Biochem. J. (1974) **141**, 675–682
Printed in Great Britain

Calcium and Thiol Reactivity of Human Plasma Clotting Factor XIII

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(*Received* 27 *February* 1974)

1. The reaction of iodoacetate, 2-chloromercuri-4-nitrophenol and 5,5′-dithiobis-(2-nitrobenzoate) with thrombin-cleaved Factor XIII (i.e. Factor $XIII_a$) was accompanied by enzyme inhibition. 2. The reaction with iodoacetate and 5,5′-dithiobis-(2-nitrobenzoate) was absolutely dependent on Ca^{2+}, and the rate of reaction increased with the Ca^{2+} concentration up to very high, non-physiological concentrations. 3. 2-Chloromercuri-4-nitrophenol reacted with Factor $XIII_a$ in the absence of Ca^{2+}, but at a much slower rate. 4. Stopped-flow methods were used to quantify the reaction with 5,5′-dithiobis-(2-nitrobenzoate) because of the Ca^{2+}-dependent dissociation of Factor $XIII_a$ (a'_2b_2) and subsequent aggregation of the a' chains into turbid precipitates. 5. The 3-carboxy-4-nitrothiophenolate released was consistent with the reaction of 2 thiol groups/molecule of Factor $XIII_a$. The isolated b chains of Factor XIII did not react with either of the chromophoric reagents. This indicated that the a' chains of Factor $XIII_a$ were responsible for the thiol reactivity of the enzyme. 6. The Ca^{2+} dependence of the enzyme inhibition by these thiol reagents was very dependent on protein concentration. This is discussed in relation to the Ca^{2+}-induced dissociation of Factor $XIII_a$. 7. The acceptor substrate, casein, decreased the Ca^{2+} concentration required for enzyme inhibition by both the mercurial and the aromatic disulphide compounds. Dansylcadaverine did not affect Ca^{2+} dependence of inhibition.

In the terminal stage of blood clotting thrombin converts fibrinogen into fibrin (Bettelheim & Bailey, 1952) and Factor XIII into plasma transglutaminase, usually known as Factor $XIII_a$ (Lorand & Jacobsen, 1964). Factor $XIII_a$ cross-links the fibrin gel by introducing γ-glutamyl-ε-lysyl amide bridges (Pisano *et al.*, 1968). This cross-linking is essential for normal haemostasis and wound-healing (Duckert *et al.*, 1960), probably because of the decreased rate of clot fibrinolysis (Bickford & Sokolow, 1961) and the greater mechanical strength of the clot (Gormsen & Silvertsen, 1964; Tyler, 1969).

The enzyme is mechanistically similar to the guinea-pig liver transglutaminase; both enzymes have steady-state kinetics consistent with a Ping Pong acyl-enzyme mechanism, and both absolutely depend on Ca^{2+} for activity (Folk, 1969; Chung & Folk, 1972). Folk & Cole (1966) showed that the activity of the liver enzyme is associated with a reactive thiol group and they isolated the thiol–enzyme intermediate. The liver enzyme differs from the plasma enzyme in that it does not require thrombin and is much less specific as regards the glutamine acceptor substrate (Matačić & Loewy, 1966). Loewy *et al.* (1961) showed that Factor $XIII_a$ can be inactivated by compounds known to inhibit thiol groups, such as iodoacetate, *p*-chloromercuribenzoate and Ag^+ ions. This inactivation occurred in the presence of Ca^{2+} and substrate, but it was not established whether the susceptibility to inactivation was due to Ca^{2+}, the substrate or both. The experiments reported here were designed to investigate and quantify this effect by using iodoacetate and two chromophoric thiol reagents, 5,5′-dithiobis-(2-nitrobenzoate) and 2-chloromercuri-4-nitrophenol.

Human plasma Factor XIII has an a_2b_2 structure, and only the mass of the a subunits is altered by thrombin treatment ($a \rightarrow a'$). This led to the hypothesis (Schwartz *et al.*, 1971*b*) that catalytic activity is associated with the a and not the b subunits. This hypothesis was supported by the observation (Cooke & Holbrook, 1974*b*) that Ca^{2+} causes the Factor $XIII_a$ tetramer to dissociate to give a soluble inactive protein containing only b chains, and a second protein containing only a' chains which is enzymically active. In the present paper the thiol reactivity of the Factor XIII subunits was examined. The finding that only the

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a chains have a thiol group that is reactive, or whose reactivity depends on Ca^{2+}, is consistent with this hypothesis.

Materials and Methods

2-Chloromercuri-4-nitrophenol, prepared by the method of McMurray & Trentham (1969), was a gift from Dr. C. H. McMurray (Biochemistry Department, Veterinary Research Laboratories, Ministry of Agriculture, Stormont, Belfast, N. Ireland). 5,5'-Dithiobis-(2-nitrobenzoate) was supplied by Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Iodoacetic acid was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. and recrystallized from *n*-heptane until white.

