1965

# The Kinetics of the Thrombin Catalyzed Fibrinogen Fibrin Conversion

James Ross
*Loyola University Chicago*

# THE KINETICS OF THE THROMBIN CATALYZED FIBRINOGEN FIBRIN CONVERSION

by

James Ross


A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Master of Science

June

1965

# VITA

James Ross was born in Manchester, England on 19th. July 1928 and attended schools in the Manchester area until 1946.

From August 1946 to November 1948 the author served with the British Army, where he became a sergeant ammunition examiner and saw service in North Africa.

From 1949 to 1955 he attended the Royal College of Advanced Technology, Salford, as a part-time student. In 1954 he was awarded the Associateship of the College and in 1956 passed examinations for the Associateship of the Royal Institute of Chemistry.

While a part-time student, he was employed as a research assistant by the British Rayon Research Association, Wythenshawe, Manchester and later as an Assistant Technical Officer in the Chemical Engineering Department of Imperial Chemical Industries Ltd., Dyestuffs Division, Manchester.

In 1957 he emigrated to Canada and was employed as a development chemist by Merck and Co. Ltd., Valleyfield, Quebec. In 1959 he moved to Shawinigan Chemicals Ltd., Shawinigan, Quebec where he was employed as a development chemist and later as production supervisor of the polyvinyl chloride resin unit.

In 1961 he married Marielle Lafontaine of Shawinigan, Quebec and in February 1963 became the proud father of his first son.

He began graduate studies in the Department of Biochemistry and Biophysics of Loyola University, Chicago in September 1963, where he was a Graduate Teaching Assistant from 1963 to 1965.

## ACKNOWLEDGEMENT

I wish to thank Dr. F.W. Pairent for introducing me to the problem, for the practical help he has given and for the illuminating and lively discussions he has led.

My wife deserves special thanks for the sacrifices she has made, the hardship she has put up with and for the innumerable ways in which her help has made this study possible.

I also wish to thank the members of the faculty of the Department of Biochemistry and Biophysics, who have greatly contributed to my education.

J.R.

TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

## APPENDIX

# CHAPTER I

# THE CHEMISTRY OF THE FIBRINOGEN - FIBRIN CONVERSION

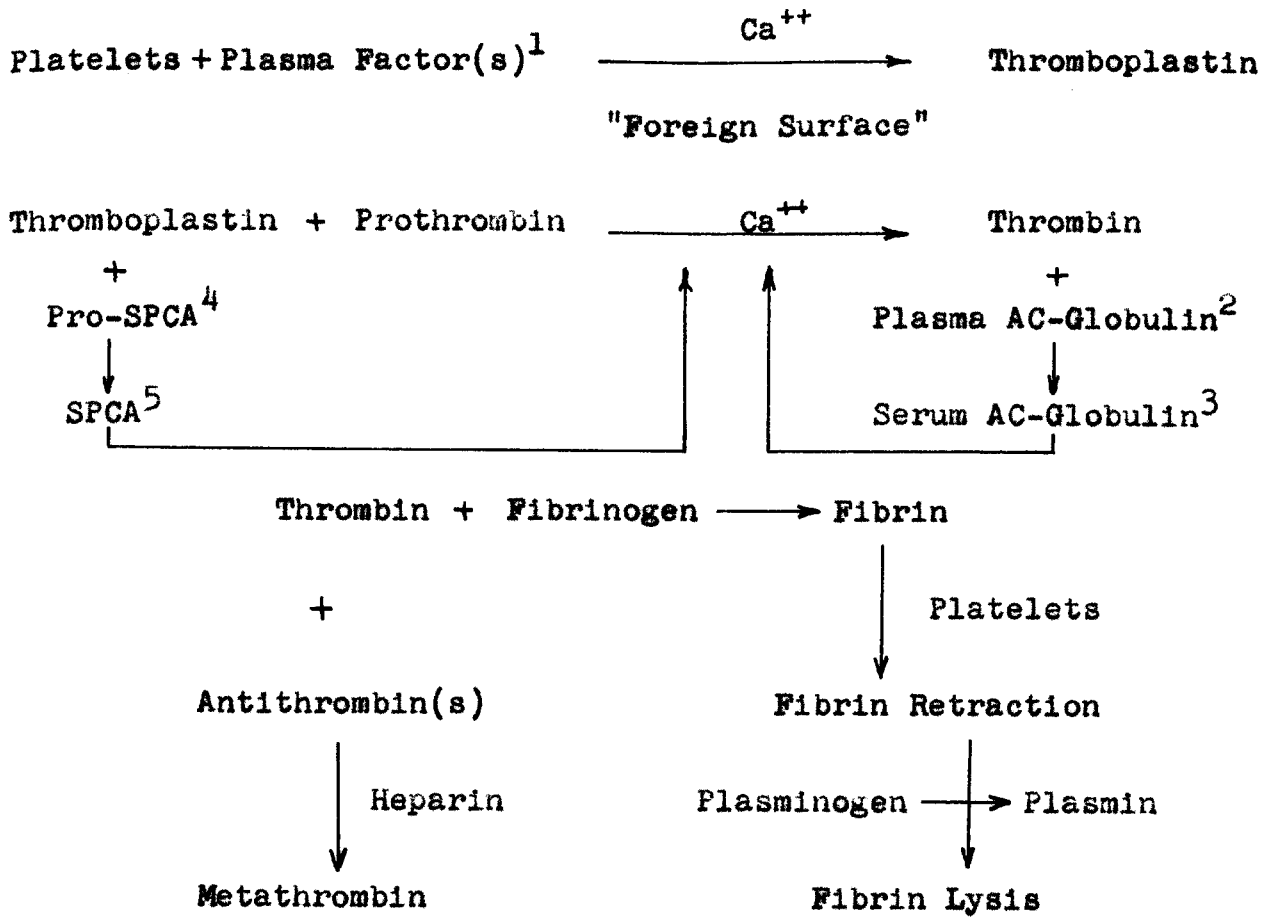
## I. RELATION TO BLOOD COAGULATION

The formation of the fibrin clot is the culmination of a series of complex reactions involved in the process of blood coagulation. The major components of blood which are involved in coagulation are listed in Table I. Procoagulents are substances which bring about coagulation; anticoagulents are substances which prevent coagulation or dissolve the clot once it is formed. There are many proposed theories of blood coagulation (11), one of which is shown in Fig. I. There appears to be considerable disagreement on the role of the various factors involved and even on the nomenclature, as indicated by the variety of synonyms. However, it can be said that, *in vivo*, reactions take place which ultimately lead to the production of thrombin. When thrombin becomes available, it converts fibrinogen to fibrin to prevent hemorrhage. After bleeding has stopped, subsequent reactions eliminate thrombin and lyse the fibrin clot. While fibrinogen exists in normal blood, thrombin is present only as its inactive precursor, prothrombin.

## TABLE I

## COMPONENTS OF NORMAL HUMAN BLOOD CONCERNED WITH COAGULATION

| PROCOAGULANTS | ANTICOAGULANTS |
|---|---|
| Fibrinogen | Antithrombin |
| Platelets | Lipoprotein Antithromboplastin |
| Calcium | Heparin cofactor |
| Precursor of Serum prothrombin Conversion accelerator | Plasminogen |
| Prothrombin | |
| Accelerator globulin | |
| Antihemophilic globulin | |




Synonyms: (1) Antihemophilic Globulin; Thromboplastinogen; Thrombocytolysin; Plasma Thromboplastin.
(2) Thrombogen; Prothrombin Accelerator; Labile Factor; Factor V; Proaccelerin; Fraction "A"; Plasmakinin; Prothrombokinase.
(3) Factor VI; Accelerin.
(4) Precursor of Serum Prothrombin Conversion Accelerator; Proconvertin; Co-factor V; Co-thromboplastin; Factor VII; Thrombokinase.
(5) Serum Prothrombin Conversion Accelerator; Convertin.

Fig. I Modern scheme of blood coagulation (1).

The coagulation process therefore consists of 1) conversion of prothrombin to thrombin and 2) the subsequent action of thrombin on fibrinogen to produce fibrin. Under normal physiological conditions the latter reaction starts as soon as a trace of thrombin is produced, that is, before the first reaction is complete.

This thesis is concerned with a study of the kinetics of the thrombin - fibrinogen reaction carried out with isolated and purified thrombin and fibrinogen so that complications arising from the prothrombin to thrombin conversion are eliminated.

## II. THROMBIN

The enzyme thrombin is not present in normal circulating blood. Instead, normal plasma contains the precursor prothrombin at a concentration of about 70 mg/l. (22), i.e. about 0.14% of the plasma proteins. Hence, any fractionation procedure designed to obtain purified prothrombin must permit the separation of this trace amount of enzyme precursor from large quantities of other plasma proteins.

Prothrombin is generally obtained from bovine plasma by isoelectric precipitation, absorption on $Mg(OH)_2$, elution, ammonium sulfate fractionation and a second isoelectric precipitation (53). This can be followed by absorption of non-prothrombin materials on $BaCO_3$. Other methods depend on absorption of prothrombin

from plasma on $BaSO_4$, ammonium sulfate fractionation, isoelectric precipitation and Seitz filtration to remove factor VII (63). Modifications of these methods are now generally used in which the purified prothrombin is chromatographed to further "modify" the prothrombin. The word "modify" is used rather than purify because the thrombin obtained from activation of chromatographed prothrombin is apparently different from non-chromatographed prothrombin, and because the C-terminal amino acids of such prothrombins differ (52). Amberlite IRC-50 (XE-64) has been used for this chromatography (41) and the prothrombin obtained (termed "resin" prothrombin) has increased specific activity due to the removal of materials which would later be absorved and eluted under the same conditions as thrombin. Diethylamino ethyl cellulose (DEAE) can also be used with apparently similar results to those obtained with IRC-50 (52). The preparations obtained from DEAE chromatography have been called "DEAE-prothrombin".

In vivo activation of prothrombin to give thrombin is far from being a simple process (Fig. I). It appears however that the enzymes(s) required for this process is part of the prothrombin molecule (50) and that all prothrombin preparations contain some thrombin as such. Other blood factors increase both the rate and the degree of activation of prothrombin viz. $Ca^{++}$, platelet factors, Ac-Globulin, lipids, proconvertin, convertin and possibly other substances (50,66).

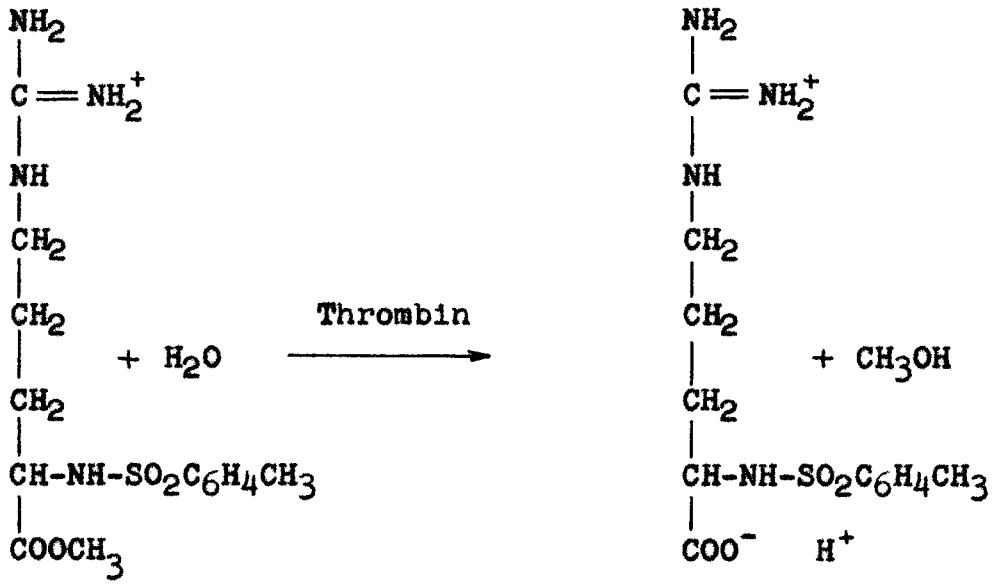
Enzymatic Properties of Thrombin

There are close similarities between thrombin and trypsin in their action on synthetic substrates e.g. p-toluenesulfonyl-L-arginine methyl ester (TAMe), (56) lysine ethyl and methyl esters (47). Thrombin, like trypsin is inhibited by diisopropylfluorophosphate (DFP) and the amino acid sequence near the DFP binding site is identical for the two enzymes (19). This plus the fact that of the approximately 3000 peptide bonds in fibrinogen, those hydrolyzed by thrombin involve only arginine (see Fig. IV), leads to the conclusion that thrombin is specific for arginine containing bonds.

The activity of thrombin has traditionally been determined by ascertaining the time required for an aliquot to clot a standard solution of fibrinogen. In the clotting time assay, an aliquot whose thrombin activity is to be determined is mixed with a solution of fibrinogen. Two "clotting time" ($t_c$) end points have been used; the time for gelation of a solution held quiescent after mixing, or the appearance of compacted strands of fibrin in an agitated solution. An assay system containing fibrinogen as its only added colloid component was first used and a unit of thrombin was defined as that amount which produced a clot in 15 seconds (61,65). When a pooled plasma was used as a reference standard, day to day variations in plasma prothrombin activity

were observed. This variation was reduced by adding gum acacia to the mixture. Assay conditions were extensively re-examined (54) and a set of conditions was derived for temperature, pH, gum acacia concentration and ionic strength which form the basis for all current assay systems including those specified by the National Institutes of Health (40). The variation of clotting time with thrombin concentration using the above method is shown in Fig. XIII (Chapter III). This figure demonstrates that the relation between clotting time and thrombin concentration is non-linear.

A more convenient method of thrombin assay is to measure the rate of hydrolysis of the synthetic substrate p-tosyl L-arginine methyl ester (TAMe) by the enzyme (56). The reaction catalyzed is illustrated in Fig. II. This hydrolysis has been followed by direct potentiometric titration (13,49). The unit adopted when using this method is that amount of thrombin which will hydrolyse 0.1 μmole TAMe per minute from 1 ml. of 0.01 M TAMe at pH 8 in 0.15 M KCl at 25°C. A modification of this method is based on the fact that p-tosyl L-arginine has a higher absorbance at 247 mu. than does TAMe (23). The rate of hydrolysis can thus be followed by measuring the rate of increase in absorbance at 247 millimicrons (mu). Possible objections to the use of TAMe as a substrate for assay purposes are that it may not be applicable to the determination of thrombin purity since other enzymes hydrolyse TAMe (e.g. trypsin, chymotrypsin) and that the ratio of




p-Toluenesulfonyl-L-arginine methyl ester (TAMe)

p-Toluenesulfonyl-L-arginine (TA)

Fig. II. Enzymatic hydrolysis of TAMe by thrombin (56).

esterase activity to clotting activity has been reported to vary for different thrombin preparations (51,53).

## III. FIBRINOGEN

Fibrinogen is one of the constituents of blood plasma. It is present at a concentration of about 2 g/l and represents about 4% of the plasma proteins (8). From the point of view of its solubility properties, fibrinogen may be regarded as a globulin since it is insoluble in water but soluble in dilute salt solution.

Fibrinogen can be obtained from plasma on a large scale by ethanol fractionation. Fibrinogen is precipitated at low temperatures (Cohn fraction 1) by bringing the concentration of ethanol to 8% after removal of prothrombin and the precursor of serum prothrombin conversion accelerator by adsorption on $BaSO_4$ (8,25). The fibrinogen can then be refractionated to remove insoluble globulin impurities by control of pH, ionic strength and temperature, followed by precipitation of fibrinogen from the supernatant by means of ammonium sulfate (25).

Purified fibrinogen solutions, when acted on by thrombin, usually contain some material that can not be transformed into a fibrin clot. Such material is known as the non-clottable fraction (NC) and could be either non-fibrinogen protein, denatured fibrin-

## TABLE II

## VALUES OF PHYSICAL CHEMICAL PROPERTIES OF HUMAN AND BOVINE FIBRINOGEN (48)

| Property | Value |
|---|---|
| Intrinsic viscosity, 100 ml./g. | 0.25 |
| Partial specific volume, $\bar{v}$, ml./g. | 0.71-0.72 |
| Sedimentation constant, $S_{20,w}$, Svedberg units | 7.7-7.9 |
| Translational diffusion coefficient, $D_{20,w}$, (Fick units) | 2.0 |
| Rotational diffusion coefficient, $\theta_{20,w}$, $sec^{-1}$ | 40,000 |
| Refractive increment, dn/dc, at 436 mμ, ml./g. | 0.197 |
| Principal refractive indices, $n_1, n_2, n_3$ at 544 mμ. | 1.64,1.69,1.69 |
| Molecular weight, M, g./mole. | 330,000-340,000 |
| Molecular length, A. | 500-600 |
| Axial ratio | 5 (prolate ellipsoid) |

ogen, fibrinopeptides or fibrin monomer and polymers in equilibrium with clot. The per cent non-clottable is usually taken as being a measure of fibrinogen purity and is determined by clotting a fibrinogen solution with thrombin, removing the clot and measuring the absorbance (at 280 mμ) of the supernatant.

Except for the most recent observations, most physical chemical studies have been carried out on fibrinogen preparations which were quite impure by the clottability criterion. Edsall (12) has discussed some of the problems involved in the interpretation of the physical chemical parameters of fibrinogen and Scheraga and Laskowski (48) have reported a set of parameters which they regard as being the best values. These are shown in Table II. Human and bovine fibrinogen appear indistinguishable by most physical chemical criteria, the only exception being electrophoretic mobility. The electrophoretic mobility of bovine fibrinogen is slightly higher than human fibrinogen (9) this result being compatible with the N-terminal amino acid composition shown in Fig. III. Although fibrinogen and fibrin (as fibrin monomer) are indistinguishable with respect to viscosity, light scattering, sedimentation behaviour and flow birefringence (48), they are different chemically as will be shown in section IV.

## IV. GENERAL ASPECTS OF THE THROMBIN - FIBRINOGEN REACTION

When thrombin is added to fibrinogen in an appropriate buffer, the fibrinogen solution is converted to a fairly rigid gel, the fibrin clot. During the course of the reaction the initially clear reaction mixture increases in turbidity as shown in Fig. X (Chapter III). The clot also becomes rigid after a time and increases as the reaction proceeds, paralleling the increase in turbidity. From Fig. X it is seen that the turbidity increases sharply at the clotting time, $t_c$. Other properties of the solution, such as the viscosity or the amount of recoverable fibrin, show a marked increase at $t_c$. The value of $t_c$ is found to be markedly dependent on pH and ionic strength. The clotting time becomes infinite (i.e. no clot forms) at pH's lower than 5 and greater than 10 and attains very low values (i.e. rapid clot formation) in the neutral pH region. It has been shown also that at a given pH, $t_c$ is increased by an increase in ionic strength (58).

Thrombin causes an initial alteration in the fibrinogen molecule without changing its hydrodynamic properties (25). When thrombin was incubated with bovine fibrinogen at pH 4.85, no clotting occured. However, when the solution was brought to neutral pH, the value of $t_c$ was much lower than for a corresponding mixture which had not been exposed to the low pH treatment. Furthermore, the clotting time of the reneutralized mixture decreased

with increasing incubation time at pH 4.85. It was thus concluded that at pH 4.85, fibrinogen (F) was converted to another species, fibrin monomer (f) and that the further conversion of f to a fibrin clot was inhibited at pH 4.85. The species F and f were indistinguishable by viscosity and sedimentation experiments.

While fibrinogen and fibrin show similar hydrodynamic behaviour and presumably therefore have the same size and shape, they do have different electrophoretic mobilities (38). The difference in electrophoretic mobility indicates that fibrinogen and fibrin monomer may differ in amino acid composition and/or net charge and is compatible with the findings that the N-terminal residues of bovine fibrinogen and fibrin (and hence presumably f) differ in that fibrinogen has N-terminal tyrosyl and glutamyl residues whereas fibrin has instead tyrosyl and glycyl residues (3). This difference was attributed to the liberation of peptide material (fibrinopeptide) from fibrinogen during the course of the clotting process. The fibrinopeptide was later isolated from the supernatant solutions of fibrin clots and found to contain the N-terminal glutamyl residue missing from fibrin (5,34,35).

Fibrinopeptide is not an artifact of the method, nor is it originally present as such in either fibrinogen or thrombin. The amount of non-protein nitrogen present in the clot supernatant has been found to rise sharply with the time of incubation of thrombin and fibrinogen, and then level off at about 3% of the

total nitrogen present (35). The initial rapid rate of production of non-protein nitrogen parallels the rate of clotting and the asymptotic level of the curve, maintained for more than 18 hours, indicates that the production of non-protein nitrogen is not due to clot breakdown (fibrinolysis), but is a characteristic feature of the enzymatic action of thrombin on fibrinogen. The production of non-protein nitrogen does not stop at $t_c$. The development of non-protein nitrogen has also been demonstrated in solvents (0.3 M KCl pH 5, 1 M NaBr pH 5.3, 2 M NaBr at pH 5.7) where no polymerization occurs thus eliminating the possibility that the peptide is split off during the subsequent polymerization of f (29).

The overall first step in the fibrinogen - fibrin conversion can be represented by

$$F \xrightarrow{T} f + P \qquad 1.$$

Where F = fibrinogen, T = thrombin, f = fibrin monomer and P = fibrinopeptide.

The subsequent fate of f was studied by following the ultracentrifuge patterns of thrombin-fibrinogen-hexamethylene glycol mixtures with time (59). Hexamethylene glycol prevents the formation of the fibrin clot. Such mixtures in the ultracentrifuge showed the presence of a fast ($S_{20,w}$ = 25) and a slow

($S_{20,w}$ = 8) component. The slow component was indistinguishable from fibrinogen and the fast component was identified as intermediate polymers. As the reaction time in the glycol increased, the relative area of the fast and slow peaks increased and decreased respectively, indicating the conversion of fibrinogen into intermediate polymers. Other inhibitors, such as 0.5 M LiBr, 1 M NaBr and 1 M urea, can be used to demonstrate the existence of intermediate polymers in thrombin - fibrinogen mixtures or in fibrin solutions (10).

The particle length of the polymers just prior to the gel point is dependent on pH and it appears as though there is a significant number of relatively long particles before the gel point between pH 6 and 10 and shorter ones outside this range. Since the sedimentation constants of elongated polymers of constant width would not be very dependent on the length and hence not separable in the ultracentrifuge, the two peaks present in the ultracentrifuge pattern are indicative of equilibria between various n-mers, rather than of two distinct species.

Flow birefringence data indicate that the intermediate polymers dissociate on dilution, showing that the reaction in which intermediate polymers are formed from fibrin monomer is reversible (45). This step may be written as

$$nf \longrightarrow f_n \qquad 2.$$

If it is recognized that intermediate polymers must be bound into the fibrin network, a final step can be written as

$$m(f_n) \rightleftharpoons \text{fibrin} \qquad 3.$$

An equilibrium is indicated here because it has been shown (31) that the solubility of fibrin clots increases with increasing temperature.

The reaction scheme for the fibrinogen - fibrin conversion can be summarized as

$$\begin{array}{lll} \text{Step 1. proteolysis} & F \xrightarrow{T} f + P & \\ \text{Step 2. polymerization} & nf \longrightarrow f_n & 4. \\ \text{Step 3. clotting} & m(f_n) \longrightarrow \text{Fibrin} & \end{array}$$

The above individual steps will now be discussed individually.

## V. STEP 1: PROTEOLYSIS

The proteolysis step involves the formation of fibrin monomer from fibrinogen with the simultaneous release of fibrinopeptide material. Bettleheim (4) found that this fibrinopeptide

material can be separated into two fractions, peptides A and B, peptide A travelling faster toward the anode than peptide B on paper electrophoresis at pH 4.15. Peptide A contains N-terminal glutamic acid whereas peptide B contains no detectable N-terminal group.

The stoichiometry of the release of peptides A and B is shown in Fig. III. For example, bovine fibrinogen contains six chains, two each having N-terminal glutamic acid and tyrosine respectively and two chains with no detectable N-terminal group, this being due to the fact that the N-terminal group of peptide B is N-acetylated (see Fig. IV). Papain and snake venom enzyme split two bonds on bovine fibrinogen with the release of only two moles of peptide A and produce a clottable material (7), whereas thrombin can split 4 bonds with the release of two moles of peptide A and two moles of peptide B. In view of this it seems as though the release of peptide B is not required for clotting and may be an extraneous action of thrombin not pertinent to the clotting process. This is obviously an important point and needs to be answered.

