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Amine Specificity in Transpeptidation. Inhibition of Fibrin Cross-Linking*

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ABSTRACT: A systematic survey was made of some 25 amines, including several newly synthesized ones, to assess their inhibitory effect in the enzymatic cross-linking of fibrin. Compounds were selected from the point of view of different basicities and side-chain substitutions.

Best inhibitors contained a pentamine or hexamine residue attached to an apolar group, toluene, or naphthalene. *N*-(5-Aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide, the most potent inhibitor so far, was shown to act as a substrate for the cross-linking enzyme. It became incorporated into fibrin, serving as a fluorescent label for the acceptor cross-linking sites of this protein. Incorporation of the fluorescent amine showed saturation kinetics with an apparent K_M of 1.6×10^{-4} M (at a fixed initial fibrin concentration of 5.45×10^{-6} M). This may be compared with a value of 3×10^{-3} M for the incorporation of [^{14}C]glycine ethyl ester (Lorand, L., and Ong, H. H. (1966b), *Biochemistry* 5, 1747). The ratio of Michaelis constants for these two amines is in good agreement with their relative potencies in inhibiting the cross-linking of fibrin.

The concept that cross-linking of fibrin[1] occurred through an enzyme-catalyzed transpeptidation (Lorand *et al.*, 1962) between selected amino groups (now shown to be those of ε-lysine; Lorand *et al.*, 1966a) of one protein molecule and carbonyl group (γ-glutamyl; Lorand and Ong, 1966a) of another (see Added in Proof), led us to examine the influence of synthetic amines ($H_2N \cdot R$) on the reaction. Many of the compounds proved to be powerful inhibitors of cross-linking which is illustrated here (eq 1) by the formation of a dimer

$$H_2N \cdot F \cdot CO \cdot Y + H_2N \cdot F' \cdot CO \cdot Y \xrightarrow[H_2N \cdot R]{e\backslash} H_2N \cdot F \cdot CO \cdot NH \cdot F' \cdot CO \cdot Y + HY \quad (1)$$

through a single amide bond. It is important to point out that amines do not seem to interfere at all with the reversible "self-assembly" of fibrin, that is, clot formation; they specifically inhibit only the subsequent cross-linking reaction between fibrin units which, in the absence of amines, would yield structures insoluble in

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[1] Abbreviations used: F, fibrin; e or e·SH, the cross-linking enzyme, *i.e.*, the thrombin-activated fibrin-stabilizing factor (FSF*); Y, the leaving group in fibrin cross-linking, presumed to be NH_2; dansyl, 1-dimethylamino-5-naphthalenesulfonyl; Cbz, benzyloxycarbonyl; Ts or tosyl, *p*-toluenesulfonyl; TAME, N^{α}-tosyl-L-arginine methyl ester.

1% monochloroacetic acid. The extent to which fibrin (under cross-linking conditions in the presence of amines) remains soluble in the acid, may be taken as an index of inhibition. Since cross-linking of fibrin is the last step in the sequence of normal blood coagulation in vertebrates (solid lines in Figure 1), amines which prevent this reaction represent a novel class of blood coagulation inhibitors.

Interestingly enough, the very same amines inhibit clot formation itself in *Homarus* plasma (broken line in Figure 1). Hence, in this system (using a muscle extract as enzyme), delay of clotting time is a measure of the inhibitory power of the added amines (Lorand *et al.*, 1963).

During the past 5 years, we have examined a large number of amines both for inhibition of cross-linking in the isolated bovine fibrin system and for delay of clotting time of *Homarus* plasma (see also Lorand and Jacobsen, 1964; Lorand, 1965; Lorand *et al.*, 1965; Bruner-Lorand *et al.*, 1966a; Lorand and Ong, 1966a,b).

In this paper, the inhibitory properties of various classes of amines are systematized from the point of view of varying basicities, without appreciably changing the size of substituents, and from the point of view of similar basicities, but changing the size and character of substituent R groups.

Experimental Section

Fibrinogen was obtained from bovine fraction I (Armour; Laki, 1951) and was stored in the deep freeze as a 1% protein solution in 0.05 M Tris–0.1 M sodium chloride, adjusted to pH 7.4 with hydrochloric acid.

Thrombin was purified from Bovine Thrombin Topical (Parke Davis) by the chromatographic procedure of Rasmussen (1955).

The *cross-linking substrate fibrin* was obtained by the method of Donnelly *et al.* (1955), with the exception that 1 mM iodoacetate was added to fibrinogen 0.5 hr before clotting with thrombin. The fibrin solution (*ca.* 1.5% in protein) in 1 M sodium bromide, adjusted to pH 5.4 with acetic acid, was stored in ice.

The precursor of the cross-linking enzyme (*i.e.*, fibrin-stabilizing factor) was fractionated from frozen oxalated bovine plasma (Pentex) with the use of ammonium sulfate and heat treatment (Loewy *et al.*, 1957). By itself, this material showed negligible cross-linking activity unless preactivated by thrombin (Lorand and Konishi, 1964a). For the incorporation experiment shown in Figure 9, the activated factor was purified on Bio-Gel P-200 (Konishi and Lorand, 1966).

The bioassay for the inhibition of cross-linking of bovine fibrin is based on a multistage procedure (Lorand *et al.*, 1962; Lorand and Jacobsen, 1964). (a) Activation of 0.4 ml of the partially purified fibrin-stabilizing factor (1.2 mg of protein) was carried out over a period of 10 min, after the addition of 0.5 ml of 0.1 M cysteine, 0.5 ml of 1 mM calcium chloride, and 0.1 ml of thrombin containing 2 NIH units. The factor concentration used in these experiments was about tenfold above the threshold or unit activity; the latter being defined as that concentration which, in the absence of inhibitors, would

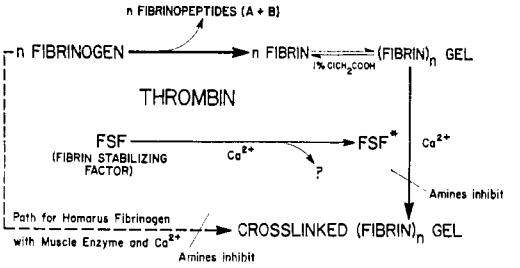


FIGURE 1: Outline of the reaction sequence in clot formation.

render 50% of the fibrin insoluble in 1% monochloroacetic acid under the experimental conditions given below. (b) Quenching of activation by thrombin was accomplished by introduction of 0.2 ml of 0.25 M TAME, a competing substrate (Sherry and Troll, 1954). (c) The inhibitor solution (0.5 ml) was then added to provide the final concentrations in the clotting mixtures shown in the abscissas of the graphs. (d) Cross-linking was initiated by the vigorous admixing of 0.3 ml of fibrin (4.5 mg of protein) and it was allowed to proceed for 30 min. Gel formation itself ensued in all instances within a few seconds after addition of fibrin. (e) The cross-linking reaction was terminated by shaking up the gels with 2.5 ml of 2% monochloroacetic acid which was presumed to disperse those portions of the clots which did not cross-link. (f) The acid-insoluble cores, remaining after about 18 hr, were centrifuged, washed with 0.9% saline and with water, then digested to measure protein contents which are shown on the ordinates of the graphs.

