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Labeling of Amine-Acceptor Cross-Linking Sites of Fibrin by Transpeptidation*

L. Lorand and H. H. Ong

ABSTRACT: The transpeptidating enzyme of blood plasma which cross-links fibrin can also be used to introduce specific tracers into fibrin itself. Three known cross-linking inhibitors (glycine ethyl ester, hydroxylamine, and hydrazine) were shown to become incorporated to the extent of 1 mole/100,000 g of bovine fibrin. The sedimentation behavior of the modified protein indicates that glycine ethyl ester prevented cross-linking of fibrin by blocking mostly monomeric units.

The incorporation of amine substrates into fibrin can be taken as evidence for the existence of a fibrinyl–enzyme intermediate in the transpeptidation reaction with the cross-linking enzyme. Of the protein derivatives prepared by the enzymatic transpeptidation, fibrin hydroxamate seems to be best suited for further identification of the acceptor carbonyl groups in the polymerizing centers of fibrin. Since the number of acceptor sites in fibrin seems to equal that of the peptide bonds broken by thrombin in fibrinogen, it is suggested that the uncovering of these sites occurs as a result of the fibrinogen–fibrin transition (*i.e.*, through the release of the fibrinopeptide fragments). This is supported by the finding that, in the presence of the cross-linking enzyme, fibrinogen incorporates glycine ethyl ester at a much slower rate than fibrin.

The physiological cross-linking of fibrin is thought to occur (Lorand *et al.*, 1962; Lorand and Jacobsen, 1964; Lorand, 1965) by a transpeptidating mechanism in which donor amino groups of one fibrin (F) molecule react with acceptor carbonyl functions (COY) of another (F'). Dimerization between two of the protein molecules could thus be written as

$$H_2NFCOY + H_2NF'COY \longrightarrow H_2NFCONHF'COY + HY$$

Extended in both directions, this reaction represents the last event in normal blood clotting, and is catalyzed by FSF*[1] (Lorand and Konishi, 1964a; Konishi and Lorand, 1966). It also illustrates the ultimate bio-

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[1] Abbreviations used: FSF*, thrombin-activated fibrin stabilizing factor; GEE, glycine ethyl ester; TAME, N^{α}-*p*-tosyl-L-arginine methyl ester.

synthetic control for building protein structures by covalent bonds.[2]

As expected from transpeptidation, the cross-linking reaction can be selectively inhibited by certain amines. Glycine ethyl ester, an amine which mimics N-terminal portions of fibrin itself, was actually shown (Lorand and Jacobsen, 1964) to be a substrate for FSF* in the sense that the latter would catalyze its incorporation into fibrin. Such a spurious substrate would act as a chain terminator by reducing the size of cross-linked units. Under favorable circumstances, as will be seen below, fibrin may be blocked in the monomeric stage.

The present paper examines the FSF*-catalyzed incorporation of [1-^{14}C]glycine ethyl ester into fibrin in greater details. In addition, the catalytic incorporation of hydroxylamine and hydrazine is described.

Materials and Methods

Bovine fibrinogen, fibrin, fibrin-stabilizing factor (FSF), and thrombin were obtained by procedures previously detailed (Lorand and Konishi, 1964a). In preparing fibrin for the experiments with hydroxylamine and hydrazine, 1 mM iodoacetate was added to fibrinogen 0.5 hr before clotting with thrombin in an attempt to inactivate intrinsic FSF impurities (Lorand and Jacobsen, 1958; Lorand, 1961).

