Interactions among Heparin, Cold-Insoluble Globulin, and Fibrinogen in Formation of the Heparin-Precipitable Fraction of Plasma

NICOLAS E. STATHAKIS and MICHAEL W. MOSESSON, Department of Medicine, State University of New York Downstate Medical Center, Brooklyn, New York 11203

ABSTRACT Fibrinogen and the cold-insoluble globulin of plasma (Clg) are the main protein components of the heparin-precipitable fraction of normal plasma. The interactions among these proteins and heparin were examined. Heparin formed a cold-precipitable complex with purified Clg or with mixtures of Clg and fibrinogen but not with purified fibrinogen alone. Cryoprecipitation was augmented by addition of Ca\(^{2+}\) or by selection of optimal heparin levels; it was reduced or even abolished by raising the ionic strength or pH or both, or by raising the heparin concentration above that for maximum precipitation of Clg. Fibrinogen reduced the threshold for heparin-induced Clg cryoprecipitation and, by coprecipitating with heparin and Clg, increased the amount of precipitate that formed. In contrast to the heparin-precipitable fraction of normal plasma which contained both fibrinogen and Clg, that from a patient with congenital afibrinogenemia contained Clg but lacked fibrinogen. Normal plasma depleted of Clg by immunoabsorption failed to form a heparin-induced cryoprecipitate. Thus, Clg is essential for heparin-induced cryoprecipitation to occur. Fibrinogen, as assessed by chromatographic experiments with heparin-Sepharose columns, had a considerably lower binding affinity for heparin than did Clg, suggesting that it participates in precipitate formation mainly, if not entirely, by virtue of its affinity for Clg. The region of the fibrinogen molecule accounting for its precipitation with Clg appears to be localized in the carboxy-terminal segment of the Aα-chain; fibrinogen subfractions lacking this region failed to augment cryoprecipitation of heparin-Clg mixtures and, even though such species were present in normal plasma, they failed to coprecipitate in the heparin-induced complex.

INTRODUCTION

In 1954, Thomas et al. (1) reported the occurrence of heparin-induced cryoprecipitates in plasma from endotoxin-treated rabbits. This fraction has come to be known as the “heparin-precipitable fraction of plasma.” Subsequent studies by Smith and von Korff (2) and Smith (3) showed that large amounts of cryoprecipitate were formed from plasmas of patients with certain inflammatory, infectious, and neoplastic diseases to which heparin had been added. This phenomenon was also observed when heparin was added to the plasma of normal donors, but the amount produced was usually less (3, 4). Smith and von Korff (2) found that cryoprecipitate formation was dependent upon heparin concentration and that it could be enhanced by the presence of divalent cations or reduced by increasing the temperature, ionic strength, or pH. They established that fibrinogen was a major component of this fraction. This conclusion was based upon the observations that the heparin-precipitable fraction was partially coagulable with thrombin, that a portion of it was similar to fibrinogen in electrophoretic behavior and ultracentrifugal behavior (s\(_{20,w}\) 7.9 to 8.3), and that heparin-induced cryoprecipitation could not be produced in serum. They suggested that the fibrinogen in the precipitate might have been modified in some way because purified preparations of fibrinogen were only partially cryoprecipitable in the presence of heparin. They also observed a second minor component of the fraction (s\(_{20,w}\) 12.5S) which they thought might be a form of fibrinogen, and they drew attention to its similarity to a component of normal plasma subfraction I-1 termed “cold-insoluble globulin” (5). Sub-
sequent investigations of a patient with ovarian carcinoma (6), and of plasma subfractions like fraction I-1, led to characterization of “cold-insoluble globulin”, (CIg), as a major plasma glycoprotein that is structurally and immunochemically distinct from fibrinogen and all other plasma proteins (7, 8). CIg normally circulates at plasma levels of 0.33±0.1 g/liter (7); the level in serum is somewhat lower owing to incorporation of some of it into the fibrin clot (7, 9, 10), to which it can be covalently bound by factor XIIIa (10, 11).

