amino-groups present in peptides and proteins, since even a very large excess of diketene is unable to induce appreciable conformational changes of a protein.

Riassunto. Sono state analizzate le variazioni conformazionali indotte nella ribonucleasi pancreatica di bue (RNase A) e nel lisozima del bianco di uovo dopo acetoacetilazione con differenti quantità di dichetene. La modifica dell'attività enzimatica, susseguente al trattamento con dichetene, non è attribuibile a variazioni conformazionali delle proteine considerate ma al bloccaggio selettivo di uno o piú gruppi amminici coinvolti nell'attività catalitica o nel «binding» col substrato.

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Molecular Alterations of Human Fibrinogen by Ultrasonic Frequencies

Although the fact that gaseous cavitation produced by ultrasonic frequencies has been used to disrupt plant and animal cells[1,2], and was shown to alter the physicobiochemical characteristics of human serum[3–5], little is known about its effects on the biological activities of purified proteins. After ultrasonication of human serum, SEARCY and BERQUIST[3] observed the disappearance of heat precipitable fibrinogen with no fibrin formed by thrombin.

The present report describes the formation by brief period of ultrasonication of non-clottable with an anticoagulant activity protein from highly purified 95% clottable human fibrinogen. Sedimentation data from the analytical ultracentrifuge, electrophoretic mobility on polyacrylamide gel and chromatographic behavior, indicated no noticeable changes between the native human fibrinogen and the 2 min ultrasonicated fibrinogen. The ultrasonicated fibrinogen is shown to act as an anticoagulant and as an inhibitor of thrombin activity.

Materials and methods. Human fibrinogen, Pentex lot 44 purified by series of salt precipitation and adsorption on tricalcium phosphate[6] to obtain a 95% clottable protein, sedimenting as a homogeneous single peak at a velocity of 7.35 S.

A 1% solution in physiological saline of this fibrinogen was freshly prepared and exposed to ultrasonic vibration, using Raytheon Sonic Oscillator Model DF 101, 250 W and 10 kc with an output current of 0.78 A and 60 cycles. The temperature of the solution was kept at 0–4 °C during ultrasonication. Aliquots were withdrawn after 2, 5, 15, 30 and 60 min of ultrasonication. All the aliquots were immediately freeze-dried in vacuum. Sedimentation experiments were carried out on a Spinco Model L analytical Ultracentrifuge equipped with a Schlieren system.

DEAE-cellulose of 100–200 mesh with 0.70 mEq nitrogen/g was first equilibrated in 0.005*M* phosphate-0.040*M* *tris*, of pH 8.6, then packed into a column of 35 × 2.2 cm. 300 mg of purified human fibrinogen or its 2 or 5 min ultrasonicate were dissolved in 5 ml of the phosphate-*tris* buffer and equilibrated at 4 °C against the buffer, then applied onto the DEAE-cellulose column. Elution was carried out with a gradient pH using the same buffer starting at pH 8.6 and gradually decreasing to pH 4.1 with a flow rate of 75 ml/h and 15 ml fractions were collected. Optical densities of the fractions were measured at 280 nm against distilled water as a blank in a model DU Beckman Spectrophotometer.

Disc electrophoresis was carried out on columns of polyacrylamide gel[7] containing 0.15*M* e-aminocaproic acid[8].

The clottability of the ultrasonicated fibrinogen was compared with that of fibrinogen. Solutions (2 mg/ml) of fibrinogen before and after 2, 5, 15, 30 and 60 min ultrasonicates were prepared and incubated at 36 °C. 1 ml aliquots of each were removed at various times, 1 drop of stock thrombin solution was added. The time of the appearance of a clot, or fibrin strands were noted. Thrombin clotting time[9] was determined by adding

