Bachmann, F., Duckert, F., and Koller, F. (1958), *Throm. Diath. Haem. 2*, 24.
Bell, W. N., and Alton, H. G. (1954), *Nature 174*, 880.
Bergsagel, D. E., and Hougie, C. (1956), *Brit. J. Haematol. 2*, 113.
Biggs, R., Douglas, A. S., and Macfarlane, R. G. (1953), *J. Physiol. 119*, 89.
Denson, K. W. E. (1958), *Brit. J. Haematol. 4*, 313.
Duckert, F., Yin, E. T., and Straub, W. (1960), *8th Colloq. Prot. Biol. Fluids*, Brugge, Belgium, Amsterdam, Elsevier, p. 410.
Esnouf, M. P., and Williams, W. J. (1962), *Biochem. J. 84*, 62.
Ferguson, J. H., Wilson, E. G., Iatridis, S. G., Rierson, H. A., and Johnston, B. R. (1960), *J. Clin. Invest. 39*, 1942.
Ferguson, J. H., and Wilson-Ennis, E. G. (1963), *Thromb. Diath. Haem. 9*, 62.
Flodin, P. (1961), *J. Chromatog. 5*, 103.
Flodin, P., and Killander, J. (1962), *Biochim. Biophys. Acta 63*, 403.
Flyn, J. E., and Coon, R. W. (1953), *Am. J. Physiol. 175*, 289.
Fisch, V. (1959), *Thromb. Diath. Haem. 2*, 60.
Hougie, C., Barrow, E. M., and Graham, J. B. (1957), *J. Clin. Invest. 36*, 485.
Kappeler, R. (1955), *Z. Klin. Med. 153*, 103.
King, E. J. (1932), *Biochem. J. 26*, 292.
Koller, F., Loeliger, A., and Duckert, F. (1951), *Acta Haematol. Basel 6*, 1.
Kowarzyk, H., and Marciniak, E. (1961), *Pol. Tyg. Lek. 16*, 1641.
Lowry, O. H., Rosebrough, N. I., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Macfarlane, R. G. (1961), *Brit. J. Haematol. 7*, 496.
Macfarlane, R. G. (1964), *Nature 202*, 498.
Macfarlane, R. G., and Ash, B. J. (1964), *Brit. J. Haematol. 10*, 217.
Marciniak, E., and Seegers, W. H. (1962), *Can. J. Biochem. Physiol. 40*, 591.
Mares-Guia, M., and Shaw, E. (1963), *Fed. Proc. 22*, 528.
Milstone, J. H. (1962), *J. Gen. Physiol. 45*, No. 4, pt. 2 (Suppl), 103.
Nemerson, Y., and Spaet, T. H. (1964), *Blood 23*, 657.
Papahadjopoulos, D., Hougie, C., and Hanahan, D. J. (1964), *Biochemistry 3*, 264.
Papahadjopoulos, D., Hougie, C., and Hanahen, D. J. (1962), *Proc. Soc. Exptl. Biol. Med. 111*, 412.
Papahadjopoulos, D., Yin, E. T., and Hanahan, D. J. Unpublished observations.
Pechet, L., and Alexander, B. (1960), *Fed. Proc. 19*, 64.
Proctor, R. R., and Rapport, S. (1961), *Am. J. Clin. Pathol. 36*, 212.
Ratnoff, O. D., and Davie, E. W. (1962), *Biochemistry 1*, 677.
Seegers, W. H., McClaughry, R. I., Fahey, J. L. (1950), *Blood 5*, 421.
Spaet, T. H., and Cintron, J. (1963), *Blood 21*, 745.
Straub, W. (1960), *Thromb. Diath. Haem. 4*, 451.
Straub, W., and Duckert, F. (1961), *Thromb. Diath. Haem. 5*, 402.
Streuli, F. (1959), *Thromb. Diath. Haem. 3*, 194.
Toohey, M. (1958), *J. Clin. Pathol. 11*, 56.
Weichselbaum, T. E. (1946), *Am. J. Clin. Pathol.* Techn. Suppl. *10*, 40.
Williams, W. J. (1964), *J. Biol. Chem. 239*, 933.
Yin, E. T. (1964), *Thromb. Diath. Haem.*, in press.