Human plasma Factor XIII was prepared as described by Cooke & Holbrook (1974*b*); the protein obtained by DEAE-cellulose chromatography consists of component A (Factor XIII), i.e. tetrameric a_2b_2, and component B (subunit b_2), which are resolved by Sepharose 6-B chromatography. Factor XIII was assayed by the method of Cooke & Holbrook (1974*a*), which continuously measures the increase in fluorescence as dansylcadaverine is incorporated into dephosphorylated acetylated β-casein (referred to as β-substrate). One enzyme unit gives a 1% increase in the starting fluorescence/min at 25°C. The protein concentration of Factor XIII was determined spectrophotometrically by using the extinction coefficient at 280nm of Schwartz *et al.* (1973), i.e. $E^{1\%}_{1cm} = 13.8$. The activated Factor XIII (Factor $XIII_a$) was prepared by incubation of Factor XIII (4mg/ml) with 15N.I.H. units of thrombin/ml (Leo Pharmaceutical Products, Ballerup, Denmark) in 0.1M-Tris–HCl containing 1mM-EDTA at pH7.5 for 40min at 25°C. After this time, the Factor $XIII_a$ activity had attained a steady maximum value. Factor $XIII_a$ was stored at 0°C for up to 4h until required; no loss in enzyme activity was recorded over this period.

Transient kinetic studies of the reactions between the thiol reagents and Factor XIII were carried out by using a stopped-flow spectrophotometer, similar to that described by Gutfreund (1965). The observation cell consisted of eight mixing jets and a 1cm light-path. Monochromatic light was provided by a quartz–tungsten–iodine lamp and a Bausch and Lomb grating monochromator. The wavelength drum was calibrated against the 366nm emission lines of a mercury discharge. The solution in the observation cell was 3ms old when flow stopped. Transmission of light was recorded on a Tektronix storage oscilloscope, which amplified the output of an EMI 9592 B photomultiplier. The oscilloscope traces recorded percentage transmission on the ordinate axis. Reactions were studied at room temperature (18–21°C).

Organomercurials have a very high affinity for thiol groups and the nitrophenol mercurials used in this work are highly specific chromophoric thiol reagents. Reaction between a thiol group and the mercurinitrophenol perturbs the p*K* of the phenolic hydroxyl group and produces a chromophoric change at certain pH values (McMurray & Trentham, 1969). The reaction of Factor XIII with the 2-chloromercuri-4-nitrophenol was followed at 405nm. The relative p*K* values of the free nitrophenol and the bound nitrophenol are such that the reaction causes a decrease in absorbance of the chromophore. The reaction of 5,5'-dithiobis-(2-nitrobenzoate) with thiols was monitored by the absorption at 412nm of the 3-carboxy-4-nitrothiophenolate released (Ellman, 1959).

Results

Preliminary experiments revealed that 5,5'-dithiobis-(2-nitrobenzoate) did not inhibit either Factor XIII or Factor $XIII_a$. No loss in enzyme activity or reaction with the aromatic disulphide (0.5mM) was incurred during 2h incubations at 25°C. However, addition of Ca^{2+} to Factor $XIII_a$ led to enzyme inhibition and the appearance of 3-carboxy-4-nitrothiophenolate. The same behaviour was observed for iodoacetate; inhibition only occurred after thrombin activation and in the presence of Ca^{2+}. Factor $XIII_a$ (2mg/ml) was 95% inhibited after a 2min exposure to a 30-fold molar excess of iodoacetate (0.3mM) at 25°C in 50mM-Ca^{2+}.

Ca^{2+} causes Factor $XIII_a$ to dissociate into an enzymically inactive protein consisting of *b* subunits (component B) and an enzymically active protein consisting only of *a'* subunits; the latter protein slowly forms a misty precipitate (Cooke & Holbrook, 1974*b*). Consequently, conventional spectrophotometric observations of the 3-carboxy-4-nitrothiophenolate release could not be used to quantify the thiol reactivity. We have resolved the absorbance changes caused by thiol reactivity from those due to the turbidity caused by *a'*-chain precipitation by using stopped-flow techniques, which allow one to monitor reactions within milliseconds after mixing the reactants (Gutfreund, 1965).

The initial stopped-flow experiments were carried out with Factor XIII that had not been fractionated on Sepharose 6-B and thus consisted of about 70% component A (a_2b_2) and 30% component B (b_2) as described in the Materials and Methods section. The rapid mixing of Factor XIII or Factor $XIII_a$ with 5,5'-dithiobis-(2-nitrobenzoate) indicated a lack of titratable thiol groups (Fig. 1*a*), confirming the preliminary spectrophotometric results. The effects of Ca^{2+} were observed by rapid mixing of a solution of the reagent and Ca^{2+} with a solution of Factor $XIII_a$ containing an equivalent concentration of Mg^{2+}. This mixing technique prevented changes in ionic strength

