A Study of the Inhibitory Effect of Ethylenediaminetetra-acetic Acid on the Thrombin-Fibrinogen Reaction

By T. C. BITHELL*

Medical Research Council Blood Coagulation Research Unit, Churchill Hospital, Oxford

(Received 11 November 1963)

Current theories of the mechanism of the thrombin-fibrinogen reaction are in general agreement as to the importance of Ca^{2+} ions in the formation of the intermolecular bonds responsible for the insolubility in urea and physical characteristics of plasma fibrin (Biggs & MacFarlane, 1962; Loewy, Gallant & Dunathan, 1961). The effect of Ca^{2+} ions on the rate at which fibrinogen is converted into fibrin is less clear. The slight inhibitory effect of decalcifying agents such as oxalate (Seegers & Smith, 1942) and ion-exchange resins, and the moderate acceleratory effect of bivalent cations (Owren, 1946; Ratnoff & Potts, 1954), have together suggested that Ca^{2+} ions function as a non-specific and unessential accelerator of the thrombin-fibrinogen reaction (Ratnoff, 1960).

Re-examination of this hypothesis followed the advent of potent chelating agents such as EDTA, which, because they remove ions strongly bound to proteins, produce deionization of a degree unobtainable with oxalate, citrate or ion-exchange resins. Rosenfeld & Janszky (1952) found that EDTA markedly decreased the reactivity of fibrinogen to thrombin, an effect that was reversible by Ca^{2+} ions. They concluded that this was due to the removal of Ca^{2+} ions from fibrinogen. An alternative explanation was advanced by Triantaphyllopoulis, Quick & Greenwalt (1955), who suggested the liberation of a heparin-like substance. Zucker & Borrelli (1958) concluded that EDTA removed essential Mg^{2+} ions from fibrinogen, and Godal (1960) likewise attributed the inhibitory effect of EDTA to the removal of trace ions, but concluded that Ca^{2+} rather than Mg^{2+} was the essential ion.

The general approach, preparations, experimental conditions and controls have varied widely in these studies. Many of the experiments measured the reactivity of unfractionated plasma to thrombin, with the attendant complexities, and in most instances no observations were included as to the effects of EDTA other than the binding of metal ions.

* Present address: Department of Internal Medicine, University of Utah, Salt Lake City, Utah, U.S.A.

† Abbreviation: TAME, toluene-*p*-sulphonyl-L-arginine methyl ester hydrochloride.

The studies summarized below were carried out to elucidate further the mechanism by which EDTA affects the rate at which thrombin converts purified fibrinogen into fibrin.

MATERIALS

Fibrinogen was prepared by an ethanol-glycine extraction method as described by Blombäck & Blombäck (1956). The product, designated 'fraction I-2' by Blombäck & Blombäck (1956), was dissolved in 0.3M-NaCl at a concentration of approx. 10 mg./ml. and stored at -20° . About 90-95% of the total protein in the preparations used was coagulable with thrombin, and the fibrin clots produced were soluble in 1M-urea at pH 7.2.

A commercial bovine thrombin preparation (Parke Davis Co.) was dissolved in 0.15M-NaCl at a concentration of 100 N.I.H. units/ml. and stored at -20° , and when used was further diluted in silicone-treated glassware.

Imidazole buffers were prepared as described by Mertz & Owen (1940), and concentration was expressed as total imidazole.

The salts and ion-exchange resins were of analytical grade of purity.

A 0.25M stock solution of EDTA was prepared by dissolving its disodium salt in distilled water and correcting the pH to 7.2 with 0.1N-NaOH.

TAME† was dissolved in 0.15M-NaCl.

Reagents free of cations were prepared by column deionization with Amberlite IR-120, a strongly acidic cation-exchange resin in the sodium cycle.

Glassware was scrubbed in 0.05M-EDTA, and rinsed in deionized water.

Gel-filtration was carried out with Sephadex G-50 of medium porosity (Pharmacia).

METHODS

Fibrinogen, thrombin, TAME, buffers and salts were mixed with deionized water and NaCl, to yield a final ionic strength of 0.15 unless otherwise specified.