Fibrin-stabilizing factor, thrombin, cysteine, TAME, and the compounds tested as inhibitors were made up in 0.05 M Tris–0.1 M sodium chloride buffer adjusted to pH 7.4 with hydrochloric acid. The experiments were performed at room temperature (~24°).

Tests for inhibiting Homarus blood clotting were carried out in a manner previously described (Lorand *et al.*, 1963). In 10 × 75 mm culture tubes, 0.2 ml of the pH 7.4 Tris–sodium chloride buffer was mixed with 0.1 ml of 0.2 M calcium chloride and 0.1 ml of *Homarus* tissue coagulin (0.9 mg of protein) obtained from the tail muscle. Then, 0.1 ml of the inhibitor (in various dilutions with the Tris buffer) was added. The clotting reaction was initiated by the introduction of 0.2 ml of citrated *Homarus* plasma (containing 3.4 mg of protein), and gel formation was noted by tilting of the tubes; all at room temperature (~24°).

In order to avoid nonspecific inhibitory effects, all amine compounds were used at concentrations less than 10 mM in either test. Furthermore, in an effort to obviate possible variations in the bioassays, inhibitors grouped in any one graph were assayed simultaneously, using the same batch of reaction components.

Methodology for incorporating the fluorescent tracer 5-dansylamidopentamine into fibrin by the cross-linking enzyme (see Figure 9) was similar to that used earlier in connection with the radioactive substrate, glycine ethyl ester (Lorand and Jacobsen, 1964; Lorand and Ong, 1966b). The cross-linking enzyme, however, was preactivated by thrombin and was freed from the latter by gel filtration (Konishi and Lorand, 1966). Thus, a unique

feature of the present experiments was the absence of thrombin during amine incorporation; nor was there a need for including TAME to quench thrombin activity. The reaction mixtures comprised 0.5 ml of 0.01 M calcium chloride, 0.5 ml of 0.1 M cysteine (pH 7.4), 0.5 ml of the thrombin-activated fibrin-stabilizing factor (12.5 units, 67 μg of protein), 0.7 ml of the 5-dansylamidopentamine in various dilutions with 0.05 M Tris–0.1 M sodium chloride of pH 7.4, and 0.3 ml of fibrin (4.5 mg of protein dissolved in 1 M sodium bromide at pH 5.4). Incorporation was stopped by the vigorous admixing of 2.5 ml of 2% monochloroacetic acid 15 min following the addition of fibrin. Clots at the higher initial 5-dansylamidopentamine concentrations (1.42–4.96 mM; full circles in Figure 9) dissolved completely, while some monochloroacetic acid insoluble residues, amounting to about 10% of the fibrin, remained in the others (0.14–0.71 mM initial concentration of amine; open circles in Figure 9). All proteins were collected after adding 5 ml of 14% trichloroacetic acid, and the centrifuged sediments were freed of noncovalently trapped amine by repeated washing (4 × 10 ml) with 7% trichloroacetic acid. The latter was removed by extraction with 2 × 10 ml of ethanol which also served to eliminate the last traces of fluorescence not bound to protein. After evaporating ethanol by air, the protein precipitates were dissolved by the addition of 2 ml of 10 M urea (buffered with 0.2 M Tris at pH 8) and 2 ml of 1% sodium dodecyl sulfate. These solutions were used for measuring fluorescence intensities (Aminco–Bowman spectrophotofluorimeter; λ_A 360 mμ; λ_F 520 mμ) and for estimating protein concentrations by absorbancy at 280 mμ.

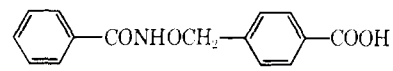
Fluorescence intensities were converted into concentrations of 5-dansylamidopentamine by calibrating the fluorescence of known concentrations of this compound in the 5 M urea–0.5% sodium dodecyl sulfate solvent. It should be mentioned that addition of fibrin (at concentrations similar to that used in the incorporation experiments) to such solutions of the amine did not change the calibration curve, showing that the mere presence of protein would not perturb the nature of fluorescence of the dansyl group. (Of course, it cannot be assumed *a priori* that covalently incorporated 5-dansylamidopentamine would also be unperturbed.)

The extinction coefficient of a 1% solution of fibrin in the 5 M urea–0.5% sodium dodecyl sulfate solvent was virtually indistinguishable from the literature value of $\epsilon^{1\%}_{280\ m\mu}$ 15.12 for fibrinogen (Johnson and Mihalyi, 1965). Protein concentrations were computed on the basis of this figure.

Finally, our calculations assume an approximate molecular weight of 330,000 for the acceptor substrate, fibrin. This is derived by subtracting 3% for the loss of fibrinopeptides (Lorand, 1951) from the 340,000 mol wt fibrinogen (Shulman, 1953).

Synthesis of Inhibitors

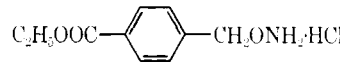
Ethyl α-(Aminoxy)-p-toluate Hydrochloride. First, *O*-(*p*-carboxybenzyl)benzohydroxamic acid was prepared as follows. Benzohydroxamic acid (0.01 mole) was dissolved in 20 ml of 50% (v/v) ethanol, containing (0.02 mole) of sodium hydroxide. To this 0.01 mole of α-bromotoluic acid was added, followed by refluxing for 6 hr. The solution was then evaporated and the residue was redissolved in 10 ml of water. After acidification by a few drops of concentrated hydrochloric acid, a solid separated which was filtered and recrystallized from ethyl acetate, mp 208–210°. Structure I was also



I

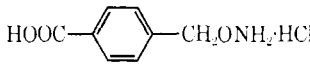
consistent with the infrared spectrum. *Anal.* Calcd for $C_{15}H_{13}NO_4$: C, 66.40; H, 4.86; N, 5.17. Found: C, 66.46; H, 4.97; N, 5.32.

