Albumin Modulates Lateral Assembly of Fibrin Polymers: Evidence of Enhanced Fine Fibril Formation and of Unique Synergism with Fibrinogen†

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ABSTRACT: We identified a new property of human albumin. It enhances formation of fine fibril (or leptofibril) structures during fibrin gelation, and by nephelometric and electron microscopic measurements, this property is independent of and synergistic with that of fibrinogen. We examined fibrin aggregation using physiologic temperatures and pH and albumin:fibrin concentration ratios below those at which the known accelerating effect on fibrin aggregation occurs. An albumin concentration dependent decrease in gel turbidity maxima was consistently demonstrable in buffers containing or lacking (2–5 mM) $CaCl_2$. This decrease was shown to be induced by albumin preparations which had been exposed to 2 mM ethylenediaminetetraacetate disodium salt (EDTA), dialyzed, and tested in EDTA-free buffer. A delay in the onset of aggregation was also shown in calcium-lacking buffers by use of either reaggregating fibrin or fibrinogen aggregated with low (0.01–0.05 unit/mL) thrombin concentrations. Rates of fibrin aggregation as well as those of fibrinopeptide release were not affected by albumin, and the decrease in gel absorbance was demonstrable when solubilized fibrin was reaggregated at all final fibrin concentrations (0.2–4 μM) examined. Computed from wavelength dependence turbidity measurements (1 μM fibrin, $I = 0.20$), albumin decreased the average mass:length ratio from 8.24×10^{11} to 4.26×10^{11} daltons/cm, or from that of an approximately six to a three protofibril-thick strand. It also decreased the mean fibril radius from 48.5 to 36.4 nm but had no effect on fibril density. Electron microscopic measurements of cross-sectional fibril widths, performed on sections of glutaraldehyde-fixed gels, disclosed differences between albumin-containing and control gels which were significant by χ^2 analysis ($P > 0.001$). Fibril groups of 7–20- and 21–40-nm width together comprised 77% of fibrils formed in the presence of albumin ($n = 251$) compared to 30% of controls ($n = 309$). Conversely, coarser fibrils of 41–60- and 61–97-nm width together comprised 23% of fibrils formed in the presence of albumin and 70% of controls. This albumin effect was demonstrable by use of different monomeric albumin preparations including defatted, undefatted (unexposed and exposed to 60 °C, 10 h), chromatographically [gel exclusion and (diethylaminoethyl)cellulose] pure, *S*-(carboxymethyl)albumin, and *S*-(*N*-ethylsuccinimidyl)albumin. Chromatographically isolated albumin oligomers lacked this property, suggesting that a specific site(s) on albumin was (were) required. Spin-labeled albumin displayed no change in electron (para)magnetic spin resonance spectral measurements during its inhibition of fibrin, indicating no perturbation on albumin conformation in the vicinities of Cys-34 and of fatty acid binding sites. Albumin concentration dependence was similar when fibrin was reaggregated in buffers containing or lacking fibrinogen, indicating that the two inhibitors interacted independently with fibrin. Fibrinogen/albumin mixtures at different concentration ratios induced a characteristically synergistic decrease in gel absorbance. In sharp contrast to albumin solutions, albumin/fibrinogen solutions permitted expression of this albumin property at albumin concentrations (e.g., 556 μM) which approached those in normal plasma, and it was undiminished in the presence of protease-free (10–30% v/v) analbuminemic serum and plasma. Preincubation of albumin with isolated fibrinogen fragment D_1 also resulted in synergistic inhibition, but albumin had no effect on the dose-dependent fibrin aggregation inhibition displayed by isolated fibrin fragment E_{1-3}. Certain fibrinogen:albumin ratios designed to induce maximal inhibition yet permit gelation in the presence of either alone prevented gelation of buffer-diluted fibrin monomers. Aliquots from these which were dried and negatively stained on formvar-coated grids disclosed strands of 5–17-nm width (i.e., one to two protofibrils thick), most displaying a 60–250-nm approximate length. The amounts of ^{131}I-labeled coagulable fibrin which remained soluble in fibrinogen solutions ($I = 0.24$) were increased by albumin. Thus, albumin increases the critical fibrin monomer/oligomer concentration at which gelation occurs and enhances fibrinogen-dependent fibrin solubility. The results imply that albumin interacts with a specific site(s) on fibrin not directly participating in fibrin assembly, inducing its effect by steric hindrance. We conclude that albumin enhances formation of leptofibril-rich gel domains when other plasma factors (e.g., the presence of fibrinogen or fibrin/fibrinogen fragments near the intravascular thrombus periphery) favor formation of such structures. Available evidence indicating decreased permeability implies that such gel domains limit efflux rates from the intrathrombus environment and from intra- to extravascular space. We postulate that, relative to pachyfibril, leptofibril structures permit less hindered access to binding sites in the fibrin gel and thus present maximal numbers of such sites for interacting proteins and cell receptors.

Fibrinogen, an extended dimeric molecule about 45 nm long which displays a multidomainal (Hall & Slater, 1959; Weisel et al., 1985) structure, is converted to fibrin through release of the small amino-terminal peptides A from α and B from β chains by cleavage of Arg–Gly bonds at positions 16–17 and 14–15, respectively [for a review, see Doolittle (1984)]. Fibrin monomers thus formed aggregate spontaneously (Ferry, 1952) by binding of the activated central (E) domain of one with the outer (D, each molecule has two) domain of another

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molecule (Hantgan & Hermans, 1979; Fowler et al., 1981). A half-staggered, two-molecule-thick, early oligomer (or protofibril) results. Linear growth of protofibrils is followed by poorly understood branching and coarse fibril formation which leads to the appearance of a visible gel. The formed structures display a bimodal coarse/fine fibril distribution (Shah et al., 1982). Polymerization is inhibited by fibrinogen (Donnelly et al., 1955; Shainoff & Page, 1962; Belitser et al., 1968; Bang & Chang, 1974) since its central domain is not activated and only its D domains participate (Marder & Shulman, 1969; Knoll et al., 1984). This explains the failure of fibrin to form insoluble polymers in the presence of high molar excess of fibrinogen in plasma (Shainoff & Page, 1962; Kierulf, 1973).

Three general effects by other proteins on the biologic properties of fibrin have been described. One is acceleration[2] of fibrin aggregation originally described in plasma (Ratnoff, 1954) and more recently shown to be induced by albumin (Blombäck & Okada, 1983; Wilf et al., 1985), plasminogen (Garman & Smith, 1982), ovalbumin, IgG, and hemoglobin (Wilf et al., 1985). The second general effect is a decrease in fibrin turbidity, attributable to formation of finer fibrin mesh, induced by thrombospondin (Bale et al., 1985, 1986), actin (Janmey et al., 1985), certain IgG subfractions (Gabriel et al., 1983), and histidine-rich glycoprotein (Leung, 1986). The third effect relates to a group of different interactions which result either directly or indirectly in a functional modification of fibrin. Among these are factor XIIIa induced gelation without release of fibrinopeptides (Kanaide & Shainoff, 1975; Blombäck et al., 1985), fibrin-enhanced activation of plasminogen by tissue (Thorsen et al., 1972) and by human vascular activator (Wallén, 1977), fibrin-enhanced thrombin activation of factor XIII (Greenberg et al., 1985; Lewis et al., 1985), and enhanced covalent stabilization of fibrin by a direct effect of albumin on factor XIIIa (Galanakis & Chung, 1984). Reports of the accelerating effect in normal plasma (vide supra) raise the possibility that this modulates the well-known fibrinogen-dependent aggregation inhibition, solubility (Shainoff & Page, 1962; Kierulf, 1973), and metabolic clearance (Lee & McCluskey, 1962) of fibrin. Moreover, little is known of the effect of the plasma environment on the assembly of fibrin gel structure. The possibility that demonstrable effects on fibrin by isolated proteins are modified or not expressed in the presence of other plasma proteins has not been explored. For these reasons, we investigated the effect of a broad range of albumin:fibrin ratios on the gel structure of fibrin aggregated in the presence and absence of fibrinogen and in diluted analbuminemic serum and plasma. Albumin:fibrin ratios below those at which the accelerating effect on aggregation was shown yielded a second albumin effect, a modest but consistent decrease in fibrin turbidity, and this formed the subject of the present report.

Experimental Procedures

Fibrinogen Fractions and Fragments. Fibrinogen fractions band I, I-4, I-2, and I-9, which are ≥98% coagulable (Mosesson et al., 1972; Galanakis et al., 1978), and a coagulable fraction I-9D (Mosesson et al., 1974) from a plasmic digest equivalent to early fragment X (Marder et al., 1969) were isolated as previously described. All isolates were dialyzed in 0.3 M NaCl and tested fresh or stored at −20 or −45 °C, and all displayed the albumin effect to be described. Band I fractions often displayed minor precipitates on freeze–thawing. These were presumed to reflect the presence of fibrin since they were electrophoretically (SDS–PAGE) identical with fibrinogen but displayed appreciably lower peptide A content by HPLC assays (Kehl et al., 1981). Accordingly, plasma was either mixed with an equal volume of distilled water at 4 °C overnight or dialyzed in PO_4, pH 6.8, I = 0.07, 4 °C. Any cryoprecipitate which formed was discarded prior to fractionation. This modification yielded isolates with diminished or no insoluble material on freeze–thawing. Two band I fractions were subjected to frontal elution DEAE-c chromatography (Mosesson & Finlayson, 1963) to remove trace protein contaminants such as factor XIII. These did not differ from other fractions (above) in susceptibility to albumin inhibition. Band I fractions were routinely employed unless otherwise stated. To remove bound Ca^{2+}, fibrinogen or albumin solutions were dialyzed in 2 mM EDTA overnight and then in EDTA-free (3 times changed) buffer. Isolated plasmic fragments D_1 and E from fibrinogen (Rupp et al., 1982) and E_{1-3} from cross-linked fibrin (Olexa et al., 1981) were prepared and electrophoretically identified as originally described. The following absorbance, at 280 nm ($A^{1\%}_{1cm}$), coefficients were assumed: fibrinogen or fibrin, 15.5 (Mosesson & Sherry, 1966); albumin, 5.3 (Finlayson, 1975); fragments D_1, 20.8, and E, 10.2 (Marder et al., 1969). Preparations of fibrinogen and fibrin or their proteolytic fragments were routinely examined for purity by SDS–PAGE (9% and 3.5%) gels (Weber & Osborn, 1969), DTT-reduced or unreduced, as applied previously (Galanakis et al., 1978).