Smith and von Korff’s original speculation (2) regarding the presence of CIg in the heparin-precipitable fraction has recently been supported. It has been shown, on the basis of electrophoretic and immunochemical analyses, that CIg is a major protein component of the heparin-induced plasma cryoprecipitate of patients with certain dermatological diseases (12). Similar studies of dermatologic patients (13), although not employing immunochemical identification of CIg, have also permitted the same conclusion. However, it is not yet clear what role CIg plays in formation of the precipitate or what the relationship is between heparin-induced cryoprecipitates and those that form spontaneously in the cold without requiring addition of heparin (the latter type are popularly termed “cryo-fibrinogen”). Many studies since the classic report of Shainoff and Page (14) have indicated that most spontaneously occurring plasma cryoprecipitates contain fibrin in a complex with fibrinogen and perhaps with other proteins as well (6). On the other hand, studies of the heparin-precipitable fraction from dermatologic patients (15) indicated that fibrinogen, but not fibrin, was a component of this type of cryoprecipitate.

To clarify some of the questions raised by studies cited above, we have undertaken a systematic investigation of the nature of plasma cryoprecipitates. The studies to be described in this report were designed to elucidate the mechanism of formation of the heparin-precipitable fraction of plasma. We have focused particularly upon the interactions taking place among the major components of this fraction: CIg, fibrinogen, and heparin.

METHODS

Preparation and analysis of fibrinogen and CIg. Fibrinogen subfractions I-2, I-4, I-8, and I-9 were prepared from outdated human acid citrate dextrose plasma as previously described (16, 17). Investigations of the amino terminal acids of these fractions (17, 18) have shown traces, at most, of glycine, an amino acid indicating the presence of fibrin in the preparation. The thrombin coagulability of each fraction was >99%. Purified human CIg was prepared by a modifica-

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1 Abbreviation used in this paper: CIg, cold-insoluble globulin.
CIG-depleted plasma prepared by either procedure behaved in a similar way in the experiments to be described.

Procedures involving heparin. Several preparations of heparin were used and included a liquid preparation for intravenous administration (Na salt, Upjohn Co., Kalamazoo, Mich.); specific anti-coagulant activity 100 U. S. Pharmacopeia U/mg and two heparin preparations in powder form (Li salt, grade IV, 158 U. S. Pharmacopeia U/mg, and Na salt, grade I, 158 U. S. Pharmacopeia U/mg, Sigma Chemical Co., St. Louis, Mo.). The heparin preparation used in any given experiment was the Upjohn product unless otherwise indicated.

The rate and extent of cryoprecipitation of plasma samples or mixtures of CIG and fibrinogen were monitored spectrophotometrically in 1-ml cuvettes as follows: CIG-fibrinogen mixtures (initial volume usually <0.2 ml) were adjusted to a final volume of 1 ml with 0.05 M sodium phosphate—0.1 M NaCl buffer, pH 7.2 (I, 0.2); plasma samples were diluted 1:2 with this buffer. For experiments involving determination of the effect of Ca2+, a 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.2 buffer (I, 0.15) was used. The filled cuvettes were placed in a Gilford spectrophotometer (model 240; Gilford Instrument Laboratories Inc., Oberlin, Ohio). The temperature of the jacketed cuvette chamber was maintained at 2°C, and the formation of precipitate (i.e., increase in light scattering) followed by absorbance measurements at 350 nm. The readings obtained for heparin-containing mixtures were corrected by using as a blank a sample of the same mixture incubated under the same conditions without added heparin. Elevations in blank readings during the course of an experiment were small (0.030 or less). The heparin-induced precipitate forming during the first 5 h in the absence of Ca2+ was too fine to appreciate the presence of any discrete particles; there was no tendency for the precipitates to settle out unless subjected to centrifugation. Maximal light scattering under most experimental conditions usually developed within 1 h and always within 3 h of incubation. Unless otherwise specified, we reported only the reading taken at 2 h. The curves were very reproducible for any given set of conditions.

The heparin-precipitable fraction from fresh normal or afibrinogenemic plasma was obtained by incubating 1–10 ml of plasma in the presence of heparin at 2–4°C for 16 h. The precipitate that formed was harvested at 4°C by centrifugation at 10,000 g for 30 min, extracted three times at 4°C with 0.15 M NaCl—0.05 M Tris-HCl buffer, pH 7.2 (I, 0.2), resuspended in the same buffer, and brought into complete solution by incubation for 20 min to 2 h at 37°C. Without the addition of heparin, no such precipitate could be recovered from normal or afibrinogenemic plasma.