This compound (1 g) was refluxed for 20 min with 6% (w/w) hydrochloric acid in ethanol. The solution was evaporated and the residue was treated with 50 ml of ethyl acetate. The insoluble material could be filtered, crystallized, and recrystallized from methanol–ethyl acetate, mp 208°. *Anal.* Calcd for $C_{10}H_{14}ClNO_3$: C, 51.83; H, 6.09; Cl, 15.30; N, 6.04. Found: C, 52.06; H, 6.13; Cl, 15.57; N, 6.19, in agreement with structure II.



II

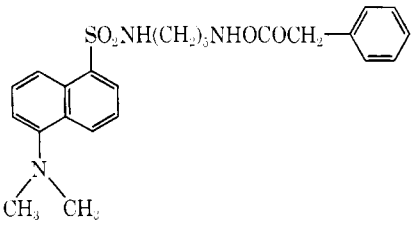
α-Aminoxy-p-toluic Acid Hydrochloride. O-(*p*-Carboxybenzyl)benzohydroxamic acid (2 g) was refluxed with 30 ml of 10% hydrochloric acid for about 90 min. The solution was evaporated to dryness *in vacuo* and the residue was extracted with four 30-ml portions of anhydrous ether. The insoluble material was then crystallized (and recrystallized four times) from methanol–ethyl acetate, mp 245°. *Anal.* Calcd for $C_8H_{10}ClNO_3$: C,



III

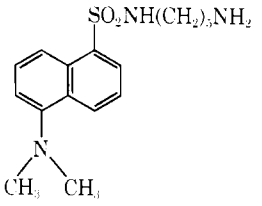
47.19; H, 4.95; Cl, 17.42; N, 6.88. Found: C, 47.29; H, 4.89; Cl, 17.30; N, 7.47.

N-(5-Aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide or 5-Dansylamidopentamine or Monodansylcadaverine. As a starting material, 5-dimethylamino-*N*-(5-(benzyloxycarbamido)pentyl)-1-napththalenesulfonamide was synthesized in the following manner. 5-Benzyloxycarbamidopentamine (2.06 g) (or mono-Cbz-cadaverine) hydrochloride (prepared by the method of Clarke *et al.*, 1959) was dissolved in 10 ml of water and stirred with 2.0 g of dansyl chloride (Pierce Chemical Co.) and redistilled triethylamine (1.60 g) in 10 ml of ether. Overnight, solid separated which, after evaporating the ether, was filtered from the aqueous layer. It was recrystallized from methanol (mp 90.5–91°) and in agreement with the structure IV. *Anal.* Calcd for $C_{25}H_{31}N_3SO_4$: C, 64.0; H, 6.76; N, 8.96. Found: C, 63.84; H, 6.61; N, 8.87. This compound (8 g) was suspended in 100 ml of methanol containing 1 ml of acetic acid and



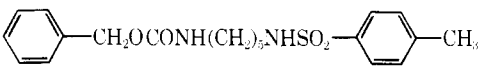
IV

1.5 g of 10% palladium–charcoal. The mixture was shaken in a Parr apparatus at 30 psi of hydrogen overnight. The catalyst was then filtered and the filtrate was evaporated to a syrup *in vacuo*. The residue was taken up in 100 ml of water and extracted repeatedly with small portions of ether. The aqueous phase was then warmed with decolorizing charcoal and filtered. On lyophilization, this filtrate gave a yellow gum which was taken up in 100 ml of water and passed through a column (35 × 350 mm) of Dowex 1-X8 resin (100–200 mesh) in the sulfate form. Elution was carried out with water and the eluate, after lyophilizing, formed a yellow solid. The product was homogeneous as judged by electrophoresis on paper, for example, in a pyridine–acetic acid buffer (10 and 4 ml, respectively, in 1 l. of solution) of pH 5.4. Only a single, cathodically migrating fluorescent band (to about 3-cm distance in 1 hr at 300 V/17 cm and 2 mA) could be seen. The compound also appeared to be homogeneous on carboxymethylcellulose thin-layer or paper chromatograms.[2] Using the above pyridine–acetate buffer, only a single fluorescent spot with an R_F of about 0.1 was obtained. Thus, even though the compound could not be made to crystallize, it could be taken to contain only a single fluorescent compound, namely, the sulfate salt of 5-dansylamidopentamine.



Accurate determination of concentrations of 5-dansylamidopentamine solutions could readily be made by measuring absorbancies at 326 mμ (in 20% (v/v) dioxane–water). Since molar extinction of the dansyl nucleus at this wavelength is virtually independent of the side chain attached to it in sulfonamido linkage (see Deranleau and Neurath, 1966), dansylamide was taken as reference which, in our hands, gave $\epsilon_{326\ m\mu}$ 4.64 × 10^6 cm^2 mole^{-1}.

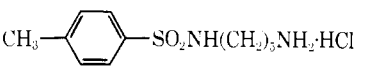
N-(5-Aminopentyl)-p-toluenesulfonamide or 5-Tosylamidopentamine or Monotosylcadaverine.[3] First *N*-(5-(benzyloxycarbonylamido)pentyl)-*p*-toluenesulfonamide was prepared as follows. Mono-Cbz-cadaverine (0.62 g), obtained by the method of Clarke *et al.* (1959) and dissolved in 5 ml of water, was treated with 0.47 g of toluenesulfonyl chloride and 0.51 g of triethylamine in 5 ml of ether. The ether phase was separated, then washed with dilute hydrochloric acid and water. After drying over anhydrous sodium sulfate, the ether solution was filtered and evaporated. The residue could be crystallized from benzene solution (mp 86–87°) as structure V. Calcd for $C_{20}N_{26}N_2O_4S$: C, 61.6; H, 6.72; N, 7.18.



V

Found: C, 61.09; H, 6.62; N, 8.00.