The thrombin time is defined as the time required, after the addition of thrombin to a fibrinogen solution, for the formation of the first visible particles of fibrin. This occurs before the gel point, and was measured in 10 mm. $\times$ 75 mm. glass tubes by using a reaction mixture composed of 0.1 ml. of fibrinogen solution and 0.4 ml. of thrombin solution. The conditions and concentrations of all reactants indicated refer to the final reaction mixture. Unless specified to the contrary these were: room temperature ($20-25^{\circ}$), thrombin

concentration 2.0 N.I.H. units/ml., pH 7.2, imidazole concentration 5 mM, and ionic strength 0.15. Except where indicated to the contrary, illustrated values for the thrombin time represent the mean of 20–100 replicate determinations, the coefficient of variation of which did not exceed $\pm 12\%$.

Total nitrogen was determined by a micro-Kjeldahl technique, with distillation into the boric acid–indicator mixture of Ma & Zuazaga (1942). The protein content of a fibrinogen solution was obtained from the total nitrogen present in the precipitate insoluble in 10% (w/v) trichloroacetic acid, after two washings with 5% (w/v) trichloroacetic acid.

Fibrinogen was determined by conversion into fibrin under the conditions described by Ferry & Morrison (1947). The fibrinogen was then washed, dried and weighed as described by Saifer & Newhouse (1954). No corrections were made for occluded non-protein nitrogen, and a nitrogen-to-protein conversion factor of 6.25 was used for both fibrinogen and fibrin.

EDTA was determined by the method of Smith & Tompsett (1958), which will detect concentrations as low as 0.05 mM.

The hydrolysis of TAME was carried out as described by Sherry & Troll (1954), and followed by titration of the acid produced with a micrometer syringe (Trevan, 1925).

The opacity of fibrinogen solutions and fibrin clots was measured in 4 ml. rectangular glass cuvettes having a 1 cm. light-path by using a Spekker photoelectric absorptiometer, with a filter (Kodak) that transmits a wavelength of approx. 420 m μ . At this wavelength, extinction is proportional to light-scatter, absorption by proteins being negligible (Ferry & Morrison, 1947).

The pH was measured with a glass electrode by using a Doran Universal model pH-meter.

RESULTS

Rate of equilibration. The preliminary experiments in Fig. 1 show the effect produced by EDTA on the thrombin–fibrinogen reaction as measured by the thrombin time. The addition of EDTA to thrombin (Expt. A in Fig. 1) did not alter the thrombin time, but when the EDTA was added to the fibrinogen (Expts. B, C, D and E in Fig. 1) a marked prolongation of the thrombin time was observed; this increased with time, reached a maximum and remained stable at this level. The thrombin time at the asymptote was dependent on the amount of EDTA (cf. Expts. B and D in Fig. 1) and the amount of fibrinogen (cf. Expts. C and D in Fig. 1) in the reaction mixture. The marked effect of temperature on the thrombin time of samples containing EDTA is apparent (cf. Expts. B and E in Fig. 1) and high EDTA concentrations at 37° resulted in greatly prolonged thrombin times that were difficult to measure accurately. Subsequent results, except where indicated, represent fibrinogen samples equilibrated with EDTA at room temperature (EDTA–fibrinogen). The time required for the thrombin time of fibrinogen containing EDTA

to reach a stable asymptote, or to equilibrate, is defined as the time elapsed after zero time until the thrombin time of the EDTA–fibrinogen mixture is within twice the s.d. of the mean of thrombin times obtained on the sample 30–120 min. after zero time. This was 8–12 min. [10.2 ± 2.2 (20)] for all samples, and was unaffected by temperature, pH, thrombin concentration and large variations in the concentration of EDTA and fibrinogen.

Effect of ethylenediaminetetraacetic acid and fibrinogen concentrations. The thrombin time of fibrinogen solutions containing EDTA depends on the amount of EDTA relative to the amount of fibrinogen. The quantitative relationships are shown in Fig. 2. The smallest EDTA concentration that produced significant inhibition ($P < 0.01$) was 0.025 mM at a fibrinogen concentration of 0.2 mg./ml. Increasing the EDTA concentration resulted in

