the other molecules studied, fragmentation, if present, was below the detection limits of the instrument (<<1 percent). Odd-electron ions have not been observed. These results suggest an unusually mild ionization process. Fourth, the relative signal strengths observed are roughly proportional to the ease of protonation of the molecule in solution. Hexane and carbon tetrachloride are readily ionized under field ionization conditions but are not ionized at all under these conditions, whereas easily protonated compounds such as pyridine give strong signals. It is of interest that the sensitivities observed are not related to ionization potentials. For example, Table 1 shows that the sensitivity for acetonitrile (ionization potential, 12.22 eV) is far better than that for benzene (9.24 eV). Finally, the presence of cluster ions of the form $(2M + H)^+$ suggests that ionization occurs from the liquid phase, since pressures in the gas phase are too low for significant dimerization. When mixtures of materials are examined, both homogeneous and heterogeneous cluster ions can be observed and the relative intensities can be related to relative basicity.

The experiment described here is a simple, reliable, and unusually mild method for producing ion beams of protonated molecules. To my knowledge, this is the first unambiguous evidence that an ion-generating mechanism independent of classical field ionization can operate under substantially the same conditions. The data suggest a mechanism based on simple field-induced cation extraction from solution—a mechanism previously invoked in organic electrohydrodynamic ionization—and provide further evidence that this mechanism can be operative in conventional field desorption experiments. It should be noted that cation extraction by a high field does not necessarily reflect equilibrium conditions in the bulk liquid since physical parameters such as temperature (*10*) may be very different in the areas of active ion emission.

It will be of considerable theoretical interest to compare ions formed by cation extraction with ostensibly the same ions formed by chemical ionization mass spectrometry (*11*), using mass-analyzed ion kinetic energy spectrometry (*12*).

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References and Notes

1. H.-R. Schulten, *Methods Biochem. Anal.* **24**, 313 (1977).
2. J. F. Holland, B. Soltmann, C. C. Sweeley, *Biomed. Mass Spectrom.* **3**, 340 (1976).
3. F. W. Röllgen and H.-R. Schulten, *Z. Naturforsch.* **30**, 1685 (1975).
4. W. Frick, E. Barofsky, G. D. Daves, Jr., D. F. Barofsky, D. Chang, K. Folkers, *J. Am. Chem. Soc.* **100**, 6221 (1978).
5. H.-R. Schulten and H. D. Beckey, *Org. Mass Spectrom.* **6**, 885 (1972).
6. H. J. Veith, *Tetrahedron* **32**, 2825 (1977).
7. J. F. Mahoney, A. Y. Yahiku, H. L. Daley, R. D. Moore, J. Perel, *J. Appl. Phys.* **40**, 5101 (1969).
8. B. P. Stimpson and C. A. Evans, Jr., *J. Electrostat.* **5**, 411 (1978).
9. F. W. Röllgen and K. H. Ott, *Z. Naturforsch. Teil A* **33**, 736 (1978).
10. J. M. Crowley, *J. Appl. Phys.* **48**, 155 (1977).
11. B. Munson, *Anal. Chem.* **49**, 772A (1977).
12. R. G. Cooks, J. H. Beynon, R. M. Caprioli, G. R. Lester, *Metastable Ions* (Elsevier, Amsterdam, 1973), pp. 42–44.
13. K. Watanabe, T. Nakayama, J. R. Mottl, *J. Quant. Spectrosc. Radiat. Transfer* **2**, 369 (1962).