Incorporation of [1-^{14}C]glycine ethyl ester into fibrin was studied at pH 7.5 and 20° (room temperature) as a function of time and of initial glycine ester concentration. The reaction mixtures comprised 0.5 ml of 1 mM calcium chloride, 0.5 ml of 0.1 M cysteine (dissolved in a mixture of 0.05 M Tris and 0.1 M sodium chloride, adjusted to pH 7.5 with hydrochloric acid), 0.4 ml of FSF (56 μg of protein in Tris), and 0.1 ml of thrombin (2 NIH units in Tris). After allowing 10 min for the activation of FSF (to FSF*), 0.2 ml of 0.25 M N^{α}-*p*-tosyl-L-arginine methyl ester (TAME, dissolved in Tris) was added to terminate thrombin action and 0.5 ml of radioactive glycine ethyl ester in Tris solution (187 cpm/mμmole) was admixed to the desired concentration. Then 0.3 ml of fibrin solution (4.3 mg of protein in 1 M sodium bromide, pH 5.4) was introduced vigorously. After various time intervals, the reaction was stopped by stirring with 2.5 ml of 14% trichloroacetic acid. The protein precipitate was centrifuged and washed with 7% trichloroacetic acid (about 10 × 20 volumes) over a period of 3 days to remove radioactivity not bound to the protein. After transferring the proteins into clean tubes, a final wash was carried out with water which was checked to see whether extraneous radioactivity in the supernatants had indeed been reduced to background level. The protein precipitates were then taken up in 2 ml of 0.6 N sodium hydroxide; aliquots were used for protein determination (Hirsch and Cattaneo, 1956) and for counting of radioactivity with a Packard liquid scintillation counter. For the latter purpose, the samples were neutralized with hydrochloric acid (aqueous phase 1 ml) just prior to mixing with 15 ml of scintillation fluid (Bray, 1960). Frequently, known amounts of radioactivity were added back to the protein samples to determine the extent of quenching by the protein. Since this never amounted to more than 10% of the counts, no correction was applied in calculating the results. The extent of incorporation was computed from a calibration curve obtained by diluting the stock isotopic glycine ethyl ester solution.

Incorporation of hydroxylamine and hydrazine into fibrin was studied as a function of time, at added amine concentration of 18 mM, under conditions similar to those employed for the incorporation of isotopic glycine ethyl ester. In a typical experiment, 12 ml of FSF (*ca.* 0.01% protein) was activated to FSF* by admixing with 15 ml of 1 mM calcium chloride, 15 ml of 0.1 M cysteine, and 3 ml of thrombin (60 NIH units). After 10 min, 6 ml of TAME (0.25 M) was added, and this was followed by the addition of 15 ml of 0.09 M hydroxylamine or hydrazine (dissolved in the Tris buffer, with pH adjusted to 7.5) and 9 ml of fibrin (1.5% protein in 1 M sodium bromide, pH 5.4). As the gel began to form it could be loosely wound around a glass rod while still in the reacting medium. At the end of the reaction period (30 min or 2 hr) the gel was pressed with the glass rod to quickly drain off the fluid, and was then placed in 80 ml of 0.15 M saline and kept at 0° overnight. After centrifugation, the protein sediments were dialyzed *vs.* water (5 × 5 l.) for 48 hr to remove unbound hydroxylamine or hydrazine. The washed proteins, derived from individual reactions, were dried separately to constant weight *in vacuo* at 0.05 mm. They were pulverized and then stored in a desiccator at 0°. In order to measure the hydroxamate and hydrazide contents, it was essential to render the proteins soluble in water. Therefore 5 mg of the dry fibrin hydroxamate or fibrin hydrazide (or simply the control fibrin) powders was incubated with 1 ml of 0.1 M ammonium carbonate containing 0.3 mg of trypsin (activity: 9700 BAEE units/mg as purchased from Mann Laboratories, N. Y.) at 25° for 24 hr.