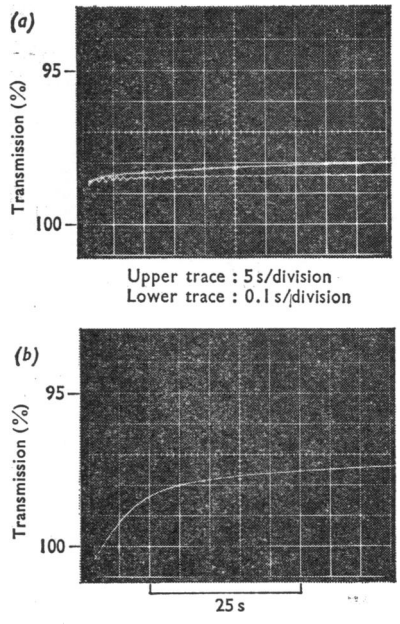

Fig. 1. *Stopped-flow spectrophotometric record of the reaction between unresolved Factor XIII$_a$ (approx. 0.25 mg/ml after mixing) and 5,5′-dithiobis-(2-nitrobenzoate) (1.25 mM) at 412 nm, 20°C*

(*a*) Reaction in the absence of Ca^{2+}. (*b*) Reaction in the presence of $CaCl_2$ (0.06M) carried out by rapid mixing of equal volumes of Factor XIII$_a$ plus $MgCl_2$ (0.12M) and 5,5′-dithiobis-(2-nitrobenzoate) plus $CaCl_2$ (0.12M). The buffer used was 0.1M-Tris–HCl, pH7.5.

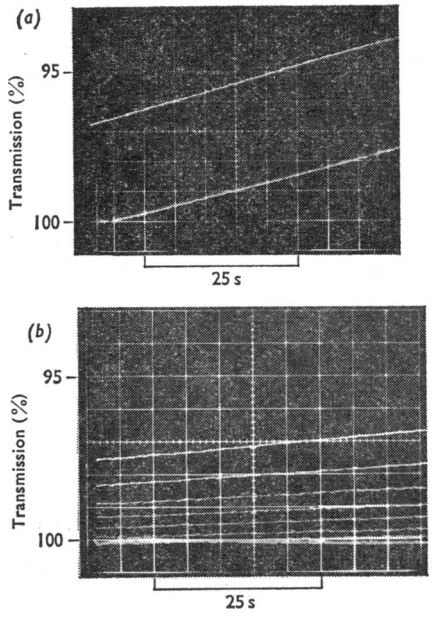

Fig. 2. *Stopped-flow spectrophotometric record of the reaction between Factor XIII$_a$ (approx. 0.25 mg/ml) and Ca^{2+}*

The reaction was carried out by rapid mixing of equal volumes of Factor XIII$_a$ plus $MgCl_2$ (0.12M) and $CaCl_2$ (0.12M), (*a*) at 330nm and (*b*) at 412nm and 20°C.

occurring as a result of mixing. Control experiments showed that Mg^{2+} cannot replace Ca^{2+} as the obligatory ion in the plasma transglutaminase reaction (Loewy, 1968), neither does it affect the thiol reactivity of Factor XIII$_a$. Fig. 1(*b*) shows that biphasic reaction profiles were obtained. The initial phase may be considered as a first-order reaction under the conditions of the experiment. The first-order rate constant calculated by the modified Guggenheim method (Sturtevant, 1937) is $0.166 s^{-1}$, and from the 5,5′-dithiobis-(2-nitrobenzoate) concentration used this corresponds to a second-order rate constant of $6.64 \times 10^{-5} s^{-1} \cdot M^{-1}$.

These experiments were repeated with Factor XIII that had been resolved into components A and B. It was discovered that only thrombin-treated component A (0.44mg/mg after mixing) in the presence of Ca^{2+} reacted with 5,5′-dithiobis-(2-nitrobenzoate). This reaction profile was similar to that shown in Fig. 1(*b*); the initial phase extinction recorded was 0.036 (at 412nm). The initial phase extinction of the reaction can be determined to a good approximation by extrapolating the apparent linear portion of the second phase to zero time. A more accurate value may be obtained by using the modified Guggenheim method (Sturtevant, 1937). The reaction of thrombin-treated component B in the presence of Ca^{2+} was similar to that shown in Fig. 1(*a*). This lack of titratable thiol groups was maintained in the presence of 60mM-$CaCl_2$ and 4M-urea.

Rapid mixing of $CaCl_2$ and Factor XIII$_a$ (component A), in the absence of 5,5′-dithiobis-(2-nitrobenzoate) (Fig. 2*a*), showed that Ca^{2+} precipitation starts to occur within the first 5s. Fig. 2(*a*) was recorded at 330nm; a change in transmission of about 2.5% occurred in the first 50s. Fig. 2(*b*) was recorded at 412nm, and since the magnitude of scatter is much less at longer wavelengths, the percentage change in transmission was smaller (approx. 0.5% in 50s, rising to 1% in 50s, after about 3min). This rate of change in transmission corresponds to the slower phase of the traces depicting the reaction of Factor XIII$_a$ with 5,5′-dithiobis-(2-nitrobenzoate). This implies that the light-scattering, associated with the *a*′-chain precipitation, is responsible for the biphasic nature of these traces (e.g. Fig. 1*b*). The Ca^{2+} dependence of the 5,5′-dithiobis-(2-nitrobenzoate) reaction with Factor XIII$_a$ is shown in Table 1.

