Albumin Indirectly Modulates Fibrin and Protofibrin Ultrastructure†

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ABSTRACT: Albumin modulation of fibrin and protofibrin coagulation parameters was studied. Cation-depleted, fatty acid free, human and bovine albumins decrease fibrin clot turbidity in a concentration-dependent manner. Albumin also inhibits the formation of protofibrin gels, induced by addition of 25 μM Zn(II) to protofibrils, though it does not bind to (proto)fibrin. In order to verify that competition for cations underlies the influence of albumin, fibrinogen was dialyzed against cation-depleted albumin. Elemental analysis indicates a redistribution of Zn(II) from the fibrinogen to the albumin compartment, and the resultant fibrin clots are less turbid. Apparently, cation-depleted albumin acts as a competitor for divalent cations. The ability of albumin to compete for available Zn(II) was also expressed in gels formed by pH-jump experiments, in which fibrin monomer, maintained soluble at pH 4.9, is induced to change phase by addition of NaOH to pH 7.4. While turbidimetric evidence indicates that individual fibrin fibers simply become thinner with albumin, scanning electron micrographs (SEM) reveal a more complex effect on ultrastructure. Though albumin does not bind to the gels, fibrin gels produced with albumin show major changes in fiber ultrastructure, particularly evident in gels formed in the presence of Zn(II). These structural modifications are discussed within the context of the "excluded volume" effect, in which "crowding" by albumin alters (proto)fibrin reactivity and ultrastructure.

The polymerization of thrombin-activated fibrinogen is a complex process (Mossesson & Doolittle, 1983), which breaks down into two distinct steps, namely, linear assembly of activated fibrin monomers into linear fibrin polymers and lateral association of different chains, leading to the formation of a three-dimensional network required for gelation (Florey, 1953, 1975).

A recent series of reports describe an experimental method for studying intermediate, polymerization processes. Essentially, it was observed that thrombin- or reptilase-induced fibrin oligomers (protofibrils) are in themselves capable of forming gels upon exposure to physiologic levels of Ca(II) (1–2 mM) or Zn(II) (25–100 μM) (Marx, 1987, 1988a–c). In contrast to the linear assembly of protofibrils which is a cation-insensitive process, it was demonstrated that the lateral assembly of protofibrils (oligomeric chains) is independently augmented by Ca(II) or Zn(II), with the latter more effective by 2 orders of magnitude. Experimentally, this was expressed as increased (proto)fibrin gel turbidity or thicker fibers observed by electron microscopy.

Thrombin-generated protofibrils, which have lost both types of fibrinopeptides, FPA and FPB, coagulate following exposure to either Ca(II) or Zn(II). By contrast, protofibrils generated with reptilase, which releases only fibrinopeptide A (FPA), coagulate only by addition of Ca(II). This has been taken to indicate that the ability of these divalent cations to individually modulate coagulation processes coincides with the release of fibrinopeptides FPA and FPB (Marx, 1988a).

In pure systems (containing only fibrinogen and thrombin, ±cations), the viscoelasticity of protofibrin gels was noted as being significantly lower than that of fibrin (Marx, 1988b). Paradoxically, it was noted that though both divalent cations increased gel turbidity and apparent fiber thickness (mass/length ratio), Ca(II) and Zn(II), respectively, increased and decreased (proto)fibrin viscoelasticity (Marx, 1988b). This suggests that lateral packing of monomers within the fibers is differently modulated by the divalent cation.

Morphologically (by SEM), the cation-induced protofibrin gels were indistinguishable from clots produced directly from purified fibrinogen, cation, and thrombin. TEM microscopy of negatively stained (proto)fibrin gels showed no changes in the linear packing of composite monomers (Marx, 1988b). These findings all indicate that the lateral association of fibrin oligomers is driven and modulated by physiologic levels of Ca(II) and Zn(II).

Blood plasma, needless to say, is composed of more than fibrinogen and electrolytes. It contains large quantities of macromolecules and metabolites, some of which modify coagulation kinetics and structure. Proteins such as albumin (Wilfe et al., 1985; Galanakis et al., 1986, 1987; Carr, 1987), fibronectin (Mosesson & Amrami, 1980), immunoglobulin (Gabriel et al., 1983), elastin (Rabaud et al., 1988), and thrombospondin (Bale & Mosher, 1986) modify the rate of gelation and the turbidity of fibrin gels. Clots formed in plasma or whole blood differ significantly from those obtained in pure systems (Carr, 1988). Turbidimetric evidence indicated that fibrin formed in plasma was more massive than that formed in purified systems (Carr, 1988).