About 1.5 g of the previous compound was taken up in 20 ml of methanol containing 0.5 ml of acetic acid and 0.25 g of 10% palladium–charcoal. After reduction with hydrogen, the mixture was filtered and the solvent was evaporated from the filtrate. The residue was taken up in water by addition of a few drops of hydrochloric acid and lyophilized. The residue was again taken up in water, then a cloudy residue formed and crystallized. This was removed by filtration and the clear filtrate was relyophilized. The product gave a mp 123.5–124.5° for structure VI. *Anal.* Calcd for $C_{12}H_{12}ClN_2O_2S$: C, 49.3;



VI

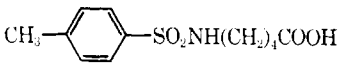
H, 7.26; N, 9.6. Found: C, 48.86; H, 7.24; N, 9.42.

N-(5-Hydroxypentyl)-p-toluenesulfonamide or 5-Tosylamidopentanol. First, 5-tosylamidovaleric acid synthesized in the following manner. 5-Aminovaleric acid (20 mg) was dissolved in 200 ml of 0.2 M sodium hydroxide to which 40.8 g of tosyl chloride in 200 ml of ether was added. After stirring of this mixture for 24 hr, the aqueous layer was separated and extracted three times with 50-ml aliquots of ether. The water phase was then adjusted to about pH 1 with hydrochloric acid

[2] The well-defined behavior of 5-dansylamidopentamine on electrophoresis or on carboxymethylcellulose chromatography forms the basis of a very simple and sensitive method for studying the transpeptidase- (*e.g.*, transglutaminase) catalyzed coupling of this compound to nonfluorescent acceptor substrates, for example, to Cbz-glutaminylglycine (Lorand *et al.*, 1966b). The coupling product gives rise to a new fluorescent spot which in electrophoresis (as described) moves towards the anode and on carboxymethylcellulose chromatography (as described) gives an R_F of about 0.4–0.5, in agreement with Cbz-NHCH(COOH)-$CH_2CH_2CONHCH_2CH_2CH_2CH_2CH_2NH$-dansyl being the fluorescent product of transpeptidation.

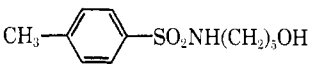
[3] Mono-*N*-acylated derivatives of 1,5-diaminopentane (such as, for example, monotosylcadaverine) might be expected to inhibit trypsin (or trypsin-like enzymes) because they contain the equivalent of a decarboxylated lysine residue. Indeed, monotosylcadaverine could be shown to inhibit trypsin competitively. An association constant (for combining of the compound with the enzyme) of 2–3 × 10^3 l. mole^{-1} was obtained, under the conditions given in Table I of the reference by Rule and Lorand (1964). It will be recalled that *p*-toluenesulfonamidobutylguanidine (or tosylagmatine), which contains the equivalent of a decarboxylated arginine residue, gave an identical inhibition constant.

which induced crystal formation. This separated product was treated with activated charcoal and was recrystallized three times from ethanol–water–2% acetic acid, mp 96.5–97.5°, structure VII.



VII

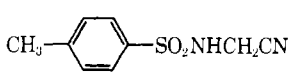
The 5-tosylamidovaleric acid (22.6 g) was dissolved slowly in a cool solution of 5.5 g of sodium borohydride in 110 ml of diglyme. To this was added slowly a solution of 50 ml of tetrahydrofuran, 21 g of borontrifluoride–etherate, and 50 ml of diglyme. The mixture was allowed to warm to room temperature and to stand for 24 hr. The excess borohydride was then destroyed by addition of ethanol and the solution was concentrated by rotary evaporator. The grey residue was taken up in 250 ml of water which was then extracted with 300 ml of ether. The ether layer was separated, washed with dilute bicarbonate solution, and finally dried over sodium sulfate. The residue, remaining after removal of ether, was induced to crystallize from methylene chloride at −60° and from a mixture of methylene chloride–hexane (60:40, v/v) at 0° to yield structure VIII, mp 52.5–53.5°. *Anal.* Calcd for $C_{12}H_{19}NSO_3$: C,



VIII

56.0; H, 7.44; N, 5.44. Found: C, 56.01; H, 7.55; N, 5.39. The product was just sufficiently soluble in the pH 7.4 Tris buffer (after slight warming) to make it possible to measure the potency of this compound at the concentrations shown in Figure 3.

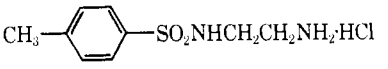
N-(2-Aminoethyl)-p-toluenesulfonamide. First, *p*-toluenesulfonamidoacetonitrile was prepared as follows. Aminoacetonitrile (3 g) was dissolved in 15 ml of water. To this 6.2 g of triethylamine and 4 g of tosyl chloride were added in 15 ml of ether. The mixture was stirred overnight, during which time a solid material separated. The system was made slightly acidic with dilute hydrochloric acid and stirred for a few minutes. The solid was filtered and the ether layer was separated from the filtrate. The ether phase was washed with dilute bicarbonate solution, dried over sodium sulfate, filtered, and evaporated. The residue was crystallized from ethanol–water and then combined with the solid which had been filtered earlier. The combined material was recrystallized three times from ethanol–water (once with charcoal decolorization), mp 140–140.5°, in agreement with structure IX. *Anal.* Calcd for C_9H_{10}-



IX

N_2O_2S: C, 51.5; H 4.82; N, 13.35. Found: C, 51.93; H, 4.90; N, 13.34.

This compound (1.15 g) was taken up in 60 ml of ethanol–hydrochloric acid (3:1, v/v) to which 0.5 g of 10% palladium–charcoal was added. After reduction with hydrogen, the catalyst was removed by filtration and the solvent was evaporated. The residue was taken up in water and lyophilized. The solid was again dissolved in water, boiled with decolorizing charcoal, filtered, and relyophilized. The product appeared to be homogeneous on thin-layer silica gel G, using ethanol as solvent and iodine vapor as indicator. The product was crystallized from methanol–ethyl acetate, mp 122–123°. *Anal.* Calcd for $C_9H_{15}ClN_2O_2S$: C, 43.11; H, 6.03; N, 11.17. Found: C, 43.34; H, 5.97; N, 11.24, in agreement with structure X.



X

ε-Aminocaproic Acid Methyl Ester Hydrochloride. The methyl esterification of ε-aminocaproic acid (Mann Research Laboratories) was carried out with 2,2-dimethoxypropane and aqueous hydrogen chloride according to Rachele (1963), and the product was crystallized from methanol–ethyl acetate, mp 124.5–125°. *Anal.* Calcd for $C_7H_{16}ClNO_2$: C, 46.3; H, 8.83; N, 7.71. Found: C, 46.24; H, 9.15; N, 7.81.