Table 1. *Effect of increasing Ca^{2+} concentration on reactivity of thrombin-activated Factor XIII with 5,5'-dithiobis-(2-nitrobenzoate)*

Reaction of thrombin-activated Factor XIII (approx. 0.25 mg/ml) and 5,5'-dithiobis-(2-nitrobenzoate) (1.25 mM) was followed spectrophotometrically at 412 nm in 0.1 M-Tris–HCl buffer, pH 7.5. The rate constants were calculated from the initial phases of the reactions.

Concn. of $CaCl_2$ (mM)	$k_{measured}$ for the initial phase (s^{-1})
2.5	0.042
7.5	0.046
15.0	0.049
22.5	0.060
30.0	0.083
45.0	0.125
60.0	0.147

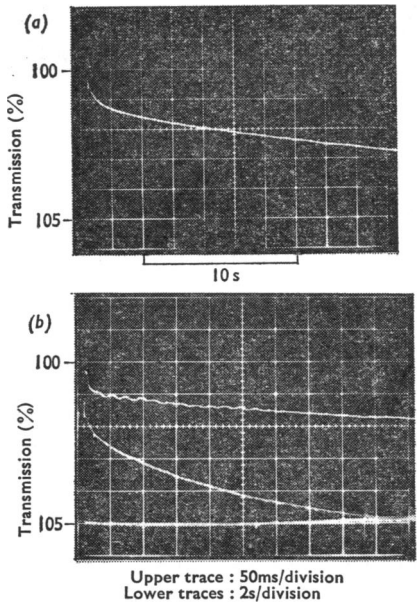

Fig. 3. *Stopped-flow spectrophotometric record of the reaction between Factor $XIII_a$ (0.44 mg of component A/ml) and 2-chloromercuri-4-nitrophenol (25 μM) at 405 nm, 20°C*

(*a*) Reaction in the absence of Ca^{2+}. (*b*) Reaction in the presence of $CaCl_2$ (0.06 M) carried out by rapid mixing of equal volumes of component A plus $MgCl_2$ (0.12 M) and mercurial compound plus $CaCl_2$ (0.12 M). The horizontal trace at 2 s/division is a record of the reaction after the completion of the initial trace at this sweep speed. The buffer used was 0.1 M-Tris–HCl, pH 7.5.

The difference in thiol reactivity of components A and B was confirmed by experiments with 2-chloromercuri-4-nitrophenol. These experiments indicated that the mercurial can react with thrombin-treated component A in the absence of Ca^{2+} (Fig. 3), but that the presence of Ca^{2+} enhanced the rate of this reaction (Fig. 3). The observed reaction between component B and mercurial was not increased by the addition of Ca^{2+} (Fig. 4). The reason for the small rapid increase (about 1%) in percentage transmission at 405 nm observed at an oscilloscope sweep speed of 50 ms/division (Figs. 3 and 4) was not discovered. It could possibly be attributed to a trace impurity or to the presence of a small amount of denatured enzyme.

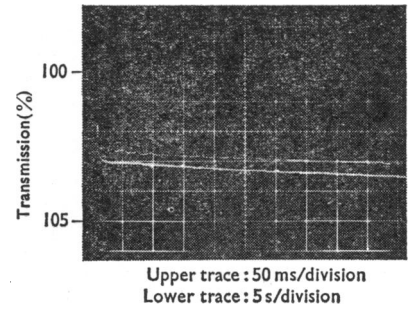

Fig. 4. *Stopped-flow spectrophotometric record which is representative of the reaction at 405 nm and 20°C between thrombin-treated component B of Factor XIII (0.36 mg/ml) and 2-chloromercuri-4-nitrophenol (25 μM) in the absence or presence of $CaCl_2$ (0.06 M)*

The reaction in the presence of Ca^{2+} was carried out by rapid mixing of equal volumes of component B plus $MgCl_2$ (0.12 M) and 2-chloromercuri-4-nitrophenol plus $CaCl_2$ (0.12 M). The buffer used was 0.1 M-Tris–HCl, pH 7.5.

Ca^{2+} dependence of the inhibition of Factor XIII by 5,5'-dithiobis-(2-nitrobenzoate)

Fig. 5 shows that high non-physiological Ca^{2+} concentrations were required to incur appreciable rates of inhibition. This agrees with the observation (Table 1) that the rate of 5,5'-dithiobis-(2-nitrobenzoate) reaction with Factor $XIII_a$ increased with Ca^{2+} concentration far in excess of normal plasma concentrations.

