably accelerate the hydrolytic degradation of this unstable compound. Hence, kinetic experiments of amine incorporation might easily give a completely spurious evaluation.

2. *The amine acceptor.* Bulk casein, which has already been extensively used as an amine incorporating substrate in conjunction with the guinea pig liver transglutaminase (21) was selected as acceptor substrate. In much of our related work, however, α-casein was used also to good advantage. Since both the extent and rate of incorporation may vary somewhat from one casein preparation to another, the values reported in this paper must be regarded as strictly pertinent only for the particular batch given. A conversion factor might be neces-

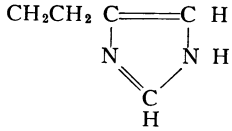
[2] Roehr Products Co., Inc., Deland, Fla.

TABLE I

Enzymatic Incorporation of Amines into Fibrin

(A) $H_2N\cdot F\cdot CO\cdot Y + H_2N\cdot F'\cdot CO\cdot Y \xrightarrow[H_2NR]{E} H_2N\cdot F\cdot CO\cdot NH\cdot F'\cdot CO\cdot Y + HY$

(B) $H_2N\cdot F\cdot CO\cdot Y + H_2NR \xrightarrow{E} H_2N\cdot F\cdot CO\cdot NH\cdot R + HY$

R	$K_{m,app}$ at $(F)_0 \sim 5 \times 10^{-6}$ mole/liter	Label	Reference
$CH_2\cdot COO\cdot CH_2\cdot CH_3$	4×10^{-3} mole/liter	^{14}C isotope	(12, 13)
$(CH_2)_5\cdot NH\cdot$dansyl	2×10^{-4} mole/liter	Fluorescent	(2, 9)
OH		Chemical	(10, 13)
NH_2		Chemical	(13)
		^{3}H isotope	L. Lorand and D. Chenoweth (unpublished results)

(*A*), cross-linking of fibrin (F and F') by the fibrinoligase (E) is inhibited by specific amines (H_2NR) which themselves become incorporated (*B*) into fibrin, blocking the γ-glutamyl acceptor groups of the latter. The amino groups in fibrin are those of ϵ-lysine and are shown to participate in single cross-linking of a dimer. The leaving HY is assumed to be ammonia; this, however, has not been unambiguously demonstrated as yet. "Dansyl" stands for the fluoresent "5-dimethylamino-1-naphthalenesulfonyl" residue.

sary to correlate findings based on different casein acceptors. An ideal acceptor would contain only a single incorporating site (or completely equivalent sites if more than one), and it would preferably belong to the growing number of proteins for which primary and tertiary structures have been or are expected to be soon determined.

B. A Coupled System for Measuring the Concentration of Fibrin-Stabilizing Factor

Essential components for the proposed analytical system are shown in Fig. 1. First, thrombin is added to the factor (isolated from human plasma) to bring about conversion of the precursor (abbreviated as FSF) to the active fibrinoligase (abbreviated as FSF*). Calcium ions and glutathione are also included. Activity of the generated enzyme is then tested by the addition of casein and the fluorescent amine. The covalent incorporation of the amine into casein is accompanied by appreciable changes in fluorescence as if the dansyl group was placed in a more hydrophobic environment. This provides the basis of a continuous rate assay (22). However, the present investigation was intended to lead to the development of a method for measuring the FSF content of human plasma so that as many as two dozen samples could be handled simultaneously in a convenient manner. Hence, a sampling methodology rather than a continuous rate assay was decided upon. Several simple ways are available for terminating the incorporation of monodansylcadaverine into casein. To mention only two, incorporation of the fluorescent compound may be effectively and instantaneously quenched by the addition of high enough concentrations of a nonfluorescent competitive amine (e.g. hydroxylamine) or simply by precipitating the proteins with either ethanol or trichloroacetic acid. In the present report, attention is paid only to the latter procedures.

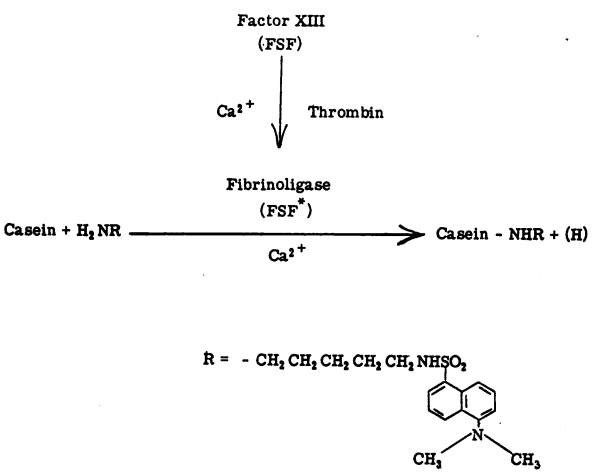


FIGURE 1 A coupled system for measuring the concentration of fibrin-stabilizing factor in terms of incorporation of a labeled amine (fluorescent monodansylcadaverine) into an acceptor protein (casein). The factor (FSF) is converted to the active transpeptidase (FSF* or fibrinoligase) by the addition of thrombin.