Specific Inhibitors and the Chemistry of Fibrin Polymerization

L. Lorand and A. Jacobsen

From the Department of Chemistry, Northwestern University, Evanston, Illinois

Received July 16, 1964; revised September 22, 1964

Our earlier proposal that the polymerization of fibrin occurs by a transpeptidating (synonymously called transamidating) mechanism predicts the existence of two types of specific inhibitors of the reaction. Amine compounds would compete with the donor end-amino (glycyl) residues of the attacking fibrin, whereas compounds with carbonylamide functions would compete with the acceptor groups of the attacked fibrin particle. Specific inhibitors of both varieties were shown to exist. The former can be typified by glycine ethyl ester, the latter by carbobenzoxy-L-asparagine amide. Glycine ethyl ester was actually shown to be incorporated into fibrin if present during the polymerization reaction. Chemical and biological implications of the selective inhibitors of fibrin polymerization are discussed.

The terminal phase of blood clotting in vertebrates consists of the following major steps which, in essence, represent the interaction of three blood proteins: fibrinogen, thrombin, and the fibrin-stabilizing factor (FSF).

$$\text{fibrinogen} \xrightarrow{\text{thrombin}} \text{fibrin} + \text{fibrinopeptide}^1 \quad (1)$$

$$n\ \text{fibrin} \underset{\text{e.g., 30\% urea}}{\overset{Ca^{2+}}{\rightleftharpoons}} \text{fibrin aggregates} \quad (2)$$

$$\text{FSF} \xrightarrow[Ca^{2+}]{\text{thrombin}} \text{FSF}^* \quad (3)$$

$$n\ \text{fibrin (or fibrin aggregates)} \xrightarrow[Ca^{2+}]{\text{FSF}^*} \text{fibrin polymer} \quad (4)$$

Step 1 describes the limited proteolysis of fibrinogen by thrombin (Lorand, 1951, 1952). The removal of fibrinopeptide[1] from the parent protein drastically alters the solubility of the product (fibrin) so that at neutral *p*H ($\mu \sim 0.15$; 20°) the latter will aggregate into a gel (step 2). This gel, however, can be reversibly dispersed (Lorand, 1948, 1950; Laki and Lorand, 1948; Mihalyi, 1950; Lorand and Middlebrook, 1952; Donnelly *et al.*, 1955) in a number of solvents such as 30% urea, 1% monochloroacetic acid, or 10% sodium bromide. In contrast, the gel produced during the

[1] After the characterization of fibrinopeptide (Lorand, 1951, 1952) another peptide, called peptide B or fibrinopeptide B, was recovered from the clot liquor (Bettelheim and Bailey, 1952). It is, however, released at a much slower rate, and its removal from the protein is definitely not a prerequisite for the reversible gelation of fibrin in step 2 (Blomback and Vestermark, 1958). It is not known whether fibrinopeptide B must be released from fibrin prior to the polymerization reaction in step 4.

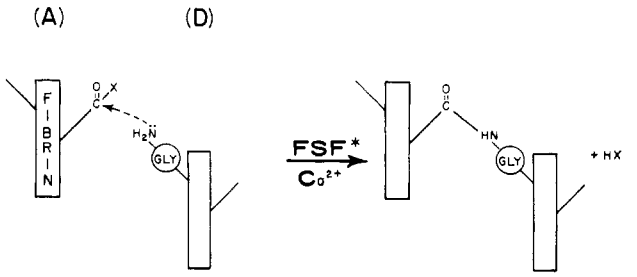

FIG. 1.—Proposed mechanism of fibrin polymerization. Only the interaction between two fibrin particles is shown. Ignoring probable intermediates between FSF* and the "acceptor" fibrin (A), the reaction would represent a nucleophilic displacement initiated by the N-terminal glycine of the "donor" fibrin (D), as marked by the broken arrow. *X* might be any of a variety of substituents (such as primary or secondary amine, alcohol, hydroxyl). The leaving group (*HX*) might either be released into the clot liquor or remain attached to the protein.