Fibrin Preparations. Fibrin was prepared as previously described (Galanakis & Mosesson, 1976) by use of human thrombin (a kind gift from Dr. J. Fenton) or batroxobin (Reptilase, Pentapharm Ltd., Basel, Switzerland). Those to be stored (−40 °C) were dialyzed in 1 mM acetic acid. Since it was experimentally established that fibrin displayed similar reaggregation inhibition by albumin whether dissolved in 500 mM Tris-HCl, pH 5.3, and 400 mM NaCl (two preparations), in 1 M KBr, pH 5.3 (one preparation), or in 40 mM acetic acid or 1 mM acetic acid, the last solvent was routinely employed. Thrombin was neutralized when necessary prior to washing the clot by a 15-min incubation with either 5 units/mL Hirudin (Sigma Chemical Co., St. Louis, MO) or 50 nM P-Pack (Calbiochem-Behring Co., San Diego, CA) for each thrombin unit per milliliter.

Fibrin Aggregation. Physiologic temperatures were employed, owing in part to the observation of the accelerating effect (Ratnoff, 1954) at such temperatures, and in part to the thermal sensitivity of fibrin assembly (Scheraga, 1983; Shainoff & Dardik, 1979). Timed measurements of fibrin aggregation were performed in a spectrophotometer equipped with temperature control and printer. For fibrin reaggregation,

[1] Abbreviations: ESR, electron (para)magnetic spin resonance; SDS–PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate containing buffer; DEAE-c, (diethylaminoethyl)cellulose (Whatman Co.); I, ionic strength; EDTA, ethylenediaminetetraacetate (disodium); r- or t-fibrin (the latter also referred to as fibrin), obtained by clotting fibrinogen with batroxobin (r) or human thrombin (t); P-Pack, D-phenyl-L-propyl-L-arginine chloromethyl ketone; KPTI, Kunitz pancreatic trypsin inhibitor; DFP, diisopropyl fluorophosphate (Aldrich Co.); DTT, dithiothreitol (Cleland's reagent, Calbiochem-Behring Co.); HPLC, high-performance liquid chromatography; cpm, counts per minute; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; for mass:length ratio calculations, c represents fibrin(ogen) concentration in grams per cubic centimeter, τ is turbidity, and λ is wavelength (nanometers).

[2] Nonprotein polymers such as dextran (Ts'ao & Krajewski, 1982), hydroxyethyl starch (Strauss et al., 1985), and poly(ethylene glycol) (Fenton & Fasco, 1974) also induce this, and dextran increased fibril cross-sectional size but decreased the fibril density, intimating previously suggested steric exclusion could not account for this acceleration (Carr & Gabriel, 1980). Also, a cationic peptide isolated from rabbit granulocytes (Carr et al., 1986) was shown to induce an accelerating effect, but whether or not this is released during normal hemostasis is not known.

fibrin solutions were diluted at least 15-fold in buffer, shown experimentally to result in no change in buffer pH following mixing. Fibrinogen mixtures with plasma or other isolated proteins were allowed to stand at room temperature for 10–30 min and at the testing temperature for 3–5 min prior to each experiment. The turbidity of fibrin gels was monitored until negligible or no further increment in absorbance occurred. High and low turbidity gels were obtained by varying thrombin concentrations (Shah et al., 1985) and ionic strength. To compute mass:length ratio, mean fiber radius, and fiber density, gels were scanned from 400 to 800 nm with a Perkin-Elmer Model Lambda 3B spectrophotometer equipped with temperature control. Plots[1] of $c/\tau\lambda^3$ vs. $1/\lambda^2$ (Carr & Hermans, 1978) yielded parallel slopes for high or low turbidity gels obtained in buffer lacking and containing albumin.

Fibrin Solubility. To radiolabel samples for solubility studies, 1 mCi of ^{125}I (New England Nuclear) was added to 100 μL of 5 μM albumin or fibrinogen in 500 mM PO_4, pH 7.5, containing two Iodobeads (Pierce Chemical Co.). After a 5–15-min incubation, free iodide was removed by passage through a Sephadex G25 (Pharmacia Fine Chemicals) column. Fibrinogen so labeled was clotted with thrombin (1 unit/mL) in unlabeled fibrinogen excess, solubilized as described above, and assayed in a γ counter. Following dilution and incubation to allow reaggregation in buffer, visible gel was carefully synerized and removed so as to express as much of the liquor as possible. The latter was centrifuged (5000*g*) to remove remaining sedimentable aggregates and passed through a 0.45-μm filter to remove unsedimentable aggregates. Alternatively, soluble fibrin was harvested at 56 °C (30 min), and the precipitate from the filtered liquor was washed 3 times in excess buffer.

Albumin Preparations. Most albumin preparations were obtained either from the New York Blood Center or from the American Red Cross and used either prior to or following heat treatment (60 °C, 10 h), and all had been isolated by the Cohn et al. (1946) procedure [for a review, see Peters (1985)]. Because the albumin effect on fibrin tended to diminish, in that increased concentrations of the same samples were needed to show the effect after storage in buffer, samples were usually dialyzed for use each time. One preparation of defatted albumin was obtained from Sigma Chemical Co. (fatty acid content less than 0.1 mol/mol of albumin). Three additional defatted albumin preparations were freshly made (Chen, 1967). To obtain albumin oligomers, freeze-dried defatted albumin stored in a humid environment (Reithel, 1963) was subjected to gel permeation chromatography using 0.01 M Tris-HCl, pH 7.4, and 0.15 M NaCl, a 60 × 1.5 cm Sepharose 6B (Pharmacia Fine Chemicals) column, and a flow rate of 30 mL/h. A fraction enriched with oligomers eluted as a minor distinct protein (280 nm) peak overlapping the beginning of the ascending portion of the albumin monomer peak and was isolated from several 15-mg application runs. The electrophoretogram of SDS-stable unreduced oligomers is shown in Figure 4, gel 7. Two chromatographic albumin isolates were kind gifts from Dr. J. S. Finlayson. One, not exposed to 60 °C, had been isolated (Cohn et al., 1946) from two donors and the other from a commercial (60 °C exposed) lot. Both preparations had been subjected to DEAE-c gradient elution chromatography (Finlayson et al., 1960) and had been obtained from pooled fractions of the ascending and center portions of the respective elution peaks. In this procedure, identifiable contaminant proteins elute in positions preceding and following these segments of the chromatogram (J. S. Finlayson, personal communication). Subfractions lacking or enriched with amber-yellow pigments were obtained from albumin, taking advantage of the observation (D. K. Galanakis, unpublished data) that when stored in self-defrosting freezers (−20 °C) and thawed undisturbed, the amber-colored moiety appears in the bottom portion of the solution, and the upper portion is visually clear. For example, the amber subfraction of a 19 mg/mL defatted albumin solution yielded an absorbance (455 nm, 1 cm) of 0.284, and its clear counterpart did not differ from the buffer blank. Since the amber-colored subfractions displayed somewhat higher albumin content, care was taken to adjust their final albumin concentrations when comparing these subfractions in fibrin aggregation experiments. Under fibrin aggregation conditions detailed in Table I, buffer B, single isolates of clear and amber-colored subfractions from the same albumin preparation did not differ in their capacity to decrease the turbidity of aggregating fibrin. Use of two different spin-labels permitted assessment of conformational changes in the vicinities of the reactive –SH group and of the hydrophobic binding sites of albumin which might have occurred during storage and during its interaction with fibrin. To probe the hydrophobic binding sites, albumin was mixed with stoichiometric quantities of the spin-label 5-doxylstearate (Molecular Probes, Junction City, OR) using published procedures (Morrisett et al., 1975) as applied by Perkins et al. (1982). Alternatively, to probe the environment of the reactive –SH group, albumin was reacted with the maleimide spin-label *N*-(2,2,5,5-tetramethyl-3-pyrrolidinyl-1-oxy)maleimide (3-maleimido-PROXYL; Aldrich Chemical Co., Milwaukee, WI) as described (Hull et al., 1975). Comparisons of all spin-labeled albumin samples with their parent or starting counterpart disclosed similar inhibitory capacity against fibrin (e.g., Figure 4, Table I). With the use of conditions described in the Figure 2A,B legend, reaction mixtures to be examined were drawn up into 100 μl of buffer in a capillary pipet sealed at one end by heat or by capillary sealant. ESR spectra were obtained on a Varian E-4 spectrometer equipped with thermostated sample chamber, by repeated scanning throughout the time course of fibrin aggregation. *S*-(Carboxymethyl)albumin was prepared (three albumin preparations) by incubation in 10 mM Tris-HCl, pH 8, overnight in refrigeration with a 100 molar excess of iodoacetic acid (3 times recrystallized in the dark in heptane followed by drying in vacuo). Unreacted compound was removed by dialysis or by desalting (vide supra). Measurement of free –SH (Ellman, 1959) was carried out by using 5,5′-dithiobis-(2-nitrobenzoic acid) (Sigma Chemical Co.) as described by Habeeb (1972). This disclosed trace or negligible free –SH following albumin exposure to this reagent, compared to 0.64 and 0.73 mol of SH/mol of unreacted albumin (two preparations). Isolated IgG and fibronectin were kind gifts from Drs. Peter Gorovic and David Amrani, respectively, and were SDS–PAGE pure.

