Papain-induced Polymerization of Fibrinogen*

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The clotting activity of papain on fibrinogen can be regulated to comprise two distinct phases. First, a gel is produced which, similarly to "thrombin-fibrin," is soluble in monochloroacetic acid (1%), urea (5 M), or sodium bromide (2 M). Later, this gel is transformed into a polymer which can no longer be dispersed by the reagents mentioned. During the first phase of the reaction, a "papain-fibrin" could be isolated which, depending on the presence or absence of sodium bromide or urea, is capable of reversible gelation just like "thrombin-fibrin." Furthermore, the isolated "papain-fibrin" can be converted to a monochloroacetic acid–insoluble polymer by further reaction with either papain or with the *thrombin-activated* form of the *fibrin-stabilizing factor* of blood plasma. This finding is in agreement with our view that the production of a monochloroacetic acid (1%)–insoluble, cross-linked fibrin clot is a transpeptidation (i.e., transamidation) reaction. The ability of papain to substitute for FSF* might be useful in the treatment of patients who, lacking in stabilizing factor, cannot otherwise form a normal acid-insoluble clot polymer. Papain can be regarded, in any case, a more complete hemostatic agent than thrombin.

In the process of blood clotting, fibrinogen can give rise to two types of gel networks, differing both in mechanical strength and in solubility behavior (Lorand, 1954). Fibrin, obtained from fibrinogen by reaction with thrombin, has the propensity (at *p*H 7 and μ:0.15) to aggregate and precipitate in the form of a gel which is soluble in 1% monochloroacetic acid, 5 M urea, or 1 M sodium bromide. As such, no covalent bonding is assumed to occur during gelation, and it is sufficient to postulate that the removal of fibrinopeptide from fibrinogen (Lorand, 1951, 1952), brought about by the limited proteolysis with thrombin, somehow alters the solubility of the parent protein. A urea- or acid-insoluble polymer (referred to as cross-linked fibrin) may be obtained, however, by reacting fibrin with the *thrombin-activated* form of the *fibrin-stabilizing factor* (FSF*)[1] in the presence of calcium ions. Recently we showed that this polymerization of fibrin, which represents the final stage of blood clotting in vertebrates, is very likely to be a transpeptidation (i.e., transamidation) reaction between the fibrin units (Lorand *et al.*, 1962).

It has been known for a long time that papain can induce fibrinogen to gel (Eagle and Harris, 1937; Steiner and Laki, 1951), and the enzyme was believed to achieve this simply by simulating the proteolytic activity of thrombin on fibrinogen. In view of the fact, however, that papain is as good a transpeptidase (i.e., transamidase) as a protease (Fruton, 1957), we thought that the action of papain on fibrinogen could be so controlled that it might replicate the effect of FSF* in producing a urea- or acid-insoluble cross-linked clot. The present paper describes how this was achieved.

Materials and Methods

Tris buffer contained 0.05 M of the base, brought to *p*H 7.5 with hydrochloric acid, and 0.1 M sodium chloride.

Sodium bromide (2 M) was dissolved in 0.1 M acetic acid and the *p*H was adjusted to 5.3 with sodium hydroxide. For dialyzing the fibrinlike intermediate this stock sodium bromide solution was twice diluted with water.

Cysteine hydrochloride and N^{α}-p-tosyl-L-arginine methyl ester (TAME) *hydrochloride* were purchased from Mann Research Laboratory and were dissolved in the Tris buffer (with adjustment of *p*H to 7.5) to give 0.1 and 0.05 M solutions, respectively.

The 0.1 M aqueous solution of *monoiodoacetic acid* (Matheson, Coleman and Bell Co.) was adjusted to *p*H 7 with the aid of sodium hydroxide.

The sodium form of *Chelex* (Bio-Rad) was equilibrated with Tris buffer and 10 ml of suspension contained 0.5 g of the resin.

Fibrinogen was purified from Armour bovine fraction I (Laki, 1951) and was finally dissolved in the Tris buffer.

Thrombin was prepared by the chromatographic procedure of Rasmussen (1955) using Parke-Davis bovine Thrombin Topical as starting material. The purified product was dissolved in Tris buffer.

Crystalline *papain* (Worthington) was diluted into Tris buffer

Fibrin-stabilizing factor (*FSF*) was prepared from oxalated bovine plasma (Lorand and Jacobsen, 1958; Loewy *et al.*, 1961).