course of normal blood coagulation is insoluble in the solvents mentioned (Robbins, 1944; Lorand, 1948; Laki and Lorand, 1948). It can therefore be regarded as a true polymeric network in which (unlike the gel-product of step 2) the particles are linked by covalent bonds. This feature is also evident from the mechanical properties of the gel (Ferry *et al.*, 1951). The actual polymerization (or cross-linking) process is due to the functioning of the fibrin-stabilizing factor (FSF), recognized to be one of the plasmaglobulins (Lorand, 1954). However, as recently shown (Lorand and Konishi, 1964a), FSF is only the precursor of the active polymerizing principle (FSF*) which arises from the former as a result of thrombin action (step 3). The actual cross-linking of fibrin to yield a urea- or acid-insoluble gel (step 4) under the influence of FSF* also requires the presence of calcium ions.[2]

After methods had been worked out for selectively studying step 4 (Lorand *et al.*, 1962), an inquiry could be initiated into the chemical basis of fibrin polymerization per se. While experiments with ill-defined systems (which incorporated all steps 1–4) produced the clearly erroneous conclusion that polymerization of fibrin was due to an exchange of disulfide groups (Loewy and Edsall, 1954; Loewy *et al.*, 1961) or to the hydrolysis of a glycoside (Laki and Chandrasekhar, 1963), study of the isolated step 4 allowed us to recognize immediately that a transpeptidating (i.e., transamidating) mechanism[3] was the crucial feature of this reaction (Lorand *et al.*, 1962). We proposed that some of the N-terminal glycine residues of one fibrin molecule reacted with suitable functions (possibly activated carbonyl) of a neighboring fibrin particle, thereby producing peptide-type bonds. The present paper provides further evidence for the operation of such a transpeptidating mechanism in fibrin polymerization.[4]

MATERIALS

[*1*-^{14}C] *Glycine ethyl ester* hydrochloride was obtained from the New England Nuclear Corporation, Boston. It was dissolved into a solution of "cold" carrier in a tris (hydroxymethylaminomethane; 0.05 M)-sodium chloride (0.1 M) buffer, adjusted to *p*H 7.5 with hydrochloric acid, to yield a 0.2 M solution of the glycine ethyl ester with a specific radioactivity of about 0.1 mc/mmole. Aliquots of this stock solution were counted in a Packard scintillation counter to construct a calibration curve between radioactivity and concentration of glycine ethylester.

Bovine *fibrin*, *thrombin*, and *fibrin-stabilizing factor* were purified by procedures previously detailed (Lorand and Konishi, 1964a).

N-Tosyl-L-arginine methyl ester (TAMe), *glycine methyl- and ethyl ester*, and L-*cysteine* (as hydrochlorides) as well as *N-carbobenzoxy glycine amide* were purchased from Mann Research Laboratories, New York. *N-Carbobenzoxy-L-asparagine* (mp 165; N: 10.5%), *N-carbobenzoxy-β-alanine amide* (mp 164; N: 12.5%), and *N-carbobenzoxy-L-asparagine amide* were products of Cyclo Chemical Corporation, Los Angeles. In view of the special significance of the last-named compound, it was recrystallized from dimethylformamide by Dr. N. G. Rule of this laboratory to give a product with a mp of 223–224° and the following elementary analysis: Found C, 54.34%; N, 15.49%; H, 5.82%. $C_{12}H_{15}O_4N_3$ requires C, 54.4%; N, 15.8%; H, 5.7%.

For use in experiments, these compounds were freshly made up in $\mu \sim 0.15$ Tris buffer with readjustment of *p*H to 7.5 by addition of sodium hydroxide.

EXPERIMENTAL AND RESULTS

The working hypothesis that fibrin polymerization is a transpeptidation reaction (Lorand *et al.*, 1962) in which some of the terminal glycine amines of one fibrin are presumed to react with carbonyl functions of a neighboring fibrin (Fig. 1) predicts the existence of at least two types of specific inhibitors of fibrin polymerization. One group would include compounds which mimic the end amino residues (glycyl) of the attacking or *donor* fibrin molecule, whereas the other would simulate the *acceptor* carbonyl function of the attacked fibrin. Finding of such specific inhibitors would indirectly support the transpeptidation mechanism proposed and would provide invaluable probes for elucidating the chemical details of the polymerization of fibrin.