The Ca^{2+} dependence of the 5,5'-dithiobis-(2-nitrobenzoate) inhibition of Factor $XIII_a$ was very sensitive to protein concentration. Fig. 6 shows the effect of incubating a fixed concentration of enzyme in 5,5'-dithiobis-(2-nitrobenzoate) and various Ca^{2+} concentrations for 10 min at 25°C, before assay. The experiments were performed over a tenfold range of protein concentrations, and indicated that the Ca^{2+} concentration required for inhibition of Factor $XIII_a$ by 5,5'-dithiobis-(2-nitrobenzoate) decreased sharply as the protein concentration was decreased. The tailing-off in the percentage inhibition achieved at higher Ca^{2+} concentrations is misleading. Fig. 5

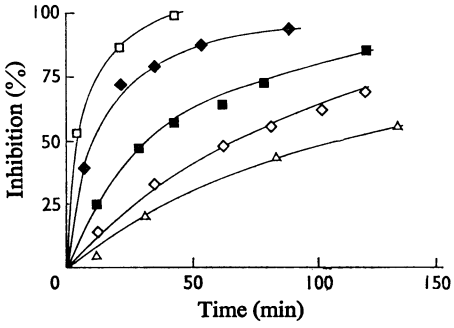

Fig. 5. *Ca^{2+} dependence of the inhibition of Factor $XIII_a$ (0.5 mg/ml) by 5,5′-dithiobis-(2-nitrobenzoate) (0.5 mM)*

Factor $XIII_a$ was incubated in the presence of 5,5′-dithiobis-(2-nitrobenzoate) and the appropriate Ca^{2+} concentration at 25°C. Portions from each incubation mixture were assayed periodically. □, 20 mM-Ca^{2+}; ◆, 15 mM-Ca^{2+}; ■, 10 mM-Ca^{2+}; ◇, 7.5 mM-Ca^{2+}; △, 5 mM-Ca^{2+}.

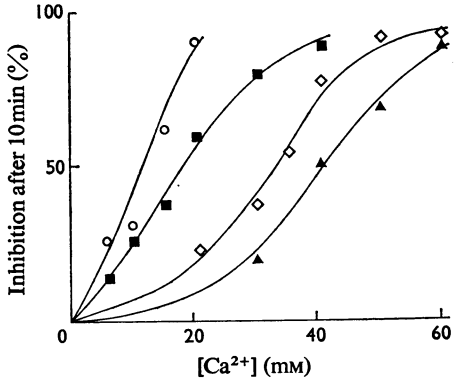

Fig. 6. *Protein-concentration dependence of the inhibition of Factor $XIII_a$ by 5,5′-dithiobis-(2-nitrobenzoate)*

Factor $XIII_a$ (○, 0.38 mg/ml; ■, 0.73 mg/ml; ◇, 2.17 mg/ml; ▲, 3.87 mg/ml) was incubated with 5,5′-dithiobis-(2-nitrobenzoate) (0.5 mM) and Ca^{2+} at 25°C. After 10 min the activity of Factor $XIII_a$ remaining at the various Ca^{2+} concentrations was assayed. The buffer used was 0.1 M-Tris–HCl, pH 7.5.

shows that the rate of inhibition increased as the Ca^{2+} concentration was increased. However, as the percentage inhibition achieved after 10 min approaches 100%, the incremental change in inhibition observed at various Ca^{2+} concentrations became smaller.

Fig. 7 shows the effect of the substrates, dansylcadaverine and casein, on the rate of inhibition of Factor $XIII_a$ by the mercurial. β-Substrate was used as the casein substrate because it does not bind Ca^{2+}

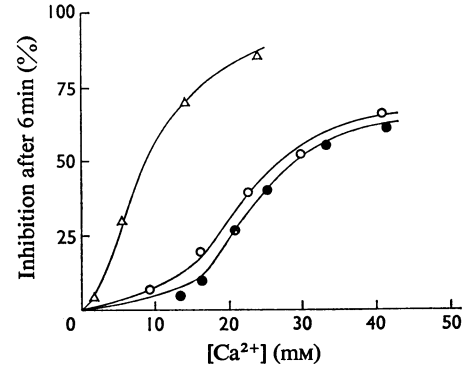

Fig. 7. *Effect of dansylcadaverine and β-substrate on the inhibition of Factor $XIII_a$ by 2-chloromercuri-4-nitrophenol*

Factor $XIII_a$ (0.5 mg/ml) was incubated with the mercurial compound (approx. 3 μM) and the various Ca^{2+} concentrations for 6 min at 25°C (●). The percentage inhibition was then determined. Parallel experiments were performed in the presence of β-substrate (△, 9 mg/ml) and dansylcadaverine (○, 4 mM).

(Cooke & Holbrook, 1974*a*). The presence of β-substrate in the incubation medium led to a marked increase in the extent of inhibition observed at each Ca^{2+} concentration, whereas the presence of dansylcadaverine had no effect. This is consistent with the Ping Pong acyl-enzyme mechanism proposed for plasma Factor $XIII_a$ by Chung & Folk (1972), in which the amine donor can only bind subsequent to binding of the glutamine acceptor substrate. The susceptibility to inhibition by 5,5′-dithiobis-(2-nitrobenzoate) was also increased by β-substrate (Fig. 8). The Ca^{2+} concentration required for 50% inhibition in 10 min was decreased from 18 to 7 mM; however, the inhibition was still absolutely Ca^{2+} dependent.