Electron Microscopic Analyses. Samples were prepared by addition of human thrombin, 1 unit/mL, to fibrinogen solutions of 340–500 μg/mL and Tris-HCl, pH 7.4, adjusted to ionic strength 0.2 with NaCl. They were incubated at 37 °C for 30–60 min and at 4 °C for an additional several hours. Following syneresis, several volumes of 3% glutaraldehyde were added to fix the gel overnight. Samples were treated with 1% osmium tetraoxide, dehydrated in ethanol, embedded with epoxy plastic (Epon 812, Electron Microscopic Science, Ft. Washington, PA), and sectioned for transmission electron microscopy. For quantitation, transparent 5-mm-square grids were superimposed on photographs (magnification 120000×) of gel sections. Structures under grid intercepts were enumerated and their widths recorded. Preparations of liquid mixtures with no visible gel were obtained as follows. Liquor

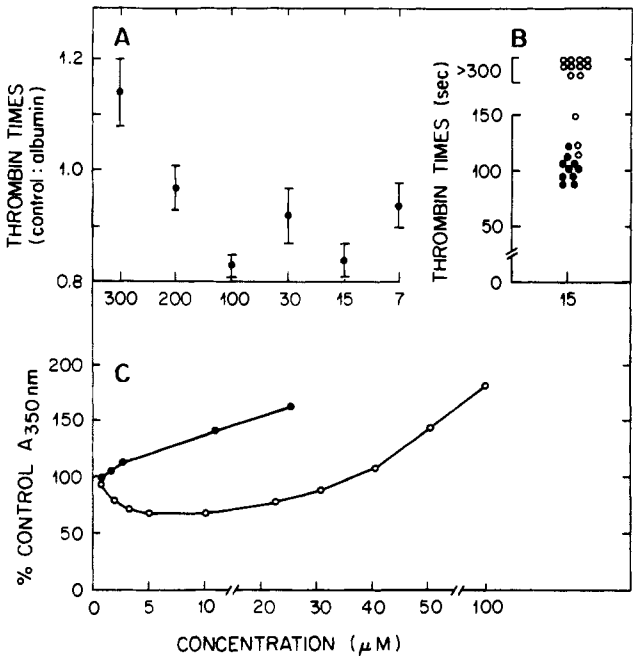

FIGURE 1: Effect of different concentrations of undefatted albumin on fibrinogen clotting times and fibrin gel turbidity. (A) Thrombin clotting time mean and range of at least four determinations are expressed as control:albumin ratios at different albumin concentrations. Human thrombin was added (0.5 unit/mL) to fibrinogen (2 μM) solutions in Tris-HCl, pH 7.4, I = 0.24, 2 mM $CaCl_2$, 37 °C. (B) Conditions as in (A) except for no $CaCl_2$ and 0.5 μM fibrinogen. Thrombin time in seconds for albumin-containing samples (open circles) and controls (closed circles) is shown. (C) Effect of increasing albumin concentrations on clot turbidity maxima (O). Each point reflects the percent of control absorbance (A), which was defined as a point on the plateau of the time course displaying no or less than 1% increment per minute. Since test and control samples were monitored until this requirement was met by both, the absorbance maxima employed were obtained at identical time points. A separate control clot, prepared in buffer lacking albumin, was measured for each albumin concentration point plotted. Fibrin (0.6 μM) was reaggregated in 25 mM Tris-HCl, I = 0.17. IgG (●) is shown as the non-albumin protein control.

was removed within 30–60 s by use of adsorbent paper from a drop of mixture on a formvar-coated 200-mesh copper grid (Ted Pella Inc., Tustin, CA). The grid was then immersed in 1% uranyl acetate (approximately 60–120 s) and the staining solution adsorbed.

Plasma and Serum. Analbuminemic serum and citrate-collected plasma were kind gifts from Dr. K. Weigand (Berne, Switzerland). These displayed trace amounts of albumin (0.25 μM) detected by immunoassay (Weigand et al., 1983), consistent with our SDS–PAGE analyses performed on undiluted samples, and 6.1 g/dL total protein. Afibrinogenemic plasma was obtained from George King Biomedical Co., Overland, KS, and was adsorbed for 60 min with (10 mg/mL) bentonite (Sigma Chemical Co.) to remove trace fibrinogen, the absence of which was shown by rocket immunoassay (Laurell, 1966). Following centrifugation, it was passed through a 0.45-μm filter (Millipore, Bedford, MA) and stored frozen. Pooled normal serum was obtained from blood bank donors, and fibrinogen/fibrin in such pools (1–3 μg/mL) was similarly removed. All serum and plasma samples used in fibrin aggregation experiments had been treated with P-Pack (50 nM), KPTI (FBA Pharmaceuticals, New York City, NY; 200 units/mL), and DFP (5 mM) for at least 30 min prior to their dialysis in buffer.

Nomenclature. The terms leptofibril (from the Greek lepto = fine) and pachyfibril (pachy = thick or coarse) are used to designate two groups of fibrin strand populations. Leptofibrils include protofibrils plus those of a diameter range intermediate between the latter and that of maximum diameter fibrils (pachyfibrils). These define all maximum and submaximum diameter structures of given measurements, with the advantage of a single rather than a double term for each group.

Table I: Effect of Different Albumin Preparations on the Absorbance (350 nm) of Fibrin Gels Aggregated Using Different Buffers at 35 °C[a]

buffer	A_{350nm} (×10³) control	A_{350nm} (×10³) albumin	% of control absorbance
A	262	181	69
B	122	42	34
C	502	354	70
D	602	495	82
E	992	789	80
F	174	52	30
G	173	126	73
H	802	521	65
I	440	544	119
J	187	277	132

[a] Determinations reflect the plateau absorbance obtained at identical time course points for each control and albumin pair. Shown is the mean of at least triplicate experiments, and reproducibility was within ±10% of the A shown. Albumin for buffers C, E, I, and J was undefatted, and all albumin samples had been dialyzed in the buffer employed. Buffers were as follows: A, citrate, 10 mM, pH 7.4, NaCl, 135 mM, thrombin, 1 unit/mL, fibrinogen, 0.6 μM, and albumin, 4 μM; B, Tris-HCl, 10 mM, pH 7.4, $CaCl_2$, 2 mM, NaCl, 200 mM, thrombin, 0.5 unit/mL, fibrinogen, 1 μM, and albumin, 4 μM; this albumin preparation lacked amber pigments (see Experimental Procedures); C, Tris-HCl, 10 mM, pH 7.4, NaCl, 150 mM, thrombin, 1 unit/mL, fibrinogen, 1 μM, and albumin, 3 μM; D, Tris-HCl, 10 mM, pH 7.4, NaCl, 135 mM, $CaCl_2$, 2 mM, thrombin, 0.005 unit/mL, fibrinogen, 1 μM, and S-(carboxymethyl)albumin, 5 μM; E, t-fibrin in 50 mM Tris–acetate, pH 5.3, and 400 mM NaCl reaggregated (1.7 μM) in 15 volumes of PO_4, 50 mM, pH 6.4, NaCl, 75 mM, and albumin, 12 μM; F and G, effect of the same albumin preparation before (G) and after (F) EDTA treatment on EDTA-treated fibrinogen; Tris-HCl, 10 mM, NaCl, 230 mM, thrombin, 3 units/mL, fibrinogen, 4 μM, and albumin, 12 μM; H, effect of EDTA-treated albumin (6 μM) on fibrin (3 μM) reaggregation; buffer, 50 mM Tris-HCl, pH 7.4, and 100 mM NaCl contained EDTA-treated fibrinogen (0.5 μM); I and J, effect of 3.5 μM albumin oligomer isolate (Figure 4, gel 7) at ionic strengths of 0.15 (I) and 0.20 (J) in buffer, pH 7.4, containing 5 mM $CaCl_2$ and fibrinogen (0.7 μM) following addition of thrombin (0.05 unit/mL).