The complete amino acid sequence of peptides A and B are shown in Fig. IV (6,18). Both peptides contain C-terminal arginine, consistent with the known specificity of thrombin, showing that arginine - glycine bonds are hydrolysed in step 1. Peptide A contains no tyrosine and peptide B contains one molecule as

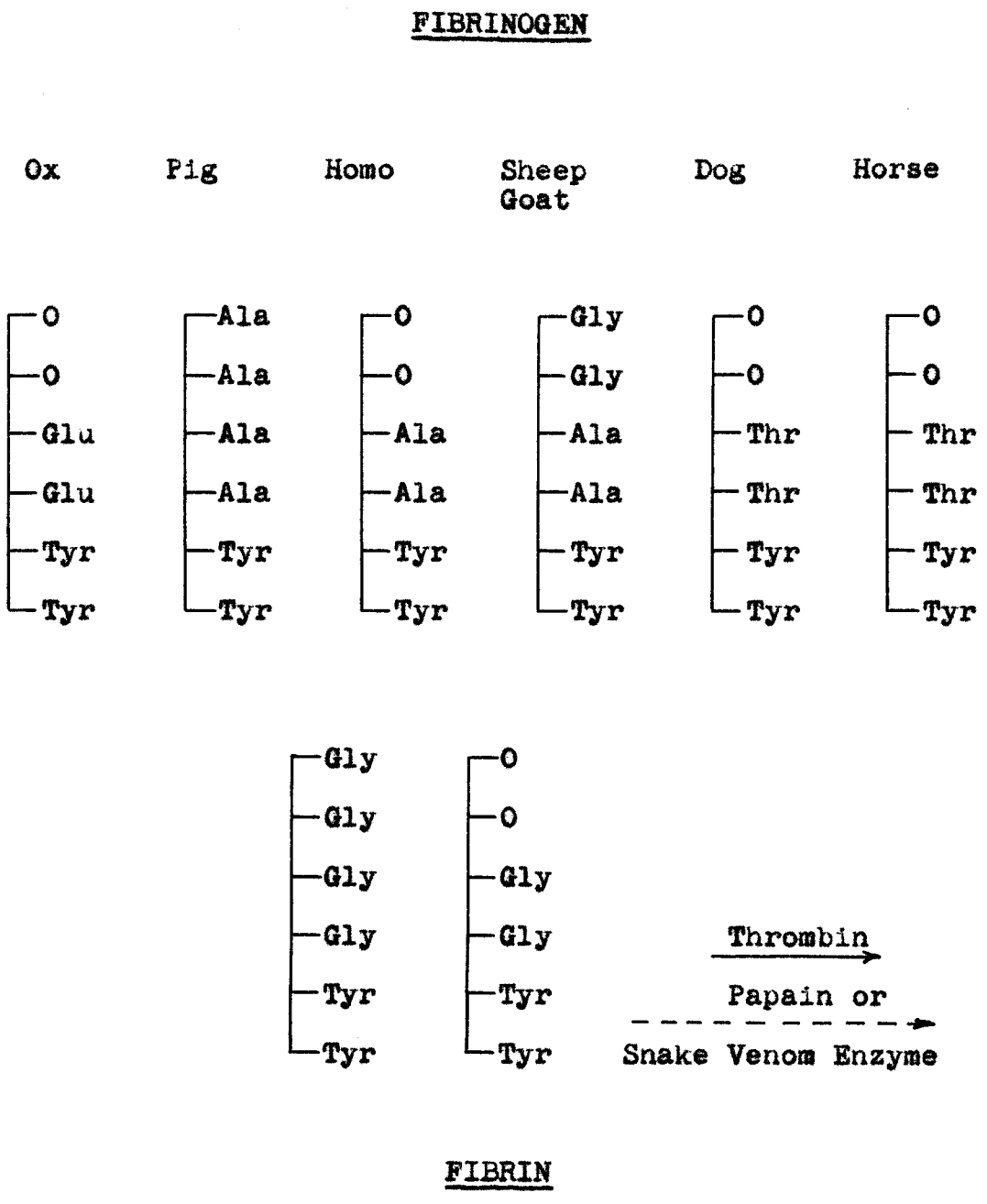



Fig. III. N-Terminal amino acids in fibrinogen and fibrin from different species. The number of chains correspond to a molecular weight of 350,000 except for horse fibrinogen, where the molecular weight is 500,000. The circles indicate chains with no detectable N-terminal groups (7).

Peptide A.

| Glu | Asp | Gly | Ser | Asp | Pro | Pro | Ser | Gly | Asp | Phe | Leu | Thr | Glu | Gly | Gly | Gly | Val | Arg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |

Peptide B.

| N.acetyl. | | | | | $SO_4$ | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Thr | Glu | Phe | Pro | Asp | Tyr | Asp | Glu | Gly | Glu | Asp | Asp | Arg | Pro | Lys | Val | Gly | Leu | Gly | Ala | Arg |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |

Fig. IV Amino acid sequences of peptides A and B. (6,17).

tyrosine-O-sulphate. Since fibrinogen concentrations are usually determined from absorbance measurements at 280 mμ, the clottability (Chapter III) in terms of absorbance measurements should be very close to 100%, because tyrosine-O-sulphate has relatively little absorbance at 280 mμ. (4).

The acidity of peptides A and B, due to the excess of free carboxyl over basic groups, is compatible with the electrophoresis experiments quoted earlier (38).

## VI. STEP 2

As mentioned earlier, sedimentation studies carried out using hexamethylene glycol to inhibit step 3 indicated that the intermediate polymers were highly asymmetrical end to end aggregates (59). Other workers (2,10,17), have shown that the intermediate polymers were polydispersed with a general size range of 3,000 to 6,000 Å. While there is heterogeneity in length, the width of the polymers seems to be fairly uniform and about twice that of the monomer, with the molecules aligned by a staggered overlapping.

In view of the polydisperse character of the intermediate polymers, step 2 should be written as a system of many fairly rapid reversible equilibria (10):

$$
\begin{array}{lll}
f_1 + f_1 \rightleftharpoons f_2 & & \\
f_2 + f_1 \rightleftharpoons f_3 & & \\
\vdots \quad \vdots \quad \vdots & & \\
f_{n-1} \quad f_1 \rightleftharpoons f_n & & 5.
\end{array}
$$

This scheme does not necessarily imply that polymerization takes place by addition of monomers only, but that the equilibrium state can be described in terms of the equilibrium constants corresponding to the above reactions, where

$$K_n = \frac{(f_n)}{(f_{n-1})\ (f_1)} \qquad \text{and } n > 1 \qquad 6.$$

The weight average degree of polymerization $(\overline{DP})_w$ is obviously a function of $K_n$. Donnelly, et al, (10) studied the effect of the concentration of protein c (g/100 ml) on $(\overline{DP})_w$ using light scattering measurements. Fig. V shows the results of these experiments. It was found that the equilibrium constants $K_n$ increased very slightly from $K_2$ to $K_7$ and then began to increase rapidly. Because of the small variation of $(\overline{DP})_w$ with c at low c, large errors are introduced and the values of the equilibrium

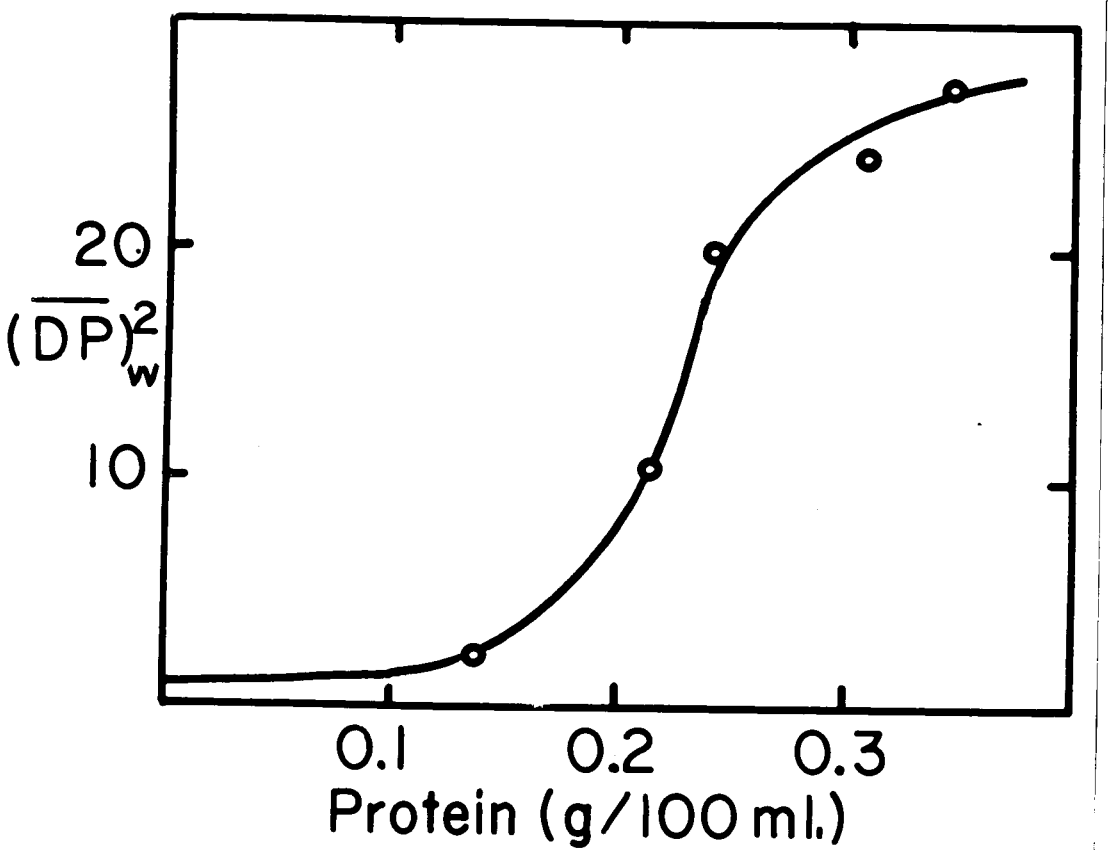



Fig. V. Plot of $(\overline{DP})^2_w$ against protein concentration (10).

constants could not be obtained. The fact that these equilibrium constants are unequal can be established by the process of reductio ad absurdum. If it is assumed that all the equilibrium constants are equal, then the following relation can be derived from definitions of $K_n$ and $(\overline{DP})_w$.

$$(\overline{DP})_w^2 = \frac{4\,Kc}{M} + 1 \qquad 7.$$

where M is the molecular weight of fibrin and $K = K_n$ for every n.

Since the data of Fig. V do not even approximate the straight line that would be expected from a plot of $(\overline{DP})_w^2$ vs c, it is established that the equilibrium constants are not equal.

The evidence presented so far suggests that the polar groups liberated in step 1 are involved in hydrogen bond formation in steps 2 and 3. Since fibrin clots and intermediate polymers are depolymerized by solvents which break hydrogen bonds it is probable that, in the absence of calcium ions and Fibrin-Stabilizing Factor (section VII), intermolecular hydrogen bonds rather than covalent bonds are formed in steps 2 and 3. At one time electrostatic effects were postulated to play a role in polymerization. In view of the ready compatibility of the hydrogen

bonding theory with experimental observations, electrostatic effects are presumed to play only a minor role in their effect on the pK's of the dissociating groups.

The acceptor groups A are considered to be available on the original fibrinogen molecule but the donor groups DH are thought to be masked by the fibrinopeptides. These donor groups become available for polymerization when the fibrinopeptide is liberated in step 1. Step 2 then involves the formation of intermolecular hydrogen bonds between side chain groups according to the equation (44):

$$DH + A \rightleftharpoons D\text{-}H\cdots A \qquad 8.$$

The non-hydrogen bonded donor and acceptor groups are themselves involved in equilibria of the type

$$\begin{aligned} DH &\rightleftharpoons H^+ + D^- \\ AH &\rightleftharpoons H^+ + A \end{aligned} \qquad 9.$$

It has been suggested that histidyl groups (64) and amino and tyrosyl groups (27) are involved in the polymerization process. Sets of histidyl groups on fibrin monomers could well function as the acceptor sites for the tyrosyl or lysyl donors and thus lead to polymerization.

The pH range of polymerization can be accounted for in terms of the above mechanisms. The tyrosyl donors (pK approximately 9.5) would lose their protons above pH 10.5 while the histidyl acceptors (pK approximately 6) would acquire protons below pH 5.5 thus enabling steps 2 and 3 to occur only in the pH 5.5 to 10.5 region.

The effect of pH on polymerization and conversely the effect of polymerization on pH can be treated quantitatively by taking into account the degree of dissociation of both DH and AH and the effects hydrogen bonding has on these dissociations (27, 62). This thermodynamic theory provides strong evidence that the polymerization of fibrin monomer takes place through a hydrogen bonding mechanism involving about 19 tyrosyl donors and about 19 histidyl acceptors. Although presumably 19 such donors or acceptors are unmasked by the release of fibrinopeptide, the actual number of hydrogen bonds formed per link depends on the pH, the average maximum being about 9 bonds, near pH 8.

An idealized schematic representation of F and f to show the functionality with respect to polymerization is shown in Fig. VI (10). These representations are schematic and do not imply that the molecules of F and f are highly asymmetrical. It is presumed that the acceptor site $A_1$ consists of 19 histidyl groups and the donor site $D_1H$ of 19 tyrosyl groups and that the auxilliary acceptor and donor sites $A_2$ and $D_2H$ contain fewer hydrogen bonding

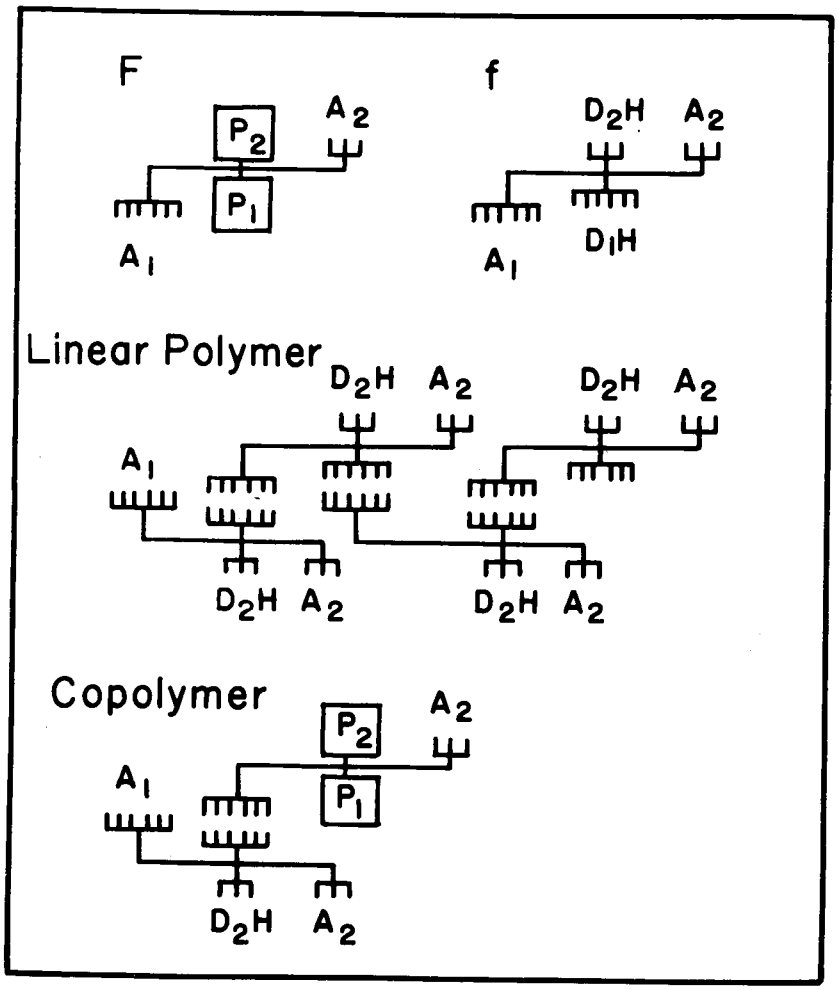



Fig. VI. Idealized schematic representation of fibrinogen (F) and fibrin monomer (f) to show the functionality with respect to polymerization. The hydrogen bond donor and acceptor groups are represented by DH and A respectively (10).

groups. When peptides A and B are liberated in step 1, the donor (or acceptor) groups are unmasked. If this representation is true then not only can f polymerize to form fibrin but there is the possibility that a copolymer can be formed (between f and F). F molecules would in this case serve as chain terminators. In the presence of thrombin these "dead ends" could be reactivated by step 1. However, Sherry, et al, (32) have thrown considerable doubt on copolymer formation. Fibrin monomer (0.18 mg/ml) prepared in 1 M NaBr, as described previously, was allowed to repolymerize in the presence of added fibrinogen (0.18 mg/ml) and the rate of clot formation followed by OD measurements. There was no increase in either rate or amount of polymerization (clot formation) over that occuring without added fibrinogen thus showing that copolymerization plays no significant role in the process when fibrin monomer and fibrinogen are present in equal amounts. The same workers also polymerized fibrin monomer in the presence of added thrombin and again no increased polymerization took place showing that thrombin plays no detectable role in the polymerization process.

## VII. STEP 3

The final step in the fibrinogen-fibrin conversion involves the aggregation of the intermediate polymers to form a gel.

In the absence of calcium ions and fibrin stabilizing factor (FSF) this gel can be reversibly dispersed in a number of solvents such as urea or NaBr (10).

In the course of normal blood coagulation or in the presence of calcium ions and FSF, the gel produced is insoluble in urea and NaBr. This has led Lorand (36) to suggest that in this case the gel should be regarded as a true polymeric network in which the polymers are linked by covalent bonds. In this reaction, FSF is only the precursor of the active polymerizing principle ($FSF^*$) which arises from the action of thrombin on FSF. The reaction also requires calcium ions.

$$FSF \xrightarrow[Ca^{++}]{\text{Thrombin}} FSF^* \qquad 10.$$

$$\text{fibrin aggregates} \xrightarrow[Ca^{++}]{FSF^*} \text{fibrin polymer} \qquad 11.$$

Lorand proposed that the crucial feature of the reaction is a transamidating mechanism in which some of the N-terminal glycine residues of one fibrin molecule react with possibly an activated carbonyl of a neighbouring fibrin molecule to form a peptide bond.

The properties of fibrin clots vary considerably between those of two extreme types referred to as fine and coarse clots by Ferry and Morrisson (16). Both types of clot are envisaged as cross linked gel structures with large amounts of liquid held in the interstices between the fibrin strands, the coarse clot being aggregated laterally to a greater extent than the fine clot.

Electron micrographs of stained specimens show alternating light and dark bands indicating a periodicity in the fibrillar structure. From measurements of the distances between dark bands, it has been suggested (20) that in polymerization, the fibrin monomers shrink to one half their length and aggregate laterally. In view of the fact that step 3 is reversible and that this reversibility can be brought about in hydrogen-bond breaking solvents, the indication is that hydrogen bonding is involved in step 3 as well as step 2.

## CHAPTER II

## KINETICS AND THERMODYNAMICS OF THE FIBRINOGEN FIBRIN CONVERSION

### 1. Kinetics

The fibrinogen - fibrin conversion is conveniently represented by the following scheme.

| | | | | |
|---|---|---|---|---|
| Step 1. | Proteolysis | $F \xrightarrow{T} f$ + Peptides | | |
| Step 2. | Polymerization | $nf \longrightarrow f_n$ | | 12. |
| Step 3. | Clotting | $m(f_n) \longrightarrow$ fibrin | | |

The fact that four peptides can be liberated in the proteolysis step (7) and that these four peptides (2 of A and 2 of B) are liberated at different rates (4) makes a complete discussion of the kinetics of even step 1 difficult. The situation is further complicated since polymerization of fibrin monomer (f) presumably starts as soon as f is produced.

Although the overall process in the clotting of even highly purified fibrinogen by highly purified thrombin is quite complex, significant progress in the understanding of some aspects

of the fibrinogen - fibrin conversion has been made in the past few years.

Waugh and Livingstone (67) investigated the kinetics of the overall reaction using bovine fraction 1. The thrombin - fibrinogen reaction was allowed to proceed to a clot, the reaction being stopped at various stages by the addition of formaldehyde (final concentration 1.8%). The supernatant from the clot was analysed for unreacted fibrinogen by U.V. absorption. From the results so obtained, ln $F_o/F$ was plotted against time. These curves exhibit an initial curvature and then a linear (first order) protion similar to the opacity curve shown in Fig. X. The authors assume that in the linear portion, which in their experiments extrapolates through the origin, the fibrin clot keeps the concentration of intermediate polymers low so that the kinetic data pertain to step 1. The linear portions of all reactions studied ($F_o$ = 0.036 to 0.36 mg/ml at pH 6.85 and ionic strength 0.15) conform to the equation

$$\ln F_o/F = \frac{0.483\ T_o}{0.051 - F_o}\ t \qquad 13.$$

where $T_o$ and $F_o$ are the initial thrombin and fibrinogen concentrations. This equation shows that the reaction is first order in

thrombin but departs from first order behaviour in fibrinogen.

The validity of the above results has been questioned by Ehrenpreis and Scheraga (14). These workers were able to show that formaldehyde, in the concentrations used by Waugh and Livingstone does not completely inhibit thrombin but does inhibit the polymerization steps. These authors maintain that because the polymerization steps are not instantaneous and because formaldehyde does not completely inhibit thrombin activity, the use of formaldehyde as a stopping agent is not valid.

Ehrenpreis and Scheraga (14) studied the kinetics of step 1 using a high concentration of TAMe (p-toluene sulfonyl L-arginine methyl ester) as a thrombin inhibitor. In these experiments, fibrinogen concentrations of 0.2 and 2.0 mg/ml, and a thrombin concentration of 0.05 u/ml were used at $25^{\circ}$, pH 6.8, ionic strength 0.15. The reaction was allowed to proceed and was stopped at various stages by adding TAMe. After TAMe addition the solutions were kept at room temperature for about 16 hours to allow steps 2 and 3 to go to completion. The supernatants from the clot were analysed for unreacted fibrinogen by U.V. absorption. The results of these experiments indicate that step 1 is first order in Fibrinogen with essentially no dependence of the first order rate constant on the initial fibrinogen concentration.

Using the same method and under the same conditions as

above, Scheraga and Ehrenpreis (46) obtained values of the first order rate constant ($k_1$) for step 1 at pH's ranging from 6 to 9. These results show that the maximum value of $k_1$ (2.5/min/TAMe unit of thrombin) occurs at about pH 8.

Kinetic data on step one in 1 M NaBr at pH 5.3 (where no polymerization occurs) were obtained by following the rate of production of fibrin monomer, again using TAMe as a thrombin inhibitor, by Ehrenpreis, et al (15). Under these conditions it was necessary to work at low temperatures (0°C), with high thrombin (100 u/ml) and fibrinogen (30 mg/ml) concentrations and with long reaction times (up to 6 days). Analysis of the data indicated that the reaction is first order with respect to both thrombin and fibrinogen. The authors concluded that step 1 appeared to be a simple bimolecular reaction to form the enzyme-substrate complex under conditions where the enzyme is not saturated.

Mihalyi and Billick have presented a kinetic analysis of the pH changes associated with the conversion of fibrinogen into fibrin (39). The method used gives a direct estimate of the hydrogen ions liberated, or taken up, during the overall reaction. This method is unable to distinguish the two polymerization steps (if indeed there is a difference) and the entire polymerization process is treated as a single step.

Using an initial fibrinogen concentration of 1.37%,

ionic strength 0.3, thrombin 1 TAMe u/ml at 25°, continuous records of pH changes with time were obtained. The recordings obtained below pH 8 indicated that hydrogen ions are released according to a first order reaction law over more than 90% of the reaction. The rate constant calculated from these results shows a steady increase from pH 5.7 to pH 8.0. Above pH 8 the pH recordings have a biphasic character. First the pH decreases, reaches a minimum and then increases and levels off before attaining the initial value, indicating the presence of two reactions, one producing the other absorbing hydrogen ions. The biphasic curves were analysed in terms of two opposing simultaneous first order reactions and the authors concluded that one reaction corresponded to the proteolysis step and the other corresponded to the polymerization step. The rate constants calculated for the two reactions are plotted against pH in Fig. VII. The rates of the proteolysis step calculated from the biphasic curves (i.e. above pH 8) fit smoothly with the points of the single reaction on the acidic side. This data also shows that the polymerization step ($k_2$) is slower than the proteolysis step ($k_1$), with a $k_1$ 2.5 times greater than $k_2$ at pH 8 and assuming that the curve can be extrapolated, $k_1$ 1.5 times greater than $k_2$ at pH 7.5.

The authors propose that hydrogen ions are involved in both the proteolysis reaction and the polymerization reactions with the net production or absorbtion of hydrogen ions depending

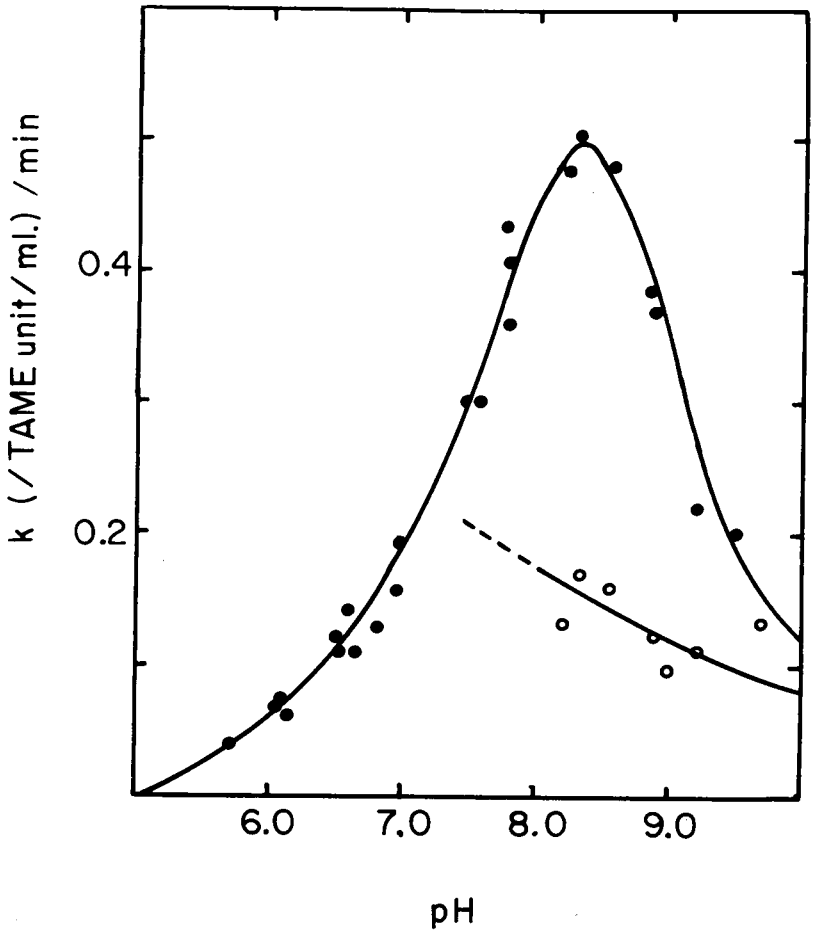



**Fig. VII.** Rate constants of the first ● and of the second ○ reactions plotted against pH. Fibrinogen concentration in the four experiments collected in this figure varied between 1.02 and 1.44%, Ionic strength 0.30, temperature 25°. (39)

on the initial pH. The reaction at the two extreme pH's of the clotting reaction can be written as follows:

| | pH 6 | | pH 10 | | |
|---|---|---|---|---|---|
| 1 | $nF \longrightarrow nf$ | | $nF \longrightarrow nf + nH^+$ | | |
| 2 | $nf \longrightarrow f_n + nH^+$ | $nf + nH^+ \longrightarrow f_n$ | | | 14. |
| Overall | $nF \longrightarrow f_n + nH^+$ | | $nF \longrightarrow f_n$ | | |

F = fibrinogen, f = fibrin monomer, $f_n$ = fibrin polymer, n = a variable number.