The donor (amine) type of inhibitors, exemplified by glycine methyl- or ethyl ester, has already been discussed elsewhere (Lorand *et al.*, 1962, 1963; see also Fig. 3, ○); thus a brief summary, given in Table I, will suffice.

The specificity of glycine derivatives is underlined by the fact that all other amino acid esters so far examined were by comparison poor inhibitors. Use of the compounds listed in Table I indicates that an unsubstituted amino function is essential for inhibition (compare glycine methyl ester with sarcosine methyl ester and glycine amide with carbobenzoxyglycine amide). Furthermore, the greater the fraction of the unprotonated amine existing at *p*H 7.5, the more effective it seems to be (compare glycylglycine with glycine). This prompted the suggestion (Lorand *et al.*, 1962), illustrated in Figure 2, that these inhibitors may function as nucleophiles and that they "may become temporarily incorporated into the clot proteins and act as transient chain terminators in the polymerization processes." With this concept in mind, the incorporation of radioactive glycine ethylester into fibrin was examined.

After we proposed the transpeptidation (i.e., transamidation) mechanism, the idea of disulfide exchange as a basis of fibrin polymerization was apparently abandoned in its favor. Loewy *et al.* (1964) in a preliminary note showed that another of the amine type

[2] Calcium ions, incidentally, are also known to accelerate the aggregation of fibrin in step 2 (Lorand and Konishi, 1964a).

[3] In a chemical sense, the word "transamidation" is synonymous with "transpeptidation" (Haurowitz, 1963).

[4] It is clear that either ammonia (Loewy *et al.*, 1964) or glycosylamine (Chandrasekhar *et al.*, 1964) could suitably serve as leaving group in the transpeptidation mechanism we proposed (Lorand *et al.*, 1962).

TABLE I
AMINE TYPE OF INHIBITORS OF FIBRIN POLYMERIZATION (AT *p*H 7.5)

Effective	Ineffective (up to 20 mM) concentration)
$H_2N \cdot CH_2 \cdot COO \cdot CH_3$	$CH_3 \cdot NH \cdot CH_2 \cdot COOCH_3$
$H_2N \cdot CH_2 \cdot COO \cdot CH_2 \cdot CH_3$	carbobenzoxy $\cdot NH \cdot CH_2 \cdot$ - $CO \cdot NH_2$
$H_2N \cdot CH_2 \cdot CO \cdot NH_2$	$H_2N \cdot CH_2 \cdot COO^- Na^+$
$H_2N \cdot CH_2 \cdot CO \cdot NH \cdot CH_2 \cdot$ - $COO^- Na^+$	
$H_2N \cdot OH$[a]	
Histamine[5]	

[a] Recent data (unpublished) suggest that some hydroxylamine derivatives, such as D-ethyl-2-amino-3-aminoxypropionate and 2,5-(diaminoxymethyl)-3,6-diketopiperazine (kindly supplied by Dr. F. C. Neuhaus of this department) are also very effective inhibitors.

of inhibitors (glycylglycine), which we described and suggested to be incorporated into fibrin (Lorand *et al.*, 1962), became protein-bound when fibrinogen was clotted under such conditions that all the overlapping steps of 1–4 would occur. Though important details of the experiment (e.g., whether glycylglycine did in fact inhibit polymerization at the fibrinogen concentration used) are yet to be published, it may well support our own.

First, in order to activate FSF to FSF*, 20 ml of 1 mM aqueous calcium chloride was mixed with 36 ml of FSF (6 mg protein) in 55 mM cysteine, and 4 ml of thrombin (80 units). Ten minutes later, thrombin activity was quenched by the addition of 8 ml of 0.25 M TAMe solution (Sherry and Troll, 1954), and soon thereafter 20 ml of the 0.2 M radioactive glycine ethylester solution was added. After this, 12 ml of fibrin (170 mg protein) in 1 M sodium bromide at *p*H (Donnelly *et al.*, 1955) was admixed. Gelation began almost instantaneously, and the gel obtained over about one and a half hours was squeezed out and dissolved by adding an equal volume of 2% monochloroacetic acid. (The fact that the entire gel dissolved in the acid provided assurance that the experiment was indeed performed under conditions where the 40 mM glycine ethyl ester prevented cross-linking of the gel. A pilot experiment, carried out in the absence of the ester, yielded a fully acid-insoluble clot, thus indicating the polymerizing potency of FSF*.) The protein was precipitated by adding 7% trichloroacetic acid (TCA), and the sediment obtained after centrifugation was taken up in about 30 ml of water. Precipitation of the protein with TCA, centrifugation of the sediment, and its redispersion in water were repeated ten times over two days, by which time the TCA-supernatant was freed from all radioactivity. The final protein sediment was dissolved in 1% monochloroacetic acid for counting radioactivity and for measuring Kjeldahl nitrogen (Chibnall *et al.*, 1943). Protein content was computed by assuming 17% N in fibrin.