In order to analyze for the originally added factor content in a complex mixture such as given in Fig. 1, the system must be tightly coupled. Clearly, operation would be best at optimal calcium and hydrogen ion concentrations and under conditions so that the factor is maximally activated by thrombin. Close to maximal velocities in regard to casein and amine concentrations are also desirable. All studies were conducted at 24°C, and a typical experiment was performed in the manner outlined. As individual variables were changed (see abscissas of Figs. 2–6), all the other components of the reaction mixtures were kept at concentrations proven to be optimal.

The first step in the procedure is the activation of 0.1 ml of purified FSF (10 "cross-linking equivalents" [14]) with 0.1 ml of thrombin; 0.05 ml of 0.2 M glutathione, 0.05 ml of 0.03 M calcium chloride, and 0.2 ml of 0.05 M Tris-hydrochloride buffer are also present. After the 10 min period allowed for the conversion of the precursor factor to the transpeptidase, 0.05 ml of calcium chloride, 0.35 ml of Tris-hydrochloride, 0.1 ml of monodansylcadaverine, and 1 ml of casein are admixed. The incorporation reaction is stopped with 6 ml of ethanol (or 2 ml of 10% trichloroacetic acid) after 30 min, though incorporation proceeds linearly up to 1 hr under the experimental conditions employed. The protein precipitate is then centrifuged and extracted with six changes (10 ml each) of an ethanol-ether (1:1) mixture. Finally, the washed precipitate is freed of ethanol-ether by gentle aspiration and is taken up after drying in 2 ml of 8 M urea–0.5% sodium dodecyl sulfate in 0.05 M Tris-hydrochloride at pH 8. Fluorescence of the clear solution is read against a 1 μM monodansylcadaverine solution, and data are given in terms of this standard. No allowance is made for possible environmental influences on the fluorescence characteristics of the covalently incorporated dye. It is considered that these are fairly well minimized by the use of the denaturing solvent system.

1. *The over-all pH optimum* for the coupled reaction, as shown in Fig. 2, appears to be about pH 7.5. This value, of course, does not represent the optimal pH for activating the factor by thrombin nor that for the enzymatic incorporation of the fluorescent amine into casein; it simply reflects the best compromise for the coupled system as a whole. The Tris buffers in both the activation and incorporation mixtures were adjusted to the pHs shown (abscissa in Fig. 2) with hydrochloric acid. 10 U of thrombin were employed for activation. 30

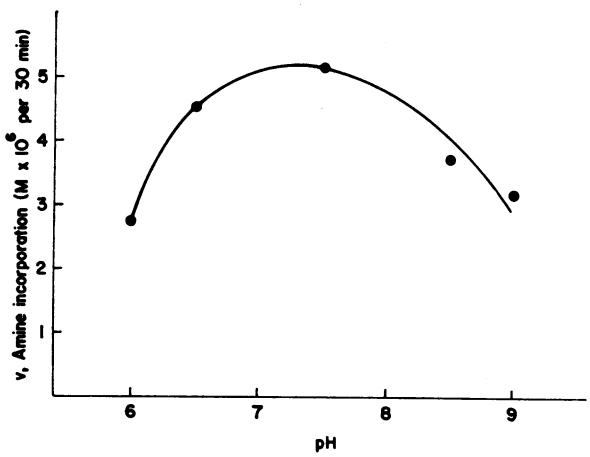


FIGURE 2 Optimal pH for the coupled assay system. v = velocity in this and in all subsequent figures.

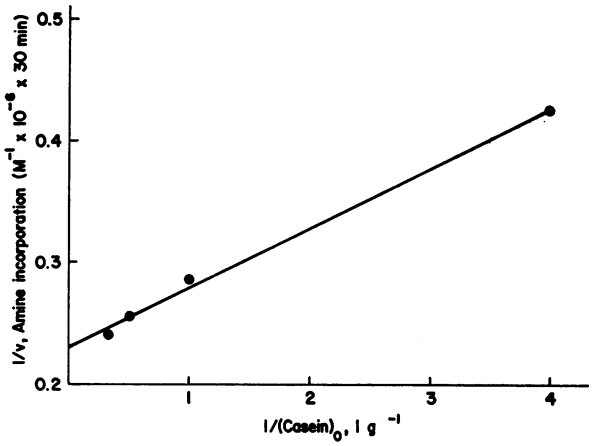


FIGURE 4 A double reciprocal plot for the relationship between the velocity of amine incorporation and initial casein concentration.

mM calcium chloride, 20 mM monodansylcadaverine, and 0.4% casein were used to make the appropriate additions.