Discussion

The experiments described here establish that the reactivity of the thiol groups of Factor XIII depends on thrombin cleavage of the zymogen and the presence of Ca^{2+}. The mercurial can react with Factor $XIII_a$ in the absence of Ca^{2+}, albeit at a slower rate. The greater reactivity of the mercurial relative to 5,5′-dithiobis-(2-nitrobenzoate) may be attributed to the fact that the bond between the cysteine residue of a protein and the 2-nitro-5-thiobenzoate group involves a dihedral angle of 90° between the carbon–sulphur bonds (Calvin, 1954). This imposes a steric restriction on the accessibility of thiol groups to 5,5′-dithiobis-(2-nitrobenzoate). However, the mercurial reaction product involves a linear mercury–sulphur–carbon arrangement (Gutfreund & McMurray, 1971). Consequently, the reactivity of the mercurial with

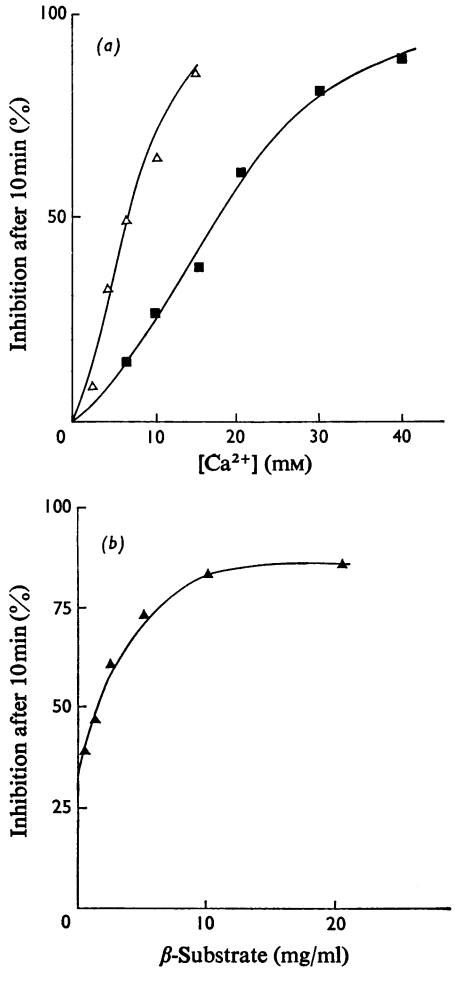

Fig. 8. *Effect of β-substrate on the inhibition of Factor XIII$_a$ by 5,5′-dithiobis-(2-nitrobenzoate)*

(*a*) Factor XIII$_a$ (0.73mg/ml) was incubated with 5,5′-dithiobis-(2-nitrobenzoate) (0.5mM) at the various Ca^{2+} concentrations, for 10min. The percentage inhibition was then determined (■). A parallel experiment was performed in the presence of 20mg of β-substrate/ml (△). (*b*) Factor XIII$_a$ (0.73mg/ml) was incubated with 5,5′-dithiobis-(2-nitrobenzoate) (0.5mM), a constant concentration of 15mM-Ca^{2+}, and various concentrations of β-substrate. After 10min, the percentage inhibition was determined.

protein thiol groups is much less sensitive to steric factors. This agrees with the observation that Ca^{2+} is required for reaction between 5,5′-dithiobis-(2-nitrobenzoate) and Factor XIII$_a$. In the case of the mercurial, Ca^{2+} is not required for the reaction, but its presence enhances the reaction rate. A similar insensitivity of mercurinitrophenols to changes in the reactivity of thiol groups of UDP-glucose dehydrogenase has been observed by Gainey *et al.* (1972).

The failure of the technique described by Swigert *et al.* (1963), designed to inactivate the Factor XIII contaminant in fibrinogen by exposure to *p*-chloromercuribenzoate, could be explained by the present findings. Since the thiol groups are only accessible after thrombin treatment of the zymogen and in the presence of Ca^{2+}, it is consistent that Schwartz *et al.* (1971*a*) found that the Factor XIII contaminant was still active after the above treatment. They concluded that fibrinogen prepared by this method is unsuitable for use in the assay of Factor XIII (Lorand *et al.*, 1962).

The close similarity between the slower phase of the biphasic reaction involving 5,5′-dithiobis-(2-nitrobenzoate), Ca^{2+} and Factor XIII$_a$ and the rate of change of transmission due to Ca^{2+} and Factor XIII$_a$ alone (Figs. 1*b* and 2*b*) indicates that the Ca^{2+}-induced aggregation of the *a′* chains occurs at the same rate in the presence or absence of enzyme inhibition [by 5,5′-dithiobis-(2-nitrobenzoate)]. This agrees with our report (Cooke & Holbrook, 1974*b*) that the turbid appearance of Factor XIII$_a$ solutions on addition of Ca^{2+} is not due to autopolymerization of transglutaminase as suggested by others (Bohn, 1970; Takagi & Doolittle, 1973).