Other Inhibitors. The other compounds tested for inhibitory activities were either obtained as gifts or purchased from the suppliers indicated. Glycine methyl and ethyl ester, glycylglycine methyl and ethyl ester hydrochlorides, and L-histidine methyl ester dihydrochloride were purchased from Mann Research Laboratories; glycineamide hydrochloride from Nutritional Biochemicals; glycylglycineamide hydrochloride and N^{α}-tosyl-L-lysine methyl ester hydrochloride from Cyclo Chemical Corp. Aminoacetonitrile hydrogen sulfate and β-aminopropionitrile[4] were obtained from K & K Laboratories; butylamine from Eastman Kodak; α-(β-aminoethyl)pyridine from Aldrich Chemicals; and histamine dihydrochloride from Fisher Scientific Co.

Results and Discussion

Prior to considering the experimental results with specific inhibitors of fibrin cross-linking, some background discussion seems to be in order. First it should be recalled that earlier work with four different amines already showed that inhibition of the cross-linking process could be attributed to the fact that these amines served as pseudo-donor substrates for the enzyme. As such, they blocked the chain of cross-linking by actually becoming incorporated into the acceptor sites of fibrin.

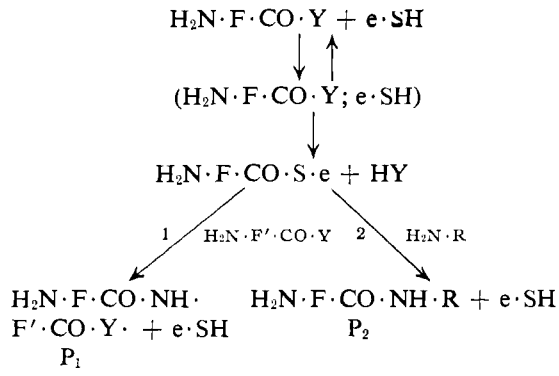
$$H_2N\cdot F\cdot CO\cdot Y + H_2N\cdot F'\cdot CO\cdot Y \xrightarrow{e} H_2N\cdot F\cdot CO\cdot NH\cdot F'\cdot CO\cdot Y + HY \quad (2)$$

[4] Inhibition of fibrin cross-linking by β-aminopropionitrile has also been observed by Pisano (1966).

$$H_2N\cdot F\cdot CO\cdot Y + H_2N\cdot R \xrightarrow{e} H_2N\cdot F\cdot CO\cdot NH\cdot R + HY \quad (3)$$

In the presence of an excess of amine ($H_2N\cdot R$), it was possible to show that blocking occurred entirely on monomeric fibrin (results of S. Tokura quoted by Lorand and Ong, 1966b). At intermediary amine concentrations, before chain termination could take place, probably a spectrum of cross-linked oligomers of fibrin would form, some of which would still be soluble in 1% monochloroacetic acid. Though it is not yet known how the various types of oligomers affect mutual solubilities, and as to what size distribution constitutes an insoluble gel, in the present work, solubility in 1% monochloroacetic acid is taken as an arbitrary criterion of the extent of cross-linking.

Knowledge on the chemistry of enzymatic transpeptidation is derived mostly from work with papain (Fruton, 1957; Smith and Kimmel, 1960; Lowe and Williams, 1964; Brubacher and Bender, 1966). Apart from being a thiol enzyme, the thrombin-activated fibrin-stabilizing factor was shown to be similar to papain in many respects (Lorand and Konishi, 1964b). Great similarities were also noted with transglutaminase (Bruner-Lorand *et al.*, 1966b) which again appears to function in a manner quite analogous to papain (Folk and Cole, 1965). Thus, if we make the likely assumption that the mechanism of fibrin cross-linking (similarly to papain-catalyzed reactions) proceeds *via* a thiol ester intermediate, competition between the natural (1) and pseudo-donor (2) substrates would probably occur at the level of aminolytic deacylation of the enzyme intermediate. The more product (P_2) that is formed in the reaction indicated by arrow 2, the less the acid-insoluble fibrin structure which arises out of P_1.



Obviously, incorporation of $H_2N\cdot R$ into fibrin through the intervention of the cross-linking enzyme fully supports this scheme. Table I lists the inhibitory amines which have already been shown to be incorporated into fibrin by the cross-linking enzyme. These represent a sufficiently varied group of compounds (both in regard to basicities and substituents) so as to permit the conclusion that most, if not all, amines which inhibit fibrin cross-linking would be similarly incorporated. Clearly then, one can postulate that an inhibitory amine must satisfy the specificity requirement of the acyl-enzyme intermediate in order to be able to combine with it (a) and that it must also be sufficiently nucleophilic to be effective in the aminolysis (b) of the intermediate.

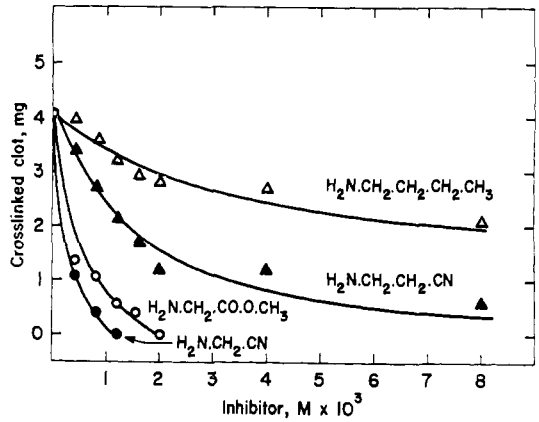


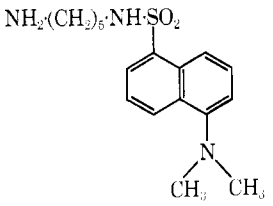
FIGURE 2: Inhibition of fibrin cross-linking (at pH 7.4) by amines of different basicities but of similar size.

$$H_2N\cdot F\cdot CO\cdot S\cdot e + H_2N\cdot R \underset{a}{\rightleftharpoons} (H_2N\cdot F\cdot CO\cdot S\cdot e;\ H_2N\cdot R) \xrightarrow{b} H_2N\cdot F\cdot CO\cdot NH\cdot R + e\cdot SH$$

Since the nature of the clotting system is such that inhibition experiments on fibrin cross-linking are essentially limited to a narrow pH range (in the neighborhood of pH 7), the $H_2N\cdot R\cdot + H^+ \rightleftharpoons {}^+H_3N\cdot R$ equilibrium should also be taken into account.