Protein determinations were carried out with the use of Folin's reagent (Hirsch and Cattaneo, 1956). Total gel protein was measured after collecting the clot on a glass rod and washing it three times in 10 ml of 0.15 M sodium chloride for a total period of about 3 hours. The acid-insoluble portion of the gels, defined as the polymeric protein core, was estimated likewise, but after allowing the clots to stand in 1% monochloroacetic acid for 15 hours (Lorand and Jacobsen, 1958).

Results

Conditions for Forming Polymeric Clots with Papain.—We have already reported (Lorand and Konishi, 1962) that when crystalline papain and fibrinogen are mixed in weight ratios of about 1:2500 a monochloroacetic acid–insoluble gel ensues which, at room temperature, persists for some hours. Figure 1 demonstrates quite convincingly that this polymerizing activity of papain is not mediated through possible FSF

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[1] Abbreviations used in this work: FSF, fibrin-stabilizing factor; FSF*, thrombin-activated form of the fibrin-stabilizing factor; TAME, *p*-tosyl-L-arginine methyl ester.

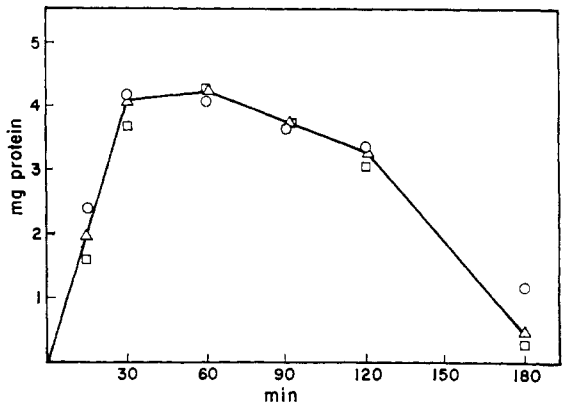

FIG. 1.—Papain-induced formation of acid-insoluble clots from fibrinogens of varying fibrin-stabilizing-factor content. Mixtures contained 0.5 ml of 1 mM calcium chloride, 0.5 ml of 0.1 M cysteine, 0.9 ml of Tris buffer, and 0.5 ml of 1% fibrinogen solution. Clotting was initiated by the addition of 0.1 ml of papain (2 μg). At the times indicated on the abscissa, 2.5 ml of 2% monochloroacetic acid was admixed. The acid-insoluble protein cores, remaining 15 hours later, were estimated, and are shown on the ordinate. In a separate assay of intrinsic fibrin-stabilizing-factor content, the native (O) and heat-treated (△, □) fibrinogens required 30 minutes, 2 hours, and 12 hours, respectively, to form acid-insoluble clots when thrombin was used to effect clotting in the presence of 20 mM cysteine (Lorand, 1962).

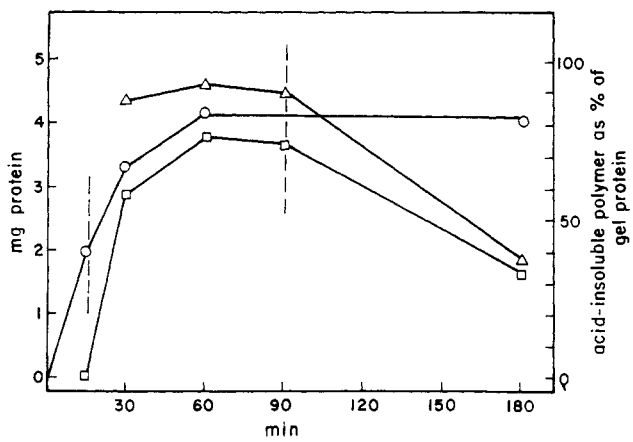

FIG. 2.—Papain-induced clotting of fibrinogen. Mixtures contained 0.5 ml of 0.1 M cysteine, 0.9 ml of Tris buffer, 0.5 ml of water, 0.5 ml of 1% fibrinogen, and 0.1 ml of papain (2 μg). At the times indicated on the abscissa, one set of mixtures (O) was analyzed for total gel proteins, and 2.5 ml of 2% monochloroacetic acid was added to the other to measure the acid-insoluble protein cores 15 hours later (□). The percentage acid-insoluble cores of the clots is shown (△) on the right-hand ordinate.

impurities which, as was shown earlier (Lorand and Jacobsen, 1958; Lorand, 1962), always contaminate fibrinogen preparations obtained by Laki's method. Repeated, moderate heat treatment is known to decrease the intrinsic FSF potency of fibrinogen preparations; yet the fibrinogens so treated behave identically with the untreated ones when papain is the clotting enzyme.