Fig. 1. Prolongation of the thrombin time by EDTA, and the rate of equilibration of EDTA and fibrinogen. In Expt. A, thrombin was made 10 mM with respect to EDTA at zero time and then added to control fibrinogen at 1 min. intervals. In Expts. B, C, D and E, EDTA was added to fibrinogen solutions at zero time, and the thrombin time of the mixture was determined at 1 min. intervals. The thrombin time of fibrinogen without EDTA, under the indicated conditions, is illustrated to the left of zero time on the abscissa. The results are representative of multiple experiments, and many intermediate points are not illustrated. Expt. A (○), 0.2 mg. of fibrinogen/ml., thrombin in 10 mM-EDTA, temp. 20°; Expt. B (■), 0.2 mg. of fibrinogen/ml., 0.1 mM-EDTA, temp. 20°; Expt. C (△), 0.4 mg. of fibrinogen/ml., 10 mM-EDTA, temp. 20°; Expt. D (●), 0.2 mg. of fibrinogen/ml., 10 mM-EDTA, temp. 20°; Expt. E (□), 0.2 mg. of fibrinogen/ml., 0.1 mM-EDTA, temp. 37°.

a linear increase in the thrombin time on a double-log scale at all fibrinogen concentrations tested. The inhibitory effect of EDTA was decreased when the fibrinogen concentration was increased from 0.2 to 0.6 mg./ml., but was altered little by further increasing the fibrinogen concentration to 3.0 mg./ml. Neither the minimum inhibitory concentration of EDTA nor the slope of the plots of Fig. 2 was affected by varying the thrombin concentration.

Effect of thrombin concentration. The thrombin times of control fibrinogen and of fibrinogen at equilibrium with EDTA were determined with various concentrations of thrombin. The resulting plots in Fig. 3 are linear on a double-log scale, and those of samples containing EDTA are parallel to those of control fibrinogen. This relationship was maintained in samples containing various concentrations of EDTA and fibrinogen, at both 22° and 37°, and at various values of ionic strength and pH.

Effect of pH, temperature and ionic strength. The extent to which variation in pH, ionic strength and temperature modify the inhibitory effects of EDTA is shown in Fig. 4. Increasing the pH from 6.2 to 7.0 (Fig. 4a) decreased the thrombin time of control samples and the samples containing EDTA equally, but above pH 7.0 the effects of EDTA

were potentiated. The thrombin time of samples containing EDTA was about 5 times as long at pH 7.8 as that at neutrality, and at pH 7.8 was about 8 times as long as that of control samples. An increase in temperature from 5° to 20° (Fig. 4c) decreased the thrombin time of both the controls and the samples containing EDTA to an equal degree, but between 20° and 37° the thrombin time of the samples containing EDTA was greatly prolonged, being about 4 times as long at 37° as that at room temperature, and at 37° being about 9 times as long as that of control fibrinogen. Increasing the ionic strength from 0.15 to 0.25 (Fig. 4b) also increased significantly the difference between the thrombin time of controls and that of samples containing EDTA. The modifying effects of pH, ionic strength and temperature were rapidly and completely reversible.

Effect of addition of bivalent ions. The effects of bivalent cations on the inhibitory effects of EDTA under various circumstances are summarized in Table 1. The slight acceleratory effect of the alkaline-earth ions Ca^{2+} , Sr^{2+} , Mg^{2+} and Ba^{2+} on

Fig. 2. Effect of EDTA concentration and fibrinogen concentration on the thrombin time of EDTA-fibrinogen. EDTA and fibrinogen were equilibrated in the indicated concentrations. The thrombin times of the various concentrations of fibrinogen without EDTA are indicated to the left of the abscissa. The final thrombin concentration in the reaction mixture was 2.0 N.I.H. units/ml. Concn. of fibrinogen: ○, 0.2 mg./ml.; ■, 0.4 mg./ml.; △, 0.6 mg./ml.; ●, 3.0 mg./ml.

Fig. 3. Effect of thrombin concentration on the thrombin time of EDTA-fibrinogen. EDTA and fibrinogen were equilibrated in the indicated concentrations and the thrombin time of the mixture was determined with the indicated thrombin concentrations. ○, 0.2 mg. of fibrinogen/ml., no EDTA; ■, 0.2 mg. of fibrinogen/ml., 0.05 mM-EDTA; △, 0.4 mg. of fibrinogen/ml., 10 mM-EDTA; ●, 0.2 mg. of fibrinogen/ml., 0.05 mM-EDTA, temp. 37°; □, 0.2 mg. of fibrinogen/ml., 10 mM-EDTA.

the thrombin time, and the precipitation of fibrinogen produced by Cd^{2+} , Ni^{2+} and Mn^{2+} ions is shown in the experiments of group 1. It is apparent that the EDTA chelates of these ions

(group 2) had little inhibitory effect, and the potency of the respective ions in modifying the inhibitory properties of high concentrations of EDTA was roughly proportional to their chelate stability constants. The ions most effective in decreasing the inhibitory effect of EDTA were Cd^{2+} , Ni^{2+} and Mn^{2+} , which precipitate fibrinogen in the absence of EDTA. Similar results were obtained when ions were added to fibrinogen already equilibrated with EDTA (group 3).