Albumin is of particular interest because it is the major protein component of blood plasma, is a widely studied model protein [for a review, see Marx (1984)], and might be expected to exert its influence in proportion to its abundance in plasma. Conflicting claims that albumin increases the rate of gelation (Wilfe et al., 1985) or decreases clotting time, optical turbidity, and fiber thickness (Galanakis & Lane, 1985) occur. Others observed that in the presence of EDTA albumin increases the magnetic birefringence of fibrin in a manner similar to that of calcium (Torbet, 1986). Turbidimetric studies indicated that albumin decreases fibrin turbidity, does not modify the kinetics of FPA release, and decreases the fibrin fiber cross

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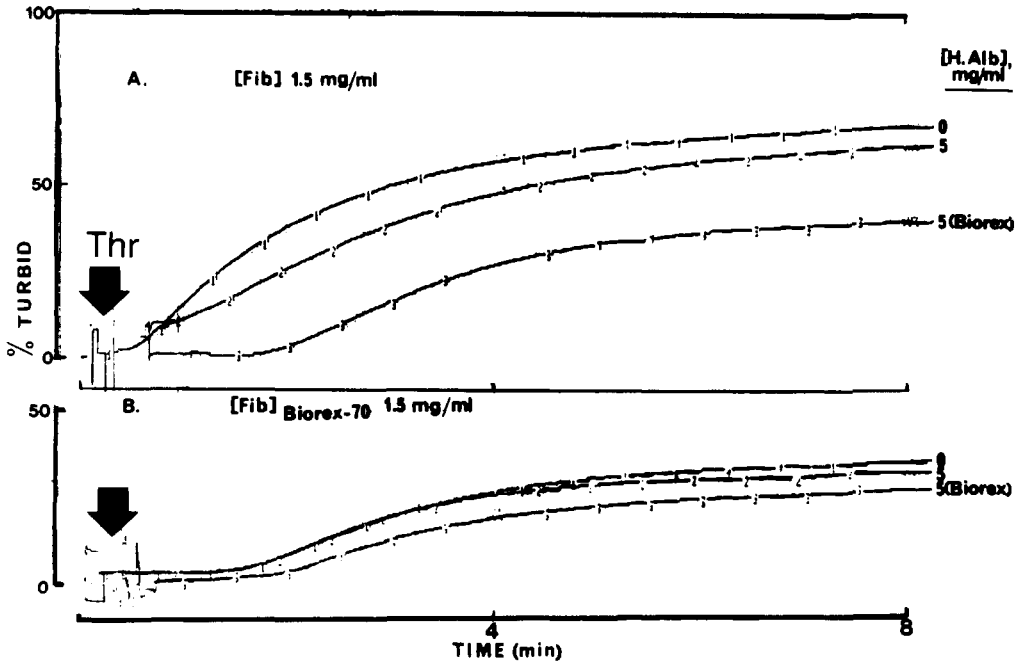

FIGURE 1: (A) Relative turbidity of thrombin-induced (0.25 unit/mL) fibrin without (top curve) and with 5 mg/mL human albumin (middle curve) and cation-depleted human albumin (lower curve). [Fib] = 1.5 mg/mL (Tris–saline buffer with no EDTA or divalent cations). (B) Same conditions but with fibrinogen also treated with cation exchange resin.

section (by TEM) (Galanakis et al., 1988). However, none of the studies considered the adventitious presence of divalent cations bound to the albumin stocks used for these experiments. Also, buffers and plasma containing EDTA or citrate were employed, and thus complicated interpretation of the results.

Within the context of examining the details of the cation-driven protofibrin gelation mechanism, it was thought worthwhile to investigate the effect of albumin. This paper describes the effect of cation-depleted albumin on fibrin and protofibrin clot turbidity and ultrastructure.

Materials and Methods

Purified human fibrinogen was from Immuno AB (Vienna) and Kabi AG (Stockholm, Sweden); bovine thrombin was Topstasin (Hoffman-La Roche, Basel, Switzerland); hirudin, Tris, and analytic-grade reagents were from Sigma (St. Louis, MO). Unless otherwise indicated, reagents and salts were diluted with 15 mM Tris–0.15 M NaCl, pH 7.4, buffer. Fibrinogen and albumin were dialyzed in Tris buffer under nitrogen at ambient temperature. Fibrinogen and albumin concentrations were determined by measuring the Abs_{280} (Zeiss PNQ 2 spectrophotometer) with a conversion factor of $E^{1\%}_{280}$ = 15 and 10, respectively.

Nomenclature. Protofibril is generated by adding thrombin to fibrinogen and inhibiting the reaction by addition of hirudin prior to gelation. In analogy to fibrin terminology, gels induced by the addition of cations to soluble protofibrils are termed *protofibrin*. The abbreviated term *(proto)fibrin* is used in analogy to *fibrin(ogen)* (Copley, 1979) and refers to either fibrin or cation-driven protofibrin gels.

(Proto)fibrin turbidity was monitored prior to, and after, addition of Ca(II) and/or Zn(II), in a four-channel P-4 aggregometer (695 nm, 37 °C; Biodata, Hatboro, PA) (Marx, 1988c). Typically, the relative turbidity of gels was set with fibrinogen for 0% turbid and fibrin (2 mg/mL), 2 mM Ca(II), and thrombin (0.25 unit/mL) for 100% turbid. Depending on the desired sensitivity and range, one could use other gels to set 100% turbid.

Albumin (fatty acid free, human and bovine) (Sigma Chemicals, St. Louis, MO), human albumin (NBC), and grade L human fibrinogen (Kabi AG, Stockholm, Sweden) were cation depleted by suspending ~250 mg of Bio-Rex 70 cation exchange resin (3.5 mequiv/mL; Bio-Rad, London) into 10 mL of protein solution, agitated 20 min, 25 °C. The suspension was centrifuged, and the supernatant protein was decanted.