Schwartz *et al.* (1971*b*) noticed that only the mass of the *a* subunit is altered when the Factor XIII zymogen is activated by thrombin ($a_2b_2 \rightarrow a'_2b_2$). They reasoned that thrombin has no action on the *b* chains and thus if there is no interaction between the subunits, they are not active. Schwartz *et al.* (1973) reported that trypsin digestion of Factor XIII$_a$ in the presence of Ca^{2+} resulted in rapid loss of activity and primary digestion of the *a′* chains; digestion in the absence of Ca^{2+} led to a slower decay in activity and primary degradation of the *b* chains. The courses of the trypsin and thrombin digestions were monitored by sodium dodecyl sulphate–polyacrylamide-gel analysis. The conclusions of Schwartz *et al.* (1971*b*, 1973) are therefore subject to the criticism that only proteolysis resulting in the removal of large polypeptides from the subunits would be detected. However, our report (Cooke & Holbrook ,1974*b*) that the isolated *b* chains obtained from crude Factor XIII by Sepharose 6-B chromatography (i.e. component B) or derived from pure Factor XIII (component A) by Ca^{2+}-induced dissociation are catalytically inactive provided direct support for this hypothesis. The Ca^{2+}-induced dissociation provides a reason for the differential trypsin digestions reported by Schwartz *et al.* (1973). The inactive nature of the *b* chains is further evidenced by the present finding that thiol reactivity is present in component A (a_2b_2) but not in component B (b_2). The extinction of the initial phase of the traces can be used to quantify the thiol reaction by using

Ellman's (1959) extinction coefficient for 3-carboxy-4-nitrothiophenolate of 1.36×10^{-4} l·mol^{-1}·cm^{-1}. Freshly prepared component A (0.44mg/ml) gave an initial phase extinction of 0.036, i.e. 2.65mM-3-carboxy-4-nitrothiophenolate; assuming that the molecular weight of component a_2b_2 is 310000 (Schwartz *et al.*, 1973) this is equivalent to 1.85mol of thiol groups/mol of a_2b_2. This suggests that only the a' subunits of Factor $XIII_a$ have the titratable thiol groups that are necessary for catalytic activity.

The results summarized in Table 1 indicate that high concentrations of Ca^{2+} are required to expose the thiol groups of Factor $XIII_a$. The normal plasma Ca^{2+} concentration is about 2.5mM (Linder & Blomstrand, 1958) and the K_m for Factor $XIII_a$ is less than 1mM (Cooke & Holbrook, 1974*a*). A solution to this problem is the sensitivity of Ca^{2+} dependence to protein concentration (Fig. 6). The concentration of Ca^{2+} required for 50% inhibition in 10min incubation with 5,5'-dithiobis-(2-nitrobenzoate) decreases from 40mM for 3.87mg of enzyme/ml to 12mM for 0.39mg of enzyme/ml. The simplest explanation for this behaviour is that the Ca^{2+}-dependent exposure of thiol is related to the Ca^{2+}-dependent dissociation of the tetrameric enzyme; this matter will be discussed at greater length in the ensuing paper (Cooke, 1974).

The essential thiol of the mechanistically related guinea-pig liver transglutaminase is protected from inhibition by glutamine acceptor substrates (Folk & Cole, 1966). Figs. 7 and 8 indicate that the acceptor substrate of the plasma enzyme enhances the susceptibility to inhibition. This means that either the essential thiol is not involved in a thiol ester–enzyme intermediate (cf. the liver enzyme), or that ligand binding affects the dissociation of the tetramer (for further discussion see Cooke, 1974). The liver enzyme is monomeric and there is no gross change in size or shape of the molecule on binding Ca^{2+} (Connellan *et al.*, 1971). The former conclusion is unlikely in view of the recent work in this laboratory (Holbrook *et al.*, 1973) on the inhibition of the plasma enzyme by iodo-[^{14}C]acetate. The label was incorporated into the a' chains, consistent with the work reported here, and it was all recovered as *S*-carboxymethylcysteine in a tetrapeptide with the sequence Gly–Gln–Cys–Trp. This is identical with the sequence isolated by Folk & Cole (1966) from guinea-pig liver transglutaminase and supports the idea of a common mechanism. Lorand *et al.* (1972) have reported that thiol esters can act as substrates and inhibitors in both transglutamination and hydrolysis by Factor XIII, thus providing further evidence for an acyl-enzyme intermediate. The stoicheiometry reported by Holbrook *et al.* (1973), i.e. 0.6mol of label incorporated/mol of a' subunit at 65% inhibition, agrees with our finding of 1 thiol group/a' subunit. The present data do not involve assumptions about the extinction of the protein in 8M-urea or after chymotryptic digestion, nor do they necessitate the extrapolation to 100% inhibition required in the labelling work. Curtis *et al.* (1973) have reported a similar labelling experiment with iodo[^{14}C]acetamide; their results indicated only 1 thiol group/tetramer, though they attributed their low incorporation to the presence of denatured enzyme in their zymogen. In a brief communication, Chung & Folk (1973) have reported a similar low stoicheiometry and postulated that the enzyme exhibits half-of-the-sites reactivity, which is not consistent with our results. A possible reason for these low stoicheiometries, apart from the need to use Factor XIII that is free of component B (excess of *b* subunits), is the need for high Ca^{2+} concentrations to achieve rapid incorporation of label at high enzyme concentrations (Table 1 and Fig. 6).