2. *Data given in Fig. 3* indicate that 3 mM calcium chloride is sufficient to allow the system to function at maximal velocity. Since the calcium ion concentrations of the activation mixtures were kept at a level previously determined to be optimal for the activation of the factor (7), this value represents the saturating requirement of the incorporating system for the cation. The experiments were performed at pH 7.5 and, apart from the variable calcium ion concentration, other additions were made as given under section IB1 above. In order to obtain a measurement in the absence of calcium, 5 mM ethylenediaminetetraacetate was added in lieu of calcium chloride.

3. *Varying the concentration of the casein acceptor in the reaction mixture,* while keeping other components, including the concentration of the fluorescent amine constant, shows apparent saturation kinetics (as illustrated by a Lineweaver-Burk type of plot in Fig. 4). It will be noticed that the 0.2–0.3% casein concentration, to be used for routine assays, is sufficiently close to that required for maximal velocity. Experimental conditions at pH 7.5, apart from variations in casein concentration, were identical with those in section IB1.

4. *The coupled reaction* displays saturation behavior also with regard to the concentration of the donor amine. As seen in Fig. 5, about 0.5 mM monodansylcadaverine yields a nearly maximal velocity; this was the initial concentration selected for use in assays with plasma. Apart from varying the concentration of monodansylcadaverine, the experiments at pH 7.5 were performed as in section IB1.

5. *Fig. 6* shows that as little as 5 NIH units of thrombin per ml could bring about complete activation

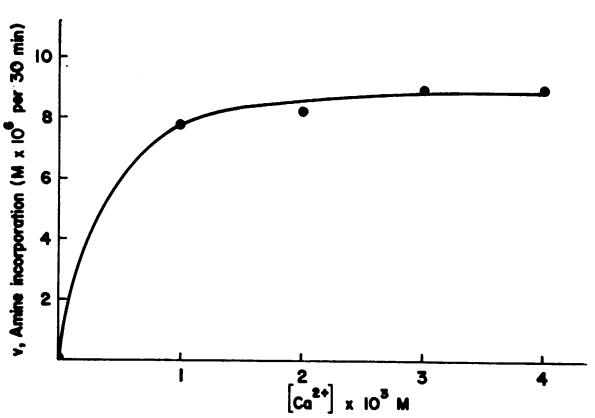


FIGURE 3 Dependence of amine incorporation on calcium ion concentration.

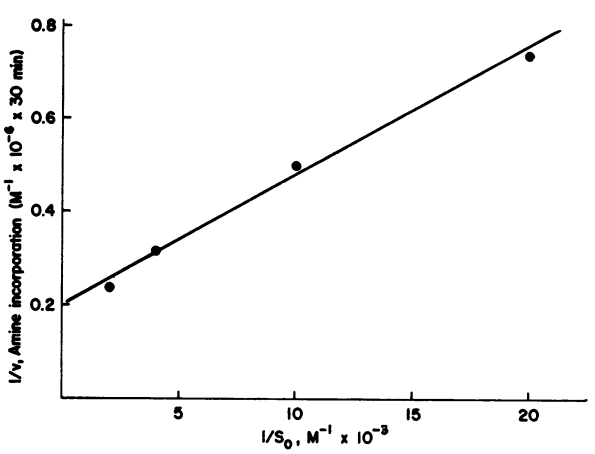


FIGURE 5 A double reciprocal plot between the velocity of incorporation and the initial concentration of monodansylcadaverine [(S)₀].

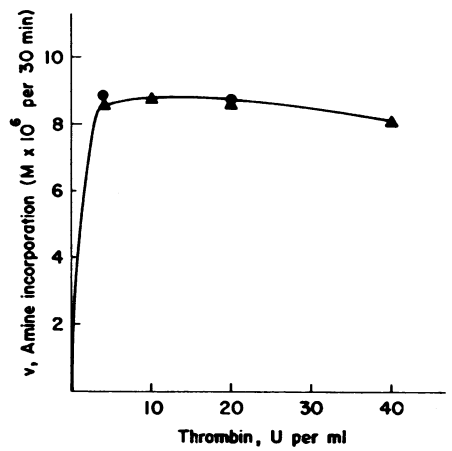


FIGURE 6 Velocity of amine incorporation as a function of thrombin concentration used for the activation of FSF. ▲, 10 min; ●, 20 min activation period.

in 10 min. The thrombin units in the activation mixture were varied, but otherwise these experiments at pH 7.5 were identical with those described in section IB1.