Estimation of hydroxymate by formation of nitrite was carried out by the procedure of Bergmann and Segal (1956) as detailed by Seifter *et al.* (1960). The 1 ml of trypsinized fibrin hydroxamate (digest of 5 mg of dry powder) was mixed with 4 ml of 6% sodium acetate, 0.5 ml of sulfanilic acid reagent, and 0.25 ml of iodine solution. The mixture was allowed to stand for 10 min. Then 0.15 ml of 0.1 N sodium thiosulfate was added to decolorize iodine and this was followed by 0.1 ml of the α-naphthylamine reagent. A bright pink coloration ensued immediately and the optical density at 520 mμ was read exactly 10 min later against a reagent blank. A reference curve was constructed with the use of acetohydroxamic acid, added in various concentrations to the trypsin digests of 5 mg of control fibrin samples. When measured in a 1-cm cell, 0.05

[2] Transpeptidation has recently been invoked also in connection with two other important biosynthetic reactions: the penicillin-sensitive cross-linking of bacterial cell wall (Wise and Park, 1965; Tipper and Strominger, 1965) and the cross-linking of collagen (Rubin *et al.*, 1963; Drake *et al.*, 1966).

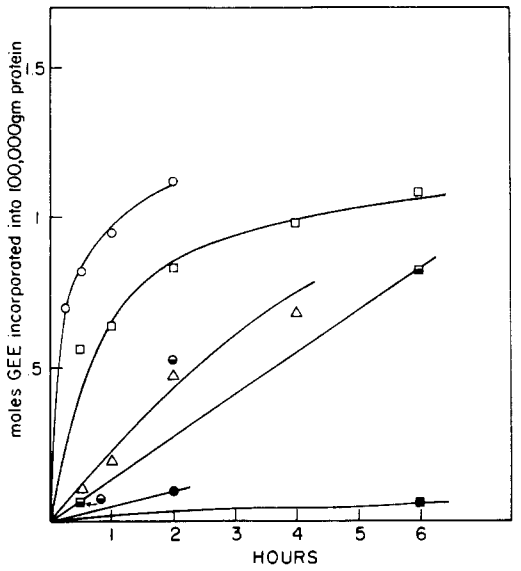

FIGURE 1: Incorporation of [1-^{14}C]glycine ethyl ester (pH 7.5, 20°) into fibrin and fibrinogen. For experimental details see text. A (○) and B (□), fibrin with 40 and 10 mM GEE, respectively, in the presence of added transpeptidase (FSF*), cysteine, and calcium ions. C (◒) and D (◪), fibrin with 40 and 10 mM GEE, in the presence of cysteine and calcium ions only. E (●) and F (■), fibrin with 40 and 10 mM GEE, in the presence of calcium ions alone. G (△), fibrinogen with 40 mM GEE and with added enzyme as in A.

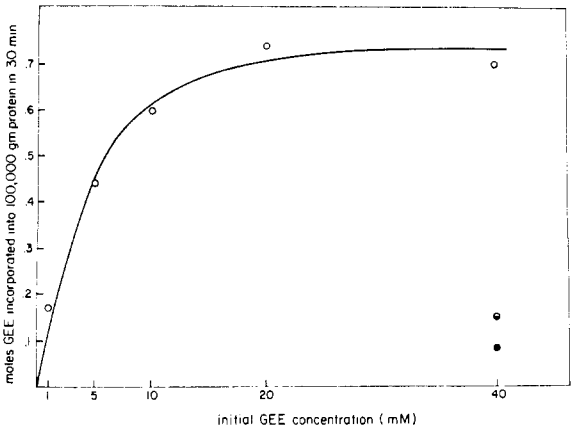

FIGURE 2: Velocity of incorporation of [1-^{14}C]glycine ethyl ester (pH 7.5, 20°), as a function of concentration of this substrate, into fibrin. For experimental details see text. (○) complete system, with added transpeptidase (FSF*), cysteine, and calcium ions. (◒) in the presence of cysteine and calcium ions only. (●) in the presence of calcium alone.

μmole of acetohydroxamic acid under these conditions gave an absorbance of 0.155.