Removal of ethylenediaminetetra-acetic acid from ethylenediaminetetra-acetic acid-fibrinogen. Table 2 summarizes representative experiments in which EDTA is removed from EDTA-fibrinogen. Prolonged dialysis sufficed to remove all measurable EDTA, and reversed its inhibitory effect, but produced an inconstant decrease in fibrinogen yield. EDTA was also removed from fibrinogen by means of an anion-exchange resin and by gel-filtration, and its inhibitory effects on the thrombin time were reversed by both methods. The slight prolongation of the thrombin time of both the control and the EDTA-containing samples after resin treatment and gel-filtration was due to the dilution from 1 to approx. 1.6 vol. produced by the buffer in the resin and gel columns, and to the retention of approx. 20 % of the fibrinogen in the resin column.

Effect on rate of opacity change. The rate at which the opacity of fibrinogen solutions increases during conversion into fibrin by small amounts of thrombin was studied. Fig. 5 shows that the effect of EDTA is primarily to delay the start of opacification. When compared with control samples 5 min. after the addition of thrombin, fibrinogen solutions containing EDTA showed no change in opacity, whereas the control had tripled in opacity and formed a dense clot. After this initial lag period, however, the opacity of samples containing EDTA increased at a rate equal to the control, and resulted in identical final opacity. Similar curves (Fig. 5) resulted when the thrombin concentration was decreased or when small amounts of TAME were added. The final opacity in all of these experiments was directly proportional to the fibrinogen concentration, and the opacity that corresponds to the visual gel point was unaffected by EDTA.

Miscellaneous. Representative experiments were repeated with a commercial human thrombin preparation and bovine fibrinogen prepared by the Blombäck & Blombäck (1956) method, with results comparable in every respect with those of the experiments mentioned above.

The effect of EDTA on the yield of fibrin was evaluated. The amount of fibrin recovered from samples containing EDTA was identical with that recovered from control samples at 1 hr. ($P < 0.01$) in all experiments, which included concentrations of EDTA up to 10 mM and fibrinogen from 0.2 to

Fig. 4. Effect of pH, ionic strength, and temperature, on the thrombin time of EDTA-fibrinogen. A solution of 0.3 mg. of fibrinogen/ml. was equilibrated with 0.5 mM-EDTA at the indicated pH (a), ionic strength (b) and temperature (c), and the thrombin time was then determined with thrombin solutions constituted so that the pH, ionic strength and temperature of the reaction mixture were the same as the equilibration mixture. The temperature of equilibration in (a) and (b) was 22°. In the concentrations used, negligible variation in ionic strength results from alteration of the $\text{H-imidazole}^+\text{Cl}^-$:total imidazole ratio. The final thrombin concentration in the reaction mixture was 2.0 N.I.H. units/ml. ●, Fibrinogen containing EDTA; ○, control fibrinogen.

Table 1. *Effect of various cations and their ethylenediaminetetra-acetic acid chelates on the thrombin-fibrinogen reaction*

Experiments are divided into three groups. The ions indicated were in the form of their chloride salts, and in group 1 were equilibrated with fibrinogen in the indicated concentrations, and the thrombin time of the mixture was determined at 22° with 1 unit of thrombin/ml. In group 2, the indicated ions were mixed with equimolar concentrations of EDTA to produce 1:1 chelates, which were then equilibrated with fibrinogen in the indicated concentrations at 37°. The thrombin time was determined at 37° with 2 units of thrombin/ml. In group 3, EDTA in the indicated concentrations and fibrinogen were first equilibrated at 37°, and then mixed and equilibrated with the indicated ion in equimolar amounts. The thrombin time was determined at 37° with 2 units of thrombin/ml. The fibrinogen concentration was 0.3 mg./ml., and the time of equilibration was 30 min. in all experiments. The thrombin time of control fibrinogen without added ions or EDTA, under the conditions specified above, is also given. Data tabulated under Na⁺/2 represent the thrombin time in the presence of EDTA alone. Appended to the Table, under each ion, are the stability or formation constants for their 1:1 EDTA chelates, as determined potentiometrically at an ionic strength of 0.1 and a temperature of 20° (Martell & Calvin, 1952, p. 537). Key: * indicates visible precipitation within 10 min., and † indicates visible precipitation within 60 min., after the addition of the ion or chelate indicated.