In the example quoted in our preliminary report (Fig. 1 in Lorand and Konishi, 1962), following the addition of papain to fibrinogen, the cross-linked polymer appeared simultaneously with the onset of gelation. However, as seen in Figures 2 and 3, careful adjustment of reaction conditions makes it possible to actually separate the gelation step from cross-linking. As indicated by the vertical broken lines in Figure 2,

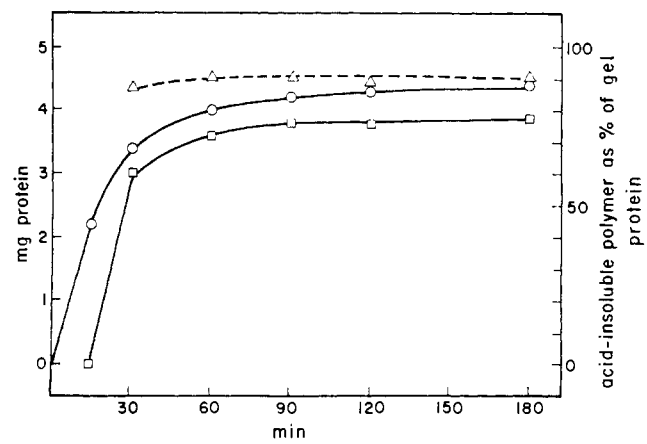

FIG. 3.—Papain-induced clotting of fibrinogen without cysteine as enzyme activator. Mixtures contained 1.4 ml of Tris buffer, 0.5 ml of water, 0.5 ml of 1% fibrinogen solution, and 0.1 ml of papain (20 μg) dialyzed for 48 hours against two changes of 30 volumes of Tris buffer at 6°. Protein analyses were carried out as in Fig. 2. O, total gel proteins; □, acid-insoluble clot residues; △, percentage acid-insoluble protein cores of clots.

three distinct zones may be discerned in time. During the early period of papain action a sizable clot is produced, which, however, is entirely soluble in monochloroacetic acid. In the following period, almost 100% of the formed gel is of the acid-insoluble cross-linked variety. Finally, presumably on account of the clot-digesting ability of papain (as is also noticeable in Fig. 1), the cross-linked polymer gives way to some acid-soluble species which still retains its gellike appearance. In the absence of cysteine as an activator (Fig. 3), papain activity was apparently so short-lived that the lack of the final digestive phase allowed the polymeric clot to persist for hours.

Isolation of a Fibrinlike Intermediate from the Fibrinogen-Papain Mixture.—Since there is a brief period during the early part of the papain-fibrinogen reaction in which, similarly to fibrin produced in a thrombin-fibrinogen mixture, the clot is of the acid-soluble type, it was thought that by interrupting the action of papain at that stage the identification of a fibrinlike protein species would become possible. This protein would be defined as being soluble in 5 M urea or 1 M sodium bromide, and reversibly forming a gel network when the concentration of the urea or sodium bromide is lowered (Lorand and Middlebrook, 1952; Donnelly *et al.*, 1955). It could be shown that, similarly to those obtained in the presence of the thrombin-FSF system, papain-produced clots fell into urea- or sodium bromide–soluble and insoluble categories. However, in contrast to the case when monochloroacetic acid was used as dispersing agent, the action of papain had to be destroyed (by addition of iodoacetate) prior to the admixture of urea or sodium bromide. Otherwise, apparently papain continues to act in these solvents and all cross-linked polymer becomes eventually digested. In accordance with the conditions described in Figure 2, the following procedure was adopted for separating the fibrinlike component from the mixture of papain and fibrinogen.