RESULTS

Studies using a broad range of albumin concentrations disclosed an inhibitory effect on aggregating fibrin (Figure 1A–C, Table I) when albumin:fibrin ratios were below those which induced the accelerating effect. Clotting times typically displayed overlaps in the range of values and the 95% confidence limits between control and albumin-containing mixtures (Figure 1A). With control clotting times of approximately 100–130 s, no clot could be detected in most albumin test mixtures, but a few values remained within the control range (Figure 1B). More readily shown was the decrease in turbidity of clots formed by the use of thrombin, or reaggregated fibrin (Figure 2), provided sufficiently low albumin concentrations were used. A delayed rise in turbidity could be shown with reaggregating fibrin, and this could also be shown with thrombin-induced aggregation.[3] Computed mass:length ratio and fibril mean diameter (Table IIB) (obtained by use of wavelength dependence measurements yielding parallel slopes;

[3] Most but not all fibrin preparations showed this delay. Albumin induced a similar delay when aggregation was initiated by low thrombin concentrations (e.g., 0.01–0.05 unit/mL). For example, fibrinogen (0.5 μM) in PO_4, pH 7, I = 0.16, 35 °C, yielded a lag in rising turbidity of approximately 6 min in the presence of (4 μM) albumin, compared to that of 3 min of the buffer control.

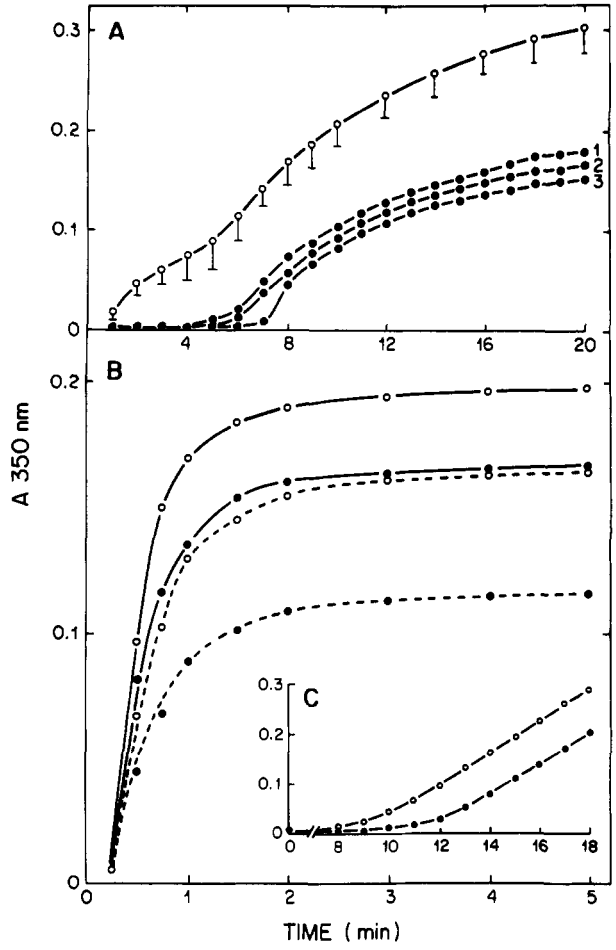

FIGURE 2: Effect of albumin (●) on the time course of fibrin turbidity at different aggregation conditions. (A) Effect of undefatted (1), defatted (2), and DEAE-c-chromatographed (3) albumin (4 μM) on reaggregating[3] t-fibrin (0.5 μM) in 80 mM PO_4 and 50 mM NaCl, pH 7, 37 °C. Brackets indicate range of triplicate buffer (○) controls. Single clot preparations, monitored for 90 min, yielded absorbances (350 nm) of 0.388 and 0.270 for control and undefatted albumin, respectively, and were examined further. Following careful syneresis, no fibrin could be discerned in the supernatant liquor by SDS–PAGE on 3.5% gels (not shown). The amounts of coagulable fibrin in another albumin test sample assessed by ^{125}I-fibrin measurements (see Experimental Procedures) were within 3% of control using these buffer conditions. (B) Effect of undefatted albumin on thrombin-induced aggregation in the presence(—) and absence (- - -) of 2 mM $CaCl_2$. To fibrinogen (3 μM) solutions (pH 7.4, I = 0.18, 35 °C), thrombin (3 units/mL) was added and mixed for 15 s. (C) Effect of defatted albumin [6 μM (●)] on reaggregation of r-fibrin (solubilized, pH 5.3; see Experimental Procedures) diluted to 1.7 μM in buffer as in (B), without $CaCl_2$.

see Experimental Procedures) were decreased by albumin. Under the conditions employed and assuming a protofibril strand value of 1.5×10^{11} daltons/cm (Carr & Hermans, 1978), albumin decreased the average mass:length ratio from that of an approximately six to that of a three protofibril-thick strand. The average fibril radius was also decreased by approximately 25%, in the presence of albumin. Fibril density values were similar, suggesting the decrease in opacity reflected differences in the number of laterally assembled protofibrils rather than in the mode of protofibril assembly. The rate of aggregation, calculated in several experiments by use of absorbance increments as shown in Figure 6, was not altered by albumin. Release rates of peptides A and B, at different thrombin concentrations, were also not affected. For example, at 0.05 unit of thrombin/mL, six separate time course determinations, encompassing release of 20–90% of the total fibrinopeptide content, yielded no differences between albumin and control clot preparations. Similarly, no differences could be discerned when individual release rates of A or B were calculated.

Table II

(A) Albumin-Induced Decrease in Fibrin Gel Turbidity (pH 7.4)[a]

condn	A_{350nm} (×10³) ± SD: albumin	control	% control
a	113 ± 12	175 ± 16	65
b	212 ± 27	293 ± 17	73
c	449 ± 13	683 ± 40	67
d	94 ± 11	167 ● 8	56

(B) Effect of 20 μM Albumin on Fibril Mass:Length Ratio (μ), Fibril Radius (r), and Fibril Density (δ)[b]

	albumin	control
μ (daltons/cm)	4.26×10^{11}	8.24×10^{11}
r (nm)	36.4	48.5
δ (daltons/cm³)	1.04×10^{22}	1.12×10^{22}

[a] Performed at two different ionic strengths, a (0.20) and b (0.15), in the presence (b) and absence (d) of 5 mM $CaCl_2$, I = 0.15, and in low (b) and high (c) turbidity gels from the same fibrinogen solution (I = 0.15). Shown are means ± standard deviation (SD) (n = 5). Determinations for conditions a, b, and c show single albumin (5 μM) preparations dialyzed and tested in 10 mM Tris-HCl, pH 7.4, containing 5 mM $CaCl_2$, and fibrinogen (band I, 0.9 μM) dialyzed in the same buffer. Determinations for condition d show five separate EDTA-treated albumin (8 μM) lots tested on one EDTA-treated fibrinogen (1 μM) preparation (see Experimental Procedures) in EDTA-free buffer (I = 0.15). Aggregation (37 °C) was induced by thrombin, 2 units/mL) for conditions a, b, and d and 0.05 unit/mL for condition c (to obtain higher turbidity gels). [b] Computed according to Carr and Hermans (1978). To obtain low turbidity gels for these measurements, fibrinogen (4 μM) was clotted in pH 7.4 buffer (I = 0.24, 5 mM $CaCl_2$ containing 200 units/mL KPTI) by thrombin (3 units/mL, 35 °C). Aggregation was monitored for 20 min, 633 nm, and gels were scanned from 400 to 800 nm as detailed under Experimental Procedures.

Inhibition, assessed as the decrease in turbidity of the formed gel, was dose dependent at a range of albumin:fibrin molar concentration ratios of approximately 0.5:1 to 5:1. As illustrated in Figure 1C, higher ratios did not induce a further decrease in absorbance. This concentration dependence was demonstrable at a broader range of albumin:fibrin ratios when increased ionic strength buffer was employed (data not shown). This albumin inhibition was more marked when increased ionic strength buffers were employed. For example, the absorbance of 2 μM fibrin (I = 0.22) decreased to 70%, 47%, and 32% of control when formed in the presence of 2, 4, and 8 μM albumin, respectively. The corresponding values for I = 0.15 were 93%, 73%, and 57%, respectively. Moreover, high ionic strength increased the maximum albumin concentration at which inhibition could be shown. When a single albumin preparation in 2 μM fibrinogen was used (0.5 unit/mL of thrombin) and I = 0.15, inhibition could not be shown at 50 μM albumin whereas at I = 0.20 inhibition could be shown even at 200 μM albumin. The dose-dependent inhibition (e.g., within or below a 5-fold albumin molar excess) was demonstrable at all fibrin (0.2–4.0 μM) concentrations, was consistently shown with different fibrinogen preparations tested (see Experimental Procedures) and was similar whether fibrin was aggregated in the presence or absence of fibrinogen (Figure 3).