II. Special problems in connection with measurements of FSF concentration in plasma

One of the main hurdles to overcome when plasma instead of a purified preparation is used as the source of the stabilizing factor is to eliminate the interference caused by fibrinogen. As thrombin and calcium ions are to be added for converting the precursor factor to the transpeptidase, the plasma fibrinogen would be transformed to a fibrin clot. Winding out the clot would remove an unknown and uncontrollable portion of the factor. Leaving the clot in the mixture would no longer permit the study of single-phase kinetics for amine incorporation. Worse yet, if one is to use casein as acceptor, fibrin would compete favorably for incorporating the amine substrate. Since the fibrinogen concentration of plasma samples from different individuals (and from the same individual at different times) may show appreciable variations, the presence of fibrin as an effective acceptor would introduce a variability not easily controlled.

On account of such considerations, for purposes of the present study, we decided to "densensitize" the fibrinogen of plasma in such a way that it would no longer clot on addition of thrombin and that it would not compete appreciably for the incorporation of the fluorescent amine. It hardly needs to be emphasized that the treatment must be gentle enough so as not to affect the FSF potential of the test plasma to a significant extent.

We find that when citrated plasma is heated at 56°C for 2.5 min in the presence of 10% (v/v) glycerol, the intrinsic fibrinogen will no longer coagulate on cooling (to 24°C) even after adding thrombin and calcium ions, provided that the concentration of glycerol is maintained. When known amounts of purified FSF, extraneously added to the plasma, are present during such treatment about 90% of the amine incorporating potential is recovered. Hence, the treatment may be assumed not to cause more than a 10% drop in real factor concentration. Amine incorporation itself is virtually unaffected by 10% glycerol.

The presence of a powerful "antithrombin" activity, not encountered in the purified system and which apparently survived the treatment described, provided the last obstacle for quantitative testing in plasma. As seen in Fig. 7, the thrombin concentration had to be raised above 20 NIH units per ml of mixture to produce maximal activation in 20 min. In order to ensure that the thrombin-neutralizing activity of plasma is saturated, addition of 50 NIH units per ml of activation mixture (including the 0.2 ml citrated test plasma) was decided upon for routine assays.

III. Rate assay for plasma

Having overcome the two special problems discussed, the assay method developed for the purified system could be readily adapted for plasma. With all variables optimized for tight coupling, the velocity of amine incorporation was shown to depend linearly on the amount of plasma tested (Fig. 8). The specific methodology is summarized in Table II.

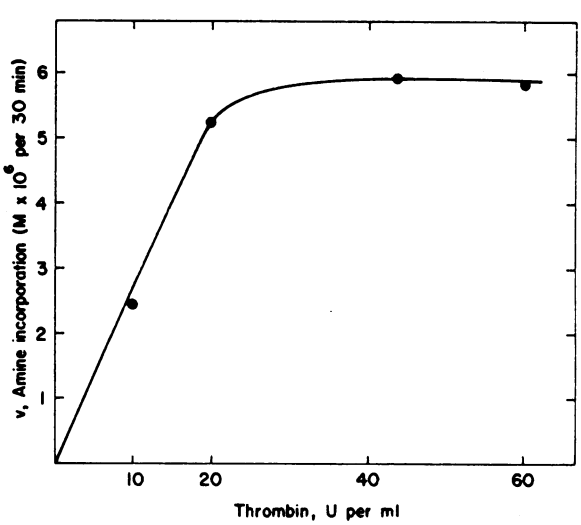


FIGURE 7 Dependence of the rate of amine incorporation on the concentration of thrombin used for activating (20 min) the intrinsic FSF in 0.2 ml of desensitized citrated plasma. For conditions, see Table II. Note that incorporation without previous activation by thombin extrapolates to zero. The latter could also be demonstrated with the use of heparin which did not inhibit the preactivated factor.