In another type of competition experiment, cation-depleted albumin (1–2 mL; 20 mg/mL) was sealed in thin dialysis tubing, suspended in a fibrinogen solution (4–6 mL; 2.2 mg/mL), and stirred continuously for 1 h (25 °C). The relative turbidity of the fibrin derived from this batch was determined at a few concentrations (0.4–2 mg/mL; 0.25 unit/mL thrombin), before and after dialysis against albumin. The fibrinogen and albumin samples were all analyzed for calcium and zinc. Results are presented which were typical of experiments carried out minimally four times.

Fibrin monomer (2 mg/mL) was prepared by adding a given volume of 0.1 N acetic acid to a fibrin clot. A small amount of protein which remains insoluble was removed and the suspension centrifuged at 5000*g*, 1 min. The pH was altered to 7.4 by adding an equivalent amount of previously calibrated 0.1 N NaOH. In a typical experiment in an aggregometer microcuvette, 100 μL of buffer or albumin was mixed with 10 μL of 2 mM Zn(II), and 100 μL of fibrin monomer was added.

SEM micrographs were prepared from 1.25% gluteraldehyde-fixed gels as previously described (Marx, 1988c). Calcium was measured with a Corning Model 940 calcium analyzer [colorimetric titration calibrated to 10 mg % (2.5 mM)] and zinc with a Perkin-Elmer atomic absorption analyzer, with samples diluted in water and calibration set with zinc standard.

Results

Effect of Albumin on Clot Turbidity. A typical tracing for turbidity development of thrombin-activated fibrinogen (2 mg/mL), without and with 5 mg/mL human albumin, is shown in Figure 1A. Pretreatment of the albumin with Bio-Rex 70 cation exchange resin results in further reduction of fibrin clot turbidity (Figure 1A, lowest curve). While some

Table I: Ca and Zn Content of Albumin and Fibrinogen before and after Exposure to Cation Exchange Resin

protein	dialysis	Ca (mg %)	Zn (μg/mL)
albumin (human, Sigma), 20 mg/mL	before	0.19	0.55
	after	0.13	0.53
albumin (human, NBC), 20 mg/mL	before	0.49	0.97
	after	0.21	0.60
albumin (bovine), 20 mg/mL	before	0.67	0.60
	after	0.15	0.50
fibrinogen, 16 mg/mL	before	0.20	0.65
	after	0.10	0.52

[a] Bio-Rex 70 pretreatment. Both proteins had been previously dialyzed against Tris buffer.

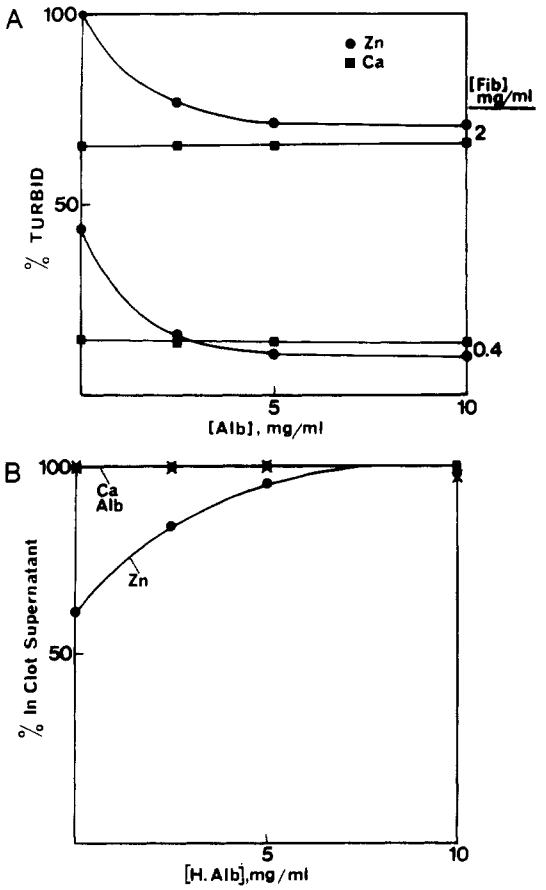

FIGURE 2: (A) Relative maximal turbidity of fibrin clot versus albumin levels. Two sets of experiments are shown with 0.4 or 2 mg/mL fibrinogen with either 2 mM Ca(II) (■) or 20 μM Zn(II) (●), clotted by addition of thrombin. (B) Relative levels of Ca(II), Zn(II), or albumin bound to the fibrin gel (2 mg/mL). Initial concentrations are 2 mM Ca(II), 20 μM Zn(II), and up to 10 mg/mL albumin.

untreated commercial albumins gave variable results, most experiments with fatty acid free albumin result in decreased clot turbidity (typical case shown in Figure 1A, middle curve). Following cation depletion by exposure to cation exchange resin, all albumin lots induce decreased clot turbidity (Figure 1B) and increased clotting time (arrow, Figure 1).

Elemental analysis of albumin, before and after Bio-Rex 70 treatment, reveals the loss of Ca(II) and Zn(II) (Table I). One can calculate that the stock 20 mg/mL albumin solutions used in these experiments contained small amounts of Ca(II), (<0.125 mM), which was reduced after treatment with Bio-Rex 70. Zn(II) is also lost by the protein after cation depletion. In is apparent that the albumin stocks contain adventitious cations which could play a significant role in determining clot turbidity and the rate of gelation.