The inhibitory activity was demonstrated by use of 11 different albumin preparations. These included defatted (three preparations), DEAE-c chromatographic (two) fractions, a fresh isolate not exposed to 60 °C, and one which had been subjected to exclusion chromatography following isolation. At decreasing temperatures (i.e., ambient and 15 °C), fibrin aggregation inhibition by albumin was always less than that at physiologic (35–37 °C) temperatures. Also, the decreased

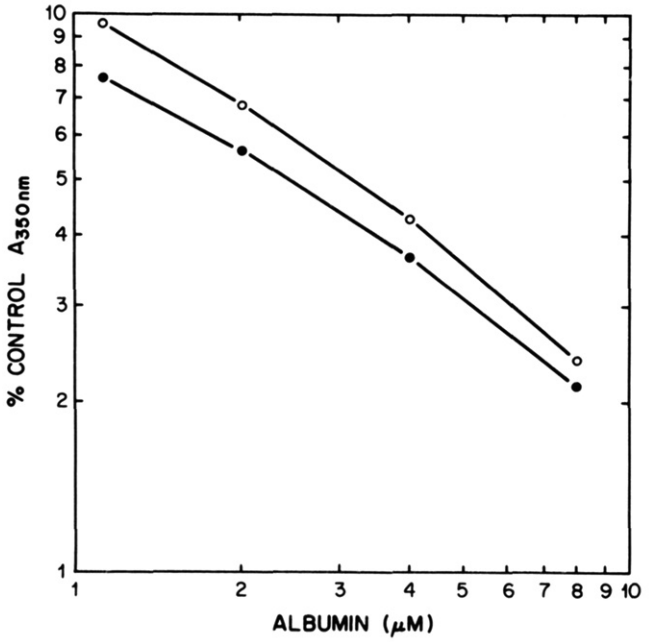

FIGURE 3: Similar albumin concentration dependent decrease in turbidity in the presence [(●) lower graph] and absence [(○) upper graph] of unconverted fibrinogen, expressed as percent of control absorbance (30 min) on double-log scale (10 = 100% on vertical axis). Fibrin (0.5 μM, lower graph) was reaggregated in 0.5 μM fibrinogen in buffer as in Figure 2B, containing 2 mM $CaCl_2$. Fibrin reaggregated in the absence of fibrinogen yielded a similar slope (not shown) in the approximate position of the upper graph. In the second series [upper graph (○)], fibrinogen (2 μM) was aggregated by addition of thrombin (1 unit/mL). Each point reflects a separate experiment with its own control. Lines are drawn between points to guide the eye; differences between the two lines are experimental and not significant.

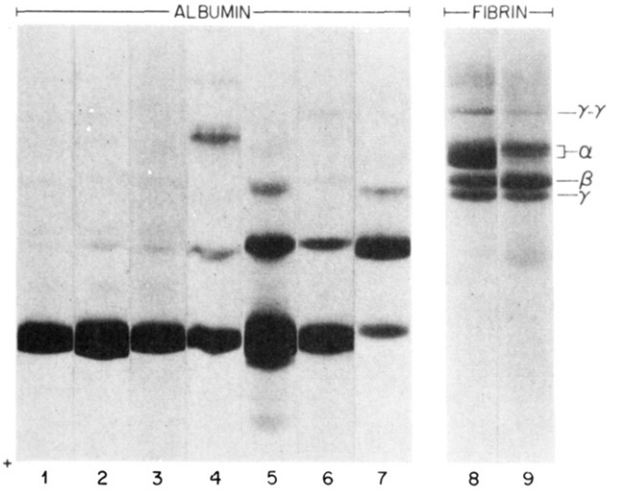

FIGURE 4: SDS–PAGE electrophoretograms of different albumin preparations (3.5% gels, lanes 1–7) and of reduced fibrin clots (9% gels, lanes 8 and 9). Defatted, undefatted, and *S*-(carboxymethyl)albumin samples are shown in gels 1, 2, and 3, respectively. Those in gels 1 and 2 displayed fibrin reaggregation inhibition, shown in Figures 2A, and that in gel 3 as shown in Table I, buffer D. Gels 4–6 show defatted albumin enriched in oligomer forms, and a chromatographic fraction from one (gel 5) preparation is shown in gel 7. Gels 8 and 9 show reduced fibrin (I-2) reaggregated as in Figure 2A, at 60 min (37 °C), displaying no proteolytic degradation in the presence of undefatted albumin (gel 8, band overlapping that of fibrin α and cathodal to that of β chains).

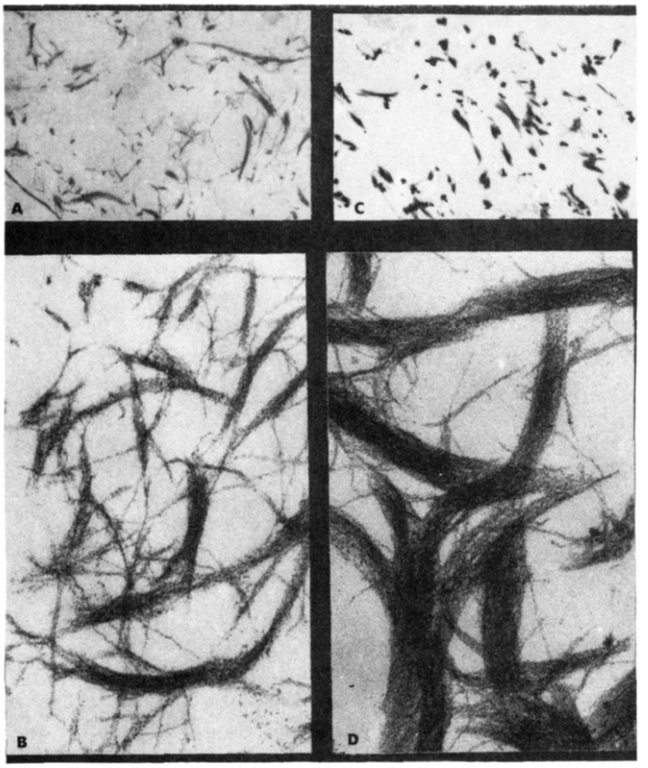

FIGURE 5: Comparison of sections of glutaraldehyde-fixed fibrin gels obtained in the presence (A and B) and absence (C and D) of defatted (10 μM) albumin, compared at magnifications of 14000× (A and C) and 66000× (B and D), respectively. Fibrinogen, 1 μM, was clotted, 37 °C, 60 min, with thrombin, 1 unit/mL, in Tris-HCl, pH 7.4, I = 0.20. Clots were washed several times undisturbed with distilled water, and portions were teased off for SDS–PAGE to ascertain that no proteolytic degradation occurred. Remaining clot was fixed in 3% glutaraldehyde overnight (see Experimental Procedures), and thin sections were cut from each of triplicate blocks.

turbidity (Tables I and IIA, Figure 2) was less pronounced and the delay in onset of aggregation was abolished (not shown) by calcium. When fibrinogen and albumin had been dialyzed in 2 or 5 mM $CaCl_2$, the albumin effect was consistently shown (Tables I and IIA) and appeared more marked than that of undialyzed samples tested in calcium-containing buffer. Moreover, the effect was displayed by EDTA-treated albumin (five preparations) tested on untreated or EDTA-treated fibrinogen (Tables I and IIA). Even so, two albumin preparations displayed loss of this property on storage (see Experimental Procedures), which could not be related to increased polymer formation, but expressed it readily in the presence of 5 mM $CaCl_2$. Oligomeric albumin forms (Table I and Figure 4) also failed to decrease fibrin turbidity, but this could not be corrected by calcium (not shown). Thus, the presence of calcium modified (i.e., usually diminished) the expression of this albumin property but did not abolish it. Also, the presence or absence of other known noncovalent contaminants of albumin such as citrate (Witwicki et al., 1982) and fatty acids (Saifer & Goldman, 1960) had no appreciable effect on this albumin property (Figure 2 and Table I).

Electron microscopic assessment of fibrin gels permitted comparison of fibril distributions by use of fibril width measurements. Ultrathin sections (triplicate blocks made from each preparation) displayed differences between control and albumin samples, illustrated in Figure 5. That is, albumin gel sections were consistently enriched with leptofibril strands, in contrast to control gels, and this was pursued by fibril population measurements. Mean cross-sectional fibril width in albumin gels was 28 ± 14 nm (SD, n = 43) compared to 57 ± 28 nm (n = 31) in controls, and width medians of these groups were 42 and 54 nm, respectively. Size distribution was calculated by subgrouping fibrils into four different width classes (Table III). Substantial differences in the fibril distributions between albumin and control gels were shown

Table III: Comparison of Distributions of Fibrils of Different Width in Albumin and Control Gel Sections[a]

fibrils (nm)	7–20	21–40	41–60	61–97
albumin, n = 251	108 (43)	85 (34)	38 (15)	20 (8)
control, n = 309	44 (14)	49 (16)	111 (36)	105 (34)

[a] Values indicate the number of fibrils measured (percents shown in parentheses) and were obtained from electron microscopy sections illustrated in Figure 5, at 120000× magnification. All fibrils measured were grouped into four subpopulations differing by width range in nanometers as shown. Included in the 61–97-nm control group are nine (3%) fibrils which ranged between 98 and 108 nm (no albumin-containing gel fibrils measured above 97 nm). Use of a transparent grid (consisting of 5-mm squares, see Experimental Procedures) permitted random identification and measurement of single structures under grid intercepts.