Hydrazine was measured with the use of *p*-dimethylaminobenzaldehyde (dissolved in ethanol–hydrochloric acid), as employed by Seifter *et al.* (1960) for gelatin. Trypsin digests (1 ml) of 5 mg of fibrin hydrazide powders were mixed with 2 ml of the dimethylaminobenzaldehyde reagent and heated for 90 min at 80–85° on a water bath. After cooling to room temperature, volumes were readjusted to 3 ml by adding 95% ethanol. Trace amounts of insoluble material often visible at this stage were removed by centrifugation. Absorbances of the solutions at 450 mμ were read *vs.* a blank made up by mixing 1 ml of water and 2 ml of the dimethylaminobenzaldehyde reagent. A standard curve was prepared with hydrazine sulfate solution at various strengths in the presence of 5 mg of control fibrin digest. Under these conditions and measured in a 1-cm cell, 0.05 μmoles of hydrazine gave an absorbance of 1.2 mμ.

Results and Discussion

The incorporation of [1-^{14}C]glycine ethyl ester (GEE) into fibrin, as catalyzed by added FSF*, is seen in Figure 1. Rates were measured with 40 mM (A) and 10 mM (B) of initial GEE concentrations. One set of controls comprised the Tris buffer, calcium chloride, cysteine, GEE (40 mM, C, or 10 mM, D), and fibrin components of the reaction. Another set contained only Tris buffer, calcium chloride, GEE (40 mM, E, or 10 mM, F), and fibrin. Controls C and D were designed so as to estimate the cysteine-sensitive stabilizing factor impurities in the fibrin preparation. They would also include the catalytic effect, if any, of cysteine itself on the reaction. Controls E and F may be taken to express the activity of stabilizing factor contaminants in fibrin, functioning without cysteine, and would also indicate whatever nonspecific uptake of GEE might have taken place.

Among the conclusions which may be drawn from the data in Figure 1, the most important one is that the incorporation of labeled GEE into fibrin is a very limited one. This certainly reflects on the specificity of the FSF*-catalyzed reaction. Although rates for incorporating GEE are quite different when 40 or 10 mM of this substrate is added, the extent of incorporation in both instances appears to be the same. When A is corrected for E (*i.e.*, A − E) and B corrected for F (*i.e.*, B − F), the net incorporation of GEE approaches about 1 mole/100,000 g of fibrin. This leveling off suggests the availability of only one amine-acceptor site/100,000 g of native fibrin. It should be emphasized that, in these experiments, incorporation of GEE into fibrin occurred while this protein existed in the gel state, close to physiological pH and ionic strength.[3] One might reasonably suppose that under different conditions the rate and perhaps even the extent of incorporation

[3] This heterogeneous system does not lend itself readily for renewing its GEE and FSF* components which might have been destroyed during the prolonged reaction. Also, possible delabeling of the GEE-fibrin has not been considered. These problems will be the subject of further investigations.