Experiment	Fibrinogen sample	Ion added ...	Control	Thrombin time (sec.)							
				Na ⁺ /2	Ba ²⁺	Str ²⁺	Mg ²⁺	Ca ²⁺	Mn ²⁺	Cd ²⁺	Ni ²⁺
Group 1	In 0.1 mM-ion In 1 mM-ion In 10 mM-ion	...	19.0	—	19.1	18.5	19.1	19.8	*	*	*
			19.0	—	19.0	19.1	18.2	17.4	†	†	†
			19.0	—	17.0	18.2	16.4	14.8	†	†	†
Group 2	In 1 mM-EDTA chelate of ion In 5 mM-EDTA chelate of ion In 10 mM-EDTA chelate of ion	...	12.6	77	12.0	14.0	12.5	12.3	12.6	12.1	13.4
			12.6	> 120	15.0	16.0	12.4	12.8	12.4	11.8	12.6
			12.6	> 120	21.9	19.5	16.6	15.0	12.6	*	12.6
Group 3	In 1 mM-EDTA, after addition of ion In 5 mM-EDTA, after addition of ion In 10 mM-EDTA, after addition of ion log (Chelate stability constant) of 1:1 chelate	...	12.6	77	16.0	12.0	14.0	12.4	11.8	11.9	11.8
			12.6	> 120	15.0	12.2	12.4	12.0	11.8	13.1	12.6
			12.6	> 120	25.5	17.0	14.6	15.0	12.9	13.5	11.8

Table 2. *Effect of removing ethylenediaminetetra-acetic acid from ethylenediaminetetra-acetic acid-fibrinogen*

Solutions of 1.5 mg. of fibrinogen/ml. were divided into paired samples, one of which was made 25 mM with respect to EDTA and equilibrated at 37°, the other, without EDTA, serving as a control. The lot of fibrinogen used in the gel-filtration experiments was different from that used in the dialysis and resin experiments. Data are tabulated after equilibration but before other treatment on the percentage (w/w) of fibrinogen coagulable and the thrombin time for each sample. All six samples were then treated as indicated. After this treatment data are tabulated on the percentage (w/w) of fibrinogen recovered, the EDTA concentration, the percentage (w/w) fibrinogen coagulable and the thrombin time of each sample. In the reaction mixture the fibrinogen concentration was 0.3 mg./ml. and the EDTA concentration was 5 mM, and the thrombin time was determined at 37°. The Amberlite was charged in the chloride cycle and washed until the pH of the effluent was 7.2. The Sephadex was washed four times. The diffuseate, eluate and wash solutions were 5 mM-imidazole buffer, pH 7.2, containing NaCl (0.15M).

Method of treatment	Before treatment			After treatment			
	Fibrinogen sample	Fibrinogen coagulable (%)	Thrombin time (sec.)	Fibrinogen recovered (%)	Fibrinogen coagulable (%)	Concn. of EDTA (mM)	Thrombin time (sec.)
Dialysis: fibrinogen was dialysed against 1000 vol. of 0.15M-NaCl at 6° for 48 hr., with constant stirring and four changes of the diffuseate	Control	91	9.5	90	95	—	10.8
	In 25 mM-EDTA	91	> 120	95	90	0	9.8
Anion exchange: fibrinogen was filtered by gravity through a column of Amberlite IR-410	Control	91	9.5	81	91	—	12.2
	In 25 mM-EDTA	91	> 120	78	93	0	12.4
Gel-filtration: fibrinogen was filtered by gravity through a column of Sephadex, and 1 ml. fractions were collected. Fibrinogen cleared the column in the 3-to-5 ml. fraction, EDTA and Ca ²⁺ ions in the 5-to-7 ml. fraction	Control	95	12.0	101	91	—	15.1
	In 25 mM-EDTA	95	> 120	95	95	0	14.5