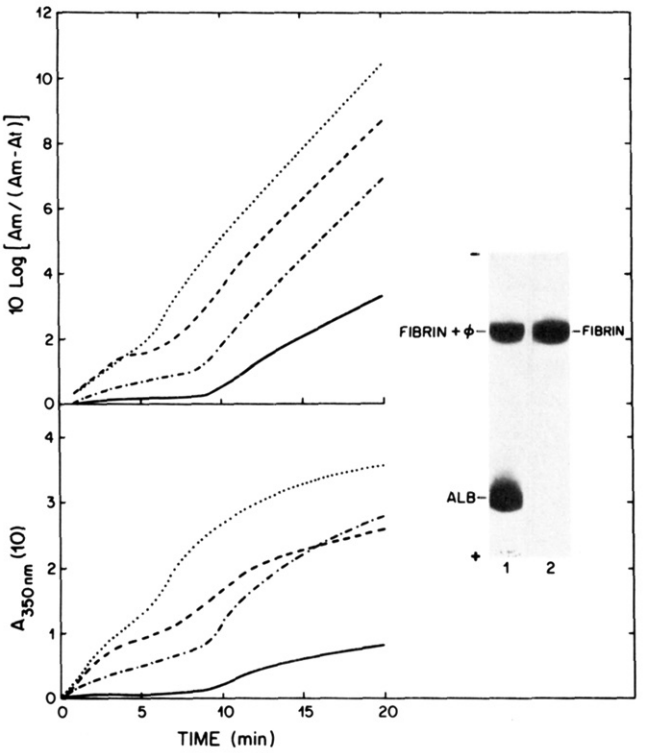

FIGURE 6: Time course of synergistic (—) inhibition of fibrin reaggregation by albumin and fibrinogen at high molar excess of the two inhibitors. Fibrin (···) was diluted 20 times to 3 μM in 50 mM Tris-HCl, pH 7.4, ionic strength 0.17, KPTI 50 units/mL, at 37 °C. Albumin (- - -), 250 μM, had been defatted fresh and dialyzed in this buffer. Albumin/fibrinogen mixtures were prepared at least 30 min prior to testing. Fibrinogen (–·–) concentration was 7 μM. The upper graphs reflect the computed rate values from a similar experiment, using 10 log $[A_m/(A_m - A_t)]$ where A_m reflects the absorbance at 60 min and A_t the absorbance at different time points. It shows a change in slope, a slower reaggregation, by fibrinogen/albumin solution as compared to their respective controls. The SDS–PAGE (3.5%) gels show the preparations used to obtain the turbidity measurements shown. For these experiments, fibrin/fibrinogen (ϕ) and defatted albumin (ALB; approximately 2.5 μg/μg of fibrin + ϕ) were incubated in buffer for 90 min at 37 °C prior to electrophoresis. They show fibrinogen/fibrin remaining intact and albumin lacking SDS-resistant oligomers. A lower albumin excess for gel 1 was necessary owing to (a) the much higher excess used in the aggregation mixtures tended to trail and obscure the positions where albumin oligomer bands migrate and (b) there was a need to apply a sufficient fibrin + fibrinogen load so that any minor (i.e., degraded) forms could be discerned.

by this comparison, and the differences proved to be significant ($P < 0.001$) by the χ^2 analysis. Albumin gels displayed a population shift toward fine (7–20 nm) and intermediate (21–40 nm) width structures. These two subgroups together accounted for 77% of strands in albumin-containing as compared to 30% in control gels. Conversely, fibrils in the two higher width (41–60 and 61–97 nm) subgroups together comprised 23% of strands in albumin-containing gels compared to 70% in control gels. No differences in fibril banding periodicity between control and albumin samples were discerned (data not shown). Thus, these direct measurements using ethanol-dried gels (see Experimental Procedures) yielded results clearly consistent with those obtained in undisturbed gels showing a reduction in mean fibril diameter by approximately half (vide supra).

Table IV: Albumin-Induced Percent Decrease in Gel Absorbance of Fibrin Reaggregated in the Presence of Fibrinogen[a]

fibrinogen	albumin	fibrinogen + albumin
71 ± 22 (43–95)	86 ● 11 (74–100)	11 ± 8 (5–27)

[a] Values are expressed as mean ± SD, with the range in parentheses, for six experiments. Buffer was 50 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$, and 150 mM NaCl, 37 °C. Fibrin concentrations ranged from 0.5 to 3 μM. Fibrinogen and defatted albumin concentrations were equimolar and 10-fold molar those of fibrin, respectively.

Comparisons of the effectiveness of albumin and fibrinogen (Figure 3) raised the possibility that their inhibitory effects are additive and led to further investigations. When their maximum inhibition was compared, increasing the molar excess of fibrinogen over fibrin resulted in a corresponding decrease in fibrin turbidity (not shown). This is in agreement with reported work (Belitser et al., 1968) but in sharp contrast to the more limited inhibition shown by albumin (Figures 1 and 2). The presence of both inhibitors (Figure 6 and Table IV) always yielded a characteristically greater than additive inhibition of fibrin reaggregation at all tested (0.2–3.0 μM) fibrin concentrations. With albumin concentrations sufficient to induce the accelerating effect in the absence of fibrinogen, the presence of fibrinogen abolished the acceleration and resulted in more marked inhibition than that of fibrinogen solutions.[4] Even when control fibrinogen and albumin concentrations were designed to have little or minimal effect on gel turbidity (Figure 6), their mixture induced lower gel turbidity, indicating the synergism. This was further explored by use of increased fibrinogen excess (a fibrinogen:fibrin ratio of 2:1 or greater) designed to achieve maximum inhibition and yet permit detectable gelation and rise in turbidity in control samples.[4] Figure 7 illustrates the results of a typical experiment. The presence of albumin resulted in no rise in fibrin turbidity and in no visible gel formation irrespective of the time (at least 4 h) of incubation of the mixture. By lowering the temperature (e.g., from 37 to 15 °C), a visible gel formed every time as shown, indicating that lack of gelation did not reflect loss of fibrin aggregability. This failure of rise in turbidity

[4] Fibrinogen/albumin solutions disclosed two additional characteristics. (a) A transient but rapid rise in reaggregating fibrin turbidity was followed by a gradual decrease and a stable plateau, and such samples displayed a visible but easily dispersible final gel. When conditions (i.e., fibrinogen and albumin excess) were sufficiently inhibitory so that gelation did not occur, the initial rise did occur, but the final turbidity plateau was typically far below the base line. This effect could be shown by fibrinogen solutions and was typically more marked in albumin/fibrinogen mixtures. No effect could be shown when isolated IgG (two preparations, 5-fold molar excess over fibrin) or isolated fibronectin (equimolar to fibrin) was substituted for albumin in fibrinogen solutions. (b) At albumin concentrations sufficiently high so as to induce the known acceleration and high turbidity of reaggregating fibrin (two sets of experiments), the presence of fibrinogen/albumin mixtures resulted in inhibition more marked than that of fibrinogen. For example, 2.5 μM reaggregated fibrin clot absorbance values were 1.367 in buffer (pH 7.4, I = 0.15, $[CaCl_2]$ = 4 mM), 1.568 in 556 μM albumin, 1.135 in 2.5 μM fibrinogen, and 0.851 in the fibrinogen/albumin mixture. Similarly enhanced inhibition by fibrinogen/albumin mixtures was shown when a different fibrin preparation (2.5 μM) was reaggregated in 10 mM PO_4, pH 7, I = 0.17, using 12 μM fibrinogen and 510 μM albumin.

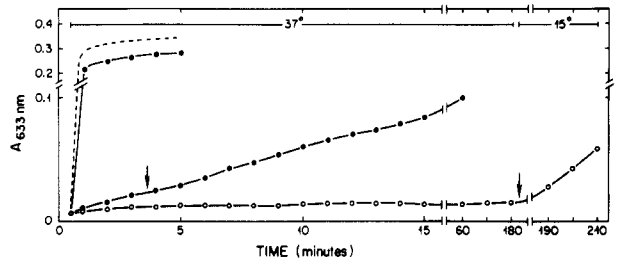

FIGURE 7: Albumin-induced failure of gelation of reaggregating fibrin in the presence of fibrinogen. Arrows denote approximate appearance of visible gel. Buffer as in Figure 6, 2 mM $CaCl_2$, 1 μM fibrin (---), in solutions containing 3 μM fibrinogen [(●) lower middle graph], 20 μM undefatted albumin [(●) upper middle graph], and both (○). Gelation was induced during the temperature transition, from 37 to 15 °C, shown by the arrow.

led to two series of related experiments. First, aliquots from two preparations which failed to form gels as a result of the albumin/fibrinogen synergistic inhibition (Figure 7) were rapidly dried on formvar-coated grids, negatively stained (see Experimental Procedures), and analyzed by electron microscopy (not shown). These disclosed the presence of fibril strands of approximately 5–17-nm width, most of which were of limited length, approximately 60–250 nm. Less frequent were fibrils of similar width but of much greater length. Assuming a protofibril width of 7 nm (Fowler et al., 1981; obtained from similarly dried and also negatively stained preparations), these measurements suggest most of the strands were one to two protofibrils thick. Second, fibrinogen-dependent fibrin solubility was investigated by measurements of the amounts of ^{125}I-fibrin remaining soluble at 37 °C (4 h) following removal of the insoluble material by syneresis, centrifugation, and filtration (see Experimental Procedures). In contrast to the buffer controls, appreciable amounts of coagulable fibrin remained soluble in albumin solutions (Table V). Fibrin soluble in fibrinogen solutions also increased appreciably when both albumin and fibrinogen were present. Although enhanced fibrinogen-dependent solubility could be shown to be dose dependent at a limited (approximately 0.3–1.5 μM) albumin concentration range, in contrast to the turbidity effect, it could only be demonstrated at high ionic strength conditions. The solubility effect appeared additive to that of fibrinogen, rather than synergistic. Thus, the combined effect of the two inhibitors (Figure 7) reflected failure of early aggregates to coalesce into a gel matrix.