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Fibrinopeptide B and Aggregation of Fibrinogen

Abstract. *Removal of fibrinopeptide B from human fibrinogen by reaction with the procoagulant enzyme from copperhead snake venom below 25°C resulted in tight aggregation of the fibrinogen, which, in turn, progressively blocked a concomitant but sluggish release of fibrinopeptide A by the enzyme. When the clots obtained at < 25°C were warmed, they dissociated into soluble aggregates and monomers. Release of fibrinopeptide A then resumed, and a secondary coagulation followed. The aggregation induced by release of fibrinopeptide B itself involves a plasmin-susceptible segment located just distal to B in the Bβ chain of fibrinogen, a segment previously shown to be of little importance in the aggregation induced by release of fibrinopeptide A.*

The formation of fibrin in the blood of vertebrates involves a proteolytic reaction in which certain NH_2-terminal segments known (*1*) as fibrinopeptides A and B (hereafter termed A and B) are cleaved from the Aα and Bβ chains (*2*) of fibrinogen by enzymic action of thrombin, with subsequent self-aggregation of the altered fibrinogen into a clot (*3*). The release of A usually precedes the release of B, and suffices to transform fibrinogen into fibrin (*4*). Herzig *et al.* (*5*) observed that coagulation of human fibrinogen by a procoagulant enzyme from the copperhead snake *Ancistrodon contortrix contortrix* depended on the release of A even though the enzyme released B beforehand. This observation focused attention on the release of A as a critical step in coagulation of human fibrinogen. In contrast, Doolittle (*6*) had observed that release of B from lamprey fibrinogen sufficed to elicit clot formation without measurable release of A. With the prospect that the disparate effects of removing B might have been due to differing temperatures rather than to the species used, we reexamined human fibrinogen. Our studies indicate that removal of B from human fibrinogen does yield a self-aggregating and clot-forming derivative that coagulates at low temperatures near 25°C as used in the studies on the lamprey (*6*), but one that dissociates into soluble aggregates and monomers at 37°C, the temperature used in the study of Herzig *et al.* (*5*). More important though, the aggregation accompanying direct removal of B from human fibrinogen provides a basis for reinterpreting an observation (*7*) that plasmin rapidly removes segments containing B without eliciting aggregation. This observation together with qualifying data lead us to conclude that the plasmin-susceptible segments located immediately behind the fibrinopeptide comprise a critical portion of the B-dependent aggregation site. Further, the aggregation effected through release of B hinders release of A while little hindrance to release of both peptides occurs when they are released in opposite order, a condition we view as adding new significance to the usual removal of A before B in the course of blood coagulation.

Materials and methods used were essentially the same as described with the copperhead enzyme (*5*) except for a modification in the processing of the venom to inactivate a fibrinolytic enzyme that had chromatographic and gel filtration characteristics differing only slightly from the procoagulant, and which occasionally destroyed the procoagulant on storage. The inactivation was carried out with 10 μM tosyllysine chloromethyl ketone (TLCK) immediately after DEAE chromatography. After treatment for 30 minutes with TLCK, fractions with procoagulant activity were dialyzed, concentrated, and purified by gel filtration as described (*5*). All reactions between the procoagulant and fibrinogen were carried out in buffer containing 0.135 M NaCl, 0.015 M tris-HCl, and 0.1 mM EDTA at pH 7.4.

Ultracentrifugation at 25°C indicated a full transformation of fibrinogen to soluble aggregates ($s_{w,20} \simeq 16S$) in association with the preferential release of B by the procoagulant, whereas concomitant but relatively sluggish release of A reached only one-fifth of completion. As

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the release of B and parallel formation of soluble aggregates approached completion, a sudden and full coagulation of the fibrinogen ensued without measurable release of additional A, the overall yield of A being the same 20 to 25 percent after prolonged incubation (10×) as had been obtained at the onset of clotting. No peptides apart from the fibrinopeptides were detected by ninhydrin or Sakaguchi reactions (Fig. 1). Both the partial release of A and its cessation concomitant with full coagulation were in contrast to experiments at 37°C (*5*) where (i) the release of A accelerated instead of stopping as the release of B approached completion, and (ii) coagulation proceeded gradually in association with the release of A subsequent to B.