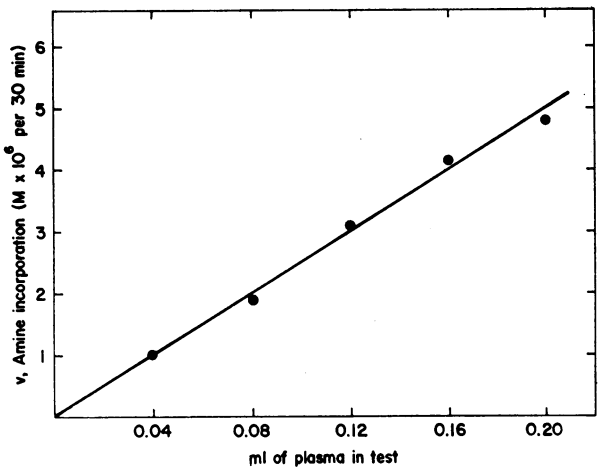


FIGURE 8 Linear relationship between rate of amine incorporation and the concentration of plasma employed in the test. For conditions, see Table II. Plasma dilutions were made with citrated saline.

TABLE II

Flow Sheet for the Amine Incorporation Rate Assay for Measuring Fibrin Stabilizing Factor (Factor XIII) in Plasma

Mix 0.2 ml of citrated plasma with 0.05 ml of 50% (v/v) aqueous glycerol in a 16×100 mm (Sorvall pyrex) test tube. Bring rapidly to 56°C and keep at this temperature for 2.5 min. Then by immersion in ice, allow it to cool to room temperature (24°C).

Add 0.05 ml of 0.2 M glutathione solution [dissolved in 50% (v/v) aqueous glycerol and adjusted to pH 7.5 with 3 N NaOH]. Activation of factor XIII is initiated by admixing 0.2 ml of thrombin [125 NIH units/ml; dissolved in 25% (v/v) aqueous glycerol and 0.02 M $CaCl_2$; pH 7.5] and it is allowed to proceed for 20 min.

Then add 0.5 ml of the synthetic amine substrate,* 2 mM monodansylcadaverine solution in 0.05 M Tris-HCl of pH 7.5 and 3 mM $CaCl_2$. The incorporation reaction is started with the addition of 1.0 ml of 0.4% casein solution; made up in a mixture containing 10% glycerol, 3 mM $CaCl_2$, 0.05 M Tris-HCl at pH 7.5 (after dialysis, the solution is clarified by centrifuging at 20,000 rpm×60 min in a Spinco L No. 30 rotor).

Amine incorporation is stopped 30 min later by adding 2 ml of 10% trichloroacetic acid. The protein precipitate is washed successively with 6×10 ml of ethanol-ether (1:1) and dried. Finally, it is taken up in a 2 ml solution of 8 M urea, 0.5% sodium dodecylsulfate in 0.05 M Tris-HCl at pH 8, for measuring the protein-bound amine.

* The same procedure could, of course, be also applied by substituting monodansylcadaverine with any of the other amine tracer substrates of fibrinoligase, such as shown in Table I. However, on account of the less favorable K_m, app, a higher concentration of these compounds (10–20 mmoles/liter) would be recommended.

For certain purposes, it may be convenient to express a given rate of amine incorporation in terms of "FSF units." It would be of further advantage to normalize these unit values for 1 ml of citrated plasma.

Using 0.2 ml of citrated plasma in a 2 ml test mixture (Table II), we decided to define units in the following manner:

FSF units per milliliter of citrated plasma

$$= \frac{2}{0.2} \times \frac{1}{3} \times \frac{1}{0.9} \left[\frac{i_T - i_B}{i_R - i_S} \right].$$

The first term accounts for the tenfold dilution of plasma in the test; the second simply serves to reduce the numerical values into a convenient range and may be considered to express units in terms of a 10 min incorporation instead of the measured 30 min; the third is to correct for the estimated 10% loss of factor during pretreatment of plasma. If units are to be expressed for original plasma and not the citrated one, another correction for dilution with the anticoagulant should be included.

From the above, it is clear that the formula for citrated plasma could be simplified as

$$3.7 \left[\frac{i_T - i_B}{i_R - i_S} \right].$$

The symbols within the brackets represent the measured fluorescent intensities (i) of the test sample (T) after 30 min of amine incorporation; of the blank (B) control mixture with only the monodansylcadaverine missing, but otherwise similarly treated; of a 1 μM reference (R) solution of free monodansylcadaverine and finally of the mixed (urea–sodium dodecyl sulfate) solvent (S) system itself.

The following relation of measured fluorescence intensities may illustrate the resolution of the test system and the relative magnitude of background fluorescence. Typically, i_B is about three times greater than i_S, and i_R is about three times that of i_B. After 30 min of incorporation, with normal plasma as the source of FSF in the test, i_T will be about five times that of i_R (or about 15 times higher than i_B). Such a distribution of fluorescence intensities gives rise to about 20 U of factor per ml of plasma. In actual experience, repeat measurements on the same plasma overlap within about 1 U.