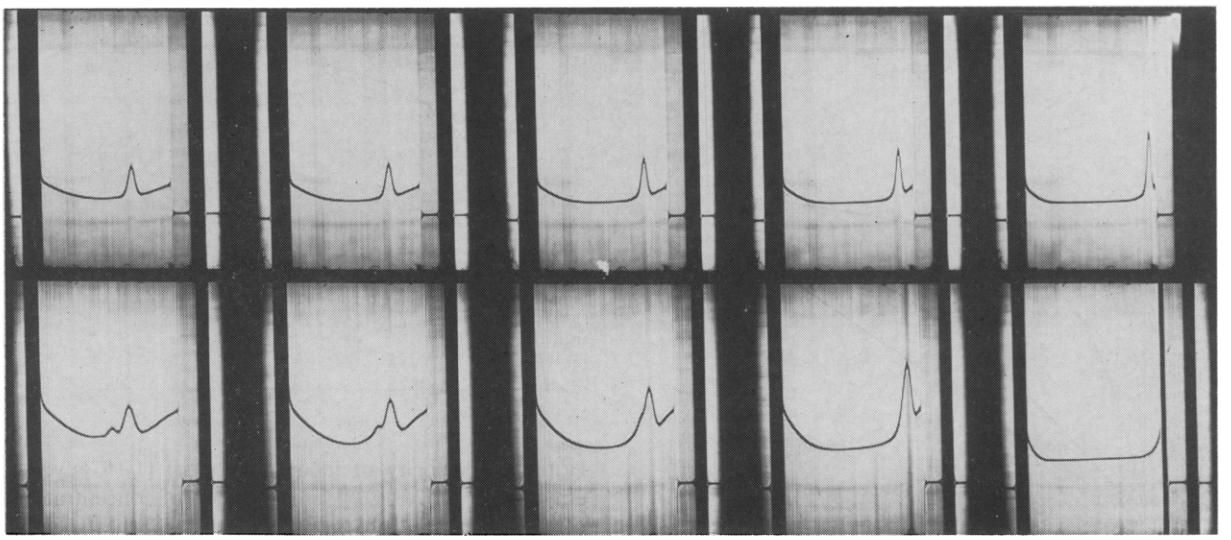

FIGURE 3: Sedimentation of fibrin (upper row) and of fibrin labeled with glycine ethyl ester (bottom row) in the ultracentrifuge. Solvent: 1 M sodium bromide at pH 5.4 with acetic acid; upper, 0.5% protein; 12-mm cell; temperature 23°; speed 59,780 rpm; 8-min intervals; bottom, 0.65% protein; 30-mm cell; temperature 23.9°; speed 50,740 rpm; 16-min intervals.

would be different, so that our measured values may not be maximal.

The finding that incorporation apparently stops with 1 mole of GEE/100,000 g of fibrin in reactions with very different initial rates would seem to argue against the notion that they are terminated only on account of premature inactivation of FSF*.

While the stability of GEE in the system is unknown, the velocity of incorporation into fibrin, at least during the first 30 min of reaction, as seen from independent data in Figure 2, is close to maximal in the 10–40 mM GEE concentration range. Thus, experiment A in Figure 1 could still have proceeded with at least two-thirds the rate if only one-quarter of the added amount of GEE survived. Clearly then, leveling off of curve A (Figure 1) cannot be ascribed to exhausting the supply of GEE. Apart from the implications just mentioned, Figure 2 shows the expected saturation behavior of the incorporating system with respect to the GEE reactant.

The apparent catalytic enhancement with 56 μg of added FSF*, when acting at 40 mM GEE concentration, was *ca.* 60-fold (ratio of initial slope of A over slope of E, in Figure 1). Without added FSF*, but in the presence of cysteine and otherwise similar conditions, enhancement was approximately fivefold (ratio of initial slope of C over slope of E, in Figure 1). If one assumes that incorporation in C was due to cysteine-sensitive stabilizing factor impurities in fibrin and that the specific activity of this impurity is identical with that of the FSF* added in A, one calculates that the 4.3 mg of fibrin present in the system could have been contaminated with 4–5 μg of the factor (*i.e.*, $^{56}/_{60} \times 5$ μg). While the former assumption is plausible and is based on earlier observations (Lorand and Jacobsen, 1958; Lorand, 1961), the latter is quite uncertain. It should also be mentioned that the above calculation presumes a linear relationship between rate of GEE incorporation and concentration of the factor.

It was of great interest to see whether fibrinogen would also have acceptor groups available for incorporating GEE when FSF* was added. Fibrinogen is the parent protein from which fibrin is derived by the scission of only 3% of the total protein (released as fibrinopeptide fragments) following limited proteolysis by thrombin (Lorand, 1951, 1952). Unless the availability of GEE-incorporating groups on the protein depends on this subtle molecular transition, fibrinogen should be as good a substrate for this purpose as fibrin. This, however, is not the case. In curve G of Figure 1, fibrinogen was taken at the same concentration as fibrin in A and otherwise under identical conditions (*i.e.*, 40 mM GEE). Yet, the rate of GEE incorporation with fibrinogen was only *ca.* one-sixteenth of that found with fibrin. Therefore, the sites available in fibrinogen for incorporating GEE are other than those existing in fibrin or they are somehow hindered in the kinetic sense. When rationalizing the action of thrombin on fibrinogen, one should clearly emphasize the importance of uncovering these sites. Since this is accomplished by a process of very limited breakdown of the protein (Lorand 1951, 1952), without the latter undergoing any major structural change (Bailey *et al.*, 1943; Hall and Slayter, 1959; Stryer *et al.*, 1963), it is not unreasonable to suppose that the GEE-incorporating sites of fibrin are close to those regions of the molecule from where the fibrinopeptides were released. At least, the number of these newly opened sites agrees well with the number of peptide bonds broken by thrombin in fibrinogen (Lorand and Middlebrook, 1952); *i.e.*, approximately four sites/400,000 g of

fibrinogen which is close to the measured molecular weight of this protein (Shulman, 1953).