The control comprised the radioactive glycine ethyl ester, fibrin, and the salt components of the experimental mixture, but no thrombin, TAMe, or FSF was added. The control gel was also dissolved in monochloroacetic acid and freed of radioactivity by repeated precipitation with TCA, exactly as with the experimental sample.

[5] The finding that a biogenic amine, like histamine, is as good an inhibitor as glycine ethyl ester suggests that the blood-clotting mechanism may act as a physiological amine-incorporating system. It also underlines the similarity between the functioning of FSF* and that of the liver transglutaminase enzyme of Clarke *et al.* (1959).

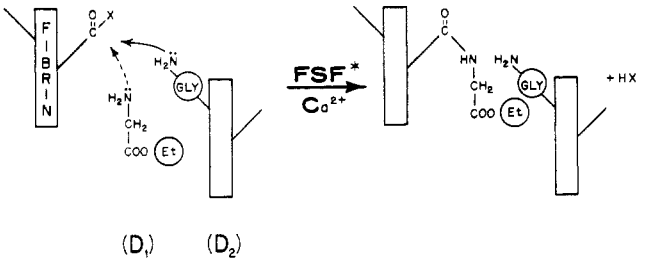

FIG. 2.—Suggested mode of action of the amine (i.e. donor) type of inhibitors of fibrin polymerization. Illustrated for glycine ethyl ester as the inhibitor. Competition between the two potential amine donors (D_1 and D_2) would inhibit fibrin polymerization as shown. At 20 mM glycine ethyl ester the concentration of D_1 is about a thousand times that of D_2.

Whereas a 1.12 mg aliquot of control fibrin showed radioactivity only at the background level (ca. 80 cpm), 2.04 mg of the fibrin, which was allowed to incubate with ^{14}C-glycine ethyl ester in the presence of FSF*, yielded 2143 cpm. According to the calibration curve with the stock radioactive ester solution, this amounted to 5.8×10^{-9} moles of glycine ethyl ester per mg of protein. Another experiment resulted in the incorporation of 4.4×10^{-9} moles of glycine ethyl ester per mg of protein. If the molecular weight of a fibrin unit (comprising two peptide chains with N-terminal residues of glycine and one chain with tyrosine [Lorand and Middlebrook, 1952]) is assumed to be 220,000, the results of the experiments described signify the incorporation of 1.2 and 0.9 moles of glycine ethyl ester per mole of fibrin, respectively.

Encouraged by the results obtained with the donor type of inhibitors, we began a search for specific inhibitors which might simulate the acceptor function of fibrin (see Fig. 1).

The experimental procedure for studying the inhibition of fibrin polymerization was identical to that described (Lorand *et al.*, 1962, 1963) in connection with the inhibitory amines, except that, on account of the relatively low solubility of some of the compounds investigated, the total volume of the polymerization mixture was increased from 2.5 to 3.0 ml. A typical experiment (Fig. 3) was carried out as follows. First, 0.5 ml of 1 mM aqueous calcium chloride, 0.5 ml of 0.1 M cysteine, 0.4 ml of FSF (90μg), and 0.1 ml of thrombin (2 units) were allowed to incubate for 10 minutes to activate the FSF. Then 0.2 ml of 0.25 M TAMe was added to quench thrombin activity. After this, 1 ml solution of the inhibitor in varying concentrations was admixed. After the addition of 0.3 ml of 1.5% fibrin solution, gelation occurred almost instantaneously. Half an hour later, the reaction was stopped by suspending the gel in an equal volume of 2% monochloroacetic acid. Protein on the acid-insoluble polymeric core of the gels was estimated 18 hours thereafter (Lorand and Jacobsen, 1958).