IV. Application of the amine-incorporating assay to a clinical case

It would now be appropriate to illustrate the application of the assay by describing some studies carried out on a patient with congenital deficiency of FSF. The patient, a 22 yr old Cuban female, had a number of bleeding episodes severe enough to require blood trans-

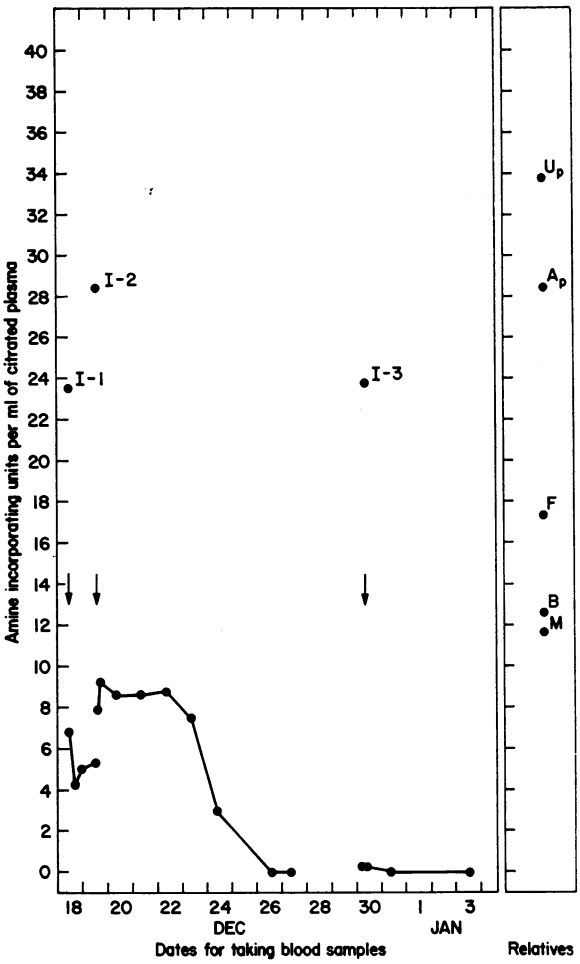


FIGURE 9 Amine-incorporating units obtained from a patient's plasma during transfusion therapy on account of a total deficiency of FSF. Infusion pool values (I-1; I-2; I-3), as well as measurements on the patient's relatives are also shown. (M, mother; B, brother; F, father; Ap, paternal aunt; Up, paternal uncle.) For experimental details, see Table II.

fusion after minor trauma. In September 1966, the deficiency was diagnosed on the basis of clot solubility in 5 M urea and 2% acetic acid. A full clinical report will be published elsewhere.[8] With the use of the new assay, the amine incorporating potential of the patient's plasma was found to be nil.

Relatives of the patient were also analyzed (right side of Fig. 9); her mother (M), brother (B), and father (F) gave low values. A paternal uncle (Up) and aunt (Ap) were on the high side of normal range.

24 individual normal frozen plasma controls yielded a mean value of 21 in the 15–29 range. It will be noticed that the three infusion pools also fall in this range. Three normal controls appeared to be excessively high (37–42 range) and three seemed to be quite low (11–12 range). Measurements with six normal fresh plasma samples gave values very similar to those found with infusion pools I-1 and I-3. Hence, it is assumed that storage of freshly frozen plasma did not cause any diminution in FSF potency.

In October 1966 the patient became pregnant for the first time, and on December 15 was admitted to the Columbia-Presbyterian Medical Center with a threatened abortion. Despite bed rest and sedation, bleeding persisted, and because of previous reports of habitual abortion without replacement therapy (23, 24), a liter of fresh frozen plasma was infused on December 18 and again on December 19. As shown in Fig. 9, FSF rose to 7 U/ml on the 18th and to 9 U/ml on the 19th. The elevation in factor level was associated with prompt cessation of bleeding. There was little decline till after December 23, but the level then rapidly dropped to 0 U. The rapid fall off in level was surprising and not consistent with previous reports of a 3 wk duration of effect and a 4–5 day half-life (23, 24). Bleeding recurred on December 30 and was unaffected by infusion of a liter of plasma which was assayed to contain 24 U/ml of FSF. No significant blood level of the factor was found. In view of these results the presence of a specific antibody to this factor was suspected, and further plasma infusion was avoided. Bleeding persisted, and complete abortion eventually occurred. Gel diffusion study of the December 30th sample of the patient's plasma showed a precipitin line with a purified FSF preparation (Fig. 10), whereas an earlier sample of plasma from September showed no precipitin line.