Because the titration ranges of the groups concerned overlap to a considerable degree, hydrogen ions are liberated by both the first and the second step when the reaction is carried out at an intermediate pH.

The first order dependence on fibrin monomer concentration of the polymerization step can be implied from the results of Sherry et. al. (32). Fibrin monomer was prepared in 1 M NaBr, as described previously. This was then allowed to polymerize at room temperature in 0.1 M phosphate buffer at pH 6 and the course of the polymerization followed by absorbance measurements at 350 mu. Initial fibrin monomer concentrations of 0.15 to 0.6 mg/ml were used and the curves shown follow a first order reaction course.

From these experimental curves, the rate constant for polymerization was calculated to be about 0.2/min at pH 6, ionic strength 0.1, room temperature (20-25°).

A summary of the kinetic constants pertaining to the proteolysis step is given in Table III.

2. Reversibility and Thermodynamics.

Laskowski, et. al. (30) have investigated the equilibrium of step 1 in 1 M NaBr, pH 5.3 at 0°, 15° and 25° under conditions similar to those used by the same group to study the kinetics of step 1. This equilibrium was investigated over a range of concentrations and temperatures by determining the concentration of f (monomer) in the reaction mixture at equilibrium, the equilibrium being approached from both sides (i.e. using both forward and reverse reactions).

By analysing for f, the degree of reaction $\alpha$, can be computed at any time during the course of the reaction,

$$\alpha = \frac{(f)}{(F) + (f)} \qquad 15.$$

where the parentheses denote molar concentrations.

## TABLE III

## SUMMARY OF KINETIC CONSTANTS FOR STEP 1

| Fibrinogen mg/ml | Temp. °C | Solvent or Buffer | Ionic Strength | pH | Thrombin* TAMe u/ml | k/min /TAMe u. | Ref. |
|---|---|---|---|---|---|---|---|
| 30 | 0 | NaBr | 1.0 | 5.3 | 100 | $1.7 \times 10^{-6}$ | (15) |
| 30 | 25 | NaBr | 1.0 | 5.3 | 100 | $5.0 \times 10^{-5}$ | (15) |
| 2.0 | 25 | Phosphate | 0.15 | 6.8 | 0.045 | 1.55 | (14) |
| 0.2 | 25 | Phosphate | 0.15 | 6.0 | 0.045 | 0.183 | (46) |
| 0.2 | 25 | Phosphate | 0.15 | 7.0 | 0.045 | 1.49 | (46) |
| 0.2 | 25 | Phosphate | 0.15 | 8.0 | 0.045 | 2.50 | (46) |
| 0.2 | 25 | Phosphate | 0.15 | 9.0 | 0.045 | 0.93 | (46) |
| 13.7 | 25 | Phosphate | 0.30 | 6.0 | 10 | 0.06 | (32) |
| 13.7 | 25 | Phosphate | 0.30 | 7.0 | 10 | 0.19 | (32) |
| 13.7 | 25 | Phosphate | 0.30 | 8.0 | 10 | 0.46 | (32) |
| 13.7 | 25 | Phosphate | 0.30 | 9.0 | 10 | 0.34 | (32) |

* 1 TAMe unit is the amount of thrombin which hydrolyzes 0.1 umole of TAMe in one minute at pH 8.0 and 25° from 0.01 M TAMe solution in 0.15 M KCl.

Fig. VIII shows a plot of $\alpha$ vs time at 0° for an initial fibrinogen concentration of 30 mg/ml and in which equilibrium was approached from both sides. Two important observations are immediately apparent; first, the values of $\alpha$ in the forward runs do not reach unity and secondly the values of $\alpha$ decrease in the reverse experiments, approaching the equilibrium values of the forward runs. These results provide strong evidence that step 1 is reversible since essentially the same equilibrium position is reached from both directions.

If step 1 is represented as

$$F \rightleftharpoons f + P \qquad 16.$$

then the equilibrium constant will be given by

$$K = \frac{\alpha_{eq}^2 F_o}{1 - \alpha_{eq}} \qquad 17.$$

If this is the case then the values of $\alpha_{eq}$ at a given temperature should be dependent on $F_o$. However, when the initial fibrinogen concentration ($F_o$) was varied from 10 to 30 mg/ml it

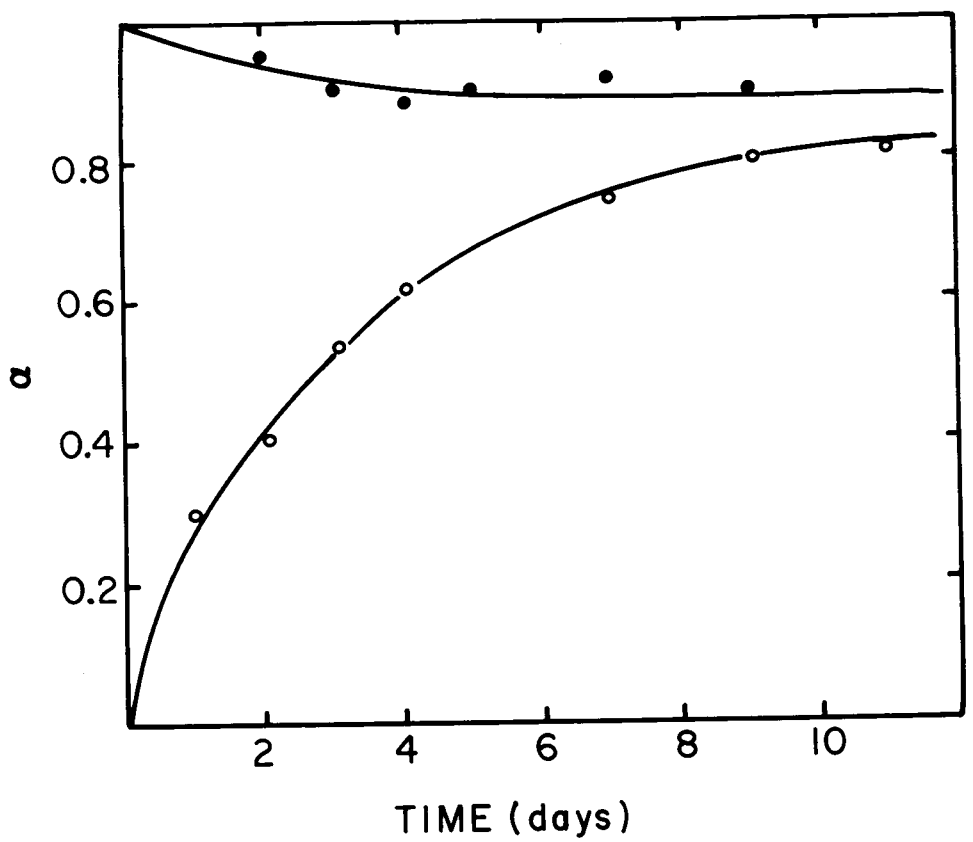



Fig. VIII. Variation of α with time for forward ○ and reverse ● runs at 0° in NaBr. $F_o$ = 30 mg/ml, thrombin = 100 TAMe u/ml. (30)

was found that the equilibrium values of $\alpha$ ($\alpha_{eq}$) were essentially independent of $F_o$. Step 1 must therefore be modified so that an equation of the form

$$K = \frac{\alpha_{eq}}{1 - \alpha_{eq}} \qquad 18.$$

will hold. That is, step 1 should be formulated in a manner such that the total number of molecules does not change. If it is assumed that P and f are associated even after hydrolysis (e.g. by side chain hydrogen bonds) then step 1 can be written as

$$F \overset{T}{\rightleftharpoons} f\ldots P \qquad 19.$$

with an additional equilibrium for the dissociation of the f...P complex

$$f\ldots P \rightleftharpoons f + P \qquad 20.$$

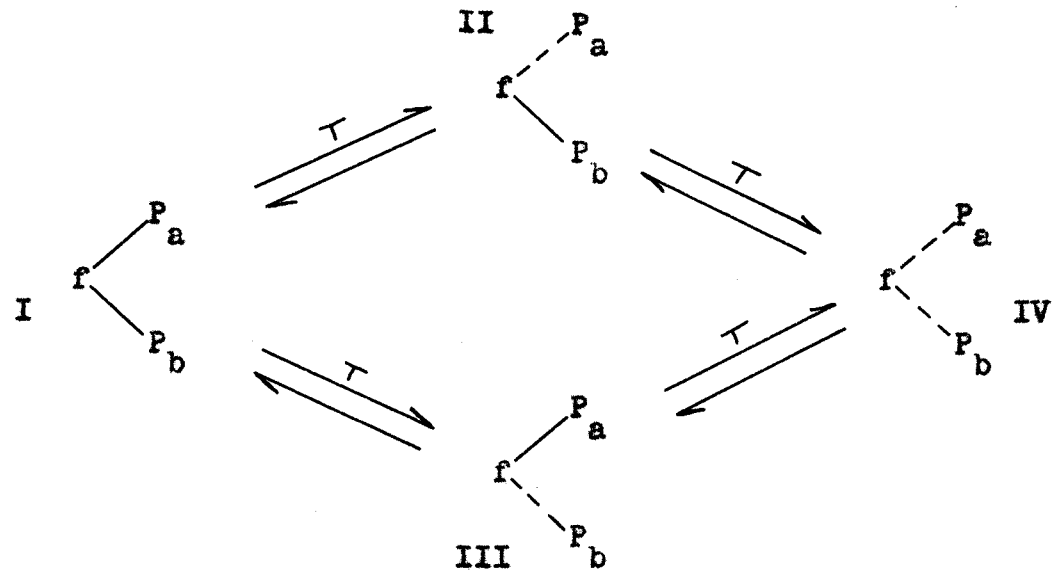
It has been shown previously (Chapter I) that at least

two types of peptide, $P_A$ and $P_B$ are involved in step 1 and that there are two molecules of each peptide per molecule of fibrinogen of 340,000 molecular weight. Taking account of only the type and not the number of peptides these equilibria can then be written as in Fig. IX. Form I is fibrinogen; forms II and III represent molecules in which a peptide bond has been hydrolysed, but the resulting peptides $P_A$ and $P_B$ are still associated with the remainder of the molecule. Form IV is a species in which two peptide bonds have been hydrolysed but both peptides are associated with f. Equations a, b and c of Fig. IX represent dissociation reactions.

Laskowski et. al. (30) also carried out similar experiments at 15° and 25° and by plotting log K vs 1/T, a value of $\Delta H^o$ = 8 Kcal/mole was obtained. From this and the values of $\Delta F^o$ obtained from the K values at the three temperatures a value of $\Delta S^o$ = 32 cal/mole/deg. was obtained.

If it is assumed that the hydrolysis of the peptide bond is accompanied by the rupture of some but not all of the side chain hydrogen bonds in the formation of the f...P complex and if all hydrogen bonds are equivalent and of the heterologous single bond type then it can be shown (28) that

$$\Delta H^o_{ob} = \Delta H^o_p + P/2\ \Delta H^o_{ij} \qquad 21.$$



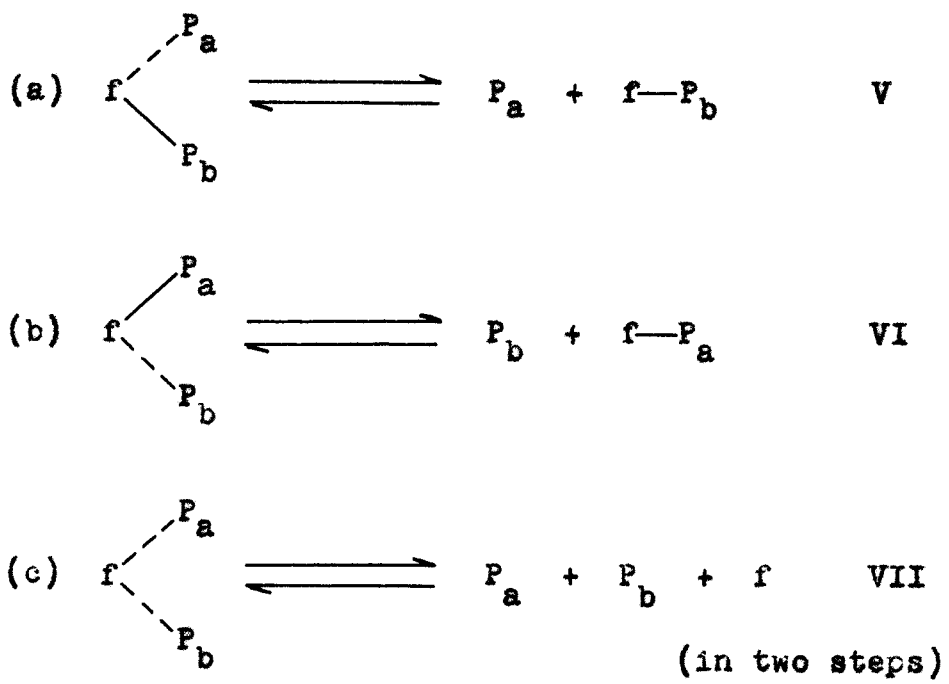




**Fig. IX.** Schematic representation of step 1. (30)

Where $\Delta H^{o}_{ob}$ is the experimentally determined enthalpy, $\Delta H^{o}_{p}$ is the value to be expected for a simple peptide, $\Delta H^{o}_{ij}$ is the enthalpy of formation of an internal hydrogen bond and p is the number of bonds involved. Using values of -1.55 and -6.0 Kcal/mole for $\Delta H^{o}_{p}$ and $\Delta H^{o}_{ij}$ and the experimental value of 8 Kcal/mole for $\Delta H^{o}_{ob}$, a value of $p = 3$ is obtained from equation (21) indicating that 3 hydrogen bonds are broken in the process. The necessity to rupture these hydrogen bonds thus provides some stability to the peptide bond to prevent the hydrolysis from going to completion.

Attempts at determining $\Delta H$ calorimetrically proved unsuccessful because of the very slow rate of the reaction at pH 5.3 and when it was determined at pH 6.1 where the reaction is faster, a value of zero was obtained. A similar result can be inferred from the data of Laki (26). A pH dependence is therefore postulated for this parameter and such a dependence would arise if the p hydrogen bonds are carboxyl-carboxyl dimer type bonds rather than heterologous single bonds.

Step 1 can therefore be regarded as involving proteolysis, with the formation of an f...P complex accompanied by the rupture of one carboxyl-carboxyl double hydrogen bond. The peptide remains associated to the fibrin monomer core even after proteolysis. Kay and Marsh (24) have shown that no optical rotation change accompanies step 1 and conclude that the groups necessary

for polymerization (step 2) appear in an unmasking or uncovering of groups rather than in a rupture of side chain hydrogen bonds when P is dissociated from the f···P complex. The fact that this dissociation is highly pH dependent thus accounts for the lack of polymerization at low pH's. The sole necessity for a thrombin induced proteolysis followed by dissociation of the peptide appears to be to liberate the donors (or acceptors) thus making them available for the polymerization reactions (step 2).

# CHAPTER III

## MATERIALS, METHODS AND PRELIMINARY STUDIES

### A. FIBRINOGEN

#### 1. Purification

Fibrinogen was purified by successive ammonium sulfate precipitation of commercially obtained bovine fibrinogen (Cohn Fraction 1) (25). Six grams of fibrinogen (Sigma F-92-B93) was dissolved in 300 ml. of 0.15 M KCl (pH 6.3), allowed to stand overnight in the refrigerator and the small amount of precipitate which formed, filtered. The solution was warmed to room temperature and then saturated ammonium sulfate solution added dropwise with stirring to bring the solution to 18,22,25,30 and 35% saturation. At each level of saturation the precipitate formed was centrifuged, (10 mins., 10,000 rpm, 25°C), drained of excess supernatant and then dissolved in the minimum amount of 0.15 M KCl. At no time did the temperature exceed 27°. To remove excess ammonium sulfate the solutions were filtered and dialysed against 5 liters 0.15 M KCl overnight in the refrigerator (4°). In some cases a gel formed in the dialysis bag which redissolved on warming to room temperature. In one case this gel was removed, dissolved and

treated as a separate fraction (P2 gel, Table IV).

During the precipitation the protein precipitated as a gel up to about 20% ammonium sulfate saturation and then precipitated as discrete particles. When the above procedure was repeated the 18-22% fraction was divided into gel and discrete particles (GP2 and P2) and treated as separate fractions. A further fraction was obtained during dialysis, as above.

These fractions were then clotted with thrombin and the % non-clottability (%NC) determined at 0.5 mg/ml fibrinogen. The %NC was also determined by the Laki method (see below) for comparison. The results obtained are shown in Table IV.

From the data of Table IV it can be seen that the starting material (Sigma F-92-B93) contained 50% protein of which 69% was clottable. Fibrinogen fractionated below 22% ammonium sulfate saturation was fairly consistent with respect to %NC and was 89-94% clottable, but that fractionated above 22% saturation was virtually non-clottable. It is also apparent that material precipitating as gel is consistently purer than material precipitating as discrete particles.

The preparations were stored either as the frozen solution or were lyophilized and stored at -20°.

2. Clottability of Fibrinogen

The purity of fibrinogen preparations is normally

## TABLE IV

## PURIFICATION OF FIBRINOGEN

| Fraction | %$AmSO_4$ Saturation | Volume ml. | Fibrinogen** mg./ml. | Fibrinogen** Total mg. | %NC* |
|---|---|---|---|---|---|
| Exp.1. | | | | | |
| Original | 0 | 300 | 10.25 | 3075 | 31.0 |
| P1 | 0-18 | 41 | 4.3 | 176 | 9.9 |
| P2 | 18-22 | 70 | 5.3 | 371 | 11.0 |
| P2gel | 18-22 | 62 | 5.6 | 348 | 6.7 |
| P3 | 22-25 | 25 | 3.4 | 85 | 65.0 |
| P4 | 25-30 | 27 | 3.3 | 89 | 101 |
| P5 | 30-35 | 29 | 3.1 | 89 | 99 |
| Supernatant | | 462 | 0.2 | 92 | 99 |
| Exp.2. | | | | | |
| Original | 0 | 300 | 10.9 | 3270 | |
| P1 | 0-18 | 65 | 6.0 | 390 | 7.1 |
| P1gel | 0-18 | 40 | 5.8 | 232 | 6.6 |
| P2 | 18-22 | 63 | 10.3 | 650 | 8.2 |
| GP2 | 18-22 | 93 | 9.8 | 910 | 5.9 |
| P3 | 22-25 | 48 | 2.2 | 106 | 53.5 |

By the Laki method the P1, P2 fractions were 4.8-3.7 %NC.

* Average of two determinations.

** Calculated on the basis of 1.60 OD-280/mg./ml. (13)

estimated by measuring the amount of a preparation that can be converted into an insoluble fibrin clot. The method most widely used and the one usually quoted in the literature when describing fibrinogen preparations is that of Laki (25).

In this method, the following mixture is prepared. 1 ml. fibrinogen solution (about 7-8 mg/ml.), 0.1 ml. buffer pH 6.1 (3 parts 0.5 M $KH_2PO_4$, 1 part 0.5 M $Na_2HPO_4$), 2.5 ml. water. To this is added 0.1 ml. thrombin solution, (Parke-Davis dissolved in water and diluted to 250 u/ml.) and the solution mixed. The clot which forms is removed after 1 hour and the OD-280 of the supernatant is measured. From this and the OD-280 of the original fibrinogen solution (converted to the same dilution as in the above method), the amount of fibrinogen taken up as insoluble clot can be estimated. When expressed as % this is the % clottability. Conversely, 100 - % clottability gives % non-clottability (%NC) of the preparation.

Although this is undoubtably a good method for comparing different fibrinogen preparations, it does not give the amount of non-clottable material found under the experimental conditions used in this study viz., pH 7.4 Tris-acetate $\mu$ = 0.15. Consequently, the %NC reported in this study, unless otherwise stated, is that determined in pH 7.4 Tris-acetate, and at the fibrinogen concentration existing in the Tris-acetate solution. This method is as follows.

Three ml. of Tris-acetate is added to a 1 cm. Beckman silica cell and the absorbance at 280 mμ. measured. Fibrinogen solution is then added and the absorbance measured again. One or two units of thrombin are then added, the solution mixed by inversion and the OD-310 is measured at 30 minute intervals. When the OD-310 has reached a maximum, (about 90-120 mins) the clot is removed using a fine copper wire loop and the OD-280 of the supernatant is measured. Necessary corrections are made to allow for Tris-acetate dilution and thrombin absorbtion. From the original and final OD-280 measurements, the %NC can be estimated.

3. Rate of Clot Formation

The rate at which the fibrin clot is formed can be estimated by measuring the rate of increase in OD-310 of a solution containing fibrinogen and thrombin. The method also gives a method of estimating the clotting time i.e. the time taken to the first appearance of clotted material.

A Beckman DU spectrophotometer and a Photovolt model 43 Linear-Log recorder was used to measure clot opacity. The fibrinogen solution is placed in a 1 cm. silica cell and the Beckman and recorder adjusted so that with the solution in the light path the recorder shows an absorbance of 0.02 or 0.03. A measured amount of thrombin is then blown into the fibrinogen solution and at the same time the recorder chart is started making the time of mixing.

The solution is mixed by inverting the cell three of four times, quickly replaced in the Beckman cell holder and the recorder pen switched on (lapsed time about 15 secs.). A record of the rate of increase in OD-310 is thus obtained. A typical record obtained in this way is shown in Fig. X. By projecting the initial sharp increase in OD-310 back to the base measurement an estimate of the clotting time ($t_c$) is obtained. Other parameters measured are $V_o$, the initial sharp increase in OD-310 and V, the slope of the tangent to the slower increases in OD-310 which passes through zero time.

4. Calibration of Clot Opacity

To obtain meaningful data from the opacity-time recordings it was necessary to calibrate the opacity of the clot at OD-310 with the amount of fibrinogen in the clot. It was most important to calibrate these two quantities when the opacity of partially formed and completely formed clots were being measured in order to establish the proportionality of clot opacity during the process of clot formation with protein concentration.

This calibration was carried out as follows. Fibrinogen at varying concentrations in pH 7.4 Tris-acetate was clotted with Parke-Davis thrombin (0.065 u/ml). The OD-310 was measured after reaching a maximum, the clot was removed with a fine copper wire

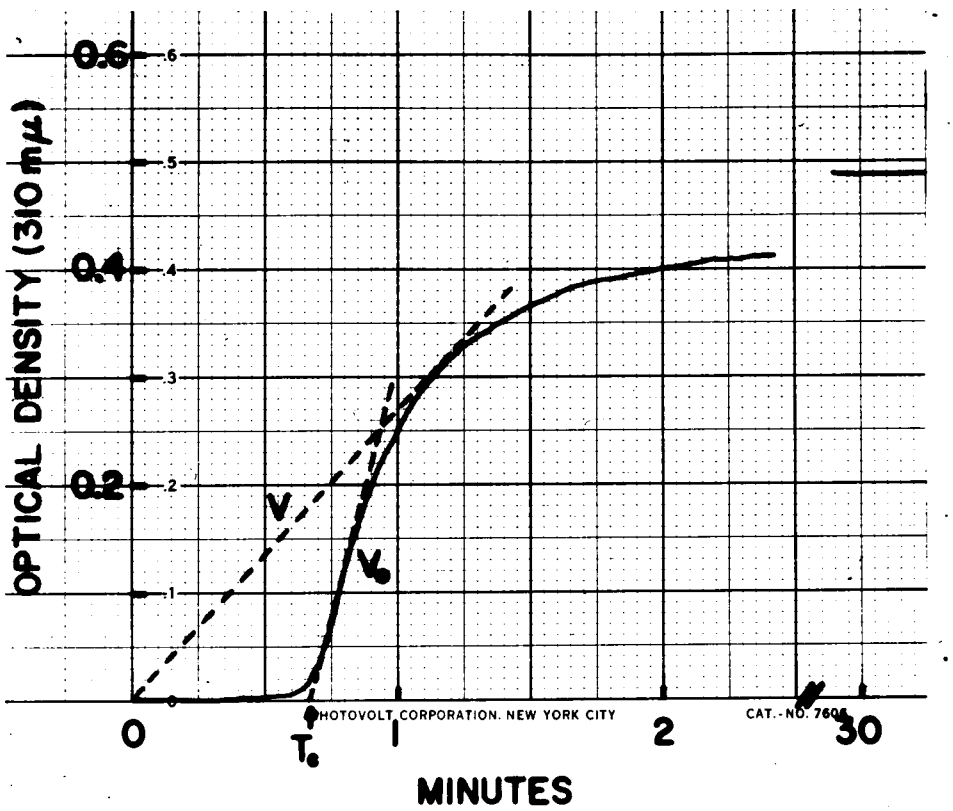



Fig. X. An actual record of the increase in opacity at 310 mμ. due to clot formation.

and the fibrinogen remaining in solution determined by measuring the OD-280. From a knowledge of the OD-280 before and after clot formation and the OD-310 of the clot, it was possible to calibrate OD-310 of the clot with the amount of fibrinogen incorporated ($\Delta$ OD-280) in the clot.