For the chromatographic experiments described below, the heparin-precipitable fraction from 140 ml fresh frozen acid citrate dextrose plasma (prepared with the Li or Na heparin preparations at a final concentration of 0.10 mg/ml) was extracted once with ice-cold 0.05 M Tris-phosphate buffer, pH 7 (I, 0.1), and then dissolved at 37°C in 0.25 M Tris-phosphate buffer, pH 7. To recover the heparin precipitated with this fraction, the dissolved fraction was diluted with water to a phosphate concentration of ~0.05 M and then applied to a diethylaminoethyl-cellulose (DE-23, Whatman, Inc., Glenwood, N. J.) column (0.9 x 30 cm) that had previously been equilibrated with 0.05 M Tris-phosphate buffer, pH 7. The column was flushed with additional 0.05 M Tris-phosphate buffer until the absorbance at 280 nm had returned to near the base line. Protein remaining adsorbed to the column was then eluted stepwise with Tris-phosphate buffers of increasing molarity to remove first the fibrinogen-enriched (0.09 M) and then the CIG-enriched (0.2 M) fractions, respectively (7, 19). After this, the column was flushed with 0.5 M NaCl to displace phosphate ions and remove any residual protein that remained. The heparin adsorbed to the column was then desorbed with 2 M LiCl (28). Heparin concentration was determined by a modification (28) of the Azure A method of Jaques et al. (29), with starting heparin as the standard. The anticoagulant activity of the heparin in the LiCl eluate peak was determined in platelet-poor citrated plasma by a thrombin time method (30); the activity, expressed in U. S. Pharmacopeia units, was based upon the reported anticoagulant activity of the starting heparin.

Heparin (Li salt) was coupled to Sepharose 4B as described by Miller-Anderson et al. (31). The amount of heparin that had been coupled was determined after hydrolysis of heparin-agarose gel samples (32) by the phenolsulfuric acid method (33) and was found to be 12 mg/ml gel. Stepwise elution chromatography on a heparin-Sepharose column (0.9 x 25 cm) was carried out at room temperature or at 3±1°C at a flow rate of 16 ml/h; fractions of 3.0±0.1 ml were collected. The following buffers were used: buffer “A,” 0.05 M Tris-HCl buffer, pH 7.2 (I, 0.05); buffer “B,” 0.10 M NaCl—0.05 M Tris-HCl, pH 7.2 (I, 0.15); buffer “C,” 0.4 M NaCl—0.05 M Tris-HCl, pH 7.2 (I, 0.45); buffer “D,” 1.0 M NaCl—0.05 M Tris-HCl, pH 7.2 (I, 1.05). For certain experiments, the pH of buffer A was adjusted to 7.6 or 8.0. After each chromatographic experiment, the column was stripped with buffer D and then reequilibrated with buffer A. This affinity column was reused several times without apparent loss of binding activity.

Electrophoretic procedures. Dodecyl sulfate polyacrylamide gel electrophoresis was performed essentially as described by Weber and Osborn (34). The final gel polymerization mixture contained 0.05 M sodium phosphate buffer, pH 7, 4 M urea, 0.1% sodium dodecyl sulfate and 3.5 or 5% acrylamide. For analyses of unreduced protein (1–2 mg/ml), samples were incubated for 2 h at 37°C in 8 M urea, 1% sodium dodecyl sulfate, 0.005 M sodium phosphate buffer, pH 7. For disulfide bond reduction, dithiothreitol (14 mM, final concentration) was included. The samples were mixed with an equal volume of 50% (vol/vol) glycerol, 0.01 M sodium phosphate buffer, pH 7, and then applied to the gels. Gels were stained with Coomassie Brilliant Blue as previously described (17). Densitometric scans of stained gels were carried out with a Gilford model 240 spectrophotometer equipped with a linear transport apparatus.

RESULTS

Cryoprecipitation of CIG and CIG-fibrinogen mixtures. Purified CIG (pH 7.2; I, 0.2) at concentrations ranging from 15 to 500 μg/ml was incubated at 2°C with heparin (0.05 mg/ml). Incubation of CIG under the same conditions without heparin did not yield any detectable precipitate. In a typical experiment (Fig. 1), heparin-induced precipitation was first detectable at a CIG concentration of about 150 μg/ml. At this level of heparin, the rate of precipitation and the amount of CIG precipitated became maximal at a CIG concentration of about 300 μg/ml.