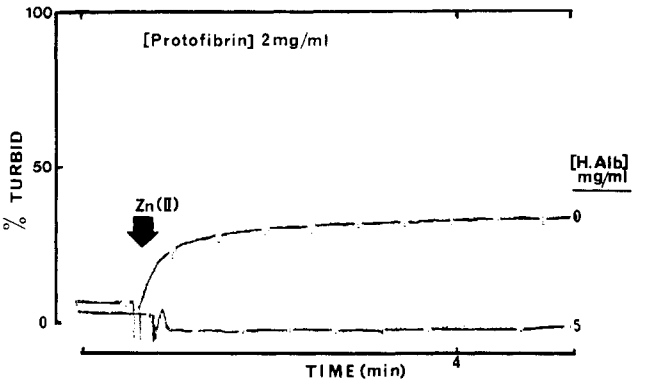

FIGURE 3: Turbidity development of protofibrin (2 mg/mL) gel induced by addition of 25 μM Zn(II) to soluble protofibril, without or with 5 mg/mL cation-depleted human albumin. Protofibril activation time (AT), 60 s; clotting time (CT), 100 s.

Table II: Competitive Dialysis of Fibrinogen and Albumin: Ca and Zn Composition before and after Dialysis[a]

protein	dialysis	mol of cation/mol of protein Ca	Zn
fibrinogen, 6.5 μM	before	9.6	3.5
	after	4.7	1.7
albumin, 74 μM	before	0.5	0.1
	after	1.9	0.2

[a] The Ca levels were determined at the limit of detection. The values are semiquantitative and are presented to indicate trends. A point worth making is that, at the albumin employed here (74 μM), it could tightly bind ($pK_D \sim 7$) equivalent levels of exogenous Zn(II) or compete for Zn(II) bound to fibrinogen ($pK_D \sim 5$) (Marx, 1988c).

One can view albumin as a competitor for available divalent cations. For example, albumin significantly inhibits Zn(II)-related clot turbidity. In the typical example shown here, 2.5 mg/mL (38 μM) cation-depleted albumin effectively negates the effect of 20 μM Zn(II) on fibrin turbidity (Figure 2A, lower set of curves). On a molar basis and in consideration of its $pK_D \sim 7$, it is not surprising that 38 μM albumin can effectively bind most of the 20 μM Zn(II). By contrast, <10 mg/mL albumin has a negligible effect on the turbidity of gels formed with 2 mM Ca(II) (Figure 2).

Effect of Albumin on Zn(II)-Induced Protofibrin. The ability of albumin to modify cation-induced protofibril gelation was evaluated. Protofibril can be induced to coagulate by 20 μM Zn(II) (Figure 3, upper curve). Cation-depleted human albumin (2.5 mg/mL) effectively prevents the Zn(II)-induced coagulation and turbidity of 1 mg/mL protofibrin (Figure 3, lower curve). Similar results were observed for a few levels of protofibrin (1–4 mg/mL), generated at a few activation times (60–100 s; CT 140 s) (not shown).

Binding of Albumin to Protofibrin. Spectroscopic analysis of the supernatant of fibrin clots, formed by 2 mg/mL fibrinogen, 0.25 unit/mL thrombin, and <50 μM Zn(II) ± 2 mM Ca(II), with or without cation-depleted human albumin (5 mg/mL), was carried out. Supernatant optical density (Abs_{280}) indicates no loss of albumin from the supernate (Figure 2B). Albumin does not bind to fibrin in the presence or absence of divalent cations. Moreover, the albumin competes with fibrin for Zn(II). Typically, for fibrin gel formed with 20 μM Zn(II), 5 mg/mL albumin increases the Zn(II) in the supernatant to more than 90% (Figure 2A).

Coagulation of Fibrinogen Dialyzed against Albumin. Fibrin turbidity significantly decreases following the dialysis against cation-depleted albumin (Figure 4). Following

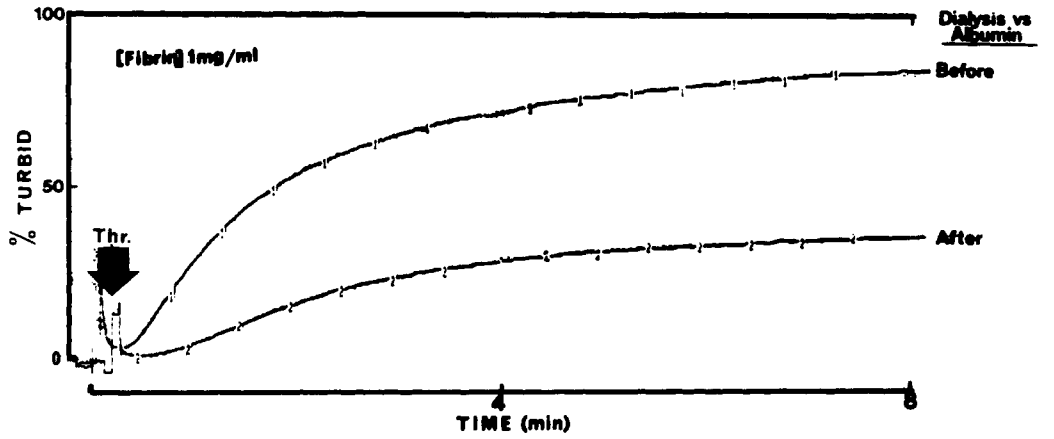

FIGURE 4: Turbidity development of thrombin-activated fibrinogen (1 mg/mL) before and after being dialyzed against 5 mg/mL cation-depleted human albumin [see Table II for redistribution of Cu(II) and Zn(II)].