Ten ml of a 2% solution of fibrinogen in Tris buffer was mixed with 19 ml of Tris buffer, 10 ml of 0.1 M cysteine in Tris buffer, and 10 ml of water. Then 1 ml of papain (20 μg diluted into Tris buffer) was added. Clotting began in about 9 minutes after the addition of the enzyme. The clot, formed during the first 5 minutes after visible gelation, was collected and squeezed out with the aid of a pair of glass rods, used as chop-

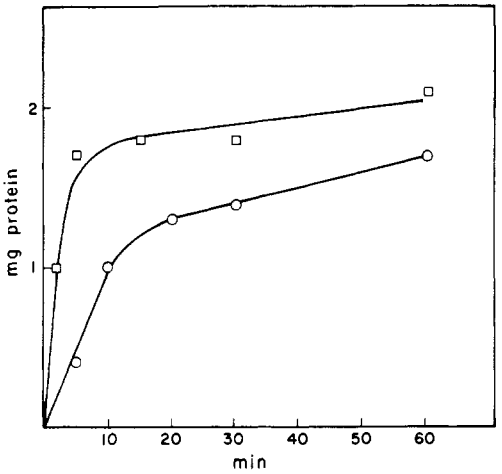

FIG. 4.—Time course of aggregation of "papain-fibrin." Mixtures contained 0.5 ml of water (O) or 1 mM calcium chloride (□), 0.5 ml of 0.1 M cysteine, 1.2 ml of Tris buffer, and 0.3 ml of 1.5% "papain-fibrin" solution in 1 M sodium bromide at *p*H 5.3. All clots were soluble in 1% monochloroacetic acid. Ordinate indicates total gel proteins of clots formed during the times specified on the abscissa.

sticks, and was immediately placed into 20 ml solution of 0.1 M iodoacetate where it was allowed to stand for a period of 1 hour. It was then transferred into a 5-ml solution of 2 M sodium bromide at *p*H 5.3 in which it readily dissolved. The solution was dialyzed against two changes of 500 ml of 1 M sodium bromide overnight at 4°. The small amount of precipitate formed during dialysis was removed by centrifugation and the supernatant was stored at 4°.

Figure 4 shows that this supernatant solution does, indeed, contain a fibrinlike substance, for reduction of the sodium bromide concentration together with raising the *p*H close to neutrality induced the formation of a gel which could be fully dispersed again in sodium bromide, urea, or monochloroacetic acid. Thus, with respect to ability to aggregate, this "papain-fibrin" parallels the behavior of "thrombin-fibrin." As in the case with the latter, calcium ions accelerate the rate of aggregation of "papain-fibrin," too.

Cross-Linking of "Papain-Fibrin" by the Further Application of Papain or of FSF.*—In order to demonstrate that the protein species isolated from the papain-fibrinogen clotting mixture was actually an intermediate toward forming the cross-linked polymer, it was necessary to show that renewed application of papain would convert it into a monochloroacetic acid–insoluble gel. Figure 5 illustrates that this could be readily accomplished and that the behavior of the intermediate on mixing with papain was what one expected from the predictions based on Figure 2.

The experiment presented in Figure 6 underlines the similarity between the cross-linking effect of papain and that of the combined fibrin-stabilizing factor–thrombin and calcium-ion system of blood. When "papain-fibrin" was allowed to regel in the presence of either papain or of the latter system, the resulting clots became insoluble in 1% monochloroacetic acid. Further, "papain-fibrin" proved to be in every respect similar to "thrombin-fibrin" in being a polymerizing substrate for FSF*. In the experiment described in Figure 7, FSF was activated first by thrombin in the presence of calcium ions to produce FSF*. Thrombin activity was then quenched by the addition of TAME (Sherry and Troll, 1954), following which "papain-fibrin" was admixed and was allowed to polymerize. Just as the polymerization of "thrombin-fibrin" by FSF* (Lorand and Konishi, 1964), that of "papain-fibrin," too, requires the presence of calcium ions.