Figure 2 shows a series of compounds which carry quite similar substituent R groups but differ in the basicity of the amines. Approximate pK_a's of the ammonium ions (NH_3^+) of these compounds range from about 5.5 for aminoacetonitrile (Soloway and Lipschitz, 1958), 7.4 for glycine methyl ester (F. J. Kézdy, personal communication), and 7.7 for aminopropionitrile (Soloway and Lipschitz, 1958) to 10.6 for butylamine (Hall, 1957). Thus, at the pH of the inhibition tests (pH 7.4), almost all of the added aminoacetonitrile would exist in the presumably effective deprotonated form as against only about 50% of the glycine methyl ester, 30% of the aminopropionitrile, and 0.1% of the butylamine. Hence, the order of effectiveness given in Figure 2 would not represent the true efficacy of these compounds. When correction is made for the degree of deprotonation and the amine concentrations are considered, rather than the concentrations of the added compounds (*i.e.*, amine plus ammonium ion) themselves, the first three seem to inhibit fibrin cross-linking approximately to the same extent. At least within this very limited series, the inhibitory index (expressed, for example, by the inverse of the amine concentration required for 50% inhibition of cross-linking) does not seem to increase with the basicity of the amine as might have been expected in terms of the known relationship between the basicities of amines and their nucleophilic

TABLE I

Compound	Label	References
$NH_2 \cdot OH$	Chemical	Lorand and Ong (1966b)
$NH_2 \cdot NH_2$	Chemical	Lorand and Ong (1966b)
$NH_2 \cdot CH_2 \cdot CO \cdot O \cdot CH_2 \cdot CH_3$	^{14}C isotope	Lorand and Jacobsen (1964) Lorand and Ong (1966b)
	Fluorescence	Lorand *et al.* (1966)

reactivities toward simple esters (Jencks and Carriuolo, 1960). There is a similarity, however, with the deacylation of furoyl-chymotrypsin by amines (Inward and Jencks, 1965) where only a very small dependence of reactivity upon basicity was noted. Hence, assistance by the enzyme must be assumed. The inhibitory index calculated for butylamine is about 10- to 20-fold higher than those for the other compounds in Figure 2. It should perhaps also be mentioned that acetonitrile (in contrast to aminoacetonitrile) is not inhibitory at all; signifying the essentiality of the amino functional group.

Figures 3–7 show the highly selective specificity requirement of the cross-linking enzyme for the substituent R group attached to the amino group. The effect of the side chain appears to be so powerful that it can compensate for a factor of about 10^3 in the degree of deprotonation of the amine itself. For example, as seen in Figure 3, 5-tosylamidopentamine is two to three times as inhibitory as glycine methyl ester in spite of the fact that, at the pH of the experiment, the effective amine concentration of the latter is about 10^3 times higher. Compared with butylamine, a compound of similar basicity, 5-tosylamidopentamine is 50–100-fold more effective. It is also of interest that 5-tosylamidopentanol, a close structural analog of 5-tosylamidopentamine, does not possess significant inhibitory properties; another illustration that the enzyme is highly specific for amines.

Figure 4 provides further examples of the intricate side chain specificity requirement for inhibition. ϵ-Aminocaproic acid or its methyl ester are essentially without inhibition, but N^{α}-tosyl-L-lysine methyl ester and 5-tosylamidopentamine are very effective indeed. 5-Dansylamidopentamine (not shown) is almost five times as inhibitory as either of the latter (Lorand *et al.*, 1966a). It would seem that the presence of a large apolar substituent, like tosyl or dansyl, on the pentamine side chain greatly enhances inhibition.

Variations of chain length in the homologous series $H_2N \cdot (CH_2)_n \cdot NH \cdot Ts$ (Figure 5) also reveal a high degree of specificity. The penta- and hexamethylene analogs are equally inhibitory; tetramethylene is slightly less effective and heptamethylene is considerably weaker. *N*-(2-Aminoethyl)-*p*-toluenesulfonamide showed virtually no inhibition.

It should be recalled that inhibition data of the type shown in Figures 3–5 provided the first significant clues for suggesting the assignment of ϵ-amino groups of specific lysine residues as donor functions in fibrin cross-linking (Lorand, 1965; Lorand *et al.*, 1966a,b).

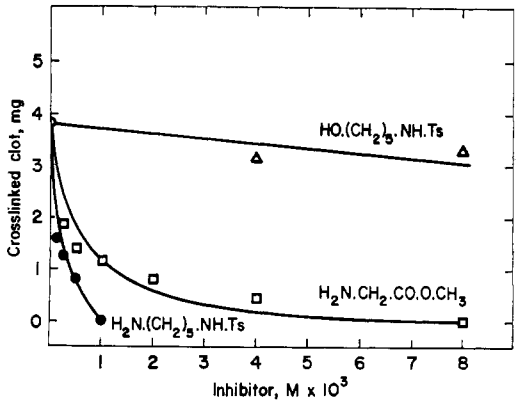


FIGURE 3: 5-Tosylamidopentamine as an inhibitor of fibrin cross-linking (at pH 7.4) is compared with glycine methyl ester and 5-tosylamidopentanol.

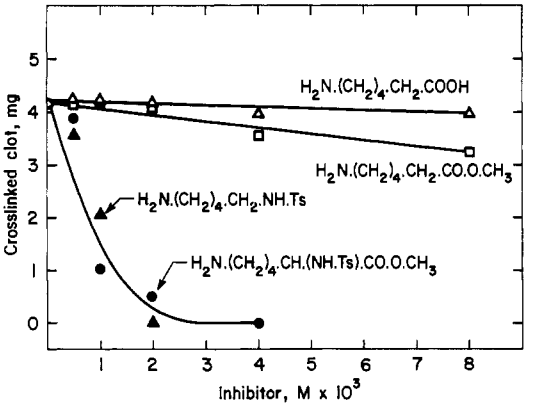


FIGURE 4: Inhibition of fibrin cross-linking (at pH 7.4) by compounds of the pentamine series: 5-tosylamidopentamine; N^{α}-tosyl-L-lysine methyl ester; ϵ-aminocaproic acid and ϵ-aminocaproic acid methyl ester.