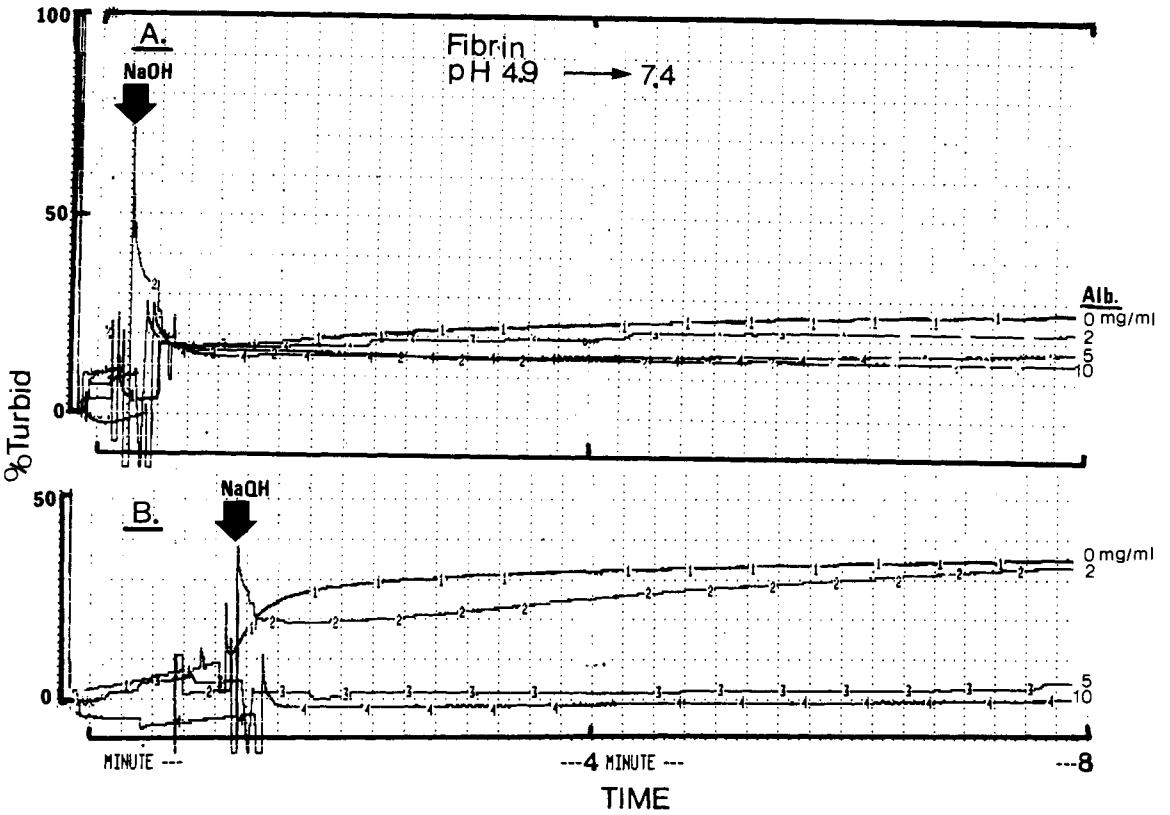

FIGURE 5: Relative turbidity (% turbid) of pH-jump experiments, in which fibrin monomer (pH 4.9) is mixed with (A) 2 mM Ca(II) or (B) 20 μM Zn(II), 0, 2, 5, or 10 mg/mL cation-depleted albumin, and NaOH (large arrow) to bring the pH to 7.4.

dialysis, the albumin gains zinc and possibly calcium, at the expense of the fibrinogen (Table II). As the albumin is sequestered within the dialysis tube, it cannot directly mediate the assembly of the fibrin gel structure. The substantial decrease of fibrin clot turbidity parallels the transfer of Zn(II) from fibrinogen to albumin.

Coagulation of Fibrin Monomer by pH Jump. Fibrin monomer, maintained at pH 4.9, can be induced to instantaneously coagulate by addition of enough NaOH to bring the pH to 7.4. The turbidity of such gels is sensitive to Ca(II) (Figure 5A) and Zn(II) (Figure 5B) in a manner similar to that previously described for fibrin and protofibrin gels. In the presence of albumin, the turbidity of fibrin monomer derived gels is decreased in a concentration-dependent manner. For example, 5 mg/mL albumin reduces the Zn(II)-related turbidity by some −30% (Figure 5B), compared to the −10% observed with 2 mM Ca(II) (Figure 5A). These results illustrate the effectiveness of albumin in competing for Zn(II).