This same type of experiment was carried out in the presence of fibrinogen free of detectable CIG (<0.2%). At fibrinogen concentrations ranging from 250 μg/ml to 6 mg/ml, heparin-induced cryoprecipitation did not
take place until after CIg had been added (Fig. 2). The threshold CIg concentration for heparin-induced cryoprecipitation was markedly lower when fibrinogen was present and the amount of precipitate that formed was greater. This behavior was a clear indication that both CIg and fibrinogen had participated in the cryoprecipitation phenomenon. However, the amount of precipitate forming at this heparin level (0.05 mg/ml) increased only up to a CIg concentration of 300 \( \mu \)g/ml, as had been the case in the previous experiment without fibrinogen (Fig. 1). This suggested that it was the amount of heparin, rather than fibrinogen, that determined the maximum amount of CIg in the cryoprecipitate. As was the case with heparin-CIg cryoprecipitates, the heparin-CIg-fibrinogen precipitate could be completely dissolved by incubation at 37°C.

**The effect of heparin concentration.** The effect of heparin concentration on precipitation of purified CIg or CIg-fibrinogen mixtures was examined further (Fig. 3). Under any given set of buffer conditions favorable to precipitation, there was always an optimal range of heparin concentration at which maximum precipitation was achieved. When the heparin concentration was raised above the optimum level, the amount of precipitate at any given heparin concentration was greater in the presence of fibrinogen. In the experiment shown, the optimal heparin concentration range for precipitation of CIg was somewhat higher than it was for the CIg-fibrinogen mixture. Because the optimum level of heparin is dependent upon the CIg concentration (Figs. 1, 2), this difference seems attributable to the fact that the CIg concentration in the CIg-fibrinogen-heparin mixture was half that of the mixture of CIg and heparin.
The effect of ionic strength, pH, and CaCl₂. The effects of ionic strength and pH on CIg cryoprecipitation were examined (Fig. 4). The amount of heparin-induced CIg cryoprecipitation decreased rapidly with increasing ionic strength or pH and was completely abolished at an ionic strength above 0.3 or at a pH above 9.0. At an ionic strength of 0.05 or lower at pH 7.0 or less, CIg was precipitated at 2°C in the absence of heparin.

The effect of CaCl₂ on cryoprecipitation was also assessed (Fig. 5). Its addition greatly increased the amount of precipitation that occurred in heparin-CIg or heparin-CIg-fibrinogen mixtures and resulted in more rapidly forming and more coarsely granular precipitates which, although ultimately soluble at 37°C, did not redissolve as rapidly as did precipitates formed without added CaCl₂. The addition of CaCl₂ had no effect on CIg solutions lacking heparin or on fibrinogen-heparin mixtures lacking CIg. Consistent with this last observation, both heparin-CIg and heparin-CIg-fibrinogen mixtures showed a similar pattern of augmented precipitation in response to increasing CaCl₂ concentration, suggesting that the CaCl₂ effect is upon the heparin-CIg interaction.

The effect of fibrinogen catabolites. Human plasma fibrinogen fractions I-8 and I-9 (16) have been shown to consist of catabolic intermediate species, virtually all of which lack the carboxy-terminal portion of the Aα-chain (17). Fraction I-4, on the other hand, is comprised of less extensively catabolized fibrinogen species characterized by the presence of a very high proportion of intact Aα-chains. Heparin-induced cryoprecipitation of CIg in the presence of these subfractions was compared (Fig. 6). In contrast to the CIg-fraction I-4 mixture which, as expected, developed a much greater amount of precipitate than did CIg alone, the cryoprecipitate forming in the CIg-fraction I-8 and CIg-fraction I-9 mixtures differed only slightly from that of CIg alone.

Cryoprecipitation of normal, afibrinogenemic, and CIg-depleted plasma. Heparin-induced cryoprecipitation was carried out on normal plasma, that from an afibrinogenemic patient, or on normal plasma depleted of CIg (Fig. 7). CIg-depleted plasma exhibited only a slight increase in light scattering during the course of the experiment, whereas afibrinogenemic plasma developed a considerable amount of cryoprecipitate. Normal plasma yielded the greatest amount. As expected, the heparin-precipitable fraction harvested from fresh normal plasma was partially coagulable with thrombin, whereas that from afibrinogenemic plasma was not.