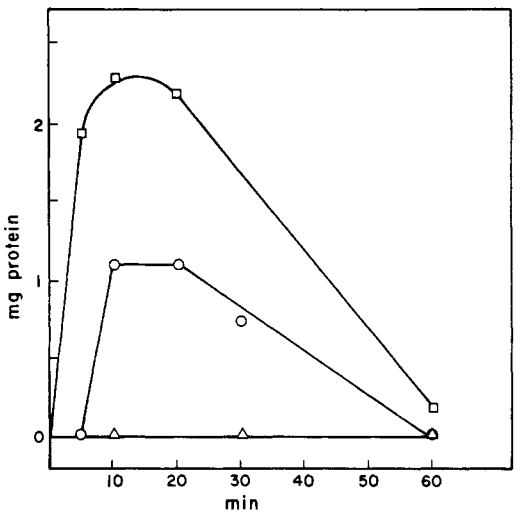

FIG. 5.—Polymerization of "papain-fibrin" by exposure to papain. Mixtures contained 0.5 ml of 1 mM calcium chloride (□ and △) or water (O), 0.5 ml of 0.1 M cysteine, 1.1 ml of Tris buffer, 0.1 ml of (1 μg) papain (□ and O) or Tris buffer (△), and 0.3 ml of 1.6% "papain-fibrin" dissolved in 1 M sodium bromide at *p*H 5.3. At the times indicated on the abscissa, 2.5 ml of 2% monochloroacetic acid was added and the acid-insoluble protein cores (shown on the ordinate) were estimated 15 hours later.

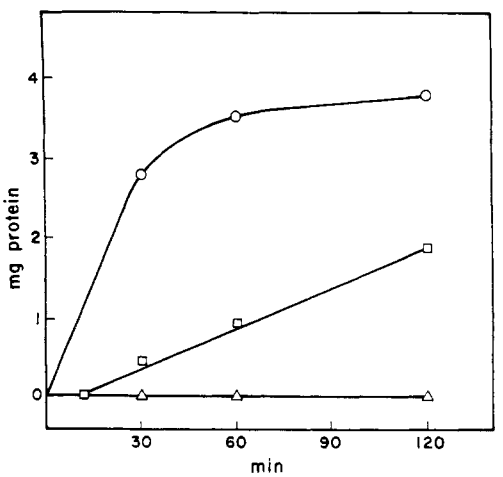

FIG. 6.—Polymerization of "papain-fibrin" by the action of papain or of the fibrin-stabilizing factor–thrombin system. Mixtures contained 0.2 mM calcium chloride, 20 mM cysteine, and 0.3 ml of "papain-fibrin" (3.8 mg) dissolved in 1 M sodium bromide at *p*H 5.3. In (O), 84 μg of fibrin-stabilizing factor and 2 units of thrombin, both in Tris buffer, were present; in (□), 0.2 μg of papain was added. Volumes were made up to 2.5 ml with Tris buffer. (△) contained neither fibrin-stabilizing factor–thrombin nor papain. The acid-insoluble protein cores of clots (ordinate) were estimated as in Fig. 5.

DISCUSSION

Our observations with regard to the clotting of fibrinogen with low concentrations of papain may be summarized by the following outline of reaction sequences:

(1) fibrinogen $\xrightarrow{\text{papain}}$ "papain-fibrin"

(2) n("papain-fibrin") $\rightleftharpoons$ aggregated "papain-fibrin" clot (1 M NaBr)

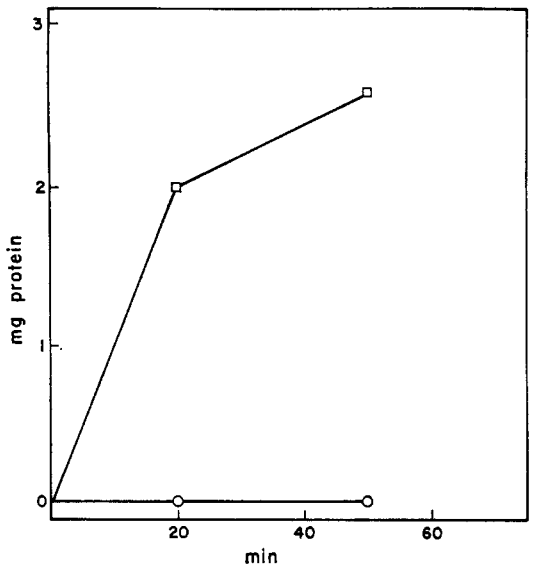

FIG. 7.—Polymerization of "papain-fibrin" by the thrombin-activated form of the fibrin-stabilizing factor in presence of calcium ions. Activation media contained 2 ml of 1 mM calcium chloride, 1.8 ml of 0.1 M cysteine, 0.1 ml of thrombin (10 units), and 0.8 ml of fibrin-stabilizing factor (0.27 μg) in Tris buffer. Following 20 minutes of incubation at room temperature, thrombin activity was quenched by the addition of 2 ml of 0.05 M TAME. Such solutions were mixed with either (□) 2 ml of Tris buffer or with (○) 2 ml of the Chelex resin suspended in Tris to remove calcium ions. The latter was then centrifuged to separate the resin. To 2.2 ml aliquots of these mixtures, containing the thrombin-activated stabilizing factor (Lorand and Konishi, 1964), 0.3 ml of a 1.8% "papain-fibrin" solution in 1 M sodium bromide at *p*H 5.3 was added. The acid-insoluble protein cores of clots (ordinate) were estimated as in Fig. 5.