SEM of Fibrin with/without Albumin. Fibrin formed in the absence of divalent cations or albumin is constructed from clearly delineated, serpentine fibers. With albumin, the fibers appear much less well-defined—it is difficult to point out individual fibers (Figure 6A,B). The gel appears to be more granulated or clustered, with fibrous features less attenuated. Visual examination of the clots conveys an unmistakable sense of ultrastructural change. It is difficult to convey in a few micrographs a sense of the altered ultrastructure, induced by albumin in fibrin. In contrast to turbidimetry which indicates that albumin simply decreases the mass/length ratios of the individual fibers, SEM indicates a more complex change in fiber ultrastructure.

SEM of fibrin formed with Zn(II) is similar. In conformity with the increased turbidity, fibers formed in a pure system in the presence of 20 μM Zn(II) are thicker, some monstrous

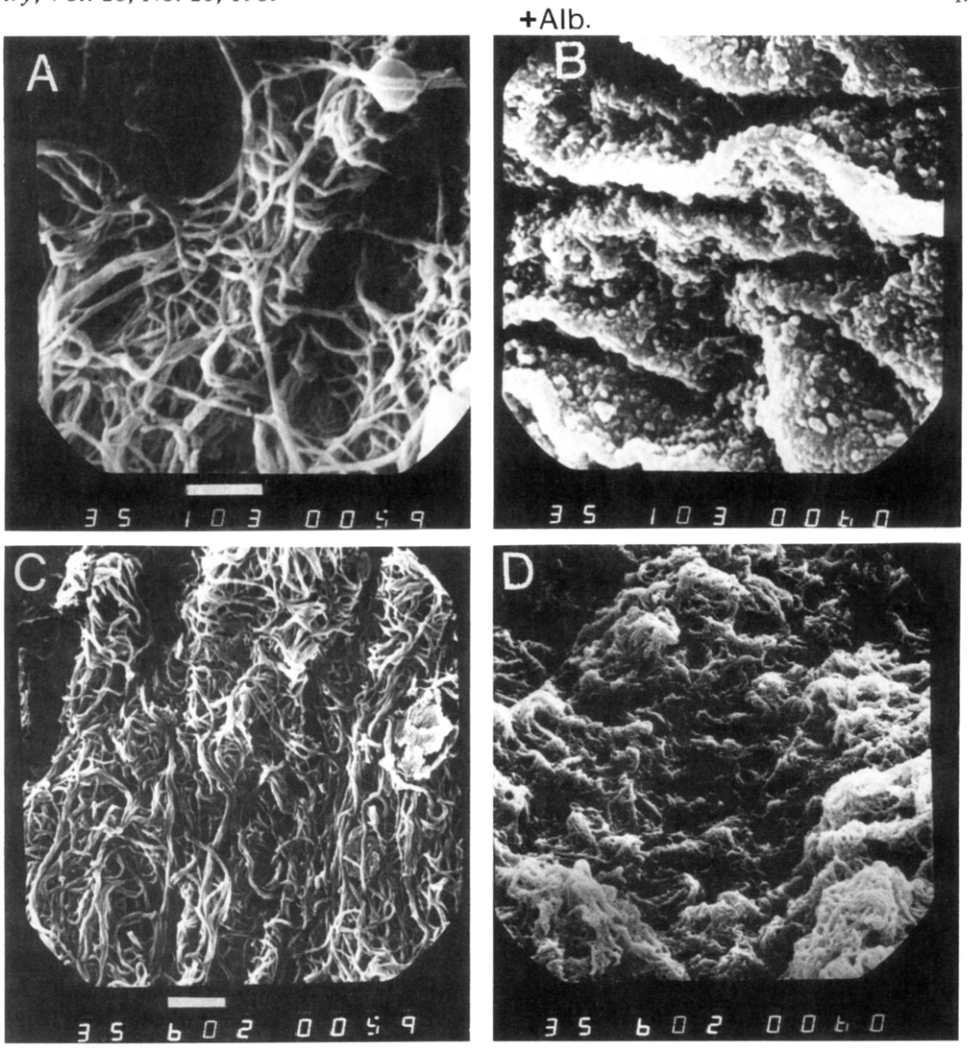

FIGURE 6: SEM (10000× and 6000×) of fibrin: 0.25 unit/mL thrombin-activated fibrinogen (1 mg/mL) clots. (A and C) No additives. (B and D) With 5 mg/mL albumin. The white bar represents 1 μm.

(Figure 7A,C), but they show clearly defined serpentine windings and supercoiling. There are individual elements where one can clearly observe the lateral alignment of multiple fiber strands which merge and diverge from one another. With albumin, it is difficult to find areas of clearly defined fibers; gels appear more granulated (Figure 7B,D). At higher magnification, the fibers also qualitatively differ. The sample with albumin has lost "definition" or "line".

The fixation and preparation of samples for SEM is a long process fraught with possibilities for modifying sample structure. However, our experience with these and other samples indicates that the micrographs describe the actual gel ultrastructures.