The amine-incorporating system could be used to demonstrate conclusively that a neutralizing inhibitor did in fact appear in the patient's plasma. When 0.1 ml of the infusion pool (I-3 in Fig. 9) was mixed with 0.1 ml of the patient's December 30th plasma specimen and tested as described in Table II, instead of the 12 U/ml expected, only 1.8 U/ml were found. Approximately, this corresponds to a neutralization of 85%. In this connection, it is worth pointing out that the only other known

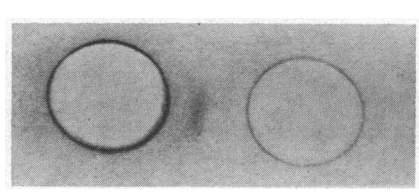


FIGURE 10 Ouchterlony diffusion test for demonstrating the appearance of an antibody against FSF in the patient's plasma of December 30. Left, 30 μl of the patient's plasma; right, 50 μl of chromatographically purified factor (about 1000 "cross-linking units" per ml).

[8] de Kiewiet, W. C., and H. L. Nossel. Data to be published.

example of a pathological inhibitor of fibrin cross-linking (25, 26) was of such a nature that it did not interfere with amine incorporation at all.

DISCUSSION

A coupled system, based on the incorporation of fluorescent monodansylcadaverine into casein, was developed for measuring FSF (factor XIII) levels in plasma. This inactive precursor is converted to a transpeptidase (fibrinoligase [FSF*]) by addition of thrombin; the generated enzyme activity is then assayed by means of amine incorporation. Mixing thrombin with plasma necessitated the differential desensitization of intrinsic fibrinogen so that it would not clot and would not thereby interfere with amine incorporation. Further, the thrombin-inactivating capacity of plasma had to be saturated to permit full conversion of the factor to the transpeptidase. Only, after these special features of the plasma test system were taken into consideration could a linear relationship be obtained between the velocity of amine incorporation and the concentration of plasma employed (Fig. 8).

The test was applied to a clinical case of hereditary total FSF deficiency. The effect of transfusion therapy was studied, and some of the patient's relatives were examined. It is evident from Fig. 9 that the mother and brother of the patient could be considered heterozygous. The father also appears to be low in FSF. The corrective powers of the 1 liter of fresh plasma infusions (I-1 and I-2) may probably be explained in terms of simple dilution (about 4-fold) in the circulation. Futility of the third transfusion (I-3) could be ascribed to the appearance of a neutralizing antibody specifically directed against the precursor form of FSF. The development of the antibody in association with uterine bleeding raises the question of maternal immunization by fetal blood. An alternative possibility is that the antibody has developed previously, and the plasma infusions provided the antigen which stimulated its reappearance. One wonders if infusion of platelets, which are also known to contain FSF (27), might not have lessened this risk.

Finally, the diagnostic potential of the amine incorporation assay might be considered. From what we already know about the fibrin-stabilizing fibrinoligase system of plasma, a number of projections can be made and differential procedures devised to distinguish between clinical manifestations which may at first seem to be similar.

In regard to results which show low levels of the factor, the following possibilities exist.

1. A heterozygous carrier state may exist for FSF deficiency, such as demonstrated in Fig. 9 for some of the relatives of the totally deficient patient.

2. An acquired reduction in the steady-state level of the factor in plasma may exist, in conjunction with a variety of diseased states (e.g. myeloma, lead poisoning [see also below], pernicious anemia, liver cirrhosis [28–30]).

3. Inhibitors to the transpeptidase (or to the precursor form of the FSF) may be present. These could be conveniently classified into three catagories. (*a*) Mercaptide-forming (mercury or lead compounds) or alkylating reagents (nitrogen mustard), if present in the circulation, might inhibit the transpeptidase by blocking functional groups. Since mercaptide formation is reversible, inclusion of the relatively high concentration of glutathione in the test (Table II) would at least minimize this kind of interference from the first class of compounds. (*b*) If a transpeptidase substrate (e.g. an amine) occurs in plasma, competition may occur against monodansylcadaverine for incorporation into casein. In order to be noticed in the test, such a compound would have to display a more favorable Michaelis constant for the transpeptidase than the fluorescent substrate [$K_{m,app}$ about 2×10^{-4} mole/liter (2, 9)] and would have to be present in sufficiently large concentrations to compete against the saturating concentrations (5×10^{-4} mole/liter) of the latter, as employed in the test. Though the possibility of such an occurrence cannot be excluded, we consider it to be very remote. In fact, a serum sample in which such a pathological inhibitor was previously demonstrated (25, 26) produced no inhibition in the amine incorporation assay. In this connection, we emphasize the importance of using the lobster clotting test (26) in addition to amine incorporation. (*c*) Antibodies to FSF, either to the precursor or to the transpeptidase, would definitely reduce the values measured with amine incorporation. Unlike the amine type of competitive inhibitor discussed above, the neutralizing effect of the antibody-containing sample can be readily demonstrated by mixture with normal plasma. With the patient's plasma discussed (Fig. 9), a 1 : 1 mixing ratio resulted in 85% neutralization. Clearly, the quantitative amine-incorporating test is sensitive enough to demonstrate much less neutralizing activity.