Release of A decreased somewhat further below 25°C, but reached a minimum at 14°C; below 14°C the peptide was released in constant (12 percent) proportion to B throughout the course of reaction and terminated at that level (Fig. 1a). The release of A in constant proportion to B and its cessation after complete release of B suggested that at the low temperatures the tightened aggregation, which was evident from coagulation of the derivative lacking B, was imposing a hindrance to the release of A. To test this possibility we examined the effect of temperature on aggregation and peptide release after removing B at low temperature. Clots formed at 14°C and held there for a period exceeding ten times that required for complete coagulation dissolved within 2 to 4 minutes when warmed to 37°C, even at fibrin concentrations of 16 mg/ml. When the dissolved clot was held at 37°C for a period comparable to the original clotting time at 14°C, clot formation began again and continued to completion. The coagulation of the dissolved fibrin was due to a resumption of the release of A by the enzyme at the elevated temperature since analysis of the fluid showed that a substantial (> 80 percent) release of A had occurred (Fig. 1a). Further, when clots were formed in a thin (1 mm) film and overlaid with buffer containing 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF) for 1 hour at 14°C to inactivate (*5*) the procoagulant prior to warming, the peptide was not released, and the dissolved protein remained completely soluble at the elevated temperature. With the enzyme inactivated, the protein could be repeatedly dissolved and again transformed to a gel by warming and chilling, provided that the gels were not compacted or stirred to form a dense coagulum. Bovine and rabbit fibrinogen also coagulated fully with release of B without appreciable loss of A (5 percent and 2 percent, respectively) by the procoagulant at 14°C, and underwent secondary coagulation through release of fibrinopeptide A after dissolution of the clots at 37°C.

Ultracentrifugation at 37°C showed that the thermally dissolved fibrin consisted of 16*S* aggregates together with 8*S* monomers (*8*) (Fig. 2). Dilution decreased the concentration of aggregates (range 1.80 to 0.2 mg/ml) while that of the monomer remained at 0.16 to 0.20 mg/ml. Monomer alone was seen at 0.1 mg/ml. These data indicated (*9*) that the equilibrium involved in the aggregation could be treated as a simple phase transition. At 25°C the concentration of monomer fell to only a trace, and at 20°C essentially all of the protein sedimented at 16*S* (Fig. 2). From the difference in monomer concentration observed at 37°C and 25°C, the enthalpy ($\Delta H°$) of transition from monomer to soluble aggregates appeared to be on the order of −20 kcal/mole, approximately one-half the calorimetrically determined value obtained by Sturtevant *et al.* (*10*) for full coagulation of fibrin lacking both A and B. The observation that substantial dissociation into monomers occurred at 37°C but not at temperatures below 20°C was in accord with the view that release of A depended on interaction of the enzyme with the fibrin in monomeric rather than aggregated form.

To test whether the aggregation might be sufficiently tight at low temperatures to block release of A by thrombin, dissolved clots were chilled from 37°C to 14°C (no PMSF added), and bovine thrombin was added as the solution began to thicken. The thrombin released almost no A at 14°C, but at 37°C did cause release (Fig. 1b). A test of the converse possibility that aggregation of fibrin lacking fibrinopeptide A might block release of B by the procoagulant gave a negative result. When purified solutions of fibrin produced by specific release of A in *p*H 5.3 buffer (*11*) were gelled by admixture with neutralizing buffer containing either the copperhead enzyme or thrombin, a full yield of B followed, regardless of temperature.