The calibration was carried out on partially formed clots by making use of the fact that at low thrombin concentrations, if a clot is removed before the reaction is complete sufficient time elapses to make an OD-280 measurement before clot can again form. Records were made of the rate of increase of OD-310, then using identical fibrinogen and thrombin solutions, the clot was removed after various lengths of time and the OD-280 quickly measured. From a knowledge of the original OD-280, the OD-280 immediately after clot removal and the OD-310 at the corresponding time from the record, calibration was possible during clot formation. Fig. XI shows the results obtained with purified fibrinogen (P1), using both the above methods, demonstrating that the clot OD-310 is directly proportional to the amount of fibrinogen in the clot and that the same calibration holds for partially formed clots. Therefore opacity measurements of the rate of clot formation can be expressed in terms of mg. fibrinogen/ml./min.

5. Extinction Coefficient of Fibrinogen

The extinction coefficient for fibrinogen has been

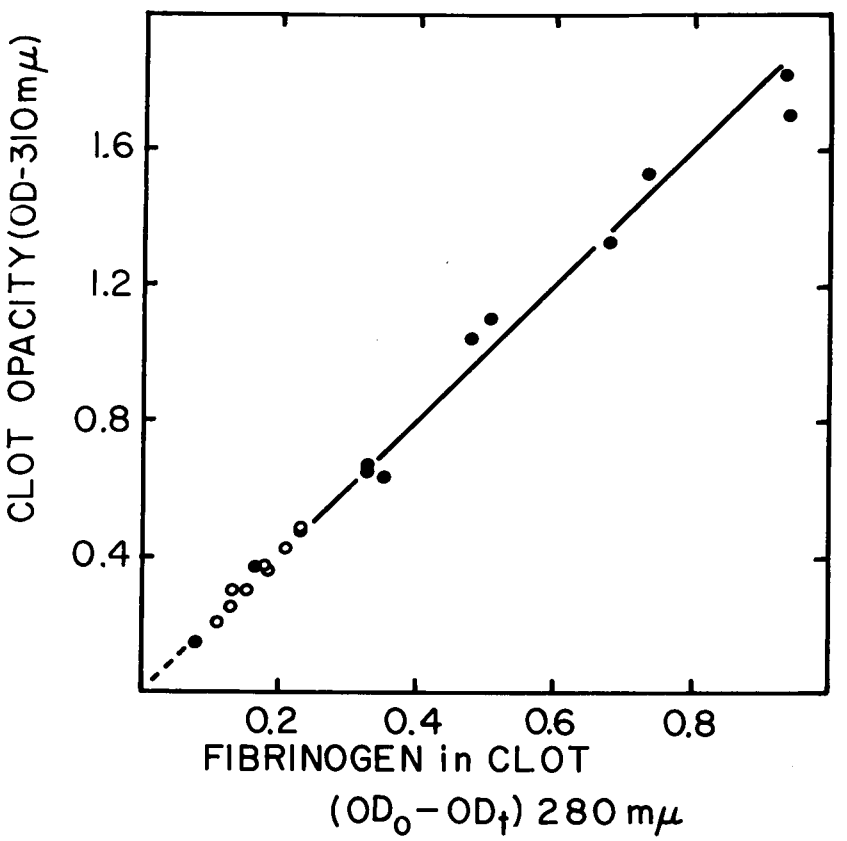



Fig. XI. Calibration of clot opacity at 310 mu. with the amount of fibrinogen in the clot. ● data from fully formed clots, o data from partially formed clots.

reported as 16.0 for 1 g./100 ml., 1 cm. cell, 280 mμ. by Ehrenpreis and Scheraga (13) 1.64 OD-280/mg./ml. by Hartley and Waugh (21) and 16.51, 1%, 1 cm, 282 mμ. by Blomback (7).

In this study a value of 1.6 OD-280/mg./ml. for fibrinogen will be assumed. Calibration of absorbance at 280 mμ. and 290 mμ. has been carried out, it being found that 1.0 OD-290 is equivalent to 1.4 OD-280.

Therefore the optical density of a solution of 1 mg. fibrinogen/ml. is equivalent to 1.6 at 280 mu. and 1.15 at 290 mμ.

## B. THROMBIN

Thrombin was purified by chromatography of Topical Parke-Davis Thrombin, (Bovine Origin). Two methods were used, one using phosphate cellulose and the other using Amberlite IRC-50 (XE-64). The thrombin obtained from the IRC-50 purification was processed through a Sephadex column in order to transfer the thrombin from the phosphate buffer to Tris-acetate buffer.

### 1. Purification

#### a) Chromatography on Phosphate Cellulose

A modification to the method of Seegers (52) was used in which the thrombin is absorbed at low ionic strength and eluted with buffers of increasing ionic strength.

The following phosphate buffers were used:-

Stock Buffer, pH 7.0, 0.10 M

5.382 g. $NaH_2PO_4.H_2O$, 21.9 g. $Na_2HPO_4.12H_2O$ dissolved and diluted to one liter with distilled water.

Buffer A: pH 7.0, 0.01 M

100 ml. Stock buffer diluted to one liter with distilled water.

Buffer B: pH 7.0, 0.05 M

500 ml. stock buffer diluted to one liter with distilled water.

Buffer D: pH 8.0, 0.30 M

2.2 g. $NaH_2PO_4.H_2O$, 101.4 g. $Na_2HPO_4.12H_2O$ dissolved and diluted to one liter with distilled water.

Buffer C: pH 8.0, 0.15 M

500 ml. Buffer D diluted to one liter with distilled water.

The cellulose phosphate used was Celu Ion P (phosphorylated Whatman cellulose), Nutritional Biochemicals Corp. Control No. 9732. Celu Ion P was stirred with buffer A using a magnetic stirrer for 10 mins. and the supernatant decanted. The process was then repeated. A 21 X 0.9 cm. column was then poured under gravity and buffer A passed at maximum rate (3.5 ml./min.) for one hour, after which time the pH of the eluent was 7.1.

The thrombin used was Topical Parke-Davis Thrombin,

(Bovine) Bio 2077, stated as being 1000 NIH units per vial, Lot 090345 A. One vial was dissolved in 2.0 ml. distilled water and a 0.1 ml. sample removed and added to 5 ml. pH 7.4 Tris-acetate buffer and assayed for thrombin activity and protein. The column was drained to bed level and the thrombin solution applied to the column. This was allowed to drain in and followed by 3 x 2 ml. washings of buffer A. The column was then connected to a reservoir and the buffer solutions passed at a rate of 15 ± 1 ml./hour. Each fraction was assayed for protein by measuring the absorbance of the effluent at 280 mμ. and for thrombin activity using clotting Method A. The buffer was changed after the elution of a peak as judged by the absorbance at 280 mμ. The column was always drained to bed level when the buffer was changed. The column was jacketed and the temperature was controlled at 25°C throughout the run. The eluate was collected in 5 ml. fractions using a Vanguard model 1000 volumatic fraction collector. About 70% of the protein was eluted after 20 ml. of buffer A, but very little (2.5%) thrombin activity appeared in this peak. Only traces of protein and thrombin were eluted with buffers B or C and only after eluting with the higher ionic strength buffer D did any activity appear.

The fractions which appeared to contain appreciable amounts of thrombin by clotting method A were assayed for esterase activity using TAMe. The purification results are shown in Table

## TABLE V

## PURIFICATION OF THROMBIN ON PHOSPHATE-CELLULOSE

| Fraction | u/ml. | Volume ml. | Total u. | OD-280 | u/OD-280* | Purification |
|---|---|---|---|---|---|---|
| Original | 1100 | 2.0 | 2200 | 0.284 | 75.7 | 1 |
| 3 | 5.0 | 5.0 | 25.0 | 2.0 | 2.5 | 0.03 |
| 4 | 6.25 | 5.0 | 31.3 | 2.0 | 3.1 | 0.04 |
| 21 | 0.9 | 5.0 | 4.5 | 0.170 | 5.3 | 0.07 |
| 38** | 18.9 | 10.0 | 189.0 | 0.052 | 364 | 4.8 |
| 39 | 11.0 | 5.0 | 55.0 | 0.023 | 480 | 6.2 |
| 40 | 2.9 | 5.0 | 14.5 | 0.003 | 970 | 12.9 |

* TAMe units per unit absorbance at 280 mu.

** Two fractions combined.

V. The data indicates that Parke-Davis Thrombin has about twice the stated (1000 NIH units) activity per vial and that the best purification was obtained by elution with the highest ionic strength buffer. Tubes 3, 4, 38, 39 and 40 were frozen and stored at -18°.

b) Chromatography on Amberlite IRC-50 (XE-64)

The method used was that of Rasmussen (43) in which the thrombin is absorbed at low ionic strength and eluted at high ionic strength.

The following buffers were used:-

Buffer A: 0.05 M Phosphate, pH 7.0

2.691 g. $NaH_2PO_4$, 4.331 g. $Na_2HPO_4$, dissolved in distilled water and made up to 1 liter.

Buffer B: 0.30 M Phosphate, pH 8.0

2.2 g. $NaH_2PO_4$, 40.33 g. $Na_2HPO_4$, dissolved in distilled water and made up to 1 liter.

The resin used was Bio-Rex 70, 200-325 mesh, sodium form, Control No. 2500, Bio-Rad Laboratories. Bio-Rex 70 was stirred with 0.3 M buffer using a magnetic stirrer for 10 mins. and the supernatant decanted. A 0.9 x 15 cm. column was then poured under gravity and washed with 100 ml. buffer B. At this point the eluate was pH 8.0. Buffer A was then passed (430 ml.),

after which the pH was 7.1. The eluate from the column was passed through a Beckman 10 mm. silica flow cell and the Beckman DU connected to a Photovolt model 43 Linear-Log recorder so that a continuous record of the eluate absorbance at 280 mμ. could be made.

The thrombin used was Topical Parke-Davis Thrombin, (Bovine) Bio 2076, Lot. 030782 A. Sixty five mg. of this material was dissolved in 2 ml. of buffer A and a 0.1 ml. sample removed and added to 1 ml. of pH 7.4 Tris-acetate and assayed for thrombin activity and protein. The column was drained to bed level, the thrombin solution applied to the column and followed by 2 x 0.5 ml. of buffer A. The column was then connected to a reservoir and buffer B passed at 7 ± 1 ml./hour. Chromatography was carried out at room temperature (27°). Fractions were collected manually on the basis of the recorded OD-280 and these fractions tested for clotting time by method A. The chromatogram is shown in Fig. XII. It can be seen from this figure that the greater part of the protein (70%) was eluted after 20 ml. and that very little thrombin activity appeared in this peak. The fourth large peak contained 37% of the thrombin activity and was collected as a single fraction (6.6 ml.). After removal of a 0.1 ml. aliquot for assay, the remainder was applied to a Sephadex gel column for buffer exchange. Chromatography on Bio-Rex 70 gave a five-fold increase in specific activity with 37% recovery of total activity.

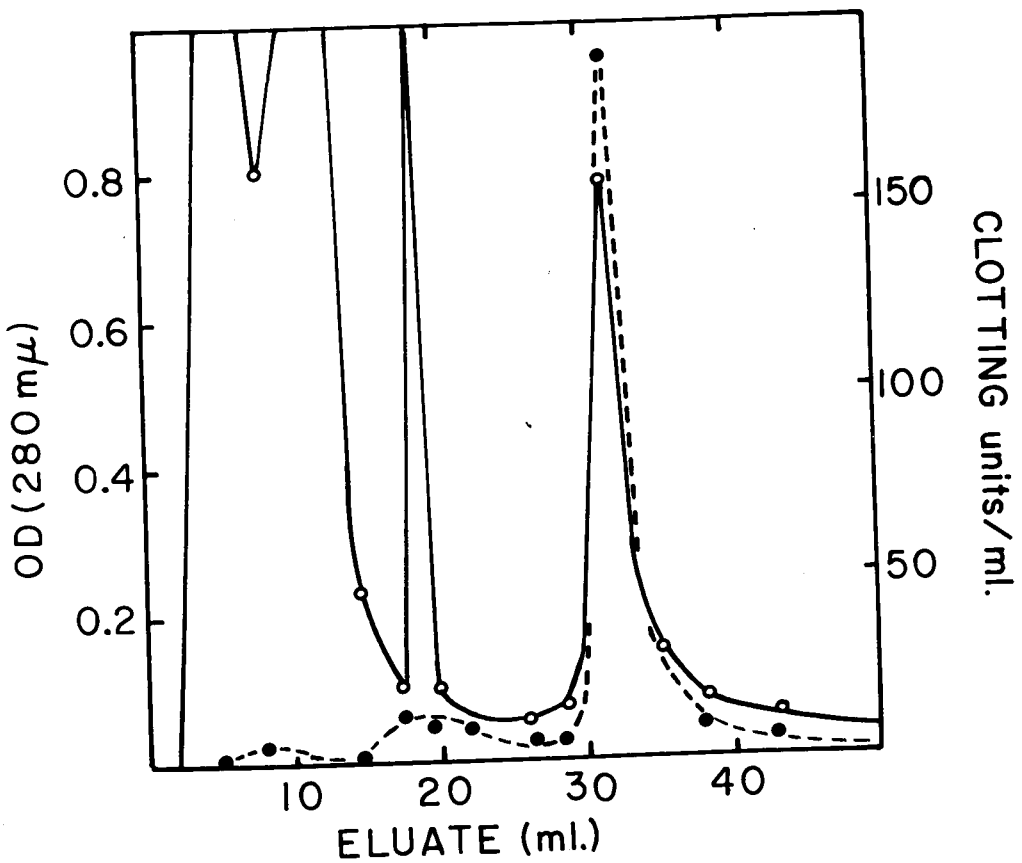



Fig. XII. Chromatography of thrombin. The solid line is OD-280 and the broken line u/ml thrombin by clotting method A.

c) Sephadex Gel Filtration

Sephadex G-50 (Fine 200/400 mesh) was used and the method was that suggested by Pharmacia Fine Chemicals (55).

Sephadex G-50 was slurried with pH 7.4 Tris-acetate ($\mu$ = 0.15). A 7.5 x 1.7 cm. (17 ml.) column was poured and Tris-acetate was passed through the column overnight. The eluate from the column was passed through a Beckman 10 mm. silica flow cell and the Beckman DU connected to a Photovolt model 43 Linear-Log recorder so that a continuous record of the absorbance at 280 m$\mu$. of the eluate could be made.

The column was drained to bed level, 6.5 ml. of the thrombin peak from the Bio-Rex 70 column was applied and followed by 2 x 0.5 ml. Tris-acetate buffer. The column was then connected to a reservoir and Tris-acetate buffer passed at a rate of 4 ± 1 ml./hour. Chromatography was carried out at room temperature ($27^{\circ}$). Fractions were collected manually on the basis of absorbance measurements and the volumes estimated by counting the number of drops of eluate per minute. Fractions of 1.3 ml. were collected and phosphate concentration was estimated by adding 2 drops of eluate to 2 drops of saturated $CaCl_2$ and comparing the precipitate formed, if any, with standards prepared from 0.01, 0.02, 0.03, 0.04, 0.05 M phosphate. The esterase activity of each fraction was measured by TAMe hydrolysis.

The purification results are shown in Table VI. Although most of the purification was obtained with chromatography on Bio-Rex 70 a further 30% increase in specific activity, with a 79% recovery of total activity, was obtained by gel filtration on Sephadex G-50.

The above fractions were frozen and stored at -20°.

2. Measurement of Thrombin Activity

a) Clotting Activity

Method A. An approximate clotting time method was used for the rapid estimation of thrombin activity in chromatographic eluates. Thrombin concentration was estimated by measuring the time to the first appearance of clotted material when 0.1 ml. of thrombin solution (eluate) was added to 0.5 ml. of fibrinogen solution (1 g. Sigma F91B-213 dissolved in 100 ml. pH 7.4 Tris-acetate $\mu$ = 0.15.

Method B. (N.I.H. Clotting Units) This was determined using the materials and methods specified by the National Institutes of Health (40).

The materials were prepared as follows:

15% Gum Acacia. Recrystallized gum acacia, 7.5 g. dissolved in and made up to 50 ml. with 0.9% NaCl.

Imidazole Buffer: 0.81 g. imidazole dissolved in 45 ml. 0.1 N HCl and made up to 50 ml. with water.

## TABLE VI

## GEL FILTRATION OF BIO-REX 70 THROMBIN ON SEPHADEX G-50

| Fraction | u/ml. | Volume ml. | Total u. | Phosphate M. | OD-280 | TAMe u/ml /OD-280 | Purification |
|---|---|---|---|---|---|---|---|
| Original* | 187.5 | 6.5 | 1220 | 0.3 | 0.319 | 590 | 1 |
| 1 | 0 | 2.0 | 0 | 0 | - | - | - |
| 2 | 0 | 2.0 | 0 | 0 | - | - | - |
| 3 | 10 | 1.2 | 12 | 0 | 0.073 | 770 | 1.3 |
| 4 | 91 | 1.2 | 109 | 0 | 0.100 | 910 | 1.5 |
| 5 | 142 | 1.2 | 170 | 0 | 0.187 | 760 | 1.3 |
| 6 | 262 | 1.4 | 366 | 0.01 | 0.340 | 770 | 1.3 |
| 7 | 173 | 1.5 | 242 | 0.02 | 0.350 | 495 | 0.8 |
| 8 | 43 | 1.5 | 65 | 0.05 | 0.220 | 195 | 0.3 |

* Single fraction obtained from Bio-Rex 70 chromatography.

Standardized Fibrinogen: Sigma F92B-93, (Bovine), Cohn Fraction 1. 1.5 g. of dried powder was dissolved in about 50 ml. distilled water and the pH adjusted to 7.2 with 0.5 M $Na_2HPO_4$ (about 2 ml.). The solution was then made up to 100 ml. with distilled water and filtered. This solution was made up for each experiment.

Titration Mixture: This was prepared by mixing together the following ingredients.

| | |
|---|---|
| 15% Gum acacia | 2 parts. |
| 1% $CaCl_2$ in 0.9% NaCl | 1 part. |
| Imidazole buffer | 1 part. |
| 0.9% NaCl solution | 5 parts. |

In the method, 0.18 ml. of the titration mixture is placed in a 12 x 75 mm. test tube. Exactly 0.06 ml. of thrombin is then added (the dilution of the thrombin will vary and must be made in 0.9% NaCl). Standardized fibrinogen, 0.06 ml., is then added with a blow-out pipette and the stop watch simultaneously. The first appearance of fibrin is the end point. When a 15 second clotting time is obtained on repeated tests at 28°C. ± 1.0°C the thrombin concentration in the final clotting mixture is 1 unit per ml. The concentration of thrombin in the original solution is 5 units per ml., since it is diluted five-fold when added to the final clotting mixture. Table A1 of the appendix gives the re-

sults obtained using purified thrombin and Fig. XIII shows the plot of clotting time against units/ml. thrombin. These results were used to calibrate the clotting and esterase activities of thrombin.

b) Esterase Activity (TAMe units)

A modification of the method of Hummel (23) was used. Thrombin activity is estimated using p-toluenesulphonyl-L-arginine methyl ester (TAMe) as substrate and the course of the hydrolysis is followed by measuring the rate of increase in absorbance at 247 mu. using a Beckman DU Spectrophotometer and a Photovolt model 43 Linear-Log recorder (chart speed 12 inches/hour).

Three ml. of 0.0015 M TAMe in pH 7.4 Tris-acetate $\mu$ = 0.15, was placed in a 1 cm. silica cuvette and the Beckman and recorder adjusted so that, with the solution in the light path, the recorder indicated an absorbance of about 0.03. A measured amount of thrombin was then added to the TAMe solution, the cuvette inverted 3 or 4 times, rapidly replaced in the Beckman cell-holder and the recorder started. The velocity of the reaction was calculated from the initial slope of the OD-247, time curves.

c) Determination of Kinetic Constants for TAMe Hydrolysis

The Michaelis-Menten equation (37) for an enzyme

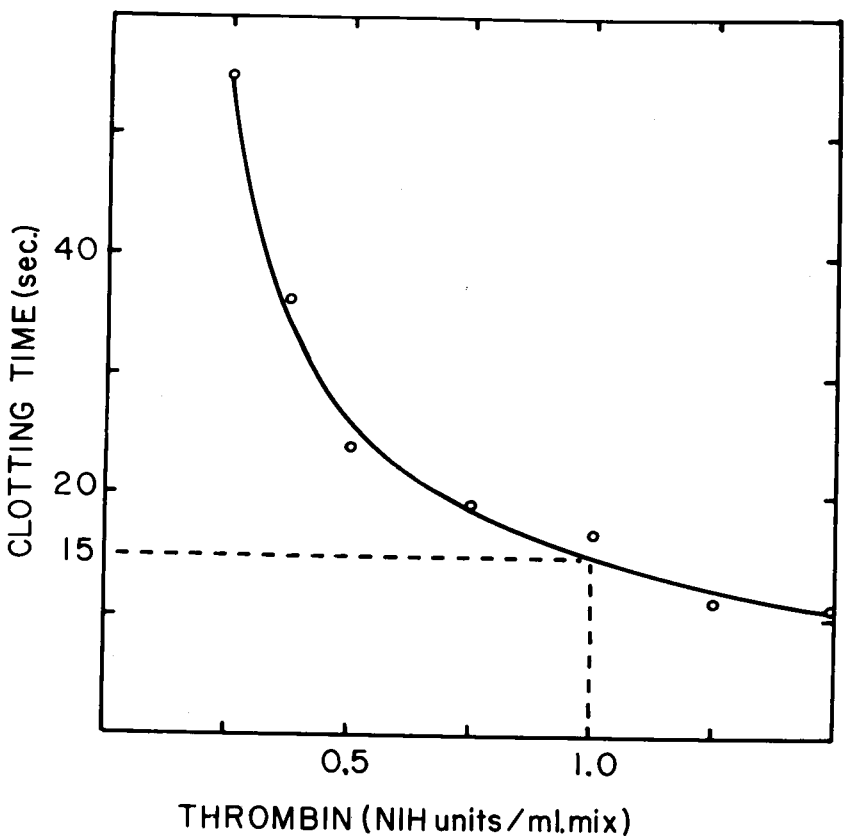



Fig. XIII. Clotting time versus thrombin concentration. Temperature 28 ± 1°, pH 7.2. By definition 1 u/ml will give a clotting time of 15 seconds.

catalyzed reaction is,

$$v_o = \frac{V_m S}{S + K_m} \qquad 22.$$

Where $v_o$ = initial velocity, $V_m$ = maximum velocity, S = substrate concentration and $K_m$ = the Michaelis substrate constant.

This equation can also be written in the form (Lineweaver-Burke) (33).

$$1/v_o = K_m/V_m \cdot 1/S + 1/V_m \qquad 23.$$

Using the method described above, values of $v_o$ were determined for Parke-Davis thrombin and for highly purified thrombin (fraction 7, Table VI) Table A2 of the appendix shows the results obtained and Fig. XIV shows a plot of $1/v_o$ against 1/S. From Fig. XIV and equation 23, $K_m$ was found to be $1.73 \times 10^{-4}$ M TAMe.

d) Definition of TAMe unit

In order to calibrate the change in optical density at

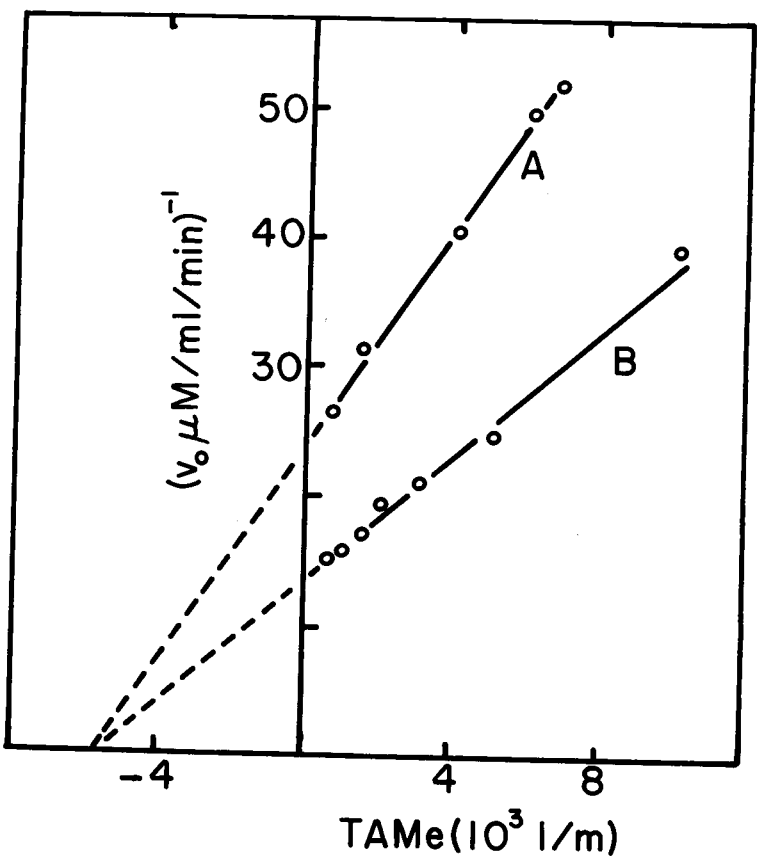



**Fig. XIV.** Liveweaver-Burke plot for the thrombin hydrolysis of TAMe. Curve A is purified thrombin, 2 u/ml., Curve B is Parke-Davis thrombin 3.5 u/ml. $K_m = 1.73 \times 10^{-4}$M. $V_m = 0.012$ $\Delta$ OD-247/min/u/ml.

247 mμ. during TAMe hydrolysis in terms of μmole/min./ml., two 0.0015 M TAMe solutions were hydrolyzed with thrombin for several days. These solutions were diluted with Tris-acetate buffer to various p-toluenesulfonyl-L-arginine (TA) concentrations and the OD-247 measured and compared to freshly prepared TAMe solutions. The extinction coefficient for TAMe is 1260 OD-247/mole/liter and for TA is 1830 OD-247/mole/liter. The change in OD-247 for the hydrolysis of 1 mole of TAMe to 1 mole of TA is therefore 1830-1260 = 570/liter. Therefore, a change of 1.00 OD-247 is equivalent to 1.75 μmole TAMe hydrolyzed/ml. Since it is shown in section 4 below that 1N1H unit/ml. of thrombin activity is equivalent to 0.0120 Δ OD-247/min., it is also equivalent to 0.021 μmole TAMe hydrolyzed/min./ml.