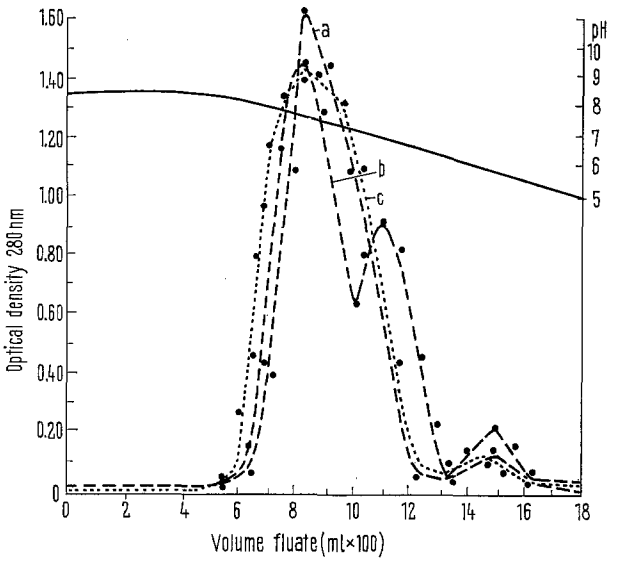

Fig. 1. DEAE-cellulose chromatography of human fibrinogen and its ultrasonicate. Elution patterns of purified human fibrinogen (a) ——— and its 60 min ultrasonicate (b) --------- and its 2 min ultrasonicate (c) ---------- from DEAE-cellulose column.

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0.1 ml of thrombin solution equivalent to 0.5 NIH units to 0.2 ml of the ultrasonicated solutions (0.5 to 1.4 mg protein/ml) and 0.2 ml of the purified fibrinogen solutions (2 mg/ml) to a test tube and measuring the time needed for the appearance of clot or fibrin thread formation at 26 °C. All solutions were made in 0.04 *M* imidazole buffered at pH 7.5.

Results and discussion. The sedimentation patterns of purified human fibrinogen and its 2, 5, 15 and 60 min ultrasonicates at protein concentration of 10 mg/ml of physiological saline with pH 7.0, yielded one single homogeneous peak, sedimenting with the velocity of $S_{20,w}$ of 7.35 *S*, 7.54 *S*, 7.15 *S*, 7.05 *S* and 7.13 *S* respectively.

When applied on DEAE-cellulose columns the purified human fibrinogen (Figure 1, curve a) as well as the 2 min ultrasonicate (Figure 1, curve c) gave one broad major peak followed by a minor small peak. The protein from the major peak of the human fibrinogen contained the clottable protein, while the protein from the major peak of the 2 min ultrasonicate contained non-clottable protein with anticoagulant activity, that is the activity of the ultrasonicate that inhibits thrombin activity which causes the coagulation of human plasma or human fibrinogen. The chromatographic pattern obtained for the 60 min ultrasonicated aliquots shown by Figure 1, curve b, suggest the presence of 2 major groups of proteins, none of which is clottable by thrombin.

Polyacrylamide gel electrophoresis suggest the hydrolysis of fibrinogen (Figure 2, tube 3) by thrombin (Figure 2, tube 1), and the hydrolysis of the 2 min ultrasonicate (Figure 2, tube 4) by thrombin (Figure 2, tube 2). Both the purified human fibrinogen and its 2 min ultrasonicate showed one protein band, while the 60 min ultrasonicate (Figure 2, tube 5) suggested 2 protein bands.

Purified human fibrinogen clotted within 150 ± 15 sec with solid clot formation. In solutions containing the 2 or 5 min ultrasonicate thin fibrin threads appeared after 350 sec which disappeared on slow gentle mixing. Solutions containing the 15, 30 and 60 min ultrasonicate gave no sign of clotting nor fibrin formation after throm-