The mere fact that fibrinogen possesses GEE-incorporating sites at all and that it can function as a substrate of the cross-linking enzyme is of considerable interest. As suggested before (Lorand, 1965; Lorand and Jacobsen, 1965), it carries the implication that, in the presence of FSF*, donor groups of fibrin might occasionally react with acceptor functions of fibrinogen (rather than with those of fibrin, *e.g.*, when the latter is produced slowly in dilute solutions) thereby forming hybrid polymers of fibrinogen and fibrin in various proportions. This may actually be the mechanism by which some cold-insoluble globulins might arise. In an abstract just published (Sasaki *et al.*, 1966), formation of stable complexes between fibrinogen and fibrin by the action of FSF* is described, which is in agreement with the above suggestion.

It should be pointed out that fibrinogen has been known to act as an amine-acceptor substrate in conjunction with the guinea pig liver transglutaminase enzyme (Clarke *et al.*, 1959). This underscores the mechanistic similarities (and even those of specificity) between the functioning of FSF* and transglutaminase.

Glycine ethyl ester prevents the cross-linking of fibrin, so that even in the presence of FSF* and calcium ions, the gel remains soluble when tested by the addition of 1% monochloroacetic acid, 5 M urea, or 1 M sodium bromide (Lorand *et al.*, 1962; Lorand and Jacobsen, 1964). Thus, in contrast to the controls without glycine ethyl ester, only relatively low oligomers of fibrin could have become cross-linked. It was clearly of interest to determine at what stage of polymerization fibrin would be blocked when conditions similar to those of A in Figure 1 were employed. An experiment with 40 mM of cold glycine ethyl ester was carried out in the manner described and, at the end of 1 hr, the gel was taken up in 1 M sodium bromide (pH adjusted to 5.4 with acetic acid). The derivatized protein was precipitated by dilution, as described for isolating fibrin (Donnelly *et al.*, 1955), and was redissolved in the sodium bromide solvent. *Ca.* 40% of the starting fibrin was recovered in this solution and it was then examined in a Spinco Model E ultracentrifuge (Figure 3). While the control fibrin showed a single peak with a sedimentation coefficient of 6.26, the fibrin which was enzymatically derivatized by glycine ethyl ester gave two peaks.[4] The major peak, with a coefficient of 6.30, was assumed to correspond to the monomeric protein; the minor peak (coefficient of 8.46) to dimers. As judged by the ratios of areas under the two peaks, the derivatized fibrin was *ca.* 77% monomeric and 23% dimeric.

Based on the behavior of other glycine derivatives acting as inhibitors of fibrin cross-linking (Lorand and Jacobsen, 1964), it may be reasonably surmised that

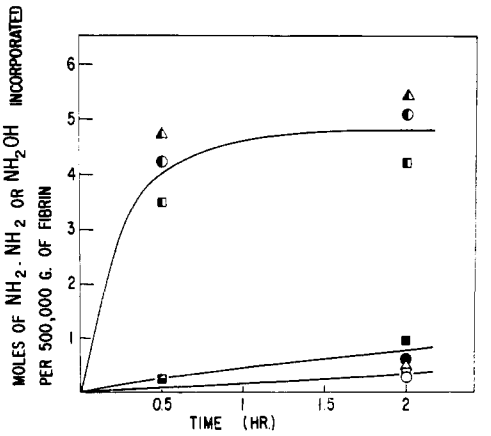

FIGURE 4: Incorporation of hydrazine or hydroxylamine into fibrin (pH 7.5, 20°). For experimental details see text. Hydroxamate determined by nitrite formation. (◐) and (◧) represents the complete system with hydrazine; (◭), complete system with hydroxylamine, (●) and (■), incorporation of hydrazine with all components of the complete system, with exception of fibrin-stabilizing factor, present; (○) and (□), fibrin and hydrazine only; (△), fibrin and hydroxylamine only.