For the purposes of this study, 1 TAMe unit of thrombin will be defined as that amount which will give a maximum velocity of 0.012 OD-247/min. (0.021 μmole/min./ml.) when the reaction is carried out in 0.0015 M TAMe in pH 7.4 Tris-acetate $\mu$ = 0.15, at 30°. A TAMe unit so defined is then equivalent to a clotting unit.

The initial velocities of the hydrolysis were determined using thrombin concentrations of 0.28 to 3.4 u/ml. Fig. XV shows maximum velocity, calculated using $K_m = 1.73 \times 10^{-4}$ M TAMe, plotted against thrombin concentration. This figure demonstrates that the reaction is first order with respect to thrombin

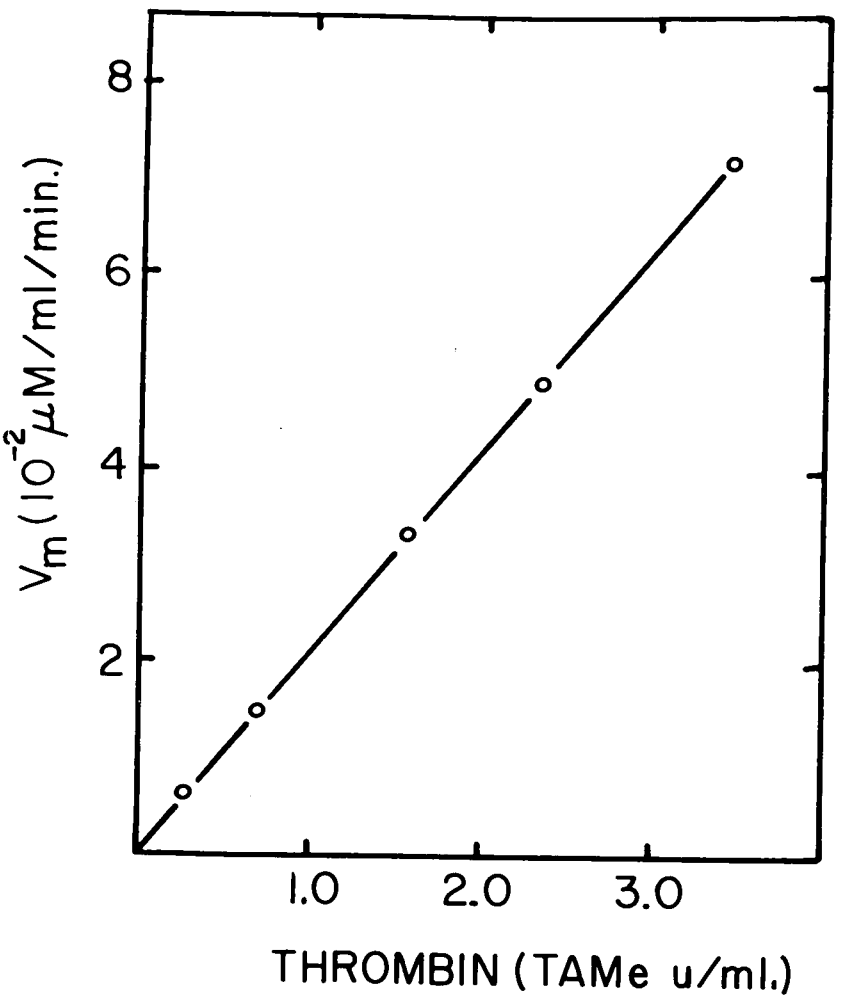



Fig. XV. Maximum velocity versus u/ml thrombin for the hydrolysis of 0.0015 M TAMe in pH 7.4 Tris-acetate.

concentration.

3. Calibration of Clotting and Esterase Activity

This calibration was carried out by using the same thrombin preparation to determine the clotting activity (by method B) and the esterase activity (by TAMe hydrolysis). The esterase activity was determined before and after the clotting time determinations and no change was detected. Typical clotting time results are shown in Table A1 and Fig. XIII. The value of the maximum velocity of TAMe hydrolysis, $V_m$, corresponding to 1 NIH Clotting unit/ml. was determined from the value of the initial velocity $v_o$ ( $\Delta$OD-247/min.) obtained using 1 NIH clotting unit/ml. for the hydrolysis and the Michaelis Menten equation in the form

$$V_m = v_o(1 + K_m/S) \qquad 24.$$

Where, in this case $K_m = 1.73 \times 10^{-4}$ M TAMe and $S = 1.5 \times 10^{-3}$ M TAMe.

The results obtained using various thrombin preparations are shown im Table VII.

## TABLE VII

## CALIBRATION OF THE CLOTTING AND ESTERASE ACTIVITIES OF THROMBIN

| Thrombin | 1 NIH Clotting unit/ml equivalent to ($V_m$) |
|---|---|
| Parke-Davis | 0.0132 OD-247/min |
| Purified on Bio-Rex 70 (Table VI) | |
| Fraction 3 | 0.0115 OD-247/min |
| Fraction 7 | 0.0117 OD-247/min |
| Fraction 8 | 0.0114 OD-247/min |
| Average | 0.0120 - 0.0006 OD-247/min |

## 4. TAMe Inhibition of Thrombin Clotting Activity

### a) Method

TAMe can be used to completely inhibit the proteolytic reaction (step 1) (14,15). Thrombin is added to the fibrinogen solution and reaction allowed to take place. At various time intervals, a small amount of concentrated TAMe solution is rapidly blown into the reaction mixture and mixed. The mixture is allowed to stand thus allowing steps 2 and 3 (polymerization) to go to completion. The resulting clot is removed and the remaining unreacted fibrinogen estimated from the UV absorption.

In actual experiments the solutions containing TAMe were left about 16 hours to allow steps 2 and 3 to take place and blanks were run in which TAMe was added before the thrombin. It was found that only when the original fibrinogen concentration was higher than 2 mg/ml did traces of clot appear in the blanks thus limiting this method to situations where the original fibrinogen concentration was 2 mg/ml. or less.

The ultraviolet absorption of TAMe interferes with that of fibrinogen at 280 mμ. but at 290 mμ., where fibrinogen still has considerable absorption, this interference is essentially negligible (14). Thus the analysis for unreacted fibrinogen can be carried out at 290 mμ. in the presence of TAMe used as the thrombin inhibitor.

The following procedure was used. Four millilitres of fibrinogen solution in pH 7.4 Tris-acetate buffer were placed in a 12 x 120 mm test tube. Then thrombin solution (usually about 50 or 100 ul) was blown in and mixed on a Vortex mixer. To stop the reaction at any given time, 0.4 ml. of 0.4 M TAMe in pH 7.4 Tris-acetate buffer was blown into the solution and again mixed on a Vortex mixer, this serving to break up the clot (if any) and distribute the TAMe throughout. For blanks, the TAMe was added first, followed by the thrombin. The completeness of thrombin inhibition by TAMe was shown by the complete absence of clotting in the blanks. The concentration of TAMe in the "stopped" solutions was 0.036 M. After adding TAMe, the solutions were kept at room temperature for about 16 hours to complete the clotting of all of the products of step 1, i.e. steps 2 and 3 were allowed to go to completion in the stopped solution. After this time any clot formed was removed using a copper wire loop or by filtration, and the OD-290 of the supernatant measured. Values of the concentration of unreacted fibrinogen were thus determined as a function of time.

The OD-290 of the fibrinogen remaining in the supernatant was calculated as follows.

1. Absorption of silica cells and pH 7.4 Tris-acetate. 12 silica cells were used throughout this work and the OD-290 against air of pH 7.4 Tris-acetate was 0.075 $\pm$ 0.003. The results given in tables are corrected for Tris-acetate absorption.

2. Absorption due to TAMe. This was measured by adding 0.4 ml. of 0.4 M TAMe in Tris-acetate to 4 ml. of Tris-acetate, the difference in OD-290 between the two giving the OD-290 due to 0.1 ml. 0.4 M TAMe/ml. solution. This was done on several occasions and was found to be 0.023 ± 0.002.

3. From the measured absorbance was deducted 0.023, for absorbance due to TAMe and the result multiplied by 4.4/4 to correct for the dilution due to the addition of 0.4 ml. of 0.4 M TAMe. This was then taken as being the OD-290 of the unreacted fibrinogen.

b) The Effect of TAMe on the Clottability of Fibrinogen

Fibrinogen solutions always contain some material, the non-clottables (NC) which remains in the supernatant after removal of the fully formed clot, whether it be non-fibrinogen protein, denatured fibrinogen or fibrin monomer and polymers in equilibrium with the clot.

The effect of TAMe on clottability was determined by clotting fibrinogen at various concentrations and then after about 3 hours, when the clot is fully formed, the solution was made 0.036 M in TAMe. This solution was then allowed to stand for a further 3 or 4 hours, the clot removed as before and the OD-290 of the supernatant measured. Experiments were also carried out 1) without TAMe and 2) adding 0.4 ml. 0.4 M KCl instead of TAMe.

These results are shown in Table A3 and are plotted in Fig. XVI. From these results it can be seen that the highest values for non-clottables are obtained in the presence of TAMe although there is a slight increase due to the presence of KCl (higher ionic strength). This would indicate that TAMe has an effect on non-clottables over and above that due to the increased ionic strength it produces. The curves for non-clottables in buffer and for non-clottables in the presence of added KCl have a common intercept on the ordinate which could be interpreted as representing the true non-clottable material. The curve for non-clottables in the presence of TAMe has an intercept on the abscissa which again points to TAMe as having some other effect on the non-clottables besides the effect due to its ionic strength. It thus appears as though some, if not all, of the non-clottable material represents monomers and polymers in equilibrium with clot rather than denatured on non-fibrinogen protein. Because of this variation, the non-clottables was determined during any actual experiment so that a reliable estimate could be obtained.

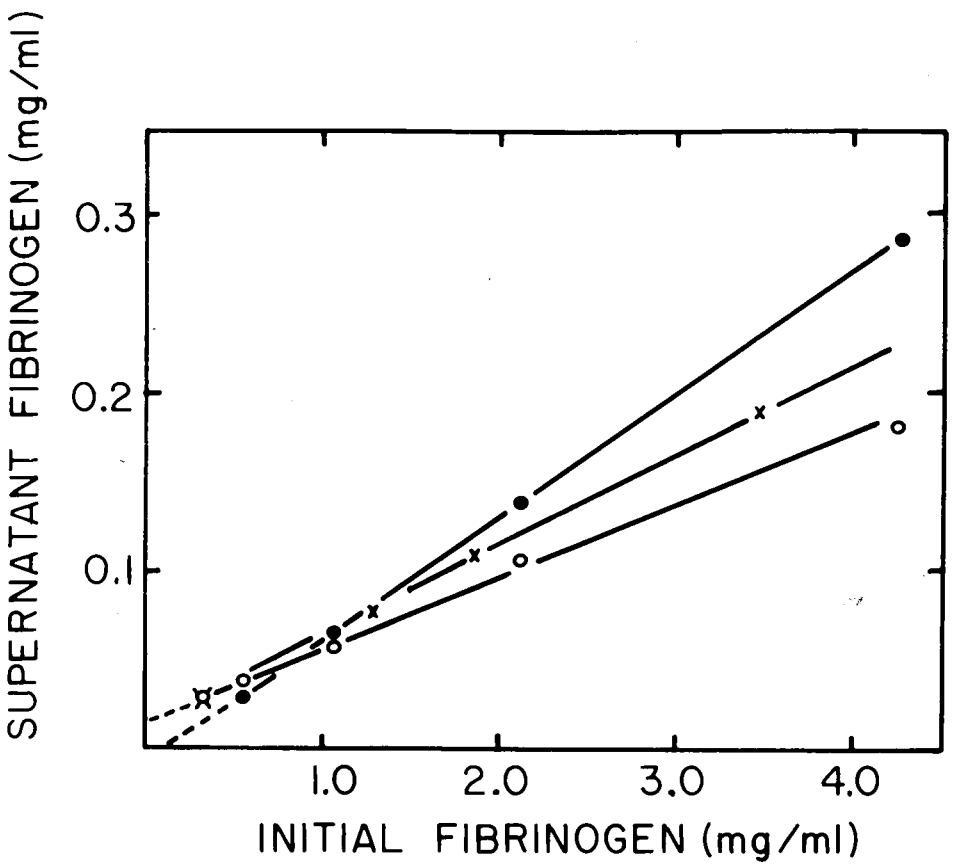



**Fig. XVI.** The effect of TAMe on non-clottables (NC) ● is NC with TAMe, × is NC with KCl, o is NC in buffer only.

# CHAPTER IV

# EXPERIMENTAL RESULTS

## 1. Determination of $K_m$ and $V_m$ for Step 1.

The thrombin catalyzed conversion of fibrinogen to fibrin monomer is a proteolytic reaction. Since other investigators (14,15,32,39,46,67) have shown this enzyme catalyzed reaction to be first order with respect to thrombin and fibrinogen at low fibrinogen concentrations, the initial velocities of reaction were measured over a wide range of fibrinogen concentrations in order to determine a Michaelis substrate constant ($K_m$) and a maximum velocity ($V_m$) for the reaction. These constants can then be compared to the action of thrombin on TAMe and other small substrates.

The initial velocity of the disappearance of fibrinogen from solutions containing varying concentrations of fibrinogen was determined by stopping the reaction with TAMe (Chapter III) at 30 and 60 sec. intervals and comparing the OD-290 of the stopped solution with the original OD-290. The apparent initial velocity is given by $(F_o-F_t)/t$ where the subscripts refer to fibrinogen concentrations at zero time and time t. The data from these experiments

are shown in Table A4, of the appendix. Determinations were made at 30 secs. and 60 secs. to verify that initial velocities were being measured. Table A4 shows that the 30 sec. and 60 sec. velocities do not agree very well (i.e. 30 sec x 2 ≠ 60 sec.) and that apparently, initial velocities were not being measured. Better agreement between 30 sec. and 60 sec. velocities is obtained, however if the value of the non-clottable protein (NC), at the fibrinogen concentration concerned, is added to these velocities. This implies that the non-clottable protein should be considered as reaction product, that is, that it is fibrin monomer or intermediate polymer. The data of Table A4, corrected for non-clottable protein are shown in Table VIII. Table A5 of the appendix shows the values of $1/v_o$ and $1/F_o$ required for a Lineweaver-Burke plot.

A Lineweaver-Burke "double reciprocal" plot of the data of Table VIII is shown in Fig. XVII all velocities being reduced to one minute and one unit of thrombin. The slope and intercept of the straight line were obtained using the method of least squares. An average maximum velocity ($V_m$) was calculated using the value of 1.9 mg./ml. for the Michaelis constant ($K_m$) obtained from the plot and the values of each of the initial velocities measured. The standard deviation (s) of $V_m$ was calculated from the same data.

## TABLE VIII

## INITIAL VELOCITIES OF PROTEOLYSIS REACTION

## CORRECTED FOR NON-CLOTTABLES

| Reaction Time mins. | $(F_o-F_t)$ From Table A4 mg/ml | NC mg/ml | Corrected for NC $(F_o-F_t)$ mg/ml | $(F_o-F_t)/t$ mg/ml/min |
|---|---|---|---|---|
| Thrombin - 0.123 u/ml. | | | | |
| 0.5 | 0.028 | 0.021 | 0.049 | 0.098 |
| 1.0 | 0.084 | 0.021 | 0.105 | 0.105 |
| 0.5 | 0.051 | 0.028 | 0.079 | 0.158 |
| 1.0 | 0.122 | 0.028 | 0.150 | 0.150 |
| 0.5 | 0.072 | 0.046 | 0.118 | 0.236 |
| 1.0 | 0.198 | 0.046 | 0.244 | 0.244 |
| 0.5 | 0.090 | 0.086 | 0.176 | 0.352 |
| 1.0 | 0.298 | 0.086 | 0.374 | 0.374 |
| Thrombin - 0.230 u/ml. | | | | |
| 0.5 | 0.109 | 0.026 | 0.135 | 0.270 |
| 1.0 | 0.172 | 0.026 | 0.198 | 0.198 |
| 0.5 | 0.161 | 0.043 | 0.204 | 0.408 |
| 1.0 | 0.305 | 0.043 | 0.348 | 0.348 |
| 0.5 | 0.260 | 0.082 | 0.342 | 0.684 |
| 1.0 | 0.517 | 0.082 | 0.599 | 0.599 |
| 0.5 | 0.330 | 0.127 | 0.457 | 0.914 |
| 1.0 | 0.580 | 0.127 | 0.707 | 0.707 |
| Thrombin - 0.50 u/ml. | | | | |
| 0.5 | 0.143 | 0.022 | 0.165 | 0.330 |
| 0.5 | 0.273 | 0.035 | 0.308 | 0.616 |

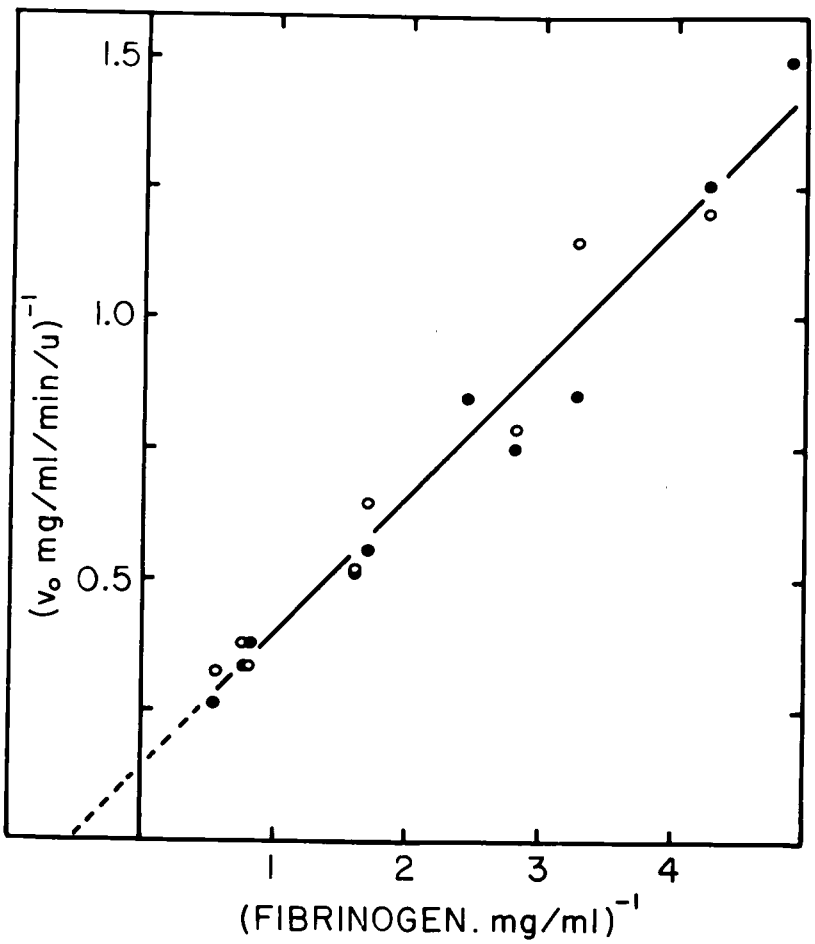



**Fig. XVII.** Lineweaver-Burke plot for the thrombin catalyzed conversion of fibrinogen into fibrin monomer.

From these results:

$K_m$ = 1.9 mg. fibrinogen/ml.

$V_m$ = 7.3 mg./min./unit. (s = 0.67)

The 95% Confidence Limits for $V_m$ are 6.0 and 8.6 mg./min./unit.

Assuming a molecular weight of 340,000 for fibrinogen,

$K_m$ = 5.6 μmoles/liter

$V_m$ = 0.021 μmoles/min./unit.

Since $K_m$ represents the concentration at which a reaction is proceeding at half maximum velocity, the above values indicate that the conversion of fibrinogen to fibrin monomer will proceed at the maximum rate of 0.01 μM/min./unit when the concentration of fibrinogen is 5.6 μM.

The values of $K_m$ and $V_m$ for the thrombin catalyzed hydrolysis of TAMe are $K_m$ = 173 μM. and $V_m$ = 0.021 μM/min./unit (Chapter III). A comparison of $K_m$ and $V_m$ for fibrinogen and TAMe reveals that there is a difference only in $K_m$. On the basis of $K_m$, the maximum velocity of reaction is attained at a fibrinogen concentration which is approximately 3% of the concentration of TAMe on a molar basis. When the reactions are proceeding at maximum velocity, however, the velocities are the same.

The Michaelis-Menten equation (37) can be written:

$$v_o = V_m F_o/(F_o + K_m) \qquad 25.$$

where $v_o$ = initial velocity,

$F_o$ = initial fibrinogen concentration.

When $F_o$ is small compared to $K_m$ this equation reduces to:

$$v_o = V_m F_o/K_m \qquad 26.$$

That is, the reaction can be represented by a first order equation with a rate constant $k = V_m/K_m$.

Using the above values of $K_m$ and $V_m$ the first order rate constant for the proteolysis step is given by

$$k_1 = 7.3/1.9 = 3.8 \text{ /min./ml./unit}$$

This value will hold true only when $F_o$ is small compared to $K_m$, e.g. when $F_o$ is 10% of $K_m$ $k_1$ will be 3.5 /min./ml./unit and when $F_o$ is 20% of $K_m$, $k_1$ will be 3.2 /min./ml./unit. Also when $F_o$

is higher than say 0.5 $K_m$, significant deviation from first order behaviour will be observed.

2. Determination of a Rate Constant for Step 1.

With $K_m$ and $V_m$ determined it is possible to arrange the fibrinogen concentration so that the proteolysis reaction can be considered as a first order reaction with a rate constant equal to $V_m/K_m$. It is necessary to study the system under these conditions so that from a knowledge of the rate constant the amount of fibrin monomer produced at any time can be calculated and comparisons between steps 1, 2 and 3 can be made.

To verify that the system follows first order kinetics up to about 0.5 mg. fibrinogen/ml. (i.e. $F_o = 0.25\ K_m$) more experiments were carried out using the TAMe inhibition method. Initial fibrinogen concentrations ($F_o$) of 0.5 mg./ml. or less and thrombin concentrations up to 0.5 u/ml. were used. The reaction was initiated by the addition of thrombin and then stopped after various intervals of time by the addition of TAMe. The amount of fibrinogen remaining at time t ($F_t$) was determined from the absorbance at 290 mμ. of the supernatant. The data from these experiments is shown in Table A-6 of the appendix. Examination of this data indicates that, in cases where relatively high thrombin

concentrations were used the values of $F_t$ reached a minimum value which was greater than expected on the basis of the non-clottable protein (NC) value. Fig. XVIII shows plots of log $(F_o/F_t)$ against time where such effects were observed. With regard to Fig. XVIII it should be noted that the origin is an experimental point and that the reaction is complete after about 1 minute (about 80% conversion), i.e. there is no further increase in log $F_o/F_t$ after 1 minute. Under these conditions therefore only the experimental points up to about 80% conversion can be used to determine a rate constant. The difference between the above minimum value of $F_t$ and the non-clottable value could be due to the fact that in the TAMe inhibition method, TAMe is added during the early stages of the reaction when the amount of clot formed is much less than maximum whereas in the NC determination TAMe is added to the reaction mixture 3 hours after the thrombin, when the clot is fully formed, i.e. in the stopped reaction, polymerization takes place in the presence of TAMe. There are two possibilities 1) TAMe affects the polymerization so that conversion of monomer to clot is less than in the system where TAMe is added after the clot is fully formed. 2) TAMe affects the equilibrium between clot and intermediate polymer, implying that a higher value for NC would be obtained if clot were left in contact with TAMe for a longer period of time than the normal 17 hours. This second possibility was checked by

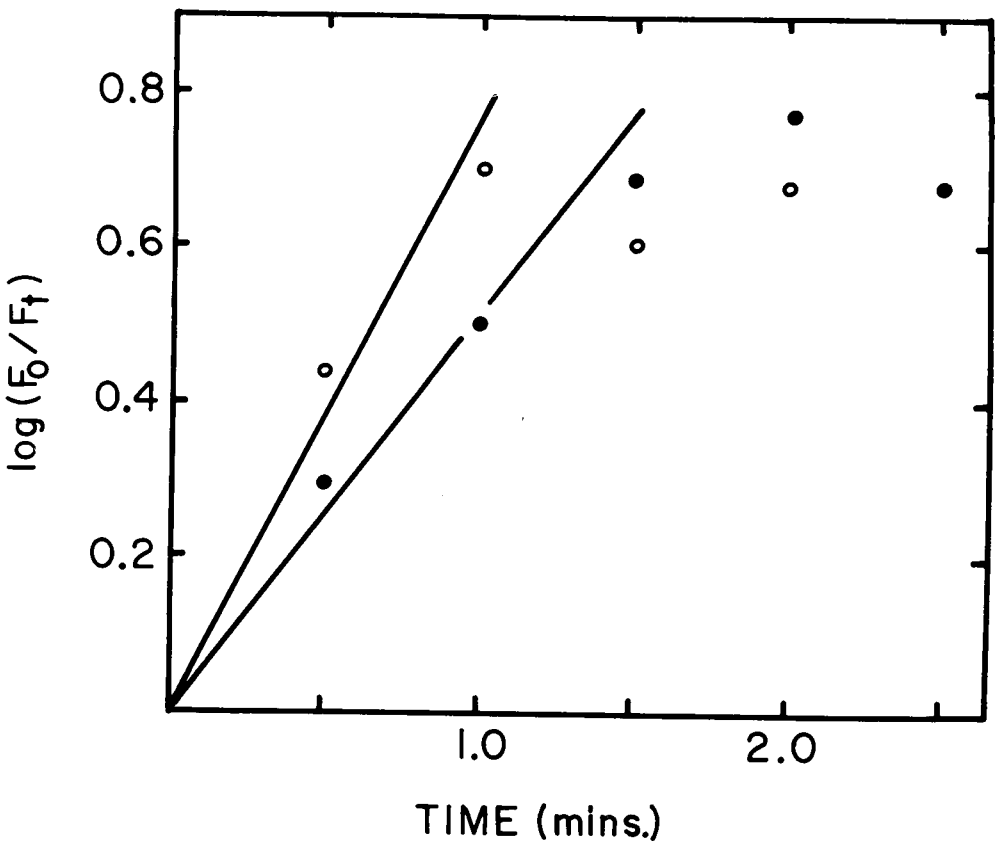



Fig. XVIII. Determination of a first order rate constant for the conversion of fibrinogen into fibrin monomer.

o Initial fibrinogen 0.14 OD-290, 0.375 u/ml thrombin.