Fig. 5. Opacity change during the conversion of EDTA-fibrinogen into fibrin. A solution of 0.4 mg. of fibrinogen/ml. was equilibrated at 22° with the indicated concentrations of EDTA and TAME. These mixtures were then transferred to a cuvette and thrombin was added at zero time to yield a final concentration, except where indicated, of 0.15 unit/ml. The change in extinction was recorded with a fibrinogen solution without thrombin as a blank. Many intermediate points are not illustrated. ○, Control fibrinogen; ■, 0.1 mM-EDTA; ●, 1.0 mM-EDTA; △, no EDTA, 0.05 unit of thrombin/ml.; □, no EDTA, 0.5 mM-TAME.

10 mg./ml., clotted under various conditions of temperature and pH. The rate of hydrolysis of TAME by thrombin was unaffected by the presence of large concentrations of EDTA under a variety of conditions.

DISCUSSION

According to current theory, the thrombin-fibrinogen reaction takes place in three phases (Scheraga & Ehrenpreis, 1958):

Enzymic:

Polymerization:

Stabilization:

Peptides are proteolytically removed from fibrinogen by thrombin to yield fibrin monomer, which then polymerizes primarily by means of intermolecular hydrogen bonds. The solubility in urea of the fibrinogen used in these experiments excludes significant contamination with fibrin-stabilizing factor (factor XIII) (Loewy *et al.* 1961), and the present experiments therefore measure only the effects of EDTA on the conversion of fibrinogen into soluble fibrin. The removal of Ca^{2+} ions may explain Godal's (1960) observations that, in unfractionated plasma, EDTA decreases the heat-resistance of fibrinogen and decreases the tensile strength and increases the solubility in urea of fibrin derived therefrom.

High concentrations of EDTA, even when incubated with thrombin for long periods at high temperatures, produce negligible effects on the thrombin time when introduced into the reaction mixture with the thrombin solution. The TAME-esterase activity of thrombin is not affected by high concentrations of EDTA, and the time-dependent inhibition produced when EDTA is added to the fibrinogen solution is not observed. The inhibitory effect of EDTA is thus not due to a direct action on thrombin, or to a competitive inhibition of this enzyme such as that produced by TAME; rather, it is due to a time-dependent alteration of the substrate fibrinogen.

These data confirm the results of others (Rosenfeld & Janszky, 1952; Triantaphyllopoulis *et al.* 1955; Zucker & Borrelli, 1958; Godal, 1960), and, together with the fact that weaker decalcifying agents do not produce a comparable impairment in fibrinogen reactivity, form the basis for the currently accepted hypothesis that EDTA alters the reactivity of fibrinogen by removing essential trace metal ions from a cation-protein complex that has intrinsic stability such that treatment with a chelating agent of high ion-binding strength for a finite period of time is necessary to remove the ions from the protein. This hypothesis is consistent with the properties of both simple complexes between cations and the polar side-chain groups of proteins (Gurd & Wilcox, 1956), and some true metalloproteins (Malmström, 1961), but is based on the assumption that the effects of EDTA are entirely due to the binding of metal ions, and that the reversal of its inhibitory effects by bivalent cations is due to a reunion of the essential ion with fibrinogen, either directly or by means of a chelate exchange reaction between the essential ion and ions that form more stable EDTA chelates (Zucker & Borrelli, 1958; Martell & Calvin, 1952, p. 231). However, EDTA may alter fibrinogen by virtue of properties other than binding metal ions, such as the chemical or electrostatic effects of its four carboxyl groups; and the reversal of its inhibitory

effects by bivalent cations could be due to the coordination of these reactive groups into a stable chelate ring. The techniques summarized in Table 2 remove all detectable EDTA from fibrinogen by means that do not involve the addition of bivalent ions or the formation of a chelate ring. In the absence of conditions capable of altering the extremely stable chelate ring of EDTA, one can conclude that all chelated ions are removed together with the EDTA, that the concurrent return of normal fibrinogen reactivity under rigidly deionized conditions cannot be attributed to the release or restoration of an essential ion, and therefore that the inhibitory properties of EDTA cannot be due to the removal of trace ions. The restoration of fibrinogen reactivity by various bivalent cations, in concentrations that by themselves have a negligible effect, must then be due to the involvement of the reactive groups of the EDTA molecule within a stable chelate ring, and the inhibitory properties of EDTA are thus apparently due to some property of the molecule in an unchelated form.