As mentioned above, compounds with an activated carbonyl function were thought to be the likeliest acceptor type of inhibitors. Since formamide, acetamide, propionamide, or carbobenzoxyglycine amide were all ineffective (Fig. 3, □), it became apparent that some specific structure was required for the inhibitory effect. For instance, the inhibitor might have to mimic a side-chain carbonylamide function, such as exists in asparagine or glutamine, of the protein. The possibility that these groups might serve as acceptors in fibrin has been emphasized previously (Lorand *et al.*, 1962; Lorand, 1963). Also, glutamine side-chains have been known to function as acceptors of amines in a number

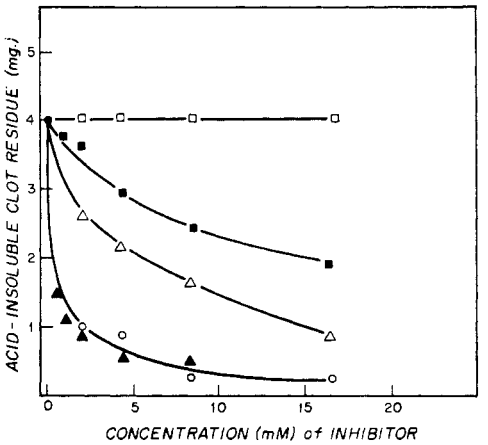

FIG. 3.—Specific amide (acceptor) type of inhibition of fibrin polymerization. For experimental procedures see text. Abscissa shows concentration of inhibitor, ordinate denotes the acid-insoluble core of fibrin gels polymerized in the presence of (□) formamide, acetamide, propionamide, or carbobenzoxyglycine amide; (■) carbobenzoxyglycine-β-alanine amide; (△) carbobenzoxyglycine-L-asparagine; (▲) carbobenzoxyglycine-L-asparagine amide. For comparison, the amine (donor) type of inhibition by glycine methyl- or ethyl ester (○) is shown.

TABLE II
AMIDE TYPE OF INHIBITORS OF FIBRIN POLYMERIZATION
(see also Fig. 3)

Effective	Ineffective
L-Carbobenzoxy·NH·CH($CONH_2$)··CH_2·$CONH_2$	$HCO·NH_2$
L-Carbobenzoxy·NH·CH(COO^-)··CH_2·$CONH_2$ Na^+	$CH_3·CO·NH_2$
Carbobenzoxy·NH·CH_2·CH_2·CO··NH_2	$CH_3·CH_2·CO·NH_2$
	carbobenzoxy··NH·CH_2·CO··NH_2

of blood proteins in conjunction with a liver enzyme (Clarke *et al.*, 1959). Table II shows the structures of those amide compounds which have been tested to date; Figure 3 expresses the extent of inhibition caused by them.

As seen from Figure 3, carbobenzoxy-L-asparagine amide proved to be as good an inhibitor of fibrin polymerization as glycine methyl- or ethyl ester. Compared with the other amides, its effect is quite specific. Since carbobenzoxy-L-asparagine and carbobenzoxy-β-alanine amide also showed appreciable—albeit lesser—inhibition, it may be tentatively assumed that the β rather than α carbonyl group constitutes the effective portion of carbobenzoxy-L-asparagine amide. The total lack of inhibition by carbobenzoxy-glycine amide also strengthens this assumption.

As a counterpart of the amine type of donor inhibitors (Fig. 2), the functioning of the amide or acceptor inhibitors is visualized as shown in Figure 4. Experiments are planned to test whether these inhibitors would also become incorporated into fibrin.

CONCLUSIONS

The proposal that the polymerization of fibrin (step 4) is a transpeptidation (i.e., transamidation) reaction (Lorand *et al.*, 1962) is supported by different lines of evidence.