● Initial fibrinogen 0.48 OD-290, 0.300 u/ml thrombin.

N.B. 1) The origin is an experimental point, 2) After about 1 minute there is no further increase in log $F_o/F_t$ i.e. the reaction is complete, 3) The value of $F_t$ when log $F_o/F_t$ is constant is the max-NC value.

determining the NC by adding TAMe 3 hours after thrombin addition and then measuring the OD-290 of the supernatant after clot had been in equilibrium with TAMe for 93 hours. The data of Table IX show the results obtained in this experiment. The OD-290 was the same after 17 hours and after 93 hours thus ruling out the possibility that in the NC determination a higher NC value would have been obtained if longer equilibration times had been used.

It has been shown in the determination of $K_m$ that more consistent results are obtained if a correction for NC is made to $F_t$. That the NC is different depending on the presence or absence of TAMe and on the point at which TAMe is added also points to the fact that some correction to $F_t$ to allow for non-clottables is necessary. As a consequence three different values of $F_t$ were used in the following determination of the rate constant, 1) the experimental value of $F_t$, corrected only for TAMe absorption and dilution (i.e. less blank) 2) experimental $F_t$ (less blank) less the value of NC and 3) experimental $F_t$ (less blank) less the minimum value of $F_t$ reached for any particular initial fibrinogen concentration. This latter correction, which will be called max-NC, was estimated from the data of Table A-6 and is shown in Table X. The NC values are shown for comparison.

## TABLE IX

## EFFECT OF EQUILIBRATION TIME ON NON-CLOTTABLES IN THE PRESENCE OF TAMe

| Initial Fibrinogen OD-290 | Supernatant* OD-290 after 17 hours | 93 hours |
|---|---|---|
| 0.68 | 0.026 | 0.031 |
| | 0.032 | 0.026 |
| 0.35 | 0.021 | 0.022 |
| | 0.023 | 0.025 |
| 0.17 | 0.012 | 0.014 |
| | 0.013 | 0.013 |

* Corrected for TAMe absorption and dilution.

## TABLE X

## MAXIMUM CORRECTION FOR NON-CLOTTABLES

| $F_o$ (OD-290) | Max-NC (OD-290) | As % of $F_o$. | NC | As % of $F_o$. |
|---|---|---|---|---|
| 0.48 | 0.100 | 21 | 0.038 | 8.0 |
| 0.24 | 0.045 | 19 | 0.025 | 10.0 |
| 0.14 | 0.030 | 21 | 0.020 | 14.0 |

Table A-7 shows the values of experimental $F_t$, $F_t$ corrected for NC and $F_t$ corrected for max-NC.

The value of the first order rate constant was determined as follows. Under the conditions used in these experiments the reaction is assumed to be first order with respect to both fibrinogen and thrombin.

$$\text{velocity} = -dF/dt = k_1TF$$

$$\log (F_o/F_t) = (k_1T/2.3)t \qquad 27.$$

Values of $k_1T$ were calculated by plotting $\log (F_o/F_t)$ against time (mins). The slope of the straight lines is then equal to $k_1T/2.3$. Table XI shows the results obtained from these

## TABLE XI

## VALUES OF $k_1T$

## (NO CORRECTION FOR INACTIVATION OF THROMBIN)

| $F_o$ OD-290 | Thrombin T. u/ml. | $k_1T$ using $F_t$ | $F_t$(NC) | $F_t$(max-NC) |
|---|---|---|---|---|
| 0.48 | 0.500 | 1.93 | 2.75 | 4.0 |
| 0.24 | 0.500 | 2.37 | 3.10 | 4.3 |
| 0.14 | 0.375 | 1.79 | 2.77 | 3.25 |
| 0.48 | 0.300 | 1.17 | 1.41 | 2.46 |
| 0.24 | 0.300 | 1.63 | 1.95 | 3.47 |
| 0.48 | 0.150 | 0.39 | 0.50 | 0.86 |
| 0.24 | 0.150 | 0.56 | 0.77 | 1.07 |
| 0.14 | 0.150 | 0.78 | 1.12 | 1.52 |
| 0.48 | 0.075 | 0.15 | 0.19 | 0.26 |
| 0.24 | 0.075 | 0.24 | 0.33 | 0.44 |
| 0.14 | 0.075 | 0.29 | 0.41 | 0.53 |
| 0.14 | 0.037 | 0.11 | 0.17 | 0.18 |
| 0.35 | 0.230 | 0.84 | 1.09 | 1.57 |
| 0.41 | 0.123 | 0.36 | 0.50 | 0.83 |
| 0.27 | 0.123 | 0.34 | 0.55 | 0.74 |

experiments and Fig. XIX shows a plot of $k_1T$ against T for the various fibrinogen concentrations used. This figure is for $k_1T$ calculated using the NC correction. Although the values of $k_1T$ are different depending on whether experimental $F_t$, NC corrected $F_t$ or max-NC corrected $F_t$ values are used, the same type of curve is obtained as can be seen from the results shown in Table XI, except that the slope of the line changes. It is also seen that, for a given thrombin concentration, the value of $k_1T$ depends somewhat on the initial fibrinogen concentration ($F_o$). Since, for a first order reaction, $k_1T$ should be independent of $F_o$ (because the ratio $F_o/F_t$ determines the slope) the reaction is apparently not first order with respect to the initial fibrinogen concentration.

However, the straight lines of Fig. XIX, when extrapolated, have a positive intercept on the thrombin axis. One possible explanation of this is that there is inactivation of thrombin by fibrinogen and that the extent of this inactivation depends on the fibrinogen concentration. The extent of thrombin inactivation is given by the value of the thrombin concentration at the intercept ($k_1T = 0$). The thrombin concentrations at these intercepts is the same regardless of the non-clottable correction made to $F_t$ and are shown in Table XII. These results indicate that the amount of thrombin inactivated appears to vary proportionately

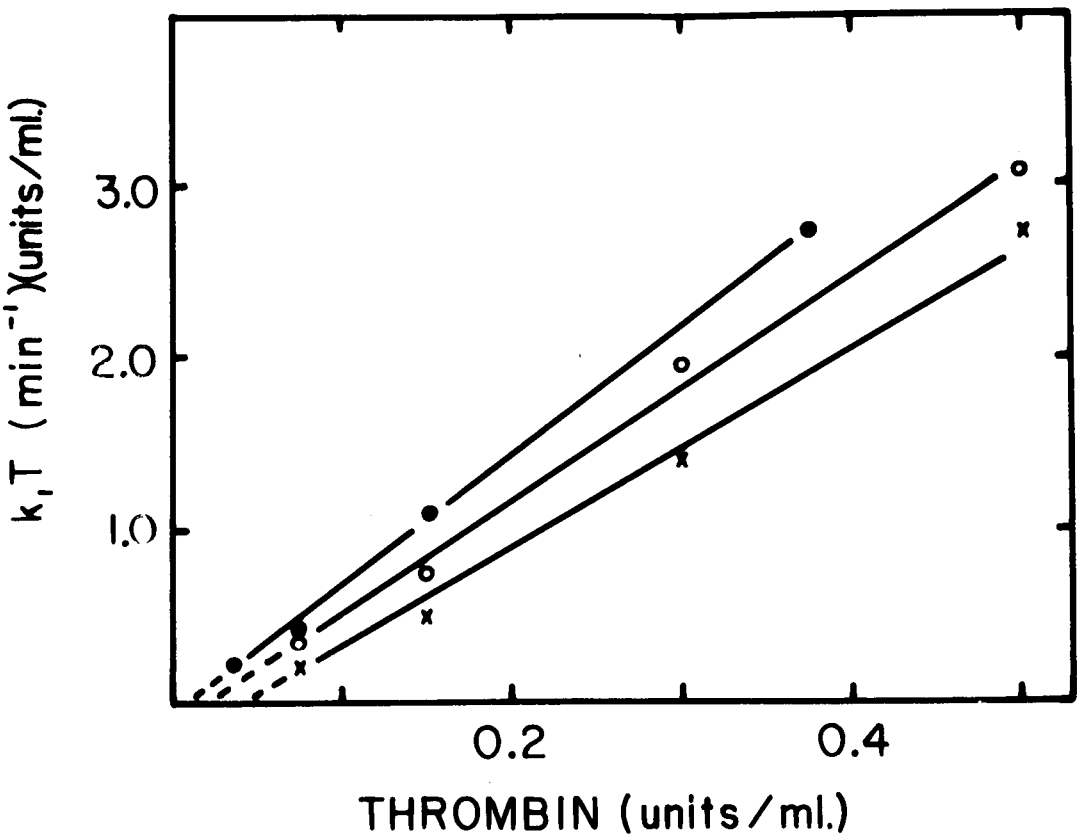



Fig. XIX. Plot of the first order rate constant ($k_1T$) against thrombin concentration. Results corrected for non-clottables.

- ● Initial fibrinogen 0.14 OD-290
- o Initial fibrinogen 0.24 OD-290
- × Initial fibrinogen 0.48 OD-290

## TABLE XII

## THE INACTIVATION OF THROMBIN BY FIBRINOGEN

| $F_o$ (OD-290) | Thrombin Inactivation u/ml. | u/ml./$F_o$ |
|---|---|---|
| 0.14 | 0.01 | 0.07 |
| 0.24 | 0.02 | 0.06 |
| 0.48 | 0.04 | 0.08 |

with fibrinogen concentration (0.07 units inactivated per 1.0 absorbance at 290 mμ).

The values of $k_1T$ from Table XI were corrected for thrombin inactivation by multiplying each $k_1T$ by the factor $T/(T - I)$ where T is the total thrombin concentration and I is the assumed thrombin inactivation from Table XII. The results are shown in Table XIII and Fig. XX is a plot of $k_1T$ versus thrombin corrected for inactivation. The last three lines of Table XIII show results calculated from some of the data used to estimate $K_m$ (Table A4). The only data used was that in which an estimate of the extent of thrombin inactivation could be made from Table XII, i.e. where $F_o$ was 0.48 OD-290 or less. These results fit very well with the other data. From these data it is seen that irrespective of the non-clottable correction made to $F_t$, $k_1T$ is a linear function of thrombin concentration and that the reaction is first order with respect to fibrinogen concentration.

Although the reaction is first order with respect to both thrombin and fibrinogen the value of $k_1$ depends on the correction made to $F_t$. These values together with the standard deviations (s) are:

## TABLE XIII

## VALUES OF $k_1T$

(CORRECTED FOR THROMBIN INACTIVATION)

| $F_o$ OD-290 | Thrombin Total u/ml. | Thrombin Corrected u/ml. | $k_1T$ using $F_t$ | $k_1T$ using $F_t$(NC) | $k_1T$ using $F_t$ (max-NC) |
|---|---|---|---|---|---|
| 0.48 | 0.500 | 0.460 | 2.10 | 3.00 | 4.35 |
| 0.24 | 0.500 | 0.480 | 2.47 | 3.23 | 4.48 |
| 0.14 | 0.375 | 0.365 | 1.84 | 2.85 | 3.34 |
| 0.48 | 0.300 | 0.260 | 1.35 | 1.63 | 2.84 |
| 0.24 | 0.300 | 0.280 | 1.75 | 2.09 | 3.72 |
| 0.48 | 0.150 | 0.110 | 0.53 | 0.68 | 1.17 |
| 0.24 | 0.150 | 0.130 | 0.65 | 0.89 | 1.23 |
| 0.14 | 0.150 | 0.140 | 0.83 | 1.20 | 1.63 |
| 0.48 | 0.075 | 0.035 | 0.32 | 0.41 | 0.56 |
| 0.24 | 0.075 | 0.055 | 0.33 | 0.45 | 0.60 |
| 0.14 | 0.075 | 0.065 | 0.33 | 0.47 | 0.61 |
| 0.14 | 0.037 | 0.027 | 0.15 | 0.23 | 0.25 |
| 0.35 | 0.230 | 0.200 | 0.97 | 1.25 | 1.80 |
| 0.41 | 0.123 | 0.090 | 0.49 | 0.68 | 1.13 |
| 0.27 | 0.123 | 0.100 | 0.42 | 0.68 | 0.91 |

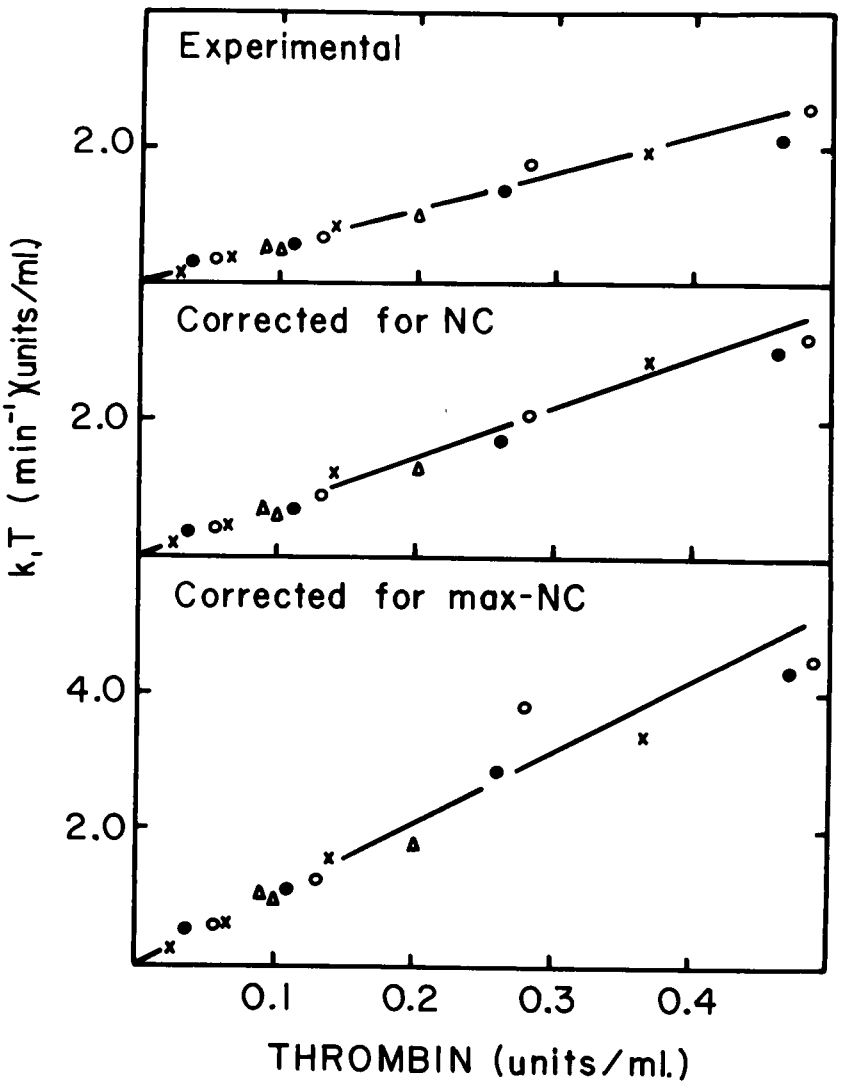



Fig. XX. Plots of the first order rate constant ($k_1T$) versus thrombin concentration corrected for inactivation.
● Initial fibrinogen 0.48 OD-290 ○ Initial fibrinogen 0.24 OD-290
× Initial fibrinogen 0.14 OD-290 △ From $K_m$ data.

| | | | |
|---|---|---|---|
| Experimental: | $k_1$ = 5.4 /min/ml/unit | s = | 1.14 |
| NC corrected: | $k_1$ = 7.3 /min/ml/unit | s = | 1.30 |
| max-NC corrected: | $k_1$ = 10.6 /min/ml/unit | s = | 1.80 |
| $V_m/K_m$ | $k_1$ = 3.8 /min/ml/unit | s = | 0.67 |

If it is assumed that there is inactivation of thrombin by fibrinogen then the difference between the value of $k_1$ from $V_m/K_m$ and that obtained from experiments specifically designed to measure $k_1$ is explained. It is seen from Fig. XX, that appropriate $K_m$ data, when corrected for thrombin inactivation, fit very well with the other data of this figure and therefore thrombin inactivation can be assumed to explain the apparent difference in reaction velocity.

Assuming that $k_1$ and $K_m$ calculated from $F_t$ corrected for non-clottables are the most reliable values, the most reliable value of $V_m$ will be given by

$$V_m = k_1 K_m = 7.3 \times 1.9 = 13.9 \text{ mg/min/unit}$$

Assuming the molecular weight of fibrinogen is 340,000, $V_m$ = 0.041 $\mu$M/min/unit.

3. Experiments on the Overall Reaction

The course of the overall reaction (F clot) was followed by means of opacity measurements at 310 mu., to obtain the experimental parameters $V_o$, V and $t_c$ (Chapter III). The correlations between $V_o$, V, $t_c$, fibrinogen and thrombin concentrations were explored. Fibrinogen and thrombin solutions of the same concentration used to obtain the rate constant $k_1$ were used to obtain values of $V_o$, V and $t_c$ from the clotting curves. These results are shown in Table XIV. Fig. XXI shows the result of plotting $V_o$ and V against thrombin concentration and Fig. XXII $V_o$ and V against fibrinogen concentration.

Fig. XXI shows that as the thrombin concentration is increased, the arbitrary rate parameters $V_o$ and V at first increase and then reach a maximum. The rate of polymerization depends on fibrin monomer concentration, and therefore at low thrombin concentrations the production of fibrin monomer will be rate limiting and the rate of polymerization will increase with thrombin concentration. At high thrombin concentrations with high rates of fibrin monomer production the rate of polymerization will be rate determining and consequently a maximum will be observed.

Since this data was obtained under conditions where the proteolysis step is first order in thrombin, the implication is

## TABLE XIV

## VALUES OF $V_o$, V AND $t_c$

| $F_o$ OD-290 | Thrombin u/ml. Total | Corrected | $t_c$ mins. | $V_o$ OD-290/min. | V OD-290/min. |
|---|---|---|---|---|---|
| 0.48 | 0.075 | 0.035 | 1.66 | 0.304 | 0.094 |
| 0.48 | 0.150 | 0.110 | 0.88 | 0.475 | 0.164 |
| 0.48 | 0.300 | 0.260 | 0.75 | 0.608 | 0.200 |
| 0.27 | 0.120 | 0.100 | 1.13 | 0.220 | 0.064 |
| 0.27 | 0.300 | 0.280 | 0.55 | 0.312 | 0.130 |
| 0.24 | 0.075 | 0.055 | 1.44 | 0.138 | 0.046 |
| 0.24 | 0.150 | 0.130 | 0.85 | 0.222 | 0.078 |
| 0.24 | 0.300 | 0.280 | 0.68 | 0.298 | 0.096 |
| 0.14 | 0.037 | 0.027 | 3.25 | 0.045 | 0.013 |
| 0.14 | 0.075 | 0.065 | 1.85 | 0.062 | 0.020 |
| 0.14 | 0.150 | 0.140 | 1.37 | 0.088 | 0.028 |
| 0.14 | 0.375 | 0.365 | 0.70 | 0.082 | 0.040 |

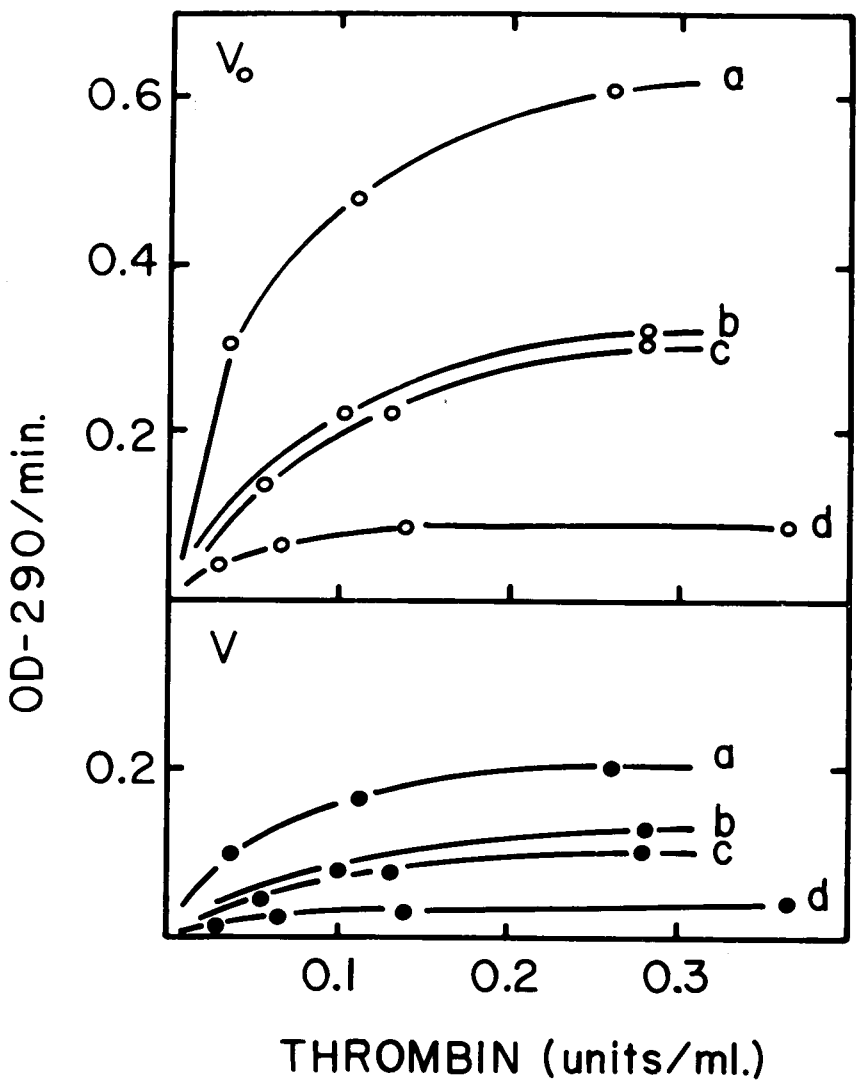



Fig. XXI. Plots of the arbitrary rate parameters $V_o$ and V against thrombin concentration (corrected for inactivation).

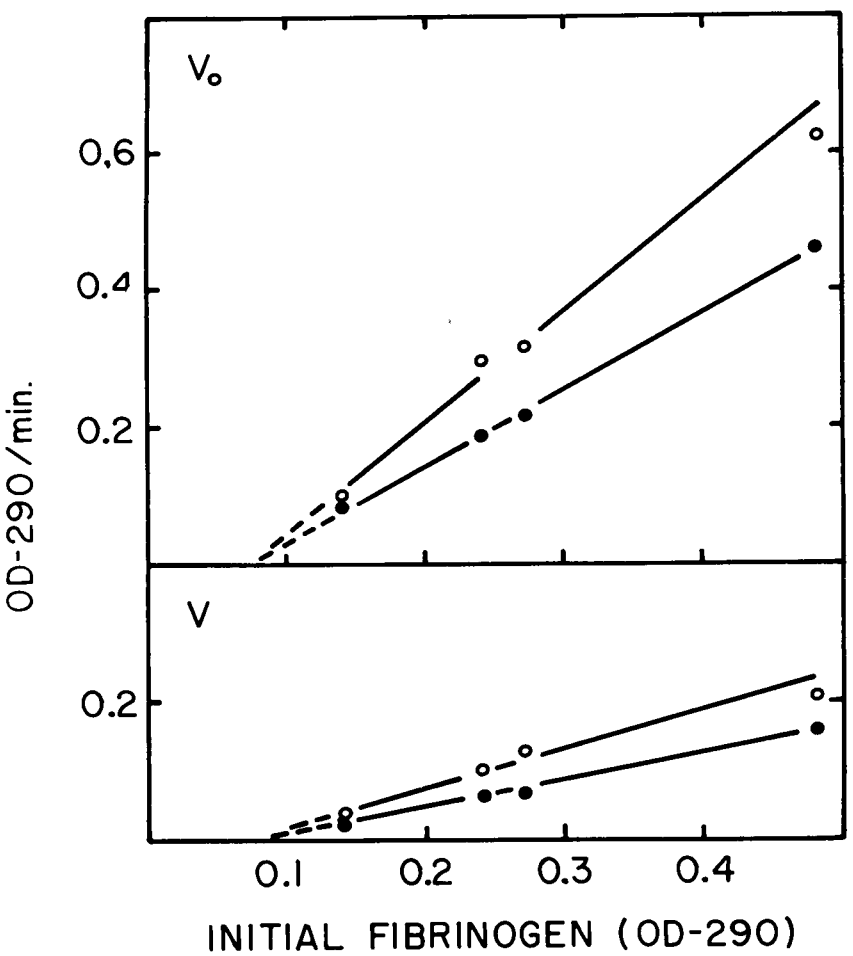



Fig. XXII. Plots of the arbitrary rate parameters $V_o$ and V against initial fibrinogen concentration.

that the polymerization step is the slower rate determining step in the overall conversion of fibrinogen to clot at pH 7.4, $\mu$ = 0.15. Because of the arbitrary nature of $V_o$ and V no estimation of the polymerization rate constant has, as yet been possible. This data is however consistent with that of Mihalyi (Chapter II), where it was shown that at pH 7.4, polymerization is slower than proteolysis.