(3) n("papain-fibrin") $\xrightarrow[(Ca^{2+})]{\text{papain}}$ crosslinked "papain-fibrin" clot

Step (1) might very well correspond to the limited proteolysis of fibrinogen by thrombin (Lorand, 1951, 1952):

$$\text{fibrinogen} \xrightarrow{\text{thrombin}} \text{fibrin} + \text{fibrinopeptide(s)}$$

It is not known as yet whether peptides identical or analogous to those removed by thrombin are actually released by papain from fibrinogen. In view of the similar specificity of papain and thrombin concerning the critical arginylglycine bonds in fibrinogen, this is a very strong possibility. However, the terminal amino groups of glycine which have been shown to appear as a result of the action of papain on bovine fibrinogen (Blomback and Yamashina, 1958) may not necessarily relate in the amino acid sequence of the protein to those produced by thrombin (Lorand and Middlebrook, 1952). In any case, it would be quite fortuitous if the urea-soluble proteins on which the end-group determinations were carried out in the papain-fibrinogen mixture would correspond to the urea-soluble "papain-fibrin" intermediate described in our work as being the counterpart of "thrombin-fibrin." As pointed out before, the action of papain apparently does not cease with the addition of urea unless the enzyme is inactivated by some other means. In the work relating to the end-groups of papain-clotted fibrinogen (Blomback and Yamashina, 1958), there is no evidence to show that such inactivation was carried out. Thus, regardless of the nature of cross-linking or state of digestion, in such circumstances the clot proteins would always be found to be soluble in urea. Furthermore, even if the enzyme were inactivated prior to the addition of the dispersing solvent, there would still be at least two periods (corresponding to the early and late phases illustrated in Fig. 2) in which the clot would be soluble in urea. Clearly, however, only the protein species existing during the early phase bears a functional relationship to fibrin. With this knowledge, a comparison between the end groups, molecular size, and other properties of "papain-fibrin" and "thrombin-fibrin" will now become possible.

Step (2) relates to the reversible aggregation of "papain-fibrin" near neutral *p*H and at moderate ionic strength (0.15). Like thrombin-produced fibrin, it aggregates, in a manner enhanced by calcium ions, to form a gel which can be dissolved in 5 M urea, 1 M sodium bromide, or 1% monochloroacetic acid.

Step (3) describes the actual polymerization of "papain-fibrin" into a clot which no longer is soluble in the solvents just mentioned. This polymerization can be effected either by papain alone or by the thrombin-activated form of the fibrin-stabilizing factor of blood. Whereas polymerization with papain is only enhanced by calcium ions, the presence of these during polymerization with FSF* seems obligatory, as in the reaction of the latter with "thrombin-fibrin." The similar effect of papain and FSF* in the polymerization step is in keeping with the view that this reaction is one of transpeptidation (i.e., transamidation) (Lorand *et al.*, 1962).

Altogether, crystalline papain alone seems to embody the combined clotting characteristics which in vertebrate blood coagulation are the properties of two separate proteins, namely, thrombin and FSF*. In this respect, therefore, papain functions similarly to the "tissue coagulin" of lobster blood clotting (Duchateau and Florkin, 1954; Lorand *et al.*, 1963).

Superimposed over the controllable clotting activity of papain, as given by steps (1)–(3), there is of course the clot-digesting property of the enzyme which (see the third phase of Fig. 2) by itself is a complex process and may lead to a total liquefaction of the clot (Steiner and Laki, 1951).

The controlled manner in which the clotting of fibrinogen may be manipulated by papain suggests a possibly useful clinical application for this enzyme. In cases of the inborn absence of fibrin-stabilizing factor, though clotting and primary bleeding times are found to be normal, the patient's clot is of the urea-soluble variety which cannot support normal wound healing. Thus severe hemorrhaging occurs (Duckert *et al.*, 1960). Based on the work described in our paper (see also Lorand and Konishi, 1962), we suggest the topical application of papain solutions of low activity (comparable to that used in the experiment given in Fig. 3) to the bleeding wounds of such patients. The ability of papain to produce a cross-linked polymer would substitute for the absence of the normal fibrin-stabilizing-factor activity. In addition to this special replacement, papain can be regarded a more complete hemostatic agent than thrombin alone.