Discussion

Albumin is the major transport protein in the blood. In addition to binding divalent cations [Ca(II), Zn(II), Cu(II), Ni(II), Hg(II), and Pb(II)], it binds many metabolites (i.e., bilirubin) and drugs via "cation bridges" (Marx, 1984). The two amino acid sequences on albumin which are relatively specific for Zn(II) are composed of imidazole with a carboxylate side chain or peptide carbonyl oxygen (Breslow, 1973). In plasma or serum, albumin binds some 66% of the available Zn(II) with $pK_D = 7.0$ (Henkin, 1975). This is some 2 orders of magnitude tighter than that of zinc binding to fibrin(ogen) ($pK_d \sim 5$) (Marx, 1988c). As exhibited by the samples used in this study, even purified albumin retains significant amounts of both Ca(II) and Zn(II) (Table I). Treatment of albumin with cation-exchange resin removes some 20% of the bound Ca(II) and ~66% of the bound Zn(II). It is within this context that we shall attempt to understand some of the effects of albumin on (proto)fibrin parameters.

Cation-depleted albumin inhibits clot turbidity in a concentration-dependent manner, achieving a plateau at a Zn/albumin molar ratio of ~1:3 (Figures 1 and 2). Similarly, Zn(II)-induced protofibrin clots and fibrin monomer derived gels (pH jump) are less turbid when formed with albumin (Figures 3 and 5). These data, which generally conform to the turbidimetric results of other laboratories (Galankis et al., 1986, 1987; Carr, 1987), are in keeping with the model of cation-driven (proto)fibrin gelation (Marx, 1988b). To a certain extent, albumin appears to modulate coagulation parameters, such as decreased (proto)fibrin turbidity, by competing with fibrinogen for divalent cations.

Albumin itself does not bind to (proto)fibrin in the absence or presence of Zn(II) or Ca(II) (Figure 2B). Such lack of albumin binding to fibrin was also observed by others (Wilfe et al., 1985). Furthermore, experiments in which cation-depleted albumin was dialyzed against fibrinogen demonstrate that albumin competes with fibrin for Zn(II) (Figure 4, Table II). Though the albumin content in the clot is necessarily nil, the dialyzed fibrinogen produces clots with lower turbidity (Figure 4). In keeping with their binding affinities, albumin effectively competes with fibrin(ogen) for available divalent cations.