The data provide substantial, although indirect, evidence that fibrinogen does not undergo an intermolecular reaction in the presence of EDTA. The inhibitory effects of EDTA are proportionately less at high fibrinogen concentrations and are potentiated by pH values above the isoelectric point of fibrinogen (pI 5.5), a range in which the presence of electronegative ions such as EDTA and increasing pH would both increase the negative charge on the fibrinogen molecule and consequently the intermolecular electrostatic repulsion (Scheraga & Ehrenpreis, 1958). Further, the opacity of fibrinogen is not altered by EDTA. The data suggest that EDTA produces a reversible intramolecular alteration of fibrinogen.

The simplicity of the techniques used in these experiments does not allow a definite conclusion as to the mechanism of this alteration. The reversible changes produced in proteins by small ionic molecules usually involve the rupture of hydrogen bonds that are responsible for the specific configuration or conformation state of the protein (Scheraga, 1963). In the thrombin-fibrinogen reaction, specific hydrogen bonds may be involved in the formation of the enzyme-substrate complex (Scheraga & Ehrenpreis, 1958) in addition to their role in the polymerization phase, but there is no information on the importance of intramolecular hydrogen bonds in stabilizing the configuration of native fibrinogen. The data are compatible with the hypothesis that EDTA, by virtue of its electronegativity, diminishes the reactivity of fibrinogen by altering intramolecular hydrogen bonds.

This hypothesis is compatible with the fact that the EDTA-fibrinogen reaction is completely and

rapidly reversible, an observation that is very difficult to reconcile with alternative possibilities such as the formation of a chelate-metalloprotein complex (Cremona & Singer, 1962), or a chemical modification of fibrinogen analogous to acetylation or iodination (Caspary, 1956). The potentiation of the inhibitory properties of EDTA by increasing ionic strength is also compatible with an alteration of hydrogen bond formation, since a variety of compounds that inhibit the thrombin-fibrinogen reaction by this means are potentiated by high ionic strength (Shulman, Herwig & Ferry, 1951). Further, the diphasic effects of temperature on EDTA-fibrinogen at equilibrium resemble the temperature-dependent configurational changes that occur in other proteins in the presence of substances that weaken stabilizing intramolecular hydrogen bonds (Klotz, 1953). EDTA may alter the hydrogen-bonding properties of specific polar side-chain groups in fibrinogen, since its inhibitory effects are altered by pH in a biphasic manner, and are potentiated between pH 7.0 and pH 7.8, a range in which the chemical properties and ionic charge of the EDTA molecule are negligibly affected (pK_3 6.2, pK_4 10.3). In this pH range, primarily α -amino and imidazole groups are titrated (pK values 6.5–8.5). The irreversible diminution of fibrinogen reactivity produced by formaldehyde exhibits a diphasic pH-dependence comparable with that of the EDTA-fibrinogen reaction, and has been attributed to the chemical modification of amino groups (Scheraga & Ehrenpreis, 1958).

Whether the alteration of fibrinogen reactivity produced by EDTA results in a sub-optimum enzymic phase, a retarded polymerization phase, or both, remains unclear. The fact that TAME hydrolysis is unaffected by EDTA establishes that EDTA does not inhibit the cleavage of peptide bonds by thrombin, but changes in the configuration of fibrinogen secondary to the effects of EDTA could retard the enzymic phase. The effects of EDTA on the opacity change during the fibrinogen-fibrin conversion are identical with those produced by small concentrations of a specific inhibitor of thrombin, i.e. TAME, or to decreasing the thrombin concentration. Moreover, the rate of polymerization of EDTA-fibrinogen, once initiated, is apparently normal, and the final opacity of the clot is unaltered; whereas inhibitors of fibrin polymerization usually diminish both the rate of opacification and the final opacity of the clot (Ferry & Morrison, 1947; Latallo, Fletcher, Alkjaersig & Sherry, 1962). These observations suggest that the change in fibrinogen reactivity produced by EDTA retards either the enzymic phase, or an early polymerization step that does not affect the light-scattering properties of the final clot.