The possible influence of the plasma protein environment on the fibrin inhibition by albumin was examined. In preliminary experiments, analbuminemic plasma (10% v/v, I = 0.15, 35 °C) displayed a 32% decrease in batroxobin-induced turbidity (633 nm) when its albumin concentration was corrected. This was albumin dose dependent and surprisingly demonstrable even at 200 μM albumin. Similarly, analbuminemic serum (10% v/v, see Experimental Procedures) acquired an inhibitory capacity (Figure 8A) comparable to that shown by diluted normal serum (or afibrinogenemic plasma) when its albumin concentration was corrected to that of the normal control. When such serum samples also contained added fibrinogen, the fibrinogen/albumin synergism against reaggregating fibrin was similarly shown (Figure 8B). These findings indicated that the native capacity of albumin to inhibit fibrin assembly either by itself or in synergism with fibrinogen is demonstrable in the presence of analbuminemic and of normal serum proteins.

Plasmic fibrinogen fragment D_1 and fibrin fragment E_{1-3} were used to assess if these fragments alter or abolish the albumin effect on fibrin. Final concentrations of each fragment were determined experimentally and were designed to assess if its preincubation with albumin alters the albumin effect against fibrin. Isolated D_1 displayed synergism with albumin against fibrin (Table VI) similar to that shown by fibrinogen (Table IV). This indicated that D_1 did not diminish the capacity of albumin to interact with fibrin and suggested D_1 itself did not interact with albumin. Fibrin fragment E_{1-3} differed in that its demonstrable inhibition[5] of fibrin aggregation was unaltered by its preincubation with albumin, indicating the albumin effect on aggregating fibrin was not expressed in the presence of this fragment. This absence of the albumin effect could not be corrected by increasing the albumin concentrations, relative to those of fibrin + fragment E_{1-3}.

Table V: Effect of Albumin on t-Fibrin Solubility[a]

	(A) soluble fibrin (%)
albumin	3.6 ± 1.8 (2–7, n = 6)
fibrinogen	13.6 ± 4.1 (8–19, n = 5)
fibrinogen + albumin	29.7 ± 3.4 (25.1–35.2, n = 10)

	(B) soluble fibrin (nM)		
fibrin concn (nM)	albumin	fibrinogen	albumin + fibrinogen
270	10	22	36
540	36	30	76
800	135	115	210
1000	120	182	240

[a] Determined by use of ^{125}I-labeled/unlabeled fibrin in a 1:1000 molar ratio which had been either dissolved at pH 5.3 (A) or prepared fresh in 1 mM acetic acid (B) prior to use. Values shown were calculated from the counts per minute (cpm) of fibrin diluted in Tris-HCl, pH 7.4, I = 0.24, containing KPTI (200 units/mL) and hirudin (5 units/mL) and allowed to reaggregate for 180 min, 37 °C. The cpm per clot sample was at least 5×10^4. The insoluble gel was removed as detailed under Experimental Procedures. Fibrinogen was equimolar to fibrin in all experiments. (A) Mean ± SD; fibrin, 1 μM; albumin, 0.7, 1.5, 3.5, 7, 15, 30, 70, 140, 210, and 350 μM. (Only the first six of these were employed for albumin controls. In experiments not shown, dose-dependent increments were obtained by use of 0.3, 0.7, and 1.5 μM albumin under these conditions.) (B) A similar series of experiments using four different fibrin concentrations and 20 μM albumin, computed in nanomolar fibrin.

Discussion

The most important result of the foregoing studies was the demonstration of synergism between fibrinogen and albumin against fibrin reaggregation. This occurred in the presence of other plasma proteins and at concentrations of fibrinogen and albumin which obtain in plasma.[4] Computed parameters from two different measurements (Tables II and III) indicated that albumin limited lateral fibril growth without affecting fibril density. The albumin effect on fibrin aggregation remained undiminished in chromatographically pure isolates, and no protease effect on fibrin was discerned to account for the results. Calcium is well-known to bind to both albumin (Pedersen, 1972) and fibrinogen (Marguerie et al., 1977; Purves et al., 1978; Nieuwenhuizen et al., 1979) and to enhance fibrin turbidity (Lorand & Konishi, 1964; Boyer et al.,

[5] Fibrinogen fragment E, well-known to lack amino-terminal segments of its α and β chains (Slade et al., 1976) and to have no effect on fibrin aggregation (Marder & Shulman, 1969), had no effect on the albumin-induced decrease in turbidity (data not shown). The major difference between fibrinogen E and fibrin E_{1-3} is that most molecules of the latter do not lack amino-terminal segments of their α and β chains and they bind to the DD dimer. That is, assessed by SDS–PAGE/gel scanning densitometry (two isolates examined), at least 80% consisted of E_1 and E_2, and these species have been shown (Olexa et al., 1981) to display intact amino-terminal segments of their α and of one (E_2) or both (E_1) β chains. Isolates of E_{1-3} were chosen for this study because they displayed inhibition of fibrin aggregation.

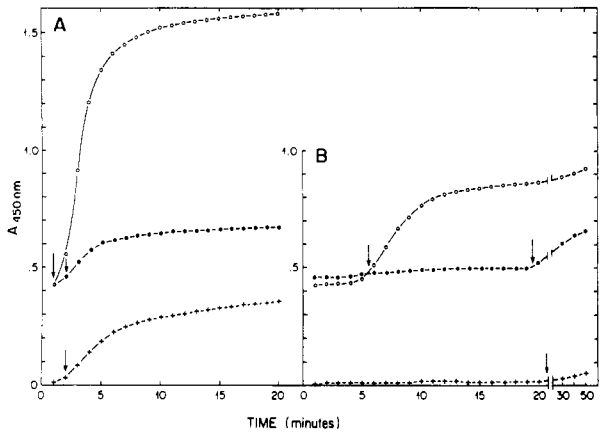

FIGURE 8: Time course of albumin inhibition of fibrin aggregation in the presence of protease-free 10% serum (see Experimental Procedures). All experiments were carried out at 35 °C in 10-fold dilutions of serum, in 25 mM Tris-HCl, pH 7.4, $I = 0.17$. Vertical arrows denote appearance of visible clot. Two samples of analbuminemic serum, one lacking and the other containing added albumin to concentrations equal to those of normal pooled serum, are compared without adjustments made for differences in total protein (normal, 6.8 g/dL; analbuminemic, 6.1 g/dL; undiluted). (A) Clot formation induced by adding batroxobin (30 μL/mL final concentration) to fibrinogen (8 μM) solutions. Analbuminemic serum without (○) and with (●) added albumin and normal (+) serum are shown. The normal serum graph shown and that of similarly treated afibrinogenemic plasma (not shown) did not differ. (B) Synergistic inhibition of fibrin (1.5 μM) reaggregation in serum by albumin and fibrinogen (3 μM, in all samples). Analbuminemic serum without (○) or with (●) added albumin and normal serum (+) are compared.

Table VI: Synergistic Decrease in Fibrin Turbidity by Albumin (ALB) and D_1 but Not by Albumin and E_{1-3}[a]

inhibitor	concn (μM)	in buffer	FGT + ALB
ALB	5	216 (92)	
D_1	0.25	172 (74)	52 (22)
	0.50	68 (35)	19 (8)
ALB	30	124 (59)	
E_{1-3}	1.3	102 (49)	96 (46)
	2.6	30 (14)	26 (12)
	2.0	315 (63)	261 (52)

[a] Values expressed as absorbance, A_{350nm} ($\times 10^3$), measurements. Computed percent, buffer controls = 100%, is shown in parentheses. Buffer controls, not shown, contained no inhibitor. Albumin concentrations were 5 and 30 μM for the D_1 and E_{1-3} experiments, respectively, as shown. FGT, fragment. For D_1, fibrin (0.5 μM) was reaggregated at pH 7.4, $I = 0.15$. For E_{1-3} (1.3 and 2.6 μM), fibrinogen (2.8 μM) ($I = 0.2$, pH 7.4, 2 mM $CaCl_2$) was clotted with 1 unit/mL thrombin. For E_{1-3} (2 μM), fibrin (2 μM) was reaggregated in 80 mM PO_4, pH 7.

1972). EDTA and citrate reportedly also bind to fibrinogen (Nieuwenhuizen et al., 1981), but our results consistently indicated that the presence or absence of calcium in buffer did not abolish this albumin effect, although removal of calcium usually served to enhance it. Results using *S*-(carboxymethyl)albumin, *S*-(*N*-ethylsuccinimidyl)albumin, or native isolated albumin implied that the single Cys-34 –SH group was not required and that unidentified covalent ligands (King, 1961) of its –SH are unlikely to account for this effect. It is also unlikely that this resulted from a nonspecific interaction, since oligomeric albumin lacked this property, intimating that the sites which interact with fibrin become inaccessible during albumin dimerization. Results using spin-labeled albumin indicated no conformational perturbations in the vicinity of its Cys-34 –SH and that of fatty acid binding sites.

The similarity of dose dependence in the presence and absence of fibrinogen compels the conclusion that albumin and fibrinogen interacted with fibrin independently. The results using fibrinogen D_1 and fibrin E_{1-3} and those showing fibrinogen/albumin synergism suggest that albumin inhibits by steric hindrance, interacting at sites allosteric to those participating directly in fibrin polymerization. That is to say, albumin does not inhibit fibrin in the presence of E_{1-3}, but it does inhibit in the presence of D_1, intimating that complementary polymerization sites residing on the respective D and E domains of fibrin do not interact with albumin. Sites elsewhere on the fibrin molecule presumably not participating in the assembly process interact in such a way, therefore, so as to inhibit formation of maximum diameter pachyfibrils. That in the presence of E_{1-3} albumin had no effect even at considerable albumin molar excess, suggests that this fragment hinders albumin from interacting with fibrin. Alternatively, possible interaction of albumin with the D domain of native fibrin remains less likely since fibrinogen had no appreciable effect on the dose dependence of the albumin inhibition and to date fibrinogen and fibrin D domains are not known to differ functionally.