Fig. XXII shows that, up to a thrombin concentration of 0.3 u/ml, as the initial fibrinogen concentration ($F_o$) is increased, $V_o$ and V increase in a linear manner. The curves extrapolate back to a positive $F_o$ value of 0.08 OD-290 where presumably the rate of clot formation is zero, i.e. no clot forms. This implies that a certain amount of fibrin monomer and/or intermediate polymer has to be present before clot will form and is another indication that some, if not all of the non-clottables should be considered as fibrin monomer and intermediate polymer in equilibrium with clot.

## CHAPTER V

## DISCUSSION

In order to simplify a theoretical treatment, the overall process can be conveniently represented as:

$$F \xrightarrow[k_1 T]{T} f \qquad 28.$$

$$nf \xrightarrow[k_2]{} f_c \qquad 29.$$

Where F = fibrinogen, T = thrombin, f = fibrin monomer, $f_c$ = fibrin polymer, $k_1 T$ = pseudo first order rate constant, $k_2$ = a rate constant assumed to be first order in fibrin monomer. In the following treatment, the letters F, T, $f_c$ and f will be taken to mean concentrations. The notation exp(x) is used for $e^x$.

The assumption that $k_2$ is first order is supported by the observations of Mihalyi and Billick (39) reported earlier

(Chapter II) in which they determined the rate constants by measuring the hydrogen ions released.

$$\left.\begin{array}{l}\text{Rate of disappearance of F} \\ \\ \text{Rate of formation of f}\end{array}\right\} = -dF/dt = k_1TF \qquad 30.$$

$$F = F_o\exp(-k_1Tt) \qquad 31.$$

$$\text{Rate of formation of } f_c = df_c/dt = k_2f \qquad 32.$$

$$\text{The rate of build-up of f} = df/dt = -dF/dt \;\; -df_c/dt \qquad 33.$$

From equations (31), (32) and (33)

$$df/dt = k_1TF_o\exp(-k_1Tt) - k_2f \qquad 34.$$

Integrating equation (34)

$$f = \frac{k_1TF_o}{k_2-k_1T}\left[\exp(-k_1Tt) - \exp(-k_2t)\right] \qquad 35.$$

Substituting equation (35) in equation (32)

$$df_c/dt = \frac{k_2 k_1 T F_o}{k_2 - k_1 T}\left[\exp(-k_1 Tt) - \exp(-k_2 t)\right] \qquad 36.$$

Integrating equation (36)

$$f_c = \frac{k_2 F_o}{k_2 - k_1 T}\left[1-\exp(-k_1 Tt)\right] - \frac{k_1 T F_o}{k_2 - k_1 T}\left[1-\exp(-k_2 t)\right] \qquad 37.$$

From this equation, $f_c$, the amount of fibrin polymer formed at any time can be calculated from a knowledge of the constants $F_o$, $k_1T$, $k_2$ and the time t.

The course of the overall reaction (F clot) can be followed by means of opacity measurements (Chapter III) and therefore the value of $f_c$ from equation (37) can be compared directly with the amount of clot formed.

The opacity-time curves and the kinetic data of Chapter IV, corrected for non-clottable protein, were used to make comparisons between $f_c$ and the amount of clot formed. Using equation (37) the amount of fibrin polymer formed at any time ($f_c$) was calculated using the appropriate experimental values of $k_1T$ (from Fig. XIX), $F_o$, T and t and a value of $k_2 = 2$.

The total amount of fibrin monomer (f) produced at any time was calculated from the first order rate equation.

$$-dF/dt = k_1 Tt \qquad 38.$$

$$F_t = F_o \exp(-k_1 Tt)$$

$$\text{and since } f = F_o - F_t$$

$$= F_o - F_o \exp(-k_1 Tt)$$

$$f = F_o \left(1 - \exp(-k_1 Tt)\right) \qquad 39.$$

Fig. XXIII shows typical examples of the experimentally determined clotting curves together with the values of $f_c$ and f calculated as shown above. The points shown on the fibrin monomer curves are the experimental values of $(F_o - F_t)$ used to estimate $k_1 T$.

In Fig. XXIII, the clotting time ($t_c$) is the point at which the absorbance first starts to increase and $V_o$ is the slope of the initial sharp increase in opacity. To explore possible correlations between the variables, the values of $f_c$ and f at $t_c$ and of $V_o$ were read from Fig. XXIII and similar curves obtained at

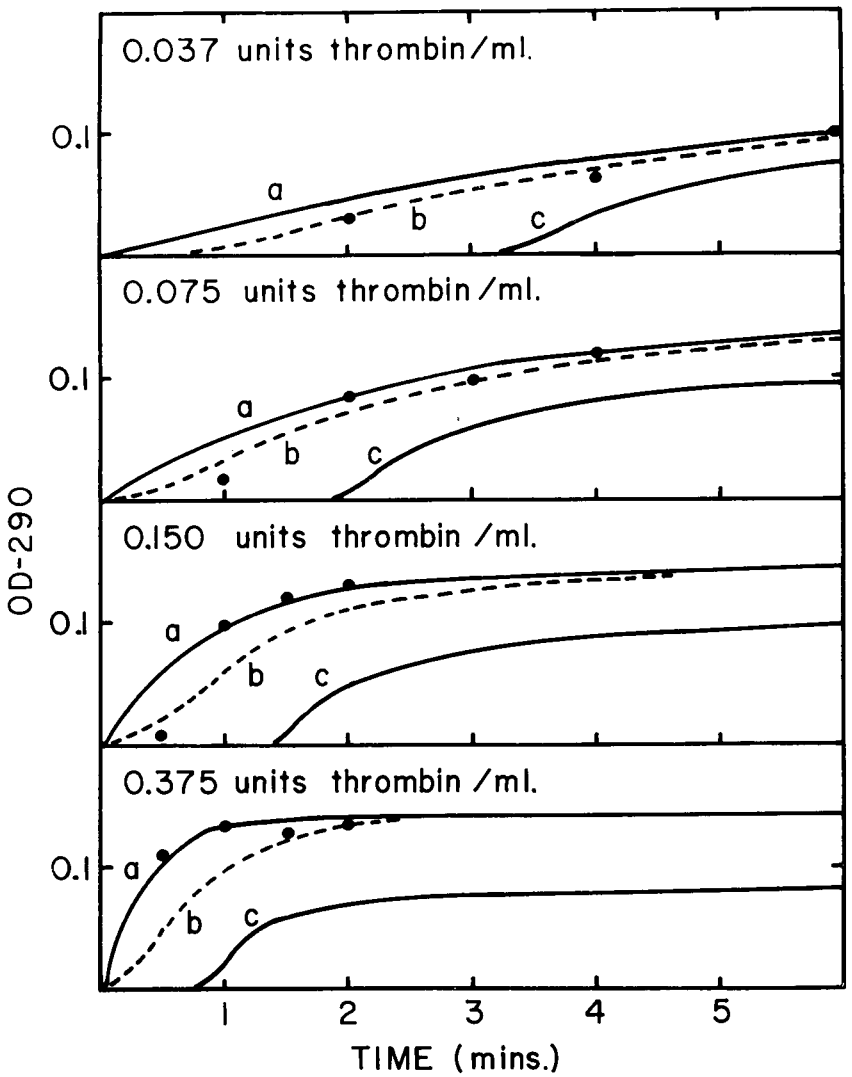



Fig. XXIII. Experimental and theoretical clotting curves. Initial fibrinogen = 0.14 OD-290. a = total fibrin monomer b = total intermediate polymer c = experimental clotting curve.

the other fibrinogen and thrombin concentrations. These results are shown in Table XV.

It is immediately apparent from Fig. XXIII that the increase in clot opacity cannot be described directly in terms of the simple model presented here. However, the data of Table XV can afford an explanation of the events leading to clot formation.

Fig. XXIV is a plot of $V_o$ against fibrin monomer (f) at $t_c$. The two straight lines of this figure join the points where the thrombin concentration is a minimum and a maximum respectively and can thus be taken as representing limiting values of $V_o$ for the fibrinogen concentrations used. These lines extrapolate to the monomer axis at f = 0.056 and 0.070 mg./ml. (0.065 and 0.080 OD-290). This range can therefore be taken to represent the concentration of f that must be present before clot can form, i.e. below f = 0.07 mg./ml., $V_o$ is zero and therefore no clot forms.

Fig. XXII of Chapter IV shows a plot of $V_o$ against $F_o$. The straight lines of this plot extrapolate to the $F_o$ axis at $F_o$ = 0.07 mg./ml. (0.08 OD-290) and similarly, this can be taken as representing the concentration of fibrinogen that must be present before clot can form.

Table XV shows that the value of $f_c$ at $t_c$ is constant at about 0.07 mg./ml. (0.08 OD-290) which, as before, can be taken as

## TABLE XV

## CLOTTING PARAMETERS

| $F_o$ OD-290 | Thrombin u/ml. | $f_c$ at $t_c$ OD-290 | f (Monomer) at $t_c$ | $V_o$ OD-290/min. | $t_c$ min. |
|---|---|---|---|---|---|
| 0.48 | 0.300 | 0.14 | 0.27 | 0.608 | 1.66 |
| 0.48 | 0.150 | 0.10 | 0.16 | 0.475 | 0.88 |
| 0.48 | 0.075 | 0.10 | 0.14 | 0.304 | 0.75 |
| 0.27 | 0.300 | 0.06 | 0.16 | 0.312 | 1.13 |
| 0.27 | 0.120 | 0.07 | 0.12 | 0.22 | 0.55 |
| 0.24 | 0.300 | 0.08 | 0.17 | 0.298 | 1.44 |
| 0.24 | 0.150 | 0.07 | 0.11 | 0.222 | 0.85 |
| 0.24 | 0.075 | 0.06 | 0.09 | 0.138 | 0.68 |
| 0.14 | 0.375 | 0.07 | 0.12 | 0.082 | 3.25 |
| 0.14 | 0.150 | 0.08 | 0.11 | 0.088 | 1.85 |
| 0.14 | 0.075 | 0.07 | 0.08 | 0.062 | 1.37 |
| 0.14 | 0.037 | 0.06 | 0.07 | 0.045 | 0.70 |

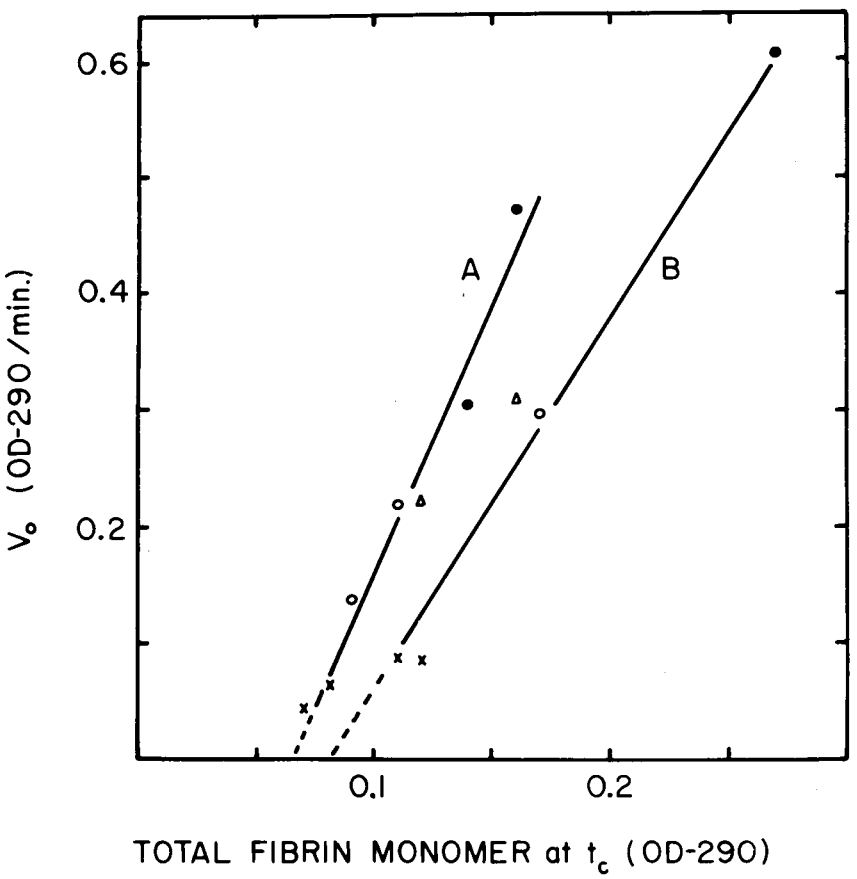



**Fig. XXIV.** Plot of $V_o$ against total fibrin monomer at the clotting time. Curve A, minimum thrombin concentration used, curve B, maximum thrombin concentration used. x , Initial fibrinogen 0.14 OD-290, o , Initial fibrinogen 0.24 OD-290, Δ initial fibrinogen 0.27 OD-290, ● initial fibrinogen 0.48 OD-290.

representing the concentration of $f_c$ that must be present before clot can form.

Summarizing the above findings.

1) Below a minimum initial fibrinogen concentration of 0.07 mg./ml., clot formation is infinitely slow i.e. no clot forms, (Fig. XXII).

2) Before a clot can form, the total amount of fibrin monomer produced has to be at least 0.07 mg./ml, (Fig. XXIV).

3) A clot will form only when $f_c$ is at least 0.07 mg./ml, (Table XV).

Taking $f_c$ to represent some intermediate polymer, it is postulated that when the concentration of $f_c$ reaches 0.07 mg./ml. insoluble polymer (i.e. clot) starts to form. This idea is supported by the findings that no clot can form unless a concentration of 0.07 mg./ml. of fibrin monomer is present and that no clot can form unless the initial fibrinogen concentration is at least 0.07 mg./ml. That is, unless the initial fibrinogen concentration is at least 0.07 mg./ml., fibrin monomer concentration cannot possibly reach 0.07 mg./ml. and $f_c$ concentration cannot possibly reach 0.07 mg./ml.

Clot formation can therefore be postulated to proceed as follows. Fibrinogen is converted into fibrin monomer and this then polymerizes to intermediate polymer. At a critical degree of

polymerization, represented by $f_c$ = 0.07 mg./ml., the polymerization of a relatively few extra monomers onto each intermediate polymer results in visible clot formation. Just prior to $t_c$ there is a high concentration of critically sized intermediate polymer. At $t_c$ the addition of a relatively few monomers onto intermediate polymer results in clot formation and therefore at $t_c$, clot formation will be rapid as is actually observed (Fig. XXIII). After this initial rapid increase in clot formation, $f_c$ concentration will be low and clot formation will be limited by the rate of polymerization of fibrin monomer which in turn depends on fibrin monomer concentration. After the initial sharp increase, clot formation should therefore parallel fibrin monomer, or $f_c$, production as is actually observed (Fig. XXIII).

Although the critical monomer, intermediate polymer concentration at $t_c$ is 0.07 mg./ml., the value of the non-clottables is less than this. The value of 0.07 mg./ml. is the concentration of intermediate polymer that must be reached before clot starts to form whereas the non-clottable is the final concentration of monomer and/or polymer in equilibrium with the clot. The equilibrium between monomer, intermediate polymer and clot will favor clot because clot is removed from solution and the equilibrium will therefore be displaced in this direction.

## SUMMARY

The fibrinogen-fibrin conversion can be represented as:

| | | |
|---|---|---|
| Step 1. | Proteolysis | $F \xrightarrow{T} f$ |
| Step 2. | Polymerization | $nf \longrightarrow f_n$ |
| Step 3. | Clotting | $m(f_n) \longrightarrow (f_n)m$ |

Where F = fibrinogen, f = fibrin monomer and T = thrombin.

By using TAMe as a thrombin inhibitor, step 1 can be isolated and studied as a proteolytic reaction. When fibrinogen is converted into a fibrin clot, some protein material, the non-clottables, always remains in solution. The assumption is made that this material is fibrin monomer and/or polymer in equilibrium with the clot and should therefore be considered as product in the reaction. Making corrections for the non-clottables, the Michaelis-Menten constant $K_m$ and the maximum velocity $V_m$, of the reaction were determined and are:

$K_m$ = 5.6 uM (1.9 mg/ml)

$V_m$ = 0.02 uM/min/unit (7.3 mg/min/unit)

Data are presented which show that, up to an initial fibrinogen concentration of 0.4 mg/ml and thrombin concentrations from 0.037 to 0.5 u/ml., thrombin is inactivated by fibrinogen (0.06 u/ml/mg fibrinogen). If thrombin inactivation is taken into account, the reaction is shown to be first order with respect to thrombin and fibrinogen up to a fibrinogen concentration of 0.4 mg/ml (i.e. $F_o$ = 0.2 $K_m$). The value of the first order rate constant, $k_1$, depends on whether or not corrections are applied for the effect of non-clottable material. Assuming that non-clottable protein is reaction product the value of this rate constant is 7.3/min/unit. The difference between this value and the value of $k_1$ = $V_m/K_m$ = 3.8/min/unit is explained by thrombin inactivation.

The course of the overall reaction (F→clot) was followed by means of opacity measurements at 310 mu. Opacity-time recordings show a lag period (the clotting time $t_c$), an initial sharp increase in OD-310 ($V_o$) and then a slower increase in OD-310. Also, an attempt was made to explain the events leading to the production of a fibrin clot by means of a model in which step 1 and step 2 are assumed to be two first order consecutive reactions.

In terms of this model, the amount of polymer produced at any time ($f_c$) is given by an equation of the form,

$$f_c = A(1-\exp(-k_1Tt)) - B(1-\exp(-k_2t))$$

Where $k_1$ and $k_2$ are the rate constants for proteolysis and polymerization and T is the thrombin concentration.

Correlations between the values of $V_o$ derived from the opacity-time recordings, the value of the total fibrin monomer produced (from the step 1 rate experiments), the values of $f_c$ obtained from the above equation and initial fibrinogen and thrombin concentrations were explored. Sufficient correlation was observed between these parameters to postulate clot formation as proceeding as follows. Fibrinogen is converted into fibrin monomer and this then polymerizes to intermediate polymer. At a critical degree of polymerization, represented by $f_c = 0.07$ mg./ml., the polymerization of a relatively few extra monomers onto each intermediate polymer results in visible clot formation. Just prior to $t_c$ there is a high concentration of critically sized intermediate polymer. At $t_c$ the addition of a relatively few monomers onto intermediate polymer results in clot formation and therefore at $t_c$, clot forma-

tion will be rapid as is actually observed. After this initial rapid increase in clot formation, $f_c$ concentration will be low and clot formation will be limited by the rate of polymerization of fibrin monomer which in turn depends on fibrin monomer concentration. After the initial sharp increase, clot formation should therefore parallel fibrin monomer, or $f_c$, production as is actually observed.

Although the critical monomer, intermediate polymer concentration at $t_c$ is 0.07 mg./ml., the value of the non-clottables is less than this. The value of 0.07 mg./ml. is the concentration of intermediate polymer that must be reached before clot starts to form whereas the non-clottable is the final concentration of monomer and/or polymer in equilibrium with the clot. The equilibrium between monomer, intermediate polymer and clot will favor clot because clot is removed from solution and the equilibrium will therefore be displaced in this direction.

# A P P E N D I X

## TABLE A1

## DETERMINATION OF CLOTTING ACTIVITY BY THE NIH CLOTTING METHOD

| Titration Mix. ml. | Thrombin ml. | Saline ml. | Thrombin Clotting units/ml. | Clotting Time seconds * |
|---|---|---|---|---|
| 3 | 0.6 | 0.4 | 1.5 | 10.6 -0.1 |
| 3 | 0.5 | 0.5 | 1.25 | 11.1 -0.4 |
| 3 | 0.4 | 0.6 | 1.0 | 17.0 -0.5 |
| 3 | 0.3 | 0.7 | 0.75 | 19.3 -0.4 |
| 3 | 0.2 | 0.8 | 0.5 | 23.7 -0.8 |
| 3 | 0.15 | 0.85 | 0.375 | 36.1 -0.3 |
| 3 | 0.1 | 0.9 | 0.25 | 54.7 -1.4 |

* Average of 3 determinations.

## TABLE A2

## HYDROLYSIS OF TAMe BY THROMBIN

| Thrombin | TAMe $Mx10^4$ | Initial Velocity OD-247/min. | $(OD\text{-}247/min)^{-1}$ | $(M.TAMe)^{-1}$ |
|---|---|---|---|---|
| Parke- | 19.40 | 0.0380 | 26.3 | 513 |
| Davis | 9.70 | 0.0370 | 27.0 | 1030 |
| 3.5 u/ml. | 6.50 | 0.0330 | 30.3 | 1540 |
| | 4.80 | 0.0290 | 34.5 | 2060 |
| | 3.20 | 0.0270 | 37.0 | 3130 |
| | 1.94 | 0.0230 | 43.5 | 5130 |
| | 0.97 | 0.0145 | 69.0 | 10300 |
| Fraction | 14.80 | 0.0215 | 46.6 | 675 |
| 7. | 7.40 | 0.0180 | 55.5 | 1350 |
| Table VI | 2.48 | 0.0140 | 71.5 | 4040 |
| 2.0 u/ml. | 1.64 | 0.0115 | 87.0 | 6100 |
| | 1.48 | 0.0110 | 91.0 | 6750 |

## TABLE A3

## EFFECT OF TAMe ON NON-CLOTTABLES

| Fibrinogen Original OD-290* | Clot Removed OD-290* | Average | Corrected** OD-290 |
|---|---|---|---|
| 0.036 M TAMe in Buffer | | | |
| 0.613 | 0.050,0.053 | 0.052 | 0.032 |
| 1.23 | 0.084,0.095 | 0.090 | 0.074 |
| 2.46 | 0.163,0.172 | 0.168 | 0.160 |
| 4.91 | 0.335,0.315 | 0.325 | 0.332 |
| 0.036 M KCl in Buffer | | | |
| 0.355 | 0.030,0.035 | 0.033 | 0.036 |
| 0.676 | 0.039,0.040 | 0.039 | 0.043 |
| 1.49 | 0.078,0.084 | 0.081 | 0.089 |
| 2.13 | 0.111,0.115 | 0.113 | 0.124 |
| 3.89 | 0.202,0.200 | 0.201 | 0.220 |
| Buffer only | | | |
| 0.355 | 0.034 | 0.034 | |
| 0.613 | 0.045,0.050 | 0.048 | |
| 1.23 | 0.065,0.069 | 0.067 | |
| 2.46 | 0.118,0.127 | 0.123 | |
| 4.91 | 0.210 | 0.210 | |

* Corrected for Tris-Acetate Blank Absorption.