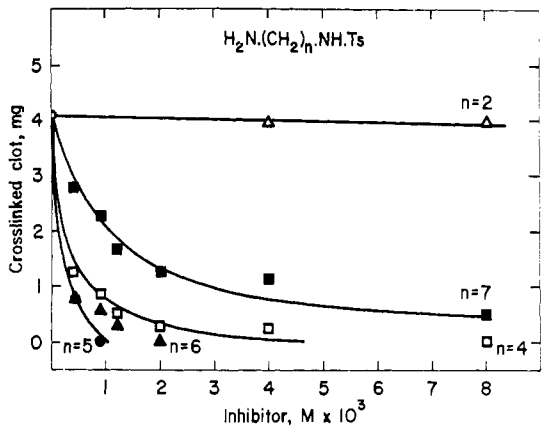


FIGURE 5: Inhibition of fibrin cross-linking (at pH 7.4) by compounds of the tosyl·$NH(CH_2)_nNH_2$ series, with n = 2, 4, 5, 6, and 7.

A series based on the α-amino groups of various glycine derivatives is shown in Figure 6. Without being able to offer a satisfactory explanation for the differences in inhibitory power, one simply recognizes the highly selective nature of the cross-linking enzyme. Glycine itself or glycylglycine would have caused practically no inhibition under conditions of this experiment. It may be assumed that the presence of a carboxylate anion in the vicinity of the amino function is deleterious for inhibition. The fact that aminoxytoluate ion is barely inhibitory, whereas aminoxytoluic acid ethyl ester is a good inhibitor (Figure 7), could also be interpreted likewise.

The fibrinogen of *Homarus* plasma appears to be the soluble equivalent of vertebrate fibrin in the sense that it is ready to undergo cross-linking by transpeptidation (Lorand *et al.*, 1963; Bruner-Lorand *et al.*, 1966b). Unlike vertebrate fibrinogen, it does not seem to need prior uncovering of its cross-linking sites by a process of limited proteolysis (Figure 1). Hence, clotting of the protein comes about solely as a consequence of reaction with a transpeptidating enzyme (tissue coagulin). The order of inhibition of this enzyme by several amines, as expressed in the delay of clotting time, fairly well parallels their effect on the cross-linking of vertebrate fibrin. An illustration of the usefulness of the *Homarus* blood clotting system is given in Figure 8, with a set of inhibitors of the histamine series. Histamine itself inhibits both cross-linking of bovine fibrin and clotting of *Homarus* plasma to an extent similar to that caused by glycine methyl ester. As seen in Figure 8, the aminoethyl derivatives of pyrazole, triazole, and pyridine show a gradual decline in inhibitory power. Interestingly, L-histidine methyl ester, differing from histamine only by the presence of a stereospecific α-carboxymethyl ester group, is completely inert.

The primary purpose of our work as described was to find, on the basis of relative potencies, some general guide lines for the design of specific inhibitors of cross-linking of fibrin, one of the important steps in blood clotting. While much of this objective was accomplished, it also became clear that the complex and even somewhat variable nature of the fibrin cross-linking and the *Homarus* clotting tests would preclude a more penetrating appraisal. Though inhibitory potencies of the compounds shown in Figures 2–7 must be related to their respective association constants with the transpeptidating enzyme, the quantitative relationship is by no means obvious.

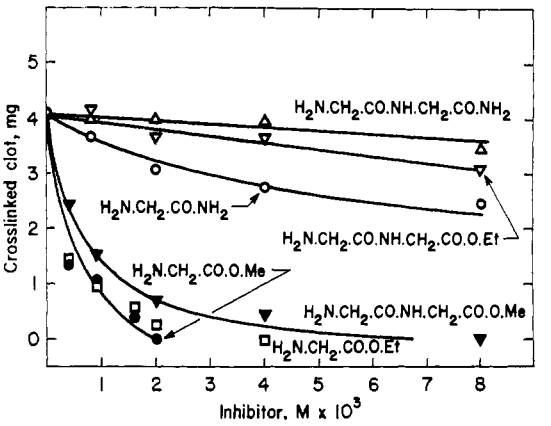


FIGURE 6: Glycine derivatives as inhibitors of fibrin cross-linking at pH 7.4.

A more quantitative estimate of inhibitory amine specificities could, no doubt, be obtained by measuring competition against the incorporation of a reference amine. Any of the compounds ([^{14}C]glycine ethyl ester, hydroxylamine, hydrazine, or 5-dansylamidopentamine; see Table I) which have been shown to be incorporated into fibrin by the cross-linking enzyme could be used as reference. The first three of these would not readily lend themselves for continuous rate assays. However, a versatile method has become available (L. Lorand and T. Urayama, to be published) on the basis of change in fluorescence which accompanies the enzyme-catalyzed incorporation of 5-dansylamidopentamine into proteins (P·CO·Y) (eq 4). Instead of fibrin, a water-soluble protein, for example, casein, is conveniently chosen as acceptor substrate, which further facilitates both ex-

$$\text{P·CO·Y} + \text{H}_2\text{N·(CH}_2)_5\text{·NH·dansyl} \xrightarrow{e} \text{P·CO·NH·(CH}_2)_5\text{·NH·dansyl} + \text{HY} \quad (4)$$

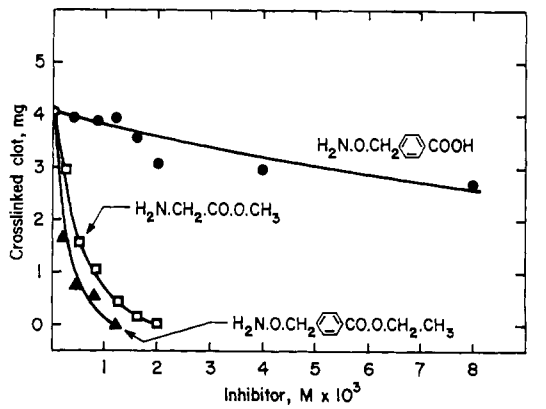


FIGURE 7: The inhibitory effect of *p*-aminoxytoluic acid ethyl ester on fibrin cross-linking (at pH 7.4) is compared with those of glycine methyl ester and of *p*-aminoxytoluic acid.