glycine ethyl ester is, indeed, incorporated through its amino groups[5] (and not primarily through its ester functions) into the protein

$$H_2NFCOY + H_2NCH_2COOEt \longrightarrow H_2NFCONHCH_2COOEt + HY$$

Nevertheless, it was essential to extend these studies to amines which would more definitely label only the acceptor polymerizing functions of fibrin. Since hydroxylamine and hydrazine are as effective cross-linking inhibitors (Lorand and Jacobsen, 1964; Lorand, 1965) as glycine ethyl ester, and since sensitive chemical methods are available for analyzing protein hydroxamates and hydrazides (Seifter *et al.*, 1960), the FSF*-catalyzed incorporations of these compounds into fibrin were studied. It is immediately apparent from Figure 4 that results with both hydroxylamine and hydrazine are in excellent agreement with those obtained by using glycine ethyl ester. No matter which of these tracers is used, *ca.* 1 mole seems to become incorporated into 100,000 g of fibrin. Furthermore, labeling with hydroxylamine and hydrazine is more certain to take place in the following manner

$$NH_2FCOY + H_2NOH \text{ (or } H_2NNH_2) \longrightarrow H_2NFCONHOH \text{ (or } H_2NFCONHNH_2) + HY$$

[4] In more recent experiments (March 1966), Dr. S. Tokura in this laboratory observed only a single peak in glycine ethyl ester derivatized fibrin which sedimented just like the parent protein.

[5] Direct chemical analysis indicates that the amino groups of GEE are involved in incorporation. The ether extract of the 8-hr acid (5.7 N HCl) hydrolysate of 1-fluoro-2,4-dinitrobenzene-derivatized (Sanger, 1945) GEE-fibrin contained <1% of the radioactivity of the hydrolysate.

Though not presented in detail, fibrin hydroxamate was also analyzed by complexation with ferric ions (Hill, 1947; Seifter *et al.*, 1960). This method was considered less reliable than the procedure based on the formation of nitrite (as given in Figure 4) because control protein samples themselves gave a rather intense coloration (corresponding to an apparent uptake of *ca.* 1 mole of hydroxylamine/250,000 g of fibrin in the blank). Nevertheless, the net incorporation of hydroxylamine in the presence of FSF* agreed well (*ca.* 1 mole/90,000 g of fibrin) with results obtained by nitrite determination.

The incorporation of amines provides strong evidence in favor of an acyl-enzyme intermediate in the FSF*–fibrin reaction. The similarities between the functioning of papain and FSF* have already been pointed out (Lorand and Konishi, 1964b). Our survey of better transpeptidating enzymes shows that, including papain and FSF*, they all fall into the category of sulfhydryl enzymes. This may be related to the fact that thiol esters react more readily with amines than do oxygen esters (Connors and Bender, 1961). It should also be recalled that acyl-papain, now an established intermediate in papain-catalyzed reactions (Smith and Kimmel, 1960; Lowe and Williams, 1964; Bender and Brubacher, 1964), is believed to undergo aminolysis in favor of hydrolysis at mildly alkaline pH values (see Fruton, 1957; Brubacher, 1965). In view of these facts, the incorporation of amines (illustrated for hydroxylamine) into fibrin with the participation of the thiol groups of FSF*, would suggest the following three-step mechanism

$$H_2NFCOY + HS(FSF^*) \rightleftharpoons [H_2NFCOY \cdots HS(FSF^*)] \downarrow H_2NFCOS(FSF^*) + HY \xrightarrow{H_2NOH} H_2NFCONHOH + HS(FSF^*)$$

In the absence of added amines (such as hydroxylamine), deacylation of the fibrinyl-enzyme compound would take place by the utilization of the donor amino groups of another fibrin molecule (H_2NFCOY), resulting in the formation of cross-links between the proteins themselves.