** Corrected for TAMe Absorbance and Dilution.

## TABLE A4

## INITIAL VELOCITIES OF THE PROTEOLYSIS REACTION

| Reaction Time t mins | Initial Fibrinogen $F_o$ mg/ml | Clot Supernatant $F_t$ mg/ml | $(F_o-F_t)$ mg/ml | $\frac{(F_o-F_t)}{t}$ |
|---|---|---|---|---|
| Thrombin - 0.123 u/ml. | | | | |
| 0.5 | 0.235 | 0.207 | 0.028 | 0.056 |
| 1.0 | 0.235 | 0.151 | 0.084 | 0.084 |
| 0.5 | 0.357 | 0.306 | 0.051 | 0.102 |
| 1.0 | 0.357 | 0.235 | 0.122 | 0.122 |
| 0.5 | 0.623 | 0.551 | 0.072 | 0.144 |
| 1.0 | 0.623 | 0.425 | 0.198 | 0.198 |
| 0.5 | 1.235 | 1.145 | 0.090 | 0.180 |
| 1.0 | 1.235 | 0.937 | 0.298 | 0.298 |
| Thrombin - 0.230 u/ml. | | | | |
| 0.5 | 0.308 | 0.199 | 0.109 | 0.218 |
| 1.0 | 0.308 | 0.136 | 0.172 | 0.172 |
| 0.5 | 0.587 | 0.426 | 0.161 | 0.322 |
| 1.0 | 0.587 | 0.282 | 0.305 | 0.305 |
| 0.5 | 1.290 | 1.030 | 0.260 | 0.520 |
| 1.0 | 1.290 | 0.773 | 0.517 | 0.517 |
| 0.5 | 1.850 | 1.520 | 0.330 | 0.660 |
| 1.0 | 1.850 | 1.270 | 0.580 | 0.580 |
| Thrombin - 0.50 u/ml. | | | | |
| 0.5 | 0.205 | 0.062 | 0.143 | 0.286 |
| 0.5 | 0.410 | 0.137 | 0.273 | 0.546 |

## TABLE A5

## VALUES OF $1/v_o$ AND $1/F_o$ FOR LINEWEAVER-BURKE PLOT

| Reaction Time mins. | $F_o$ mg/ml | Thrombin T. u/ml. | $\frac{(F_o-F_t)}{t}$ mg/ml/min | $\frac{(F_o-F_t)}{t.\ T}$ | $(F_o)^{-1}$ | $\frac{(F_o-F_t)}{t.\ T}^{-1}$ |
|---|---|---|---|---|---|---|
| 0.5 | 0.235 | 0.123 | 0.098 | 0.80 | 4.25 | 1.25 |
| 1.0 | 0.235 | " | 0.105 | 0.85 | 4.25 | 1.18 |
| 0.5 | 0.357 | " | 0.158 | 1.28 | 2.80 | 0.78 |
| 1.0 | 0.357 | " | 0.150 | 1.22 | 2.80 | 0.82 |
| 0.5 | 0.623 | " | 0.236 | 1.92 | 1.61 | 0.52 |
| 1.0 | 0.623 | " | 0.244 | 1.98 | 1.61 | 0.51 |
| 0.5 | 1.235 | " | 0.352 | 2.86 | 0.81 | 0.35 |
| 1.0 | 1.235 | " | 0.374 | 3.04 | 0.81 | 0.37 |
| 0.5 | 0.308 | 0.230 | 0.270 | 1.17 | 3.24 | 0.85 |
| 1.0 | 0.308 | " | 0.198 | 0.86 | 3.24 | 1.15 |
| 0.5 | 0.587 | " | 0.408 | 1.77 | 1.70 | 0.56 |
| 1.0 | 0.587 | " | 0.348 | 1.51 | 1.70 | 0.66 |
| 0.5 | 1.290 | " | 0.684 | 2.97 | 0.77 | 0.34 |
| 1.0 | 1.290 | " | 0.599 | 2.61 | 0.77 | 0.38 |
| 0.5 | 1.850 | " | 0.914 | 3.98 | 0.54 | 0.25 |
| 1.0 | 1.850 | " | 0.707 | 3.08 | 0.54 | 0.32 |
| 0.5 | 0.205 | 0.5 | 0.330 | 0.660 | 4.86 | 1.52 |
| 0.5 | 0.410 | 0.5 | 0.616 | 1.232 | 2.44 | 0.82 |

## TABLE A6-1

## DETERMINATION OF $k_1$ VALUES OF $F_t$

Initial Fibrinogen Concentration - 0.48 OD-290

Non-clottables - 0.038 OD-290

Max-NC - 0.100 OD-290

| | | | | | |
|---|---|---|---|---|---|
| Thrombin 0.30 u/ml. | | | | | |
| Reaction Time mins. | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 |
| $F_t$ OD-290 | 0.244 | 0.154 | 0.099 | 0.083 | 0.102 |
| $F_t$ (NC) OD-290 | 0.206 | 0.116 | 0.061 | 0.045 | 0.064 |
| $F_t$ (max-NC) OD-290 | 0.144 | 0.054 | - | - | - |
| Thrombin 0.15 u/ml. | | | | | |
| Reaction Time mins. | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 |
| $F_t$ OD-290 | 0.319 | 0.228 | 0.135 | 0.108 | 0.086 |
| $F_t$ (NC) OD-290 | 0.281 | 0.190 | 0.097 | 0.070 | 0.048 |
| $F_t$ (max-NC) OD-290 | 0.219 | 0.128 | 0.035 | 0.008 | - |
| Thrombin 0.075 u/ml. | | | | | |
| Reaction Time mins. | 2.0 | 4.0 | 6.0 | 8.0 | 10.0 |
| $F_t$ OD-290 | 0.368 | 0.304 | 0.170 | 0.149 | 0.187 |
| $F_t$ (NC) OD-290 | 0.330 | 0.266 | 0.132 | 0.111 | 0.149 |
| $F_t$ (max-NC) OD-290 | 0.268 | 0.204 | 0.070 | 0.049 | - |

## TABLE A6-2

## DETERMINATION OF $k_1$ VALUES OF $F_t$

Initial Fibrinogen Concentration - 0.24 OD-290

Non-clottables - 0.025 OD-290

Max-NC - 0.045 OD-290

| Thrombin 0.30 u/ml. | | | | | |
|---|---|---|---|---|---|
| Reaction Time mins. | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 |
| $F_t$ OD-290 | 0.105 | 0.049 | 0.041 | 0.059 | 0.054 |
| $F_t$ (NC) OD-290 | 0.080 | 0.024 | 0.016 | 0.034 | 0.029 |
| $F_t$ (max-NC) OD-290 | 0.060 | 0.004 | - | - | - |

| Thrombin 0.15 u/ml. | | | | | |
|---|---|---|---|---|---|
| Reaction Time mins. | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 |
| $F_t$ OD-290 | 0.141 | 0.077 | 0.047 | 0.041 | 0.059 |
| $F_t$ (NC) OD-290 | 0.116 | 0.052 | 0.022 | 0.016 | 0.034 |
| $F_t$ (max-NC) OD-290 | 0.096 | 0.022 | - | - | - |

| Thrombin 0.075 u/ml. | | | | | |
|---|---|---|---|---|---|
| Reaction Time mins. | 2.0 | 4.0 | 6.0 | 8.0 | 10.0 |
| $F_t$ OD-290 | 0.151 | 0.089 | 0.058 | 0.059 | 0.070 |
| $F_t$ (NC) OD-290 | 0.126 | 0.064 | 0.033 | 0.034 | 0.045 |
| $F_t$ (max-NC) OD-290 | 0.106 | 0.044 | 0.013 | 0.014 | - |

## TABLE A6-3

## DETERMINATION OF $k_1$ VALUES OF $F_t$

Initial Fibrinogen Concentration - 0.14 OD-290

Non-clottables - 0.020 OD-290

Max-NC - 0.030 OD-290

Thrombin 0.375 u/ml.

| Reaction Time mins. | 0.5 | 1.0 | 1.5 | 2.0 |
|---|---|---|---|---|
| $F_t$ OD-290 | 0.052 | 0.029 | 0.036 | 0.030 |
| $F_t$ (NC) OD-290 | 0.032 | 0.009 | 0.016 | 0.010 |
| $F_t$ (max-NC) OD-290 | 0.022 | 0.009 | - | - |

Thrombin 0.15 u/ml.

| Reaction Time mins. | 0.5 | 1.0 | 1.5 | 2.0 |
|---|---|---|---|---|
| $F_t$ OD-290 | 0.161 | 0.064 | 0.044 | 0.029 |
| $F_t$ (NC) OD-290 | 0.141 | 0.044 | 0.024 | 0.009 |
| $F_t$ (max-NC) OD-290 | 0.131 | 0.034 | 0.014 | - |

Thrombin 0.075 u/ml.

| Reaction Time mins. | 1.0 | 2.0 | 3.0 | 4.0 |
|---|---|---|---|---|
| $F_t$ OD-290 | 0.150 | 0.081 | 0.069 | 0.041 |
| $F_t$ (NC) OD-290 | 0.130 | 0.061 | 0.049 | 0.021 |
| $F_t$ (max-NC) OD-290 | 0.120 | 0.051 | 0.039 | 0.011 |

Thrombin 0.0375 u/ml.

| Reaction Time mins. | 2.0 | 4.0 | 6.0 | 8.0 |
|---|---|---|---|---|
| $F_t$ OD-290 | 0.135 | 0.103 | 0.065 | 0.056 |
| $F_t$ (NC) OD-290 | 0.115 | 0.083 | 0.045 | 0.036 |
| $F_t$ (max-NC) OD-290 | 0.105 | 0.073 | 0.035 | 0.026 |

## MISCELLANEOUS MATERIALS

Ammonium Sulfate. $(NH_4)_2SO_4$. granular, pyridine free. Analytical Reagent. Mallinckrodt Chemical Works.

Calcium Chloride. $CaCl_2$. $2H_2O$, granular. Analytical Reagent. Mallinckrodt Chemical Works.

Potassium Chloride. KCl granular. Analytical Reagent. Mallinckrodt Chemical Works.

Sodium Chloride. NaCl crystals. Analytical Reagent. Mallinckrodt Chemical Works.

Sodium Phosphate. $Na_2HPO_4$. $12H_2O$, crystals. Baker Analyzed Reagent. J.T. Baker Chemical Co.

Sodium Phosphate. $NaH_2PO_4$. $H_2O$, crystals. Baker Analyzed Reagent. J.T. Baker Chemical Co.

Tris-acetate: Tris(hydroxymethyl)aminomethane F.W. 121.14. Fisher Scientific Co.,m.pt. 170.8-171.8°., Lot 731519.

pH 7.4 Tris-acetate ionic strength 0.15 was prepared by mixing together, 150 ml. 1.0 M acetic acid and 177 ml. 1.0 M Tris. and making up to 1 liter with distilled water. pH - 7.4.

TAMe: p-tosyl L-arginine methyl ester. F.W. 378.5. Sigma Chemical Co. Lot T42B-083; Gallard Schlesinger Mfg. Co., g.s. A2644,m.pt. 143 - 146°.

Both these preparations gave identical rates of hydrolysis when hydrolysed by thrombin.

Thrombin. Fractions 3, 4, 5 and 6 of Table VI were used for clotting studies. Fractions 3, 7 and 8 were used for calibration of the clotting and esterase activities of thrombin.

Fibrinogen. Fractions P1, P2, P2gel from experiment 1, fractions P1, P1gel, P2 and GP2 from experiment 2, Table IV were used indiscriminately throughout.

# BIBLIOGRAPHY


1. Alexander, B. (1953). in Blood Cells and Plasma Proteins (J.L. Tullis, ed.), p. 75, Academic Press, N.Y.

2. Backus, J.K., Laskowski, M. Jr., Scheraga, H.A. and Nims, L.F. (1952). "Distribution of Intermediate Polymers in the Fibrinogen-Fibrin Conversion." Arch. Biochem. Biophys. 41, 354 - 366.

3. Bailey, K., Bettleheim, F.R., Lorand, L. and Middlebrook, W.R. (1957). "Action of Thrombin in the Clotting of Fibrinogen." Nature, 167, 233 - 234.

4. Bettleheim, F.R. (1956). "The Clotting of Fibrinogen II. Fractionation of Peptide Material Liberated." Biochim. et Biophys. Acta. 19, 121 - 130.

5. Bettleheim, F.R., and Bailey, K. (1952). "The Products of the Action of Thrombin on Fibrinogen." Biochim. et Biophys. Acta. 9, 578 - 579.

6. Blomback, B., and Sjoquist, T. (1960). "Studies on Fibrinopeptides from Different Species." Acta. Chem. Scand 14, 493 - 495.

7. Blomback, B., and Yamashina, I. (1958). "On the N-Terminal Amino Acids in Fibrinogen and Fibrin." Arkiv. Kemi. 12, 299 - 319.

8. Cohn, E.S., Gurd, F.R.N., Surgenor, D.M., Barnes, B.A., Brown, R.K., Derouaux, G., Gillespie, J.M., Kahut, F.N., Lever, W.F., Liu, C.N., Mittleman, D., Mouton, R.F., Schmidt, K, and Uroma, E. (1950). "A System for the Separation of the Components of Human Blood: Quantitative Procedures for the Separation of the Protein Components of Human Plasma." J. Am. Chem. Soc. 72, 465 - 474.

9. Deutsch, H.F., and Goodloe, M.B. (1945). "An Electrophoretic Survey of Various Animal Plasmas." J. Biol. Chem. 161, 1 - 20.

10. Donnelly, T.H., Laskowski, M., Jr., Nottley, N. and Scheraga, H.A. (1955). "Equilibria in the Fibrinogen-Fibrin Conversion II. Reversibility of the Polymerization Steps." Arch. Biochem. Biophys. 56, 369 - 387.

11. Dittmer, D.S. (1961). Editor. Blood and other Body Fluids. p. 231 - 236. Federation of American Societies for Experimental Biology. Washington, D.C.

12. Edsall, J.T. (1954). "Configuration of Certain Protein Molecules. An Inquiry Concerning the Present Status of our Knowledge." J. Polymer Sci. 12, 253 - 280.

13. Ehrenpreis, S. and Scheraga, H.A. (1957). "Observations on the Analysis for Thrombin and the Inactivation of Fibrin Monomer." J. Biol. Chem. 227, 1043 - 1061.

14. Ehrenpreis, S. and Scheraga, H.A. (1959). "Kinetics of the Conversion of Fibrinogen to Fibrin Monomer at Neutral pH." Arch. Biochem. Biophys. 79, 27 - 43.

15. Ehrenpreis, S., Laskowski, M. Jr., Donnelly, T.H. and Scheraga, H.A. (1958). "Equilibria in the Fibrinogen-Fibrin Conversion IV. Kinetics of the Conversion of Fibrinogen to Fibrin Monomer." J. Am. Chem. Soc. 80, 4255 - 4263.

16. Ferry, J.D., and Morrison, P.R. (1947). "Preparation and Properties of Serum Plasma Proteins VIII. The Conversion of Human Fibrinogen to Fibrin under Various Conditions." J. Am. Chem. Soc. 69, 388 - 400.

17. Ferry, J.D., Shulman, S., and Foster, J.F., (1952). "The Conversion of Fibrinogen to Fibrin IX. Further Flow Birefringence Studies on Inhibited Clotting Systems." Arch. Biochem. Biophys. 39, 387 - 394.

18. Folk, J.E., Gladner, J.A., and Levine, Y. (1959). "Thrombin Induced Formation of Co-fibrin III. Acid Degradation Studies and Summary of Sequential Evidence on Peptide A." J. Biol. Chem. 234, 2317 - 2320.

19. Gladner, J.A., and Laki, K. (1958). "The Active Site of Thrombin." J. Am. Chem. Soc. 80, 1263 - 1264.

20. Hall, C.E., and Slaytor, H.S. (1959). "The Fibrinogen Molecule: Its Size Shape and Mode of Polymerization." J. Biophys. Biochem. Cytol. 5, 11 - 16.

21. Hartley, R.W., Jr., and Waugh, D.F. (1960). "Solubility, Denaturation and Heterogencity of Bovine Fibrinogen." J. Am. Chem. Soc. 82, 978 - 986.

22. Hughes, W.L. in The Proteins. Vol. 2B., Academic Press. N.Y.

23. Hummel, B.C.W. (1959). "A Modified Spectrophotometric Determination of Chymotrypsin, Trypsin and Thrombin." Can. J. Biochem. and Physiol. 37, 1393 - 1399.

24. Kay, C.M. and Marsh, M.M. (1961). "Optical Rotatory Dispersion of the Thrombin Induced Conversion of Fibrinogen to Fibrin Monomer." Nature, 189, 307 - 308.

25. Laki, K. (1951). "The Polymerization of Proteins; the Action of Thrombin on Fibrinogen." Arch. Biochem. Biophys. 32, 317 - 324.

26. Laki, K. and Kitzinger, C. (1956). "Heat Changes During the Clotting of Fibrinogen." Nature, 178, 985 - 985.

27. Laskowski, M., Jr., and Scheraga, H.A. (1954). "Thermodynamic Considerations in Protein Reactions I. Modified Reactivity of Polar Groups." J. Am. Chem. Soc. 76, 6305 - 6319.

28. Laskowski, M., Jr. and Scheraga, H.A. (1956). "Thermodynamic Considerations of Protein Reactions II. Modified Reactivity of Primary Valence Bonds." J. Am. Chem. Soc. 78, 5793 - 5798.

29. Laskowski, M., Jr., Donnelly, T.H., Van Tijn, B.A. and Scheraga (1956). "The Proteolytic Action of Thrombin on Fibrinogen." J. Biol. Chem. 222, 815 - 821.

30. Laskowski, M., Jr., Ehrenpreis, S., Donnelly, T.H. and Scheraga, H.A. (1960). "Equilibria in the Fibrinogen-Fibrin Conversion V. Reversibility and Thermodynamics of the Proteolytic Action of Thrombin on Fibrinogen." J. Am. Chem. Soc. 82, 1340 - 1348.

31. Laskowski, M., Jr., Rakowitz, D.H. and Scheraga, H.A. (1952). "Equilibria in the Fibrinogen-Fibrin Conversion." J. Am. Chem. Soc. 74, 280 - 280.

32. Latallo, Z.S., Fletcher, A.P., Alkjaersig, N. and Sherry, S. (1962). "Influence of pH, Ionic Strength, Neutral Ions and Thrombin on Fibrin Polymerization." Am. J. Physiol. 202, 675 - 680.

33. Lineweaver, H. and Burke, D. (1934). "The Determination of Enzyme Dissociation Constants." J. Am. Chem. Soc. 56, 658 - 666.

34. Lorand, L. (1951). "Fibrino-peptide: New Aspects of the Fibrinogen-Fibrin Transformation." Nature, 167, 992 - 993.

35. Lorand, L. (1952). "Fibrino-peptide." Biochem. J. 52, 200 - 203.

36. Lorand, L. and Jacobson, A. (1964). "Specific Inhibitors and the Chemistry of Fibrin Polymerization." Biochemistry 3, 1934 - 1943.

37. Michaelis, L. and Menten, M. (1913). "Die Kinetik der Invertinwirkung." Biochem. Z. 49, 333 - 337.

38. Mihalyi, E. (1950). "Electrophoretic Investigation of Fibrin and Fibrinogen Dissolved in Urea Solutions." Acta. Chem. Scand. 4, 351 - 358.

39. Mihalyi, E. and Billick, I.H. (1963). "Transformation of Fibrinogen into Fibrin III. Kinetics of the pH Change Associated with the Clotting of Fibrinogen." Biochim. et Biophys. Acta. 71, 97 - 108.

40. Minimum Requirements: Dried Thrombin, 2nd. Revision. U.S. Dept. of Health, Education and Welfare, Public Health Service, National Institutes of Health, Bethesda, Md.

41. Miller, K.D. (1958). "Chromatographic Isolation of Plasma Prothrombin and Trans Glucosylase." J. Biol. Chem. 231, 987 - 995.

42. Miller, K.D. (1959). "Rivanol, Resin and the Isolation of Thrombin." Nature, 182, 461 - 462.

43. Rasmussen, P.S. (1955). "Purification of Thrombin by Chromatography." Biochim. et Biophys. Acta. 16, 157 - 158.

44. Scheraga, H.A. (1958). "Thrombin and its Interaction with Fibrinogen." Ann. N.Y. Acad. Sci. 75, 189 - 194.

45. Scheraga, H.A. and Backus, J.K. (1952). "Flow Birefringence in Arrested Clotting Systems." J. Am. Chem. Soc. 74, 1979 - 1983.

46. Scheraga, H.A. and Ehrenpreis, S. in E. Deutsch. Proc. 4th. Internatl. Congr. Biochem., Vienna 1958, Vol 10, Pergammon, London 1959 p. 212.

47. Scheraga, H.A., Ehrenpreis, S. and Sullivan, E. (1958). "Comparitive Kinetic Behaviour of Thrombin, Plasmin and Trypsin toward Synthetic Substrates." Nature 182, 461 - 462.

48. Scheraga, H.A. and Laskowski, M., Jr., in Advances in Protein Chemistry Vol 12. Academic Press. N.Y.

49. Schwert, G.W., Neurath, H., Kaufman, S. and Snoke, J.E. (1948). "The Specific Esterase Activity of Trypsin." J. Biol. Chem. 172, 221 - 239.

50. Seegers, W.H. (1962). "The Activation of Prothrombin." Sonderdruck aus Behringwerk-Mitteilungen 41,S 13 - 30.

51. Seegers, W.H. and Landaburu, R.H. (1951). "Esterase and Clotting Activity Derived from Purified Prothrombin." Am. J. Physiol. 191, 167 - 173.

52. Seegers, W.H. and Landaburu, R.H. (1960). "Purification of Prothrombin and Thrombin by Chromatography on Cellulose." Can. J. Biochem. and Physiol. 38, 1405 - 1410.

53. Seegers, W.H., Levine, W.G. and Shepard, R.S. (1958). "Further Studies on the Purification of Thrombin." Can. J. Biochem. and Physiol. 36, 603 - 611.

54. Seegers, W.H. and Smith, H.P. (1942). "Factors which Influence the Activity of Purified Thrombin." Am. J. Physiol, 137, 348 - 354.

55. Sephadex in Gel Filtration, Theory and Experimental Techniques, Pharmacia Fine Chemicals Inc., N.Y.

56. Sherry, S. and Troll, W. (1954). "The Action of Thrombin on Synthetic Substrates." J. Biol. Chem. 208, 95 - 105.

57. Shulman, S. (1953). "The Effects of Certain Ions and Neutral Molecules on the Conversion of Fibrinogen to Fibrin." Discussions Faraday Soc. 13, 109 - 115.

58. Shulman, S. and Ferry, J.D. (1950). "The Conversion of Fibrinogen to Fibrin II. Influence of pH and Ionic Strength on Clotting Time and Clot Opacity." J. Phys. and Colloid Chem. 54, 66 - 79.

59. Shulman, S. and Ferry, J.D. (1951). "The Conversion of Fibrinogen to Fibrin III. Sedimentation and Viscosity Studies on Clotting Systems Inhibited by Hexamethylene Glycol." J. Phys. and Colloid Chem. 55, 135 - 144.

60. Shulman, S., Katz, S. and Ferry, J.D. (1953). "The Conversion of Fibrinogen to Fibrin XIII. Dissolution of Fibrin and Inhibition of Clotting by Various Neutral Salts." J. Gen. Physiol. 36, 759 - 766.

61. Smith, H.P. Warner, E.D. and Brinkhouse, K.M. (1937). "Prothrombin Deficiency and the Bleeding Tendency in Liver Injury (Chloroform Intoxication)." J. Exptl. Med. 66, 801 - 811.

62. Sturtevant, J.M., Laskowski, M. Jr., Donnelly, T.H. and Scheraga (1955). "Equilibria in the Fibrinogen-Fibrin Conversion III. Heats of Polymerization and Clotting of Fibrin Monomer." J. Am. Chem. Soc. 77, 6168 - 6172.

63. Surgenor, D.M., Alexander, B., Goldstein, R. and Schmidt, K. (1951). "A System for the Separation of the Protein Components of Human Plasma II. The Components of the Clotting Process." J. Phys. and Colloid Chem. 55, 94 - 101.

64. Tinoco, I and Ferry, J.D. (1954). "The Conversion of Fibrinogen to Fibrin XV. Sedimentation Studies of the Polymerization of Fibrinogen at High pH." Arch. Biochem. Biophys. 48, 7 - 16.

65. Warner, E.D., Brinkhouse, K.M. and Smith, H.P. (1936). "A Quantitative Study on Blood Clotting. Prothrombin Fluctuations under Experimental Conditions." Am. J. Physiol. 114, 667 - 675.

66. Waugh, D.F., Baughman, D.J. and Miller, K.D. (1960) in The Enzymes vol. 4. p. 215.

67. Waugh, D.F., and Livingstone, (1951). "Clotting Time and Reaction Velocity in the Interaction of Bovine Fibrinogen and Thrombin." Science 113, 121 - 124.

## ABSTRACT

The fibrinogen-fibrin conversion can be represented as:

| | | |
|---|---|---|
| Step 1. | Proteolysis | $F \xrightarrow{T} f$ |
| Step 2. | Polymerization | $nf \longrightarrow f_n$ |
| Step 3. | Clotting | $m(f_n) \longrightarrow (f_n)m$ |

Where F = fibrinogen, f = fibrin monomer and T = thrombin.

By using TAMe as a thrombin inhibitor, step 1 can be isolated and studied as a proteolytic reaction. When fibrinogen is converted into a fibrin clot, some protein material, the non-clottables, always remains in solution. The assumption is made that this material is fibrin monomer and/or polymer in equilibrium with the clot and should therefore be considered as product in the reaction. Making corrections for the non-clottables, the Michaelis-Menten constant $K_m$ and the maximum velocity $V_m$, of the reaction were determined and are:

$K_m$ = 5.6 uM (1.9 mg/ml)

$V_m$ = 0.02 uM/min/unit (7.3 mg/min/unit)

Data are presented which show that, up to an initial fibrinogen concentration of 0.4 mg/ml and thrombin concentrations from 0.037 to 0.5 u/ml., thrombin is inactivated by fibrinogen (0.06 u/ml/mg fibrinogen). If thrombin inactivation is taken into account, the reaction is shown to be first order with respect to thrombin and fibrinogen up to a fibrinogen concentration of 0.4 mg/ml (i.e. $F_o = 0.2\ K_m$). The value of the first order rate constant, $k_1$, depends on whether or not corrections are applied for the effect of non-clottable material. Assuming that non-clottable protein is reaction product the value of this rate constant is 7.3/min/unit. The difference between this value and the value of $k_1 = V_m/K_m = 3.8$/min/unit is explained by thrombin inactivation.

The course of the overall reaction (F→clot) was followed by means of opacity measurements at 310 mu. Opacity-time recordings show a lag period (the clotting time $t_c$), an initial sharp increase in OD-310 ($V_o$) and then a slower increase in OD-310. Also, an attempt was made to explain the events leading to the production of a fibrin clot by means of a model in which step 1 and step 2 are assumed to be two first order consecutive reactions.

In terms of this model, the amount of polymer produced at any time ($f_c$) is given by an equation of the form,

$$f_c = A(1-\exp(-k_1Tt)) - B(1-\exp(-k_2t))$$

Where $k_1$ and $k_2$ are the rate constants for proteolysis and polymerization and T is the thrombin concentration.

Correlations between the values of $V_o$ derived from the opacity-time recordings, the value of the total fibrin monomer produced (from the step 1 rate experiments), the value of $f_c$ obtained from the above equation and initial fibrinogen and thrombin concentrations were explored. Sufficient correlation was observed between these parameters to postulate clot formation as proceeding as follows. Fibrinogen is converted into fibrin monomer and this then polymerizes to intermediate polymer. At a critical degree of polymerization, represented by $f_c$ = 0.07 mg./ml., the polymerization of a relatively few extra monomers onto each intermediate polymer results in visible clot formation. Just prior to $t_c$ there is a high concentration of critically sized intermediate polymer. At $t_c$ the addition of a relatively few monomers onto intermediate polymer results in clot formation and therefore at $t_c$, clot formation will be rapid as is actually observed. After this initial

rapid increase in clot formation, $f_c$ concentration will be low and clot formation will be limited by the rate of polymerization of fibrin monomer which in turn depends on fibrin monomer concentration. After the initial sharp increase, clot formation should therefore parallel fibrin monomer, or $f_c$, production as is actually observed.

Although the critical monomer, intermediate polymer concentration at $t_c$ is 0.07 mg./ml., the value of the non-clottables is less than this. The value of 0.07 mg./ml. is the concentration of intermediate polymer that must be reached before clot starts to form whereas the non-clottable is the final concentration of monomer and/or polymer in equilibrium with the clot. The equilibrium between monomer, intermediate polymer and clot will favor clot because clot is removed from solution and the equilibrium will therefore be displaced in this direction.

## APPROVAL

The thesis submitted by James Ross has been read and approved by three members of the faculty of Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 24th, 1965
Date

Frederick W. Paivent
Signature of Adviser