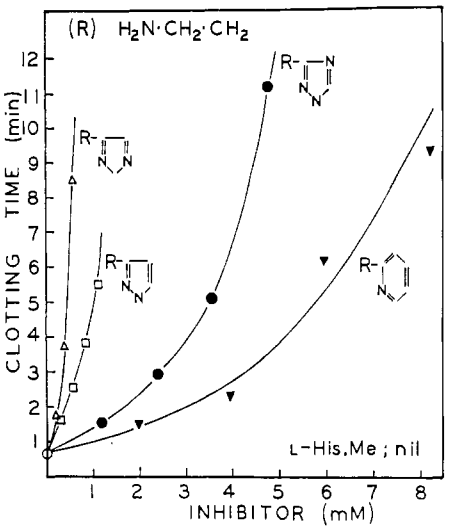


FIGURE 8: Inhibition of clotting of *Homarus* plasma by β-substituted aminoethyl derivatives.

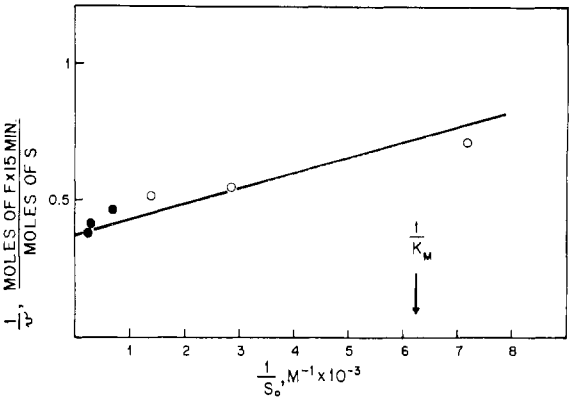


FIGURE 9: Lineweaver-Burk type of presentation of the incorporation of the fluorescent 5-dansylamidopentamine (S) into fibrin (F) at a fixed initial concentration (F_0 = 5.45 × 10^{-6} M) of the latter. Abscissa: reciprocal initial concentrations of 5-dansylamidopentamine (S_0). Ordinate: reciprocal velocities of incorporation in 15 min. For explanation, see text.

periment and interpretation. Preliminary results show that this fluorescence assay is remarkably sensitive and that it can be used as a general method for transpeptidating enzymes (*e.g.*, the thrombin-activated fibrin-stabilizing factor, guinea pig liver transglutaminase, and *Homarus* tissue coagulin). More pertinent to the present discussion is, however, the finding that several of the amines described in this paper would effectively compete against the incorporation of the fluorescent substrate. A quantitative evaluation will be the subject of a later paper.

As a substrate in transpeptidation, 5-dansylamidopentamine has yet another important characteristic which must be discussed. Using any of the transpeptidases mentioned in the previous paragraph, there is kinetic evidence to show saturation of the enzymes. This is demonstrated in Figure 9 in conjunction with fibrin as the acceptor substrate and thrombin-activated fibrin-stabilizing factor as enzyme (see also Lorand *et al.*, 1966a). It is seen that the enzymatic incorporation of the fluorescent amine into fibrin (at a fixed concentration of the latter) can be formally described by Michaelis–Menten kinetics, yielding a $K_{M\,app}$ of about 1.6 × 10^{-4} M. Incorporation of [^{14}C]glycine ethyl ester into fibrin under similar conditions gives a Michaelis constant of about 3 × 10^{-3} M (Lorand and Ong, 1966b). If the ratio of these constants is assumed to reflect the relative strength of binding of these two amine substrates to the cross-linking enzyme, 5-dansylamidopentamine appears to be bound about twenty times stronger than glycine ethyl ester. The ratio of Michaelis constants is in good agreement with the relative potencies of these compounds for inhibiting fibrin cross-linking.

Saturation kinetics in conjunction with several amine substrates have also been observed for transglutaminase (Pincus, 1966). Together with our findings, these appear to be rather rare examples for the moment, because saturation could not be achieved by a number of nucleophiles acting in the deacylation of either furoyl-chymotrypsin (Inward and Jencks, 1965) or *trans*-cinnamoyl-papain (Brubacher and Bender, 1967). Of course, some other nucleophiles which perhaps would suit the specificity requirements of these enzymes better might still give rise to saturation. In this connection, it would be of interest to examine, for example, the reactivity of *trans*-cinnamoyl-papain with 5-dansylamidopentamine.

Acknowledgment

Special thanks are due to Dr. R. P. Johnson of Abbott Laboratories, North Chicago, Ill., for the hydrochlorides of *N*-(7-aminoheptyl)-*p*-toluenesulfonamide, *N*-(6-aminohexyl)-*p*-toluenesulfonamide, and *N*-(4-aminobutyl)-*p*-toluenesulfonamide (Figure 5) and to Dr. R. G. Jones of Eli Lilly and Co. for the dihydrochlorides of 3-β-aminoethylpyrazole and 3-β-aminoethyl-1,2,4-triazole (Figure 8). Elementary analyses were carried out by Micro-Tech Laboratories, Inc., Skokie, Ill., and by Miss H. E. Beck of this Department.

Added in Proof

We have now directly demonstrated the presence of γ-glutamyl-ε-lysine in enzymatic digests of cross-linked fibrin. None of this dipeptide was found in similarly treated fibrin which was not cross-linked. Using the Beckman amino acid analyzer (custom AA-15 resin, 0.9 × 54 cm) with 0.2 N citrate buffer of pH 3.31 (66 ml/hr, 56°), the position of the reference γ-glutamyl-ε-lysine dipeptide (Cyclo) is (approximately at 283 min) located in the wide trough between the leucine (~228 min) and tyrosine (~313 min) peaks. Because of the wide separation of the latter two, the chromatography column could be loaded with more than 15 mg of protein digest to conclusively demonstrate the presence of γ-glutamyl-ε-lysine dipeptide in amounts even less than 0.05 μmole. Both fibrin and cross-linked fibrin (~50 mg) were sequentially digested (37°) with Pronase (3 × 1 mg, Calbiochem), Mn^{2+}-activated leucine aminopeptidase (2 × 0.25 mg, Worthington), and carboxy-

peptidase A (1 × 0.5 mg, Worthington), with enzyme additions made at 24-hr intervals. This digestion procedure reduced the proteins virtually to amino acids, but left the γ-glutamyl-ε-lysine dipeptide in cross-linked fibrin intact. A yield of about 1–2 moles of γ-glutamyl-ε-lysine/400,000 g of protein was obtained from the latter.

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