The incorporation of specific tracers into the polymerizing centers of fibrin, with the use of the cross-linking enzyme, is of immediate significance for the protein chemical exploration of these sites. Among the three derivatives (glycine ethyl ester, hydroxylamine, and hydrazine) of fibrin, the fibrin hydroxamate seems best suited for a direct approach. As in the case with gelatin (Gallop *et al.*, 1960), a Lossen rearrangement of the dinitrophenylated fibrin hydroxamate reveals the nature of the acceptor carbonyl functions. Both γ and β carbonyl groups of glutamine and asparagine were shown to be involved (Lorand and Ong, 1966).

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Sedimentation and Actinomycin D Binding Studies of Partially Denatured Crab dAT*

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ABSTRACT: That poly dAT of *Cancer antennarius* which has been heated and cooled possesses a partially denatured character has been shown by two additional methods, sedimentation analysis and actinomycin D inhibition of ribonucleic acid (RNA) synthesis. The once-melted dAT sediments more rapidly than does truly native dAT. Actinomycin inhibits RNA synthesis by *Escherichia coli* RNA polymerase less with the once-melted dAT template than with native dAT. The poly dAT component of *C. borealis* deoxyribonucleic acid (DNA) has been separated by the mercury-binding method. This dAT, which contains 2.5% guanine plus cytosine (GC) compared to 3.5% for *C. antennarius* dAT, behaves in a manner similar to that of *C. antennarius* dAT.

When melted and cooled, its T_m is lower and it melts over a wider temperature range than does unmelted *C. borealis* dAT. Its CsCl buoyant density is slightly increased and the actinomycin inhibition of RNA synthesis with the dAT template is lessened. The differences observed between the melted and unmelted forms are, however, less for the *C. borealis* dAT than those found for *C. antennarius* dAT probably because the former dAT has a lower GC content.

Sueoka (1961) has shown that poly dAT[1] is present in the DNA of crabs of the genus *Cancer*. The poly dAT component of the DNA of *Cancer borealis* contains about 3% GC interspersed in an otherwise alternating dAT structure (Swartz *et al.*, 1962). Sueoka and Cheng (1962a) isolated the dAT with a MAK column after heating and cooling the *C. borealis* DNA. This column separates the renatured dAT from the denatured typical major component DNA. The dAT component isolated in this way has been used as a template for DNA polymerase (Swartz *et al.*, 1962), for RNA polymerase (Goldberg *et al.*, 1962, 1963; Cheng and Sueoka, 1964), and for actinomycin binding studies (Goldberg *et al.*, 1962).

In a previous paper from these laboratories the development of a new dAT separation technique based on the preferential binding of Hg^{2+} to dAT was reported (Davidson *et al.*, 1965). It was further shown that *C. antennarius* dAT remains partially denatured after heating and cooling. This is probably due to the nonpairing of (mainly) G and C bases during renaturation. Differences were shown to exist between the native and once-melted forms by methods based upon optical density melting, electrophoresis, electron microscope, buoyant density, and *Escherichia coli* exonuclease I susceptibility.

Pochon *et al.* (1965) have also come to the conclusion that the once-melted dAT form is different from the original native form. They utilized optical density

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[1] Abbreviations used in this work: A, adenine; U, uracil; G, guanine; C, cytosine; dAB$\bar{U}$, alternating deoxyadenylate–deoxy-5-bromouridylate copolymer; dAT, alternating deoxyadenylate–deoxythymidylate copolymer; MAK, methylated albumin kieselguhr; σT, transition width in degrees centigrade between 20% below midpoint and 20% above midpoint of the OD melting profile; SSC, standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate); T_m, temperature at midpoint of the OD transition obtained from the OD melting profile; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.