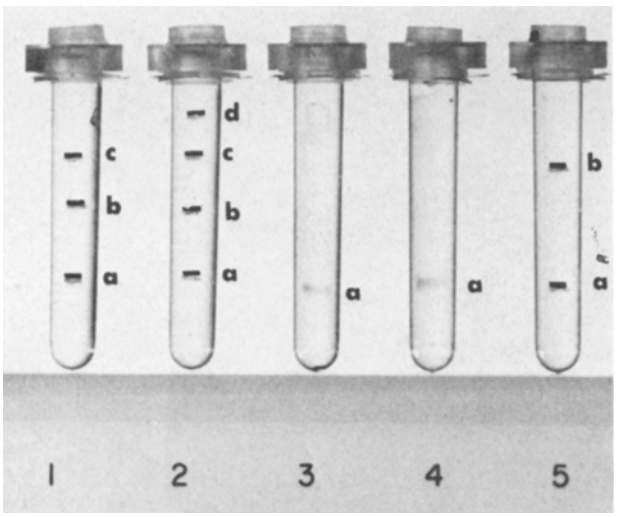

Fig. 2. Polyacrylamide gel electrophoresis. Electrophoretic behavior on polyacrylamide gel containing 0.015 *M* e-aminocaproic acid. 1. Thrombin treated purified human fibrinogen. 2. Thrombin treated 2-min ultrasonicated human fibrinogen. 3. Purified human fibrinogen. 4. Two min ultrasonicated human fibrinogen. 5. 60 min ultrasonicated human fibrinogen.

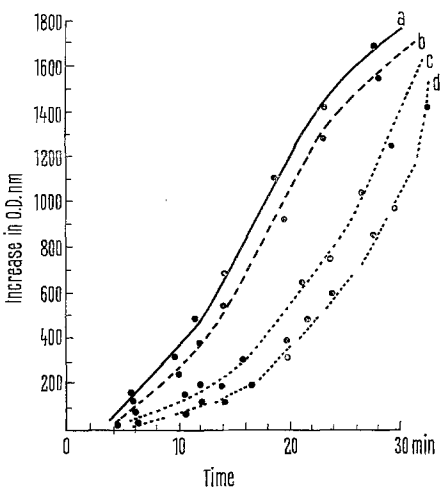

Fig. 3. Action of ultrasonicated human fibrinogen on thrombin coagulation of human plasma. Coagulation of human plasma by thrombin is followed by the increase in optical density at 350 nm. The coagulation system contained 2.0 ml tris buffer (0.1 *M*, pH 7.5), 0.2 ml freshly obtained human plasma, 0.2 ml ultrasonicated human fibrinogen (2 mg/ml in saline solution) and 0.1 ml of thrombin 6 NIH units. The ordinate shows the increase in optical density, and the abscissa gives the time in min after addition of thrombin. The effect of 0.4 mg of each of the following: a) Purified human fibrinogen (———). b) 2-min ultrasonicated human fibrinogen (———). c) 5-min ultrasonicated human fibrinogen (--------). d) 30-min ultrasonicated human fibrinogen (-- -- -- --).

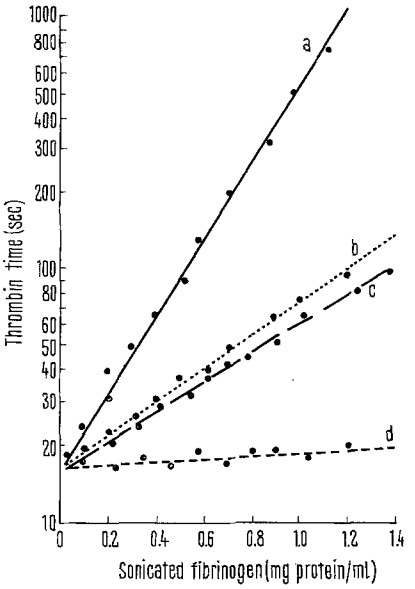

Fig. 4. Antithrombin activity of ultrasonicated human fibrinogen. The correlation between the antithrombin activity and the concentration of ultrasonicated human fibrinogen in the system described in text. The ordinate is thrombin time in sec plotted on semi-log scale, and the abscissa represents concentration of the ultrasonicated fibrinogen in mg of protein/ml of total volume. Increasing concentrations of the 2, 5, 15 and 60 min ultrasonicated with human fibrinogen gave the thrombin time curves (a) ———, (b) ·····, (c) — — —, and (d) ---------, respectively.

bin addition. The progressive increase in optical density at 350 nm observed during the coagulation of human plasma in presence of 0.4 mg of human fibrinogen (Figure 3, curve a) and in presence of 0.4 mg of the 2, 5, and 30 min ultrasonicates are shown by Figure 3, curves b, c, and d respectively. The lag phase and the slope of the curves suggest the prolongation of plasma thrombin clotting time.

The antithrombin activity of increasing amounts of the 2 min ultrasonicated fibrinogen is evident by the increase in thrombin time shown by Figure 4, curve a. Longer periods of ultrasonication of fibrinogen appears to decrease the antithrombin activity of the ultrasonicate as shown by Figure 4, curves b, c, and d.