In related experiments, the optimal heparin level for CIg cryoprecipitation of normal or afibrinogenemic plasma was evaluated. Plasma was incubated with heparin at 2°C for 16 h, the heparin-induced precipitate removed by centrifugation, and the CIg level in the supernatant plasma determined by immunosassay. The precipitation behavior of CIg in these plasmas

![Figure 4](image)

**Figure 4** The influence of ionic strength and pH on heparin-induced (0.05 mg/ml) cryoprecipitation (2°C) of CIg (225 μg/ml). In the experiments involving pH dependence, the ionic strength varied between 0.15 and 0.2 (0.05 M sodium phosphate, 0.05 M NaCl buffer). In experiments involving ionic strength dependence, pH was kept at 7.2 (0.05 M sodium phosphate buffer) while varying the concentration of NaCl.

![Figure 5](image)

**Figure 5** The influence of CaCl₂ on heparin-induced cryoprecipitation of CIg and CIg-fibrinogen mixtures in Tris-HCl, pH 7.2 buffer (I, 0.15). The CaCl₂ concentration and the concentration of each protein component in the various mixtures are indicated. Heparin concentration and temperature were those given in the legend to Fig. 1.
was similar to that observed in mixtures of heparin with purified CIg or with CIg plus fibrinogen. That is, the amount of precipitate that formed at suboptimal heparin levels increased as a function of heparin concentration, whereas precipitation was progressively decreased at heparin levels exceeding those for maximum precipitation. The optimal heparin level for CIg precipitation in normal plasma (CIg level, 310 μg/ml) was between 0.15 and 0.25 mg/ml, and resulted in precipitation of 88% or more of the plasma CIg. This was also the optimal range observed for heparin-CIg mixtures at a comparable CIg level (Fig. 3). Afibrinogenemic plasma required a somewhat higher heparin concentration (~0.50 mg/ml) for maximum precipitation; probably reflecting the relatively higher level of CIg in this plasma (540 μg/ml).

Further characterization of normal or afibrinogenemic plasma was made by dodecyl sulfate gel electrophoretic analysis of the heparin-precipitable fraction (Fig. 8). The unreduced fraction from normal plasma revealed two main protein bands corresponding to those occupied by unreduced CIg and band I fibrinogen. In contrast, the afibrinogenemic heparin precipitate lacked the major fibrinogen band. In both samples, material with a slower migration rate than the CIg band of mol wt 450,000 probably represented noncovalent aggregates of CIg (23). Reduced samples of the heparin precipitate from normal plasma showed the characteristic band pattern of fibrinogen (i.e., bands corresponding to the Aα, Bβ, and γ chains, respectively) and a major high molecular weight band corresponding to the position occupied by reduced CIg subunits (not shown). The reduced afibrinogenemic precipitate possessed only the major CIg band. Because of aggregation observed in unreduced normal plasma samples (Fig. 8), densitometric analyses were not attempted on such specimens. However, the relative proportion of CIg and fibrinogen in the precipitate from such plasma was assessed by densitometric scanning.

Analytical ultracentrifugation and dodecyl sulfate gel electrophoretic analysis of CIg preparations (8) have shown that most unreduced molecules exist as two-chain (mol wt ~220,000/chain) disulfide-bridged units (mol wt ~450,000); lower molecular weight components bearing CIg antigenic determinants have also been described (23).  

Upon dodecyl sulfate electrophoresis, unreduced fractions I-4 or I-2 are resolved into two major bands, designated I and II (35). These bands differ with respect to their Aα-chain populations. Band I contains molecules with a very high proportion of intact Aα-chains. In band II, a considerable proportion of the intact Aα-chains are replaced by a remnant chain termed Aα/4; this Aα-remnant lacks a carboxy-terminal peptide having a mol wt of approximately 13,200 (calculated from the difference in size between Aα, 70,900, and Aα/4, 57,700; [35]). Unreduced fractions I-8 and I-9 display bands with more rapid anodal migration rates than band II, this is due to the fact that the average size of the Aα-remnant chains comprising these fractions is even smaller than those of molecules migrating in band II.
Figure 8: Dodecyl sulfate polyacrylamide gel electrophoresis of unreduced samples of fibrinogen fraction I-2 (\(\Phi\)) that is appreciably contaminated with CIg, purified CIg (CIg), and the heparin-precipitable fraction (HPF) from normal (Normal) or afibrinogenemic plasma (A\(\Phi\)). Electrophoresis was carried out for 3.5 h at 6 mA/gel. The two major fibrinogen bands (bands I and II) discernible in the fraction I-2 sample are indicated. The minor bands seen in addition to the main band in the CIg specimen reflect some of the multiple molecular forms of CIg.

the same as heparin that had been subjected directly to the same chromatographic recovery procedures (151 U. S. Pharmacopeia U/mg).