As was mentioned earlier, plasmin re-

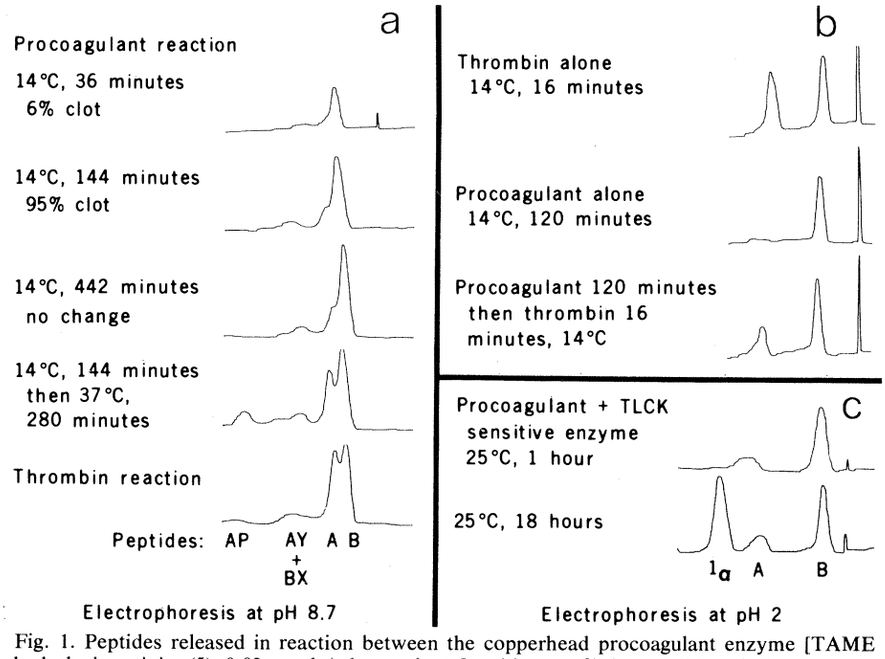

Fig. 1. Peptides released in reaction between the copperhead procoagulant enzyme [TAME hydrolysis activity (*5*), 0.03 μmole/ml per minute] and human fibrinogen (16 mg/ml). The reactions were performed with small samples (0.1 ml) in wide tubes (1.5 cm) to facilitate heat transfer without stirring; after the indicated incubations, the protein was diluted with 1 ml of 1 percent monochloroacetic acid and precipitated with 0.2 ml of 30 percent trichloroacetic acid. The supernatant was subjected to electrophoresis at *p*H 2 or at *p*H 8.7 (*5*). (a) Cessation of release of A, and its variant forms (AY and AP) after completion of clot formation and release of B and its variant BX, and then resumption of release of A after clot dissolution at 37°C. The small amount of A released during coagulation at 14°C is shown more clearly in the electrophoresis at *p*H 2 (b) where the variant forms, with exception to AP, migrate with the parent peptides. (b) Absence of appreciable effect of admixing thrombin at 14°C. The thrombin was added after warming for 2 minutes to dissolve the clot and chilling (10 minutes) until the solution began to thicken. Controls treated similarly without the addition of thrombin showed that the added release of A occurred mainly as a result of the temperature manipulation. (c) Release of the COOH-terminal Aα chain fragment ('α) by a procoagulant preparation containing the TLCK-sensitive contaminant.