EDTA also produces profound alterations in platelet morphology and rapid and irreversible inactivation of antihæmophilic globulin and accelerator globulin (Zucker & Borrelli, 1954; Mustard, 1958). These phenomena are of interest to students of blood coagulation but are poorly understood, and have been attributed, without definite proof, to the binding of trace metal ions. In view of the present data, it is possible that other mechanisms are involved.

SUMMARY

1. Ethylenediaminetetra-acetic acid reversibly inhibits the thrombin-fibrinogen reaction by means of a time-dependent diminution in fibrinogen reactivity.

2. This alteration of fibrinogen is due to some property of the EDTA molecule in an unchelated form, and is not due to the removal of trace metal ions.

3. Possible explanations for this phenomenon are discussed.

This investigation was carried out during the tenure of a Post-doctoral Fellowship from the National Heart Institute, U.S. Public Health Service. The author is indebted to Dr Ethel Bidwell for her encouragement and assistance in carrying out these experiments, and to Dr Rosemary Biggs and Dr R. G. Macfarlane for their helpful discussion and criticism of this manuscript.

REFERENCES

Biggs, R. & MacFarlane, R. G. (1962). *Human Blood Coagulation*, 3rd ed., p. 36. Oxford: Blackwell Scientific Publications Ltd.

Blombäck, B. & Blombäck, M. (1956). *Ark. Kemi*, **10**, 415.

Caspary, E. A. (1956). *Biochem. J.* **62**, 507.

Cremona, T. & Singer, T. P. (1962). *Biochim. biophys. Acta*, **57**, 412.

Ferry, J. D. & Morrison, P. R. (1947). *J. Amer. chem. Soc.* **69**, 388.

Godal, H. C. (1960). *Scand. J. clin. Lab. Invest.* **12** (suppl.), 43.

Gurd, F. R. N. & Wilcox, P. E. (1956). *Advanc. Protein Chem.* **11**, 311.

Klotz, I. M. (1953). In *The Proteins*, vol. 1, p. 727. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.

Latallo, Z., Fletcher, A. P., Alkjaersig, N. & Sherry, S. (1962). *Amer. J. Physiol.* **202**, 675.

Loewy, A. G., Gallant, J. A. & Dunathan, K. (1961). *J. biol. Chem.* **236**, 2648.

Ma, T. S. & Zuazaga, G. (1942). *Industr. Engng Chem. (Anal.)*, **14**, 280.

Malmström, B. G. (1961). *Fed. Proc.* **20**, 60.

Martell, A. E. & Calvin, M. (1952). *The Chemistry of the Metal Chelate Compounds*. New York: Prentice Hall.

Mertz, E. T. & Owen, C. A. (1940). *Proc. Soc. exp. Biol., N.Y.*, **43**, 204.

Mustard, J. F. (1958). *Amer. J. clin. Path.* **30**, 498.

Owren, P. A. (1946). *Acta med. scand.* **128** (suppl.), 194.

Ratnoff, O. D. (1960). *Bleeding Syndromes*, p. 4. Springfield, Ill.: Charles C. Thomas.

Ratnoff, O. D. & Potts, A. M. (1954). *J. clin. Invest.* **33**, 206.

Rosenfeld, G. & Janszky, B. (1952). *Science*, **116**, 36.

Saifer, A. & Newhouse, A. (1954). *J. biol. Chem.* **208**, 159.

Scheraga, H. (1963). In *The Proteins*, 2nd ed., vol. 1, p. 477. Ed. by Neurath, H. New York: Academic Press Inc.

Scheraga, H. A. & Ehrenpreis, S. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, vol. 10, p. 212.

Seegers, W. H. & Smith, H. P. (1942). *Amer. J. Physiol.* **137**, 348.

Sherry, S. & Troll, W. (1954). *J. biol. Chem.* **208**, 95.

Shulman, S., Herwig, W. C. & Ferry, J. D. (1951). *Arch. Biochem. Biophys.* **32**, 354.

Smith, D. C. & Tompsett, S. L. (1958). *J. clin. Path.* **11**, 365.

Trevan, W. J. (1925). *Biochem. J.* **19**, 1111.

Triantaphyllopoulis, D. C., Quick, A. J. & Greenwalt, T. J. (1955). *Blood*, **10**, 534.

Zucker, M. B. & Borrelli, J. (1954). *Blood*, **9**, 602.

Zucker, M. B. & Borrelli, J. (1958). *J. appl. Physiol.* **12**, 453.