R. D. C. was supported by a C.A.P.S. award in collaboration with Pfizer Ltd., Sandwich, Kent, U.K. We thank Dr. H. M. Tyler and Mr. J. T. B. Shaw of Pfizer Ltd. for the gift of dansylcadaverine, Mr. J. G. Watt and Dr. J. K. Smith of The Scottish National Blood Transfusion Association, Edinburgh, for preparing the Cohn-I fractions used in the preparation of the plasma zymogen, and Professor A. J. Zuckerman of the London School of Hygiene and Tropical Medicine for screening the Cohn-I fractions for Australia antigen.

References

Bettelheim, F. R. & Bailey, K. (1952) *Biochim. Biophys. Acta* **9**, 578–579

Bickford, A. F. & Sokolow, M. (1961) *Thromb. Diath. Haemorrh.* **5**, 480–488

Bohn, H. (1970) *Thromb. Diath. Haemorrh.* **23**, 455–468

Calvin, M. (1954) in *Glutathione* (Colowick, S. P., Lazarow, A., Racker, E., Schwarz, D. R., Stadtman, E. & Waelsch, H., eds.), pp. 1–30, Academic Press, New York and London

Chung, S. I. & Folk, J. E. (1972) *J. Biol. Chem.* **247**, 2798–2807

Chung, S. I. & Folk, J. E. (1973) *Abstr. Int. Congr. Biochem. 9th* 73

Connellan, J. M., Chung, S. I., Whetzel, N. K., Bradley, L. M. & Folk, J. E. (1971) *J. Biol. Chem.* **246**, 1093–1098

Cooke, R. D. (1974) *Biochem. J.* **141**, 683–691

Cooke, R. D. & Holbrook, J. J. (1974*a*) *Biochem. J.* **141**, 71–78

Cooke, R. D. & Holbrook, J. J. (1974*b*) *Biochem. J.* **141**, 79–84

Curtis, C. G., Stenberg, P., Chou, C.-H. J., Gray, A., Brown, K. L. & Lorand, L. (1973) *Biochem. Biophys. Res. Commun.* **52**, 51–56

Duckert, F., Jung, E. & Schmerling, D. H. (1960) *Thromb. Diath. Haemorrh.* **5**, 179–186

Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77

Folk, J. E. (1969) *J. Biol. Chem.* **244**, 3707–3713

Folk, J. E. & Cole, P. W. (1966) *J. Biol. Chem.* **241**, 3238–3240

Gainey, P. A., Pestell, T. C. & Phelps, C. F. (1972) *Biochem. J.* **129**, 821–830

Gormsen, J. & Silvertsen, U. (1964) *Thromb. Diath. Haemorrh.* **11**, 454–467

Gutfreund, H. (1965) *An Introduction to the Study of Enzymes*, pp. 117–133, Blackwell Scientific Publications, Oxford and Edinburgh
Gutfreund, H. & McMurray, C. H. (1971) *Biochem. Soc. Symp.* **31**, 39–47
Holbrook, J. J., Cooke, R. D. & Kingston, I. B. (1973) *Biochem. J.* **135**, 901–903
Linder, E. & Blomstrand, R. (1958) *Proc. Soc. Exp. Biol. Med.* **97**, 653–658
Loewy, A. G. (1968) in *Fibrinogen* (Laki, K., ed.), pp. 185–224, E. Arnold Ltd., London
Loewy, A. G., Dunathan, K., Gallant, J. A. & Gardner, B. (1961) *J. Biol. Chem.* **236**, 2644–2647
Lorand, L. & Jacobsen, A. (1964) *Arch. Biochem. Biophys.* **105**, 58–67
Lorand, L., Konishi, K. & Jacobsen, A. (1962) *Nature (London)* **194**, 1148–1149
Lorand, L., Chou, C.-H. J. & Simpson, I. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 2645–2648
Matačić, S. & Loewy, A. G. (1966) *Biochem. Biophys. Res. Commun.* **24**, 858–866
McMurray, C. H. & Trentham, D. R. (1969) *Biochem. J.* **115**, 913–921
Pisano, J. J., Finlayson, J. S. & Peyton, M. P. (1968) *Science* **160**, 892–893
Schwartz, M. L., Pizzo, S. V., Hill, R. L. & McKee, P. A. (1971*a*) *J. Clin. Invest.* **50**, 1506–1513
Schwartz, M. L., Pizzo, S. V., Hill, R. L. & McKee, P. A. (1971*b*) *J. Biol. Chem.* **246**, 5851–5854
Schwartz, M. L., Pizzo, S. V., Hill, R. L. & McKee, P. A. (1973) *J. Biol. Chem.* **248**, 1395–1407
Sturtevant, J. M. (1937) *J. Amer. Chem. Soc.* **59**, 699–702
Swigert, S., Koppel, J. L. & Olwin, J. H. (1963) *Nature (London)* **198**, 797–798
Takagi, T. & Doolittle, R. F. (1973) *Biochem. Biophys. Res. Commun.* **51**, 186–191
Tyler, H. M. (1969) *Thromb. Diath. Haemorrh.* **22**, 398–400