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The Spectrophotometric Titration of the Phenolic Groups of Horse Heart Cytochrome c*

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The ionization of the four phenolic hydroxyl groups of horse heart cytochrome c was investigated by measuring the change in the absorbance of the protein at 243 mμ as a function of *p*H. In ferricytochrome c, the ionization of the phenolic groups described a nonsigmoidal, nearly reversible titration curve with a mid-point at *p*H 12.1. Reduction of the heme iron shifted the titration curve to a higher *p*H range, elevating the mid-point to *p*H 12.6. The increase in the reduced viscosity of both ferri- and ferrocytochrome c coincided with the titration curve of the latter. In the presence of 8 M urea or after tryptic digestion, all four phenolic groups of ferricytochrome c ionized normally and reversibly with apparent *pK* values of 10.4 and 11.0, respectively. It was concluded that all four tyrosyl residues in horse heart cytochrome c are buried within the structure of the protein and that the local environment about these residues is changed upon reduction of the heme iron.

Horse heart cytochrome c has been found to be a compact, highly organized protein by hydrodynamic measurements (Atlas *et al.*, 1952; Ehrenberg and Paléus, 1955), X-ray diffraction (Arndt and Riley, 1955), and electron microscopy (Levin, 1962). With the recent elucidation of the complete amino acid sequence of this protein (Margoliash *et al.*, 1961), it is of interest to describe the tertiary structure of the protein in terms of the chemical reactivity of its functional groups. Over 90% of the ε-amino groups can be guanidinated without impairing the catalytic function of the protein (Takemori *et al.*, 1962), suggesting that the majority of the lysyl residues are located on the surface of the molecule. Photooxidation studies in the presence of methylene blue have revealed differences in the reactivity of the three histidyl residues (Nakatani, 1960). In this communication, the structural environment of the four tyrosyl residues is examined by measuring the change in the absorbance of the protein at 243 mμ as a function of *p*H under a variety of conditions.

EXPERIMENTAL

Cytochrome c.—Cytochrome c was purified from horse heart by the method of Keilin and Hartree (1952) and chromatographed on Amberlite CG-50 according to the method of Margoliash (1957). Only the protein eluted with 0.25 M ammonium acetate (fraction 1) was used in these experiments. The purified protein had an $A_{550(red)}/A_{280(oxid)}$ of 1.29 and was found to be monodisperse by chromatography on Sephadex G-25 (Margoliash and Lustgarten, 1962). The concentration of cytochrome c was determined spectrophotometrically by measuring the absorbance at 550 mμ in the presence of sodium dithionite, using an ε of 27.7×10^3 (Margoliash and Frohwirt, 1959).

The ferrocytochrome c used in the viscosity measurements was prepared from the oxidized form of the protein by addition of 0.01 M sodium dithionite. In the spectrophotometric studies, however, the ferrocytochrome c was prepared from the oxidized form by catalytic hydrogenation immediately before use. About 2 mg of platinum dust was added to 40 ml of a dilute solution of ferricytochrome c, about 1.4×10^{-5} M, in 0.25 M acetate, *p*H 6.8. This mixture was placed in a stoppered Erlenmeyer flask fitted with inlet and outlet tubes and stirred magnetically. A gentle stream of N_2 was directed over the surface of the solution for 5 minutes, followed by introduction of H_2 for 5 minutes, and N_2 for another 5-minute period. The catalyst was then quickly removed by centrifugation and the supernatant placed under N_2.

Hemopeptide.—A purified hemopeptide obtained from a peptic digest of Bombyx cytochrome c was a gift from Prof. Hans Tuppy. This hemopeptide is identical with the peptic hemopeptide of horse heart cytochrome c except for the replacement of the lysyl residue by an arginyl residue (Tuppy, 1957). The concentration of hemopeptide was determined spectrophotometrically by measuring the absorbance at 410 mμ using an ε of 9.3×10^4 (Paléus *et al.*, 1955).

Chemicals.—All chemicals used in this investigation were of reagent grade. Trypsin was purchased from Sanabo, Vienna, and freed of chymotryptic activity by incubation in 0.06 N HCl at 37° for 24 hours. Chro-

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