4. Low amine incorporation values might occur with plasma samples with an unusually high titer of "antithrombin" activity. Hence, the thrombin concentration used in the test (Table II) may not saturate the system and ensure complete conversion of the precursor factor to the transpeptidase. We consider this a trivial possibility which could easily be recognized by measuring amine incorporation at two different concentrations of plasma (see Fig. 8, e.g. 0.1 and 0.2 ml). Deviation from linearity (towards lower values) at higher plasma concentration would indicate a greater anti-

thrombin titer than the test was designed for. Apparently normal controls seem to display an extra mode of distribution around relatively high values (see Results, section IV). Such values could, indeed, reflect a steady-state level of FSF higher than usual. On the other hand, there is a distinct possibility that certain plasma samples may contain another transpeptidating enzyme in addition to the FSF* which is generated by thrombin. Amine incorporation with the use of thrombin (Table II) would represent the combined activity of both enzymes. If the presence of another transamidase is suspected, a control without thrombin will be necessary. As shown in Fig. 7, normal plasma displays no amine-incorporating activity in the absence of thrombin; only the precursor is present. The enzyme is produced transiently (7, 28) after the appearance of thrombin. As a transpeptidase, FSF* is similar in its mode of action to other enzymes such as liver transglutaminase and muscle tissue coagulin (31). The three enzymes are interchangeable to a great extent but are not identical. All three are calcium ion–dependent, inhibited by sulfhydryl-blocking reagents and by amines (2) and can be assayed by the method given in Table II. The liver and muscle enzymes, however, do not depend on thrombin activation and their activities (E′) could be measured in a simplified incorporation system with monodansylcadaverine (H_2NR) and casein:

$$\text{casein} + H_2NR \xrightarrow[Ca^{2+}]{E'} \text{casein-HNR} + (H).$$

Since thrombin is not required, heat desensitization of fibrinogen is unnecessary, and glycerol could also be omitted. Otherwise, the test could be performed either as outlined in Table II, or by a continuous rate assay which depends on the enhancement of fluorescence intensity accompanying the incorporation of the amine into casein (22). The trichloroacetic acid precipitation, subsequent washing, and analytical steps (see Table II) are obviated by the latter method.

If such transglutaminase enzymes unexpectedly appear in plasma, they may indicate liver or muscle tissue damage. The amine-incorporating assay with the omission of thrombin could then become a major tool for monitoring such conditions.

Clearly, the presence of a transglutaminase in plasma must be regarded as potentially dangerous. A variety of exchange reactions would occur on γ-glutaminyl residues of a number of proteins (P) in an uncontrolled manner (21):

$$PCONH_2 + H_2NR \rightleftharpoons PCONHR + NH_3.$$

The amine partner (H_2NR) could be another protein molecule, resulting in promiscuous cross-linking (even of the hybrid type). Naturally occurring amines (histamine, noradrenaline [21]) or therapeutically administered compounds (isoniazid [26, 32]) could become covalently incorporated into carrier proteins with possible far-reaching consequences such as autoimmunizing antigen production (see e.g. reference 33).

The fact that, in the course of blood coagulation, FSF (factor XIII) becomes enzymatically active only after the appearance of thrombin (3, 7) may indicate that even this enzyme, which appears to be of more restricted specificity than transglutaminase, cannot be safely tolerated in plasma. Indeed, similar to the removal of thrombin by antithrombin factors, a special mechanism may exist for removing FSF* from the circulation so that it would appear only for a very short time during coagulation. Our assay makes it possible to investigate this question quantitatively.

Another possible reason for apparent high amine incorporation values could be the occurrence of unusual protein acceptors in plasma which also are able to incorporate monodansylcadaverine. Normally, in the absence of casein (Table II), such background incorporation is negligible. Should such a situation be suspected, a control without casein would be necessary.

In the foregoing discussion, an attempt was made to illustrate possible applications of molecular findings in a meaningful manner to an interesting area of biochemcial medicine. Of course, the new assay could also be useful in studying FSF levels in plasma under various physiological conditions such as neonatal life or pregnancy, for example.

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