Brief period of ultrasonication, at 0–4 °C of purified human fibrinogen resulted in the loss of clottability of the protein, and the appearance of an anticoagulant and anti-thrombin activities. Since the two minutes ultrasonicate of the purified human fibrinogen produced no noticeable changes in the sedimentation rate, electrophoretic mobility and elution from DEAE-cellulose when compared with the purified native fibrinogen, it is suggested that the clottability of human fibrinogen depends on the conformational status of the protein molecule.

Résumé. Placée 2 min sous l'influence de la vibration ultrasonique, la fibrinogène humaine se transforme en une protéine qui ne se fige pas et fonctionne donc comme un anticoagulant. Les propriétés physico-chimiques de la protéine, après action ultrasonique, ressemblent fortement aux propriétés originales de la fibrinogène humaine en voie de sédimentation; mouvement électrophorétique et adsorption sur la cellulose DEAE.

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Posthypertonic Hemolysis in a Sucrose System

The hemolysis which occurs after freezing blood appears to be mainly due to the increase in solute concentration when water separates out as ice during freezing and to the reverse event during thawing[1]. Studies of posthypertonic hemolysis may therefore be of great interest for blood-preservation[2–5].

SÖDERSTRÖM[6] first explained the mechanism of posthypertonic hemolysis in an unfortunately rather neglected paper as being due to salt entering the cell in the hypertonic phase and the cell then reacting to the subsequent isotonic phase in a way analogous to a normal cell reacting to a very hypotonic medium and designated it 'paradoxical hypotonic hemolysis'. By measuring chloride concentrations, he also showed that an uptake of salt occurs in a hypertonic medium. ZADE-OPPEN[3] pointed out that, in view of the rapid passage of water across the cell membrane, a driving force for net solute influx will not occur until a certain concentration in the medium has been exceeded, i.e. that which is just sufficient to shrink the cell to a minimal volume. Further, he calculated that, in order to explain his experimental results on a basis of the SÖDERSTRÖM hypothesis, it was required that the solute permeability should increase with increasing external solute concentration. In an almost simultaneous publication MERYMAN[5] came to very nearly the same conclusions as regards the mechanism of posthypertonic hemolysis and the increase in solute permeability. Both ZADE-OPPEN[2–4] and MERYMAN[5] used electrolyte media and both[2–5] also noted that similar effects were obtained when sucrose was used to produce hypertonicity. Although it has long been known that posthypertonic hemolysis is obtained after exposure to hypertonic non-electrolyte solutions (TAKEI[7] used glucose, VALDIVIESO and HUNTER[8] used sucrose), little is known about the possible difference in the effects of electrolytes and non-electrolytes. This communication describes quantitatively the effect of sucrose under certain conditions and attempts to compare this effect with the effect of NaCl in a similar system, so far as such a comparison is possible.

Human red cells were washed in a buffered isotonic NaCl solution. To an isotonic cell suspension (0.25 ml) first a hypertonic solution (1 ml) was added and then, after a certain time interval, a large volume (20 ml) of isotonic solution. Hemolysis was estimated as the percentage of Hb liberated into the medium. The procedure is given in detail elsewhere[3]. When sucrose was used to vary the tonicity, the electrolyte concentration was kept constant at the isotonic level.

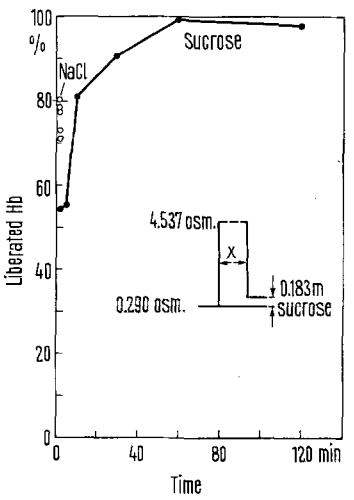

Fig. 1. Posthypertonic hemoglobin liberation after varying times of hypertonicity. Tonicities were varied with sucrose (filled circles) or with sodium chloride (open circles). The inset is intended as a short description of the sucrose experiment. NaCl was used at the same osmolalities.

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