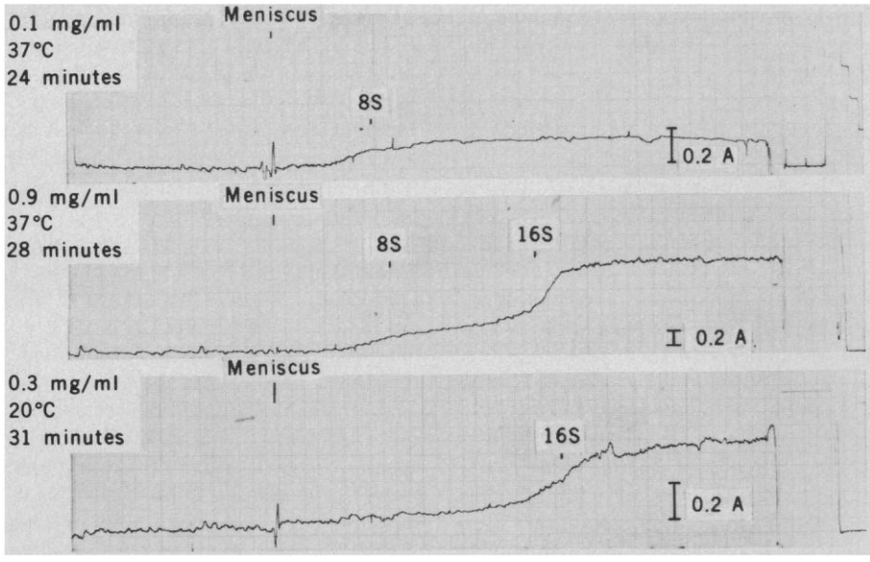

Fig. 2. Ultracentrifuge patterns demonstrating the dissociation of soluble aggregates into monomers at 37°C. Fibrinopeptide B was removed from fibrinogen with the copperhead procoagulant enzyme at 14°C as in the experiments of Fig. 1, and the resultant clot was dissolved by warming to 37°C with 10 μM PMSF added to inactivate the enzyme. The fibrin was then diluted and examined in the ultracentrifuge at concentrations and temperatures indicated at the left of the diagrams. The diagrams portray scans of absorbancy at 280 nm (ordinate) for the location and measurement of boundaries formed by protein sedimenting in the centrifugal field (left to right). The position of boundaries corresponding to monomers with sedimentation coefficient ($s_{w,20}$) of 8*S*, and aggregates sedimenting at 16*S* are indicated in the diagram. The 16*S* aggregates seen in the moderately concentrated solution (central pattern) disappeared because of full dissociation into monomers on dilution to 0.1 mg/ml at 37°C (upper pattern). At 20°C, all of the protein sedimented as 16*S* aggregates, even at high dilution (lower pattern). The vertical scale bar indicates 0.2 absorbancy units.

moves fibrinopeptide B along with other segments without eliciting aggregation of human fibrinogen or destroying its coagulability by thrombin. Since direct removal of B as effected with the copperhead enzyme elicits aggregation, a critical segment must have been destroyed or lost in the reaction with the plasmin. The removal of B from fibrinogen by plasmin arises through cleavage at points somewhat distal to the fibrinopeptide in the span of amino acids 21 to 42 located in the β portion of the Bβ chain (*7, 12, 13*). Although the COOH-terminal region of the Aα chain undergoes considerable degradation during release of the Bβ fragments by plasmin, loss of the critical segment would have to be either coupled to the loss of B as with release of the Bβ fragments or fully precede it to block aggregation. A small segment from the extreme COOH-terminal region of the Aα chain is released at a very rapid rate (*7, 12*); but neither this nor other Aα chain cleavages fully precede release of the Bβ fragments (*7*). Thus, we contend that the critical region is contained at least in part in the β segments of the Bβ fragments released by plasmin.

Additional evidence that the COOH-terminal region of the Aα-chain is not critical to the aggregation accompanying direct release of B was obtained from experiments with copperhead procoagulant contaminated with the TLCK-sensitive protease from the venom. Electrophoretic analyses showed that, when the TLCK treatment was omitted in purifying the enzyme, the Aα chains within clots formed by the procoagulant underwent degradation in a manner (*13*) similar to their degradation by plasmin, while the β and γ chains were left intact in the clot. Furlan *et al.* (*14*) observed similar degradation of the Aα chains with their procoagulant preparation. Analysis of the soluble fragments in trichloroacetic acid (Fig. 1c) showed the expected amount of B released in clotting, an equimolar amount of peptide with mobility equal to the COOH-terminal Aα chain fragment observed (*7*) in degradation by plasmin, and a small amount of A only slightly greater than that released in the initial clot formation by the procoagulant enzyme. The slight release of A indicated that the peptide remained resistant to release by the procoagulant, and that little dissociation of the clot accompanied degradation of the Aα chains. Further, the clots proved refractory to dissolution by warming alone, and therefore appeared more tightly bound than the original clot formed with Aα chains intact. Clots dissolved without difficulty on acidification to *p*H 5.3 as used for dissociation of regular fibrin, and release of A by the procoagulant proceeded to completion at that *p*H.