After polymerization there is a marked decrease in the number of N-terminal residues of glycine in fibrin and no new amino end-groups appear. Interestingly, N-terminal glycines are common to all vertebrate fibrins hitherto examined, regardless of whether they

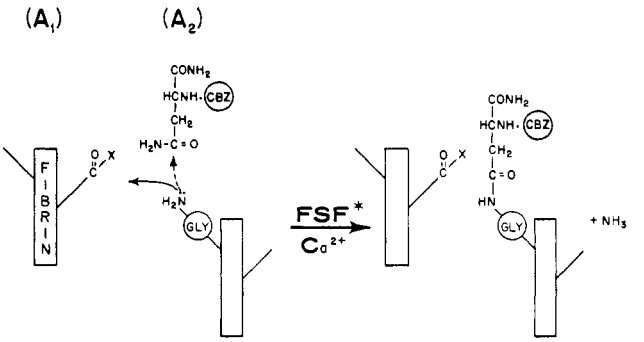

FIG. 4.—Suggested mode of action of the amide (i.e., acceptor) type of inhibitors of fibrin polymerization. Illustrated for carbobenzoxy-L-asparagine amide, with its β-carbonylamide function, acting as an inhibitor. Competition between the two potential acceptors (A_1 and A_2) would inhibit fibrin polymerization as shown. At 20 mM carbobenzoxy-L-asparagine amide the concentration of A_1 is at least one thousand times less than that of A_2.

are obtained by clotting fibrinogen with homologous thrombin, as in our investigations (Lorand and Middlebrook, 1952, 1953; Doolittle *et al.*, 1963), or by using a heterologous enzyme (Blomback and Yamashina, 1958; Blomback and Sjoquist, 1960). Thus, the impression is gained that, apart from the removal of fibrinopeptide and the concomitant lowering of protein solubility, the main action of thrombin on fibrinogen (step 1) lies in producing the terminal amines of glycine which can then specifically participate in the subsequent polymerization reaction (step 4).

Papain, a known transpeptidase (i.e., transamidase), and FSF* were shown to be fully interchangeable in polymerizing a fibrin derivative into urea- or acid-insoluble gels (Lorand and Konishi, 1964b). It is clear that the earlier belief that papain produced a urea-soluble clot from fibrinogen (Steiner and Laki, 1951) was based on work with overdigested samples of fibrin.

The prediction and discovery of specific inhibitors of fibrin polymerization, as described in the present paper (see also Lorand *et al.*, 1962, 1963), provides perhaps the strongest support for the operation of a transpeptidating mechanism. It must be emphasized that these inhibitors selectively interfere only with the polymerization (or cross-linking) of fibrin (i.e., step 4). They do not affect the gelation of fibrin and therefore must not be confused with known inhibitors of fibrin aggregation (step 2; Shulman and Ferry, 1951).

As expected from the concept of transpeptidation (Fig. 1), specific inhibitors of fibrin polymerization fall into two categories: amines and amides which presumably compete with the donor and acceptor cross-linking functions of the protein, respectively. The actual finding of such inhibitors, typified by glycine ethyl ester and carbobenzoxy-L-asparagine amide, for example, lends further credence to the assumption that, during polymerization, the amino groups of glycine of one fibrin molecule react with a side-chain amide function of a neighboring protein particle.

The clear demonstration that glycine ethyl ester, as suggested earlier (Lorand *et al.*, 1962), functions as an inhibitor by being incorporated into fibrin is of major significance. It provides means for selectively labeling the acceptor polymerizing function of fibrin and makes the chemical exploration of these reactive sites now possible in as specific a manner as, for example, diisopropyl-fluorophosphonate allowed the elucidation of the active centers of some hydrolytic enzymes (Jansen *et al.*, 1949). Conversely, the discovery of the amide type of inhibitors is expected to make feasible the introduc-

tion of a specific label into the donor sites of the protein.

Furthermore, two complementary types of specific inhibitor compounds having been obtained, the possibility exists for constructing simple chemical model systems of fibrin polymerization and studying the action of FSF* on synthetic substrates. For example, the following reaction should be considered:

carbobenzoxy-L-asparagine amide + glycine ethyl ester $\xrightarrow[Ca^{2+}]{FSF^*}$ carbobenzoxy-L-α-amidoaspartylglycine ethyl ester + NH_3

This and similar reactions are now under study in our laboratory.

Finally, the possible biological usefulness of the specific polymerization inhibitors must be pointed out. When added to blood, these compounds do not interfere with the onset of normal clotting time, but merely delay the appearance of the acid-insoluble character of the gel. As such, they favor the proteolytic digestion of the clot (Lorand and Jacobsen, 1962). In addition to the amine type of cross-linking inhibitors, the spectrum of such possible useful compounds now extends to the specific amide type of inhibitors.