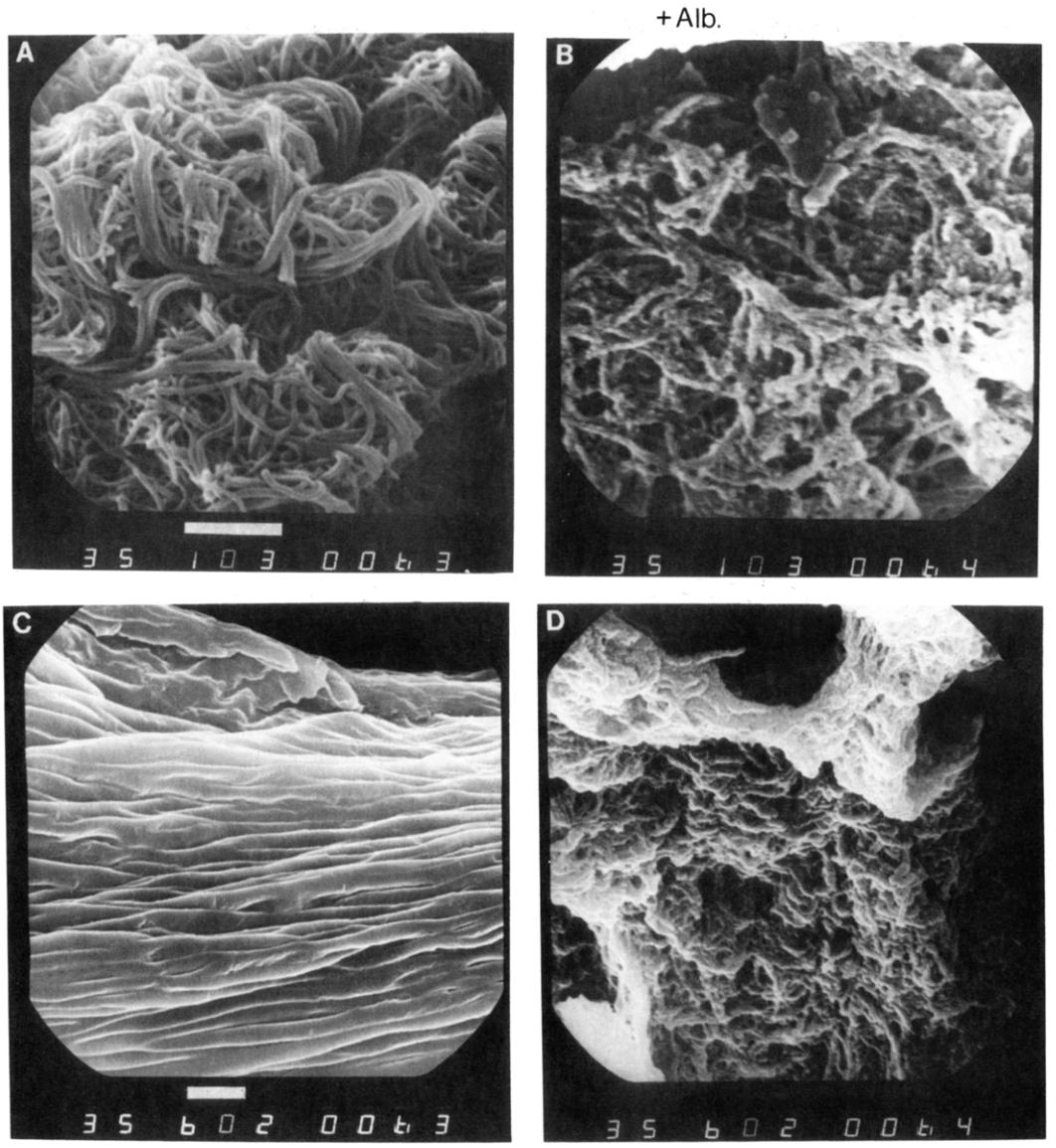

FIGURE 7: SEM (10000× and 6000×) of fibrin (as Figure 6) with 25 μM Zn(II). (A and C) No additives. (B and D) With 5 mg/mL albumin. The white bar represents 1 μm.

Competition for divalent cations underlies some of the ability of albumin to modulate fibrin and protofibrin gelation. This is evident with respect to Zn(II)-containing (proto)fibrin gels and is also apparent in the pH-jump experiments. Albumin is much more effective in decreasing the turbidity of fibrin monomer clots formed in the presence of Zn(II) than in the presence of Ca(II) (Figure 5). Consonant with its general role as a cation transport protein and depending on its preparatory history, albumin can act as either a competitor for or donor of divalent cations. One can infer that the adventitious cation composition of human and bovine albumin preparations can vary from lot to lot and that this can influence coagulation parameters. Table I summarizes the total cation binding data, which is also expressed as moles of cation per mole of protein. The little adventitious divalent cation bound to albumin which is removed by the resin has a significant effect on fibrin turbidity (Figure 1). Table II summarizes the redistribution of divalent cations following the dialysis of one protein versus the other. At the molar quantities employed here, albumin can be a significant competitor for divalent cations bound to fibrinogen. This may not be too important for Ca(II), which makes its effects felt on coagulation in the millimolar range, but can be significant for Zn(II), which is effective in the micromolar range.

Fibrin gels formed without or with cation-depleted albumin show distinct, qualitative ultrastructural differences. Pure fibrin gels exhibit fine, delineated serpentine fibers (Figures 6A,C and 7A,C). With albumin, gel appearance is somehow coarser; finer fibers are not evident (Figures 6B,D and 7B,D). The appearance of the gel fibers is qualitatively altered and is not simply a thickening or thinning, as observed upon addition of Zn(II) to pure systems (Marx, 1987). The changes induced by relatively low concentrations of albumin on fibrin gels cannot be translated into simple structural parameters (such as fiber mass/length), as the ultrastructural changes appear to be beyond those expected for simple competition for divalent cations. Therefore, light scattering at any wavelength or geometry cannot be extrapolated from pure systems to fiber ultrastructure of gels formed with albumin. Conversely, with albumin, it can be problematic to calculate fibrin(ogen) concentration on the basis of relative or absolute turbidity, as in plasma clots.

Possibly the "excluded volume" effect (Minton, 1981) might rationalize the observed effects of albumin on fibrin ultrastructure. This concept has previously been applied to the reactivity of the enzyme glyceraldehyde-3-phosphate dehydrogenase (Minton & Wilf, 1981) and to the solubility of hemoglobin S (Ross & Minton, 1979). In a "crowded" system,

a nonideal correction (Γ) needs to be applied to reaction parameters, such as the forward rate (k_o) or the equilibrium (K_o) constants, as in eq 1 and 2.

$$k = k_o\Gamma \text{ (rate)} \quad (1)$$

$$K = k_o\Gamma \text{ (binding equilibrium)} \quad (2)$$

The excluded volume effect has also been applied to the interactions of proteins localized in membranes (Grasberger et al., 1986) and the force–length relationship of molecular chains (Gao & Weiner, 1987). The idea here is that changes in the reactivity of monomeric (fibrin or protofibril) components may be ascribed to the fractional volume (Φ) occupied by other macromolecules (i.e., albumin) in the system. In the (proto)fibrin system, it is possible that, above and beyond competition for divalent cations, crowding by albumin makes its effect felt on the conformation of fibrin monomers, ultimately manifested as changes in fibrin architecture. The molar ratios at which albumin exerts crowding are expressed at surprisingly low levels. For example, 10 mg/mL (0.15 mM) albumin significantly modifies the structure of fibrin gel derived from the pH-jump experiments, again most strongly in the presence of Zn(II) but also with Ca(II) in vast molar excess (panels A and B of Figure 5, respectively). To the best of our knowledge, the excluded volume principle has not been previously applied to polymerization reactions or ultrastructural changes. At this time, it remains an interesting option which needs to be evaluated on a wider experimental basis.

To conclude, we have examined the gelation process from three different directions. We have demonstrated that the coagulation parameters of thrombin-activated fibrinogen, of cation-driven protofibril, and of fibrin monomer (pH jump) are all similarly sensitive to divalent cations and that albumin's ability to modulate gels is partly dictated by competition for divalent cations. Though albumin does not itself bind, (proto)fibrin gels formed with albumin differ structurally from those formed without. This might have bearing on the kinetics and ultrastructure of normal and pathological blood coagulation.

Acknowledgments

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Registry No. Zn, 7440-66-6; Ca, 7440-70-2.

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