The present data permit the conclusion that albumin modulates the size distribution (i.e., leptofibril:pachyfibril ratio) of fibril structures during assembly of the fibrin gel, along with other proteins in the plasma environment. On the one hand, gel turbidity has been shown to be increased, and clotting times shortened, by albumin, by serum (Blomback & Okada, 1983), and by whole plasma (Ratnoff, 1954). That such pachyfibril-rich gels resulted by use of batroxobin in analbuminemic serum (present data) implies the "b" epitope (exposed by release of B peptide) is not a critical determinant of lateral aggregation in plasma.[6] Consistent with this is evidence (Blomback et al., 1978) that in naturally formed whole blood clots only one-third of B peptide had been cleaved.

On the other hand, decreased turbidity can be induced by fibrinogen (Belitser et al., 1968; present data) by fibrinogen/albumin synergism (present data), by histidine-rich glycoprotein (Leung, 1986), and by increased thrombin concentrations (Ferry & Morrison, 1947; Buchanan et al., 1977; Carr & Hermans, 1978; Nelb et al., 1976; Hantgan & Hermans, 1979; Shah et al., 1985) attainable in normal plasma (Denson & Biggs, 1972). Intracellular proteins, thrombospondin (Bale et al., 1985), and actin (Janmey et al., 1985) have also been reported to enhance formation of low turbidity gels. These are released during cell injury, and thrombospondin has been found in plasma (Dawes et al., 1983) and actin in serum (Thorstesson et al., 1982). Increased leptofibril:pachyfibril ratio can be envisioned at the intravascular peripheral margins of thrombi where fibrinogen remains unconverted and albumin is abundant. Also, very low thrombin concentrations which yield coarse clots in buffer (Shah et al., 1985) are unlikely to do so in whole plasma, owing to thrombin inhibitors and to unconverted fibrinogen. That formation of pachyfibrils in analbuminemic plasma is inhibited by albumin is consistent with this view. Thus, even when thrombin concentration gradients favor formation of coarse gels, the plasma environment itself appears to modulate gelation conditions so as to permit formation of leptofibril-rich domains, particularly, at the intravascular thrombus periphery.

[6] There is evidence that the "b" epitope serves to reinforce or stabilize the fibrin gel (Shen et al., 1977; Shainoff & Dardik, 1983). This is indicated by comparisons of r- and t-fibrin, showing more tight binding by an order of magnitude (Shainoff & Dardik, 1983) of t-fibrin oligomers, and by the clearly more recoverable deformability (Müller & Ferry, 1984) of t-fibrin gels. Also, that only one of every three b epitopes is exposed hours after formation of thrombus in native whole blood (Blombäck et al., 1978) suggests this is sufficient to stabilize leptofibril structures prior to or concurrent with covalent stabilization by factor XIIIa.

The physiologic role of leptofibril structures remains poorly understood. Decreased permeability of leptofibril-rich gels (Blombäck & Okada, 1982; Blombäck et al., 1984; Shah et al., 1982) may limit protein efflux rates from the intravascular space, and wound edema is largely attributable to such an efflux (Grega et al., 1986). Slowed fluid egress may also serve to reduce the rate of loss of important free solutes from the intrathrombus environment, particularly those which appear released from fibrin during its gelation (e.g., thrombin; Kaminski & McDonagh, 1983) or stabilization (e.g., Glu and Lys plasminogen; Sakata et al., 1984). Several lines of evidence raise the possibility of another leptofibril function, suggested by Bale et al. (1985) in relation to the fibrin/thrombospondin interaction. That is, leptofibril structures may provide increased numbers of sites available for interactions with other proteins such as Glu or Lys plasmin (Suenson & Thorsen, 1981) and platelet receptors (Tangan et al., 1971; Dahll et al., 1983; Harfenist et al., 1985). Incorporation of fibronectin, thrombospondin (Bale et al., 1985), and α_2-anti-plasmin (Sakata & Aoki, 1980) in plasma clots is increased substantially in the presence of factor XIIIa, intimating an exclusion effect by aggregating fibrin. This is consistent with evidence that factor XIIIa stabilization decreases Glu and Lys plasminogen and plasmin incorporation in plasma clots (Sakata et al., 1984) and that fibrin monomer bound thrombin (Liu et al., 1979; Wilner et al., 1981) is released during gelation (Kaminski & McDonagh, 1983). Factor XIIIa induced stabilization of fibrin (Greenberg et al., 1985; Lewis et al., 1985), also enhanced by albumin (Galanakis & Chung, 1984), occurs rapidly in vivo (Finlayson & Aronson, 1974) and is especially critical to elasticity or "recoverable deformability" (Müller et al., 1984) of leptofibril structures. A recent report by Ciano et al. (1986) suggests a leptofibril role in the macrophage/fibrin (Colvin & Dvorak, 1975; Sherman & Lee, 1977; Gonda & Shainoff, 1982) interaction. Ciano et al. (1986) described macrophage migration through fibrin gels optimal at 1–3 thrombin units/mL while it was inhibited at lower thrombin concentrations (i.e., 0.1 unit/mL), presumably yielding pachyfibril-rich gels. That no enhancement occurred at further increasing thrombin or fibrin concentrations is unexplained and may reflect an optimum range of fibril population density required for macrophage migration, as the authors intimated, since these appeared to yield the expected leptofibril-rich gels.

Enhancement by albumin of fibrinogen-dependent fibrin solubility (present data) suggests that maximum solubility of fibrin in plasma is increased by albumin. Failure to show albumin binding to fibrin (Blombäck & Okada, 1983)[7] intimates an interaction much weaker than that of soluble fibrinogen/fibrin. Considering evidence (Shainoff & Dardik, 1979, 1980) that under physiologic conditions soluble fibrin/fibrinogen complexes are highly dissociable, disaggregation early in the time course of our fibrin/fibrinogen/albumin mixtures[4] as well as failure of gelation (Figure 7) of early polymers is consistent with enhancement of this dissociability by albumin. This raises the possibility of an albumin role in the relative rarity of intravascular thrombi in noncapillary vessels in disseminated intravascular coagulopathy (Mina et al., 1974). That is, in addition to the presumed role of increased fibrinolysis and that of the circulatory shear rates on fibrin deposition (Baumgartner & Sakariassen, 1985), increased circulating fibrin(ogen) fragments which are themselves inhibitory (Marder & Shulman, 1969) may act synergistically with albumin. Iioka et al. (1984) described an inverse relationship between circulating concentrations of albumin and fibrin complexes in some nephrotic syndrome patients. The two findings may reflect unrelated expressions of the same disease, since albumin was only moderately decreased. Hemostatic anomalies have not been described in the 23 reported analbuminemic probands (Russi & Weigand, 1983; Berger et al., 1985). However, detectable albumin concentrations were found (e.g., approximately 1–50 μM) when sufficiently sensitive assays were employed (Russi & Weigand, 1983).

[7] Using conditions detailed in Table IV, we could not show ^{125}I-albumin binding by fibrin–Sepharose chromatography (three unreported experiments), and radiolabeled albumin was quantitatively (>95%) recovered from undisturbed fibrin gels by washing with excess buffer and changing the buffer daily 3 times.

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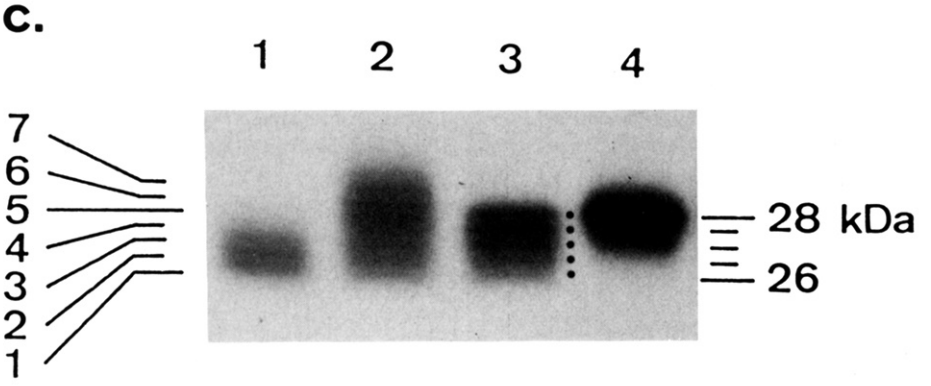
CORRECTIONS

Concerted Phosphorylation of the 26-Kilodalton Phospholamban Oligomer and of the Low Molecular Weight Phospholamban Subunits, by Juerg Th. Gasser, Michele P. Chiesi, and Ernesto Carafoli*, Volume 25, Number 23, November 18, 1986, pages 7615–7623.

Page 7618. Pertinent details of Figure 2c were lost during the printing process. An enlarged, clearer version is as follows:



Interaction of Cholera Toxin with Ganglioside G_{M1} Receptors in Supported Lipid Monolayers, by Robert A. Reed, Jairajh Mattai, and G. Graham Shipley*, Volume 26, Number 3, February 10, 1987, pages 824–832.

Page 827. During the printing process, Figures 2 and 3 were reversed. The captions are correct as printed.