Acknowledgment

This investigation was supported by a Public Health Service Career Program Award (HE-K6-3512) and by a grant (HE-02212) from the National Heart Institute, National Institutes of Health.

References

Bettelheim, F. R., and Bailey, K. (1952), *Biochim. Biophys. Acta 9*, 578.

Blomback, B., and Sjoquist, J. (1960), *Acta Chem. Scand. 14*, 493.

Blomback, B., and Vestermark, A. (1958), *Arkiv Kemi 12*, 173.

Blomback, B., and Yamashina, I. (1958), *Arkiv Kemi 12*, 299.

Chandrasekhar, N., Osbahr, A., and Laki, K. (1964), *Biochem. Biophys. Res. Commun. 15*, 182.

Chibnall, A. C., Rees, M. W., and Williams, E. F. (1943), *Biochem. J. 37*, 354.

Clarke, D. D., Mycek, M. J., Neidle, A., and Waelsch, H. (1959), *Arch. Biochem. Biophys. 79*, 338.

Donnelly, T. H., Laskowski, J., Jr., Notley, N., and Scheraga, H. A. (1955), *Arch. Biochem. Biophys. 56*, 369.

Doolittle, R. F., Lorand, L., and Jacobsen, A. (1963), *Biochim. Biophys. Acta 69*, 161.

Ferry, J. D., Miller, M., and Shulman, S. (1951), *Arch. Biochem. 34*, 424.

Haurowitz, F. (1963), The Chemistry and Function of Proteins, New York, Academic, 403.

Jansen, E. F., Nutline, M. D., Jang, R., and Balls, A. K. (1949), *J. Biol. Chem. 179*, 189.

Laki, K., and Chandrasekhar, N. (1963), *Nature 197*, 1267.

Laki, K., and Lorand, L. (1948), *Science 108*, 280.

Loewy, A. G., Dahlberg, J. E., Dorwart, W. V., Jr., Weber, M. J., and Eisele, J. (1964), *Biochem. Biophys. Res. Commun. 15*, 177.

Loewy, A. G., Dunathan, K., Kriel, R., and Wolfinger, H. L., Jr. (1961), *J. Biol. Chem. 236*, 2625.

Loewy, A. G., and Edsall, J. T. (1954), *J. Biol. Chem. 211*, 829.

Lorand, L. (1948), *Hung. Acta Physiol. 1*, 192.

Lorand, L. (1950), *Nature 166*, 694.

Lorand, L. (1951), *Nature 167*, 992.

Lorand, L. (1952), *Biochem. J. 52*, 200.

Lorand, L. (1954), *Physiol. Rev. 34*, 742.

Lorand, L. (1964), *Thromb. Diath. Haemorrh., Suppl. 13*, 45.

Lorand, L., Doolittle, R. F., Konishi, K., and Riggs, S. K. (1963), *Arch. Biochem. Biophys. 102*, 171.

Lorand, L., and Jacobsen, A. (1958), *J. Biol. Chem. 230*, 421.

Lorand, L., and Jacobsen, A. (1962), *Nature 195*, 911.

Lorand, L., and Konishi, K. (1964a), *Arch. Biochem. Biophys. 105*, 58.

Lorand, L., and Konishi, K. (1964b), *Biochemistry 3*, 915.

Lorand, L., Konishi, K., and Jacobsen, A. (1962), *Nature 194*, 1148.

Lorand, L., and Middlebrook, W. R. (1952), *Biochem. J. 52*, 196.

Lorand, L., and Middlebrook, W. R. (1953), *Science 118*, 515.

Mihalyi, E. (1950), *Acta Chem. Scand. 4*, 344.

Robbins, K. C. (1944), *Am. J. Physiol. 142*, 581.

Sherry, S., and Troll, W. (1954), *J. Biol. Chem. 208*, 195.

Shulman, S., and Ferry, J. D. (1951), *J. Phys. & Colloid Chem.* (now *J. Phys. Chem.*) *55*, 135.

Steiner, R. F., and Laki, K. (1951), *Arch. Biochem. Biophys. 34*, 24.