The binding site, which is unmasked by removal of A has been shown (*15*) to lie in the NH_2-terminal domain of the fibrinogen molecule. It may be right at the amino terminus of fibrin as suggested by the observation (*16*) that tri- and tetrapeptides containing the Gly-Pro-Arg (glycine-proline-arginine) sequence of the α chain terminus have an inhibitory effect on aggregation of fibrin. Although no inhibition has been obtained (*16*) with the peptide Gly-His-Arg-Pro (His, histidine) corresponding to the β chain terminus, our study shows that a binding site exists at the β chain terminus. The aggregation that accompanies removal of B is abolished when the contiguous segment of the β chain is removed in the degradation of fibrinogen by plasmin. Moreover, the qualified observation that plasmin destroys the B-dependent aggregation site without destroying the A-dependent site indicates that the two peptides mask separate sites.

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References and Notes

1. L. Lorand, *Nature (London)* **167**, 992 (1951); F. R. Bettelheim, *Biochim. Biophys. Acta* **19**, 121 (1956).
2. B. Blombäck and A. Johnson, *Thromb. Diath. Haemorrh. Suppl.* **51**, 251 (1971).
3. H. A. Scheraga and M. Laskowski, Jr., *Adv. Protein Chem.* **12**, 1 (1957); T. C. Laurent and B. Blombäck, *Acta Chem. Scand.* **12**, 1875 (1958); K. Laki and J. A. Gladner, *Physiol. Rev.* **44**, 127 (1964).
4. B. Blombäck, M. Blombäck, I. M. Nilsson, *Thromb. Diath. Haemorrh.* **1**, 3 (1957); J. R. Shainoff and I. H. Page, *J. Exp. Med.* **116**, 687 (1962).
5. R. H. Herzig, O. D. Ratnoff, J. R. Shainoff, *J. Lab. Clin. Med.* **76**, 451 (1970).
6. R. F. Doolittle, *Biochem. J.* **94**, 735 (1965).
7. J. R. Shainoff, B. Lahiri, F. M. Bumpus, *Thromb. Diath. Haemorrh. Suppl.* **39**, 203 (1970); B. Lahiri and J. R. Shainoff, *Biochim. Biophys. Acta* **303**, 161 (1973); A. Z. Budzynski, V. J. Marder, J. R. Shainoff, *J. Biol. Chem.* **249**, 2294 (1974).
8. G. Shulman and J. D. Ferry, *J. Phys. Colloid Chem.* **55**, 135 (1951).
9. M. A. Lauffer, *Entropy Driven Processes in Biology* (Springer-Verlag, New York, 1975), pp. 1-5 and 84.
10. J. M. Sturtevant, M. Laskowski, Jr., T. H. Donnelly, H. A. Scheraga, *J. Am. Chem. Soc.* **77**, 6168 (1955).
11. H. Kanaide and J. R. Shainoff, *J. Lab. Clin. Med.* **85**, 574 (1975).
12. M. W. Mosesson, J. S. Finlayson, D. K. Galanakis, *J. Biol. Chem.* **248**, 7913 (1973); B. A. Cottrell and R. F. Doolittle, *Biochem. Biophys. Res. Commun.* **71**, 754 (1976); R. F. Doolittle, K. G. Cossman, B. A. Cottrell, S. J. Friezner, J. T. Hucko, T. Takagi, *Biochemistry* **16**, 1703 (1977).
13. S. V. Pizzo, M. L. Schwartz, R. L. Hill, P. A. McKee, *J. Biol. Chem.* **247**, 663 (1972).
14. M. Furlan, T. Seelich, E. A. Beck, *Thromb. Haemostas.* **36**, 582 (1976).
15. B. D. Kudryk, D. Collen, K. R. Woods, B. Blombäck, *J. Biol. Chem.* **249**, 3322 (1974).
16. A. P. Laudano and R. F. Doolittle, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3085 (1978).
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