Adsorption of CIg to a heparin affinity column. To better delineate the temperature-dependent nature of heparin-CIg complex formation and to investigate other variables, such as ionic strength and pH, that influence its formation, we studied the adsorption behavior of CIg on a heparin-Sepharose column at 4°C and at room temperature. Under the buffer and temperature conditions we used, there was no significant binding of CIg or fibrinogen to Sepharose alone. However, with a heparin-Sepharose column, approximately 87% of the CIg (1.2 mg) applied to the column under the same conditions (1.5 mg protein; buffer A; 4°C) only 7% was retained.

Binding experiments were also carried out with normal plasma applied to the heparin-Sepharose column in buffer A (Fig. 9). Under the experimental conditions illustrated, 13% of the absorbance of the plasma, including 54% of the CIg and 2–4% of the fibrinogen, was retained by the column. The fibrinogen and CIg were subsequently recovered in the buffer C eluate peak. Dodecyl sulfate gel electrophoresis of material from this peak revealed numerous bands. One of these, a relatively weak staining band, had an anodal migration rate corresponding to that of CIg in reduced or unreduced samples. No further attempt was made in these experiments to characterize other bands in this fraction.

**Discussion**

Several previous studies, including that on a patient with ovarian carcinoma (6), have suggested that the main nonclottable component of the heparin-precipitable fraction, first clearly described by Smith and von Korff (2), would prove to be CIg. Indeed, Fyrand and Solum (12) and Matsuda et al. (13) have recently provided evidence that this is the case; our present studies confirm their conclusion. Fyrand and Solum did not specifically direct their attention toward defining the nature of the interactions of the main components of this fraction, but based upon the reported affinity of fibrinogen or fibrin for heparin (42, 43), they suggested that it is the interaction between fibrinogen and heparin that is crucial for formation of the fraction (12), and that CIg is included.
in this precipitate only by virtue of its affinity for fibrinogen. Their finding that CIg levels were not always elevated in patients manifesting increased amounts of heparin precipitate (44) also implied a secondary role for CIg in its formation. Nevertheless, our present investigations provide compelling evidence for a different sequence of interactions among the components of the heparin-precipitable fraction. Most importantly, they indicate that CIg, not fibrinogen, plays the central role in mediating the formation of a heparin-induced plasma cryoprecipitate as represented by the following formula:

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\text{heparin} + \text{CIg} \xrightarrow{22^\circ C} \text{heparin} \cdot \text{CIg complexes} \xrightarrow{2^\circ C} \text{heparin} \cdot \text{CIg precipitate.}
\]

The stoichiometry of such a reaction remains to be fully evaluated but we can calculate from our present data (such as in Fig. 3) that heparin molecules are present at ~30-fold molar excess or more relative to CIg (assumed mol wt of heparin, 11,000, [45]; CIg, 450,000, [8]) when optimal conditions for cryoprecipitation are reached. However, in our experiments related to determination of the anti-coagulant activity of heparin in the heparin-induced cryoprecipitate, only 11–12% of the amount added was recovered in the precipitate. Furthermore, preliminary results of heparin-induced cryoprecipitation of plasma using a tritiated heparin preparation (117 U. S. Pharmacopeia U/mg, generously provided by Dr. Grant Barlow, Abbott Laboratories, N. Chicago, Ill.) indicate that at optimal heparin concentrations, only 5–8% of added radioactivity is precipitated. These findings therefore suggest that the heparin:CIg ratio in the cryoprecipitate itself is in the range of 1.5–2.

Fibrinogen is neither essential for heparin-induced cryoprecipitation to occur nor does fibrinogen by itself form a cold-precipitable complex with heparin under the conditions we used. It is incorporated in the heparin-induced cryoprecipitate, and augments the process mainly, if not entirely, by virtue of its affinity for CIg. Furthermore, consistent with the findings of Smith and von Korff (2), the presence of divalent cations like Ca^{++} was found to augment the rate and

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amount of cryoprecipitate formation in mixtures of heparin with Clg or with Clg plus fibrinogen. The apparent lack of effect of Ca\(^{++}\) upon heparin-fibrinogen mixtures thus emphasizes in another way the central importance of the Clg-heparin complex.

Our binding experiments with heparin-Sepharose columns indicate that complex formation between Clg and heparin is more dependent upon pH and ionic strength than it is upon temperature. In addition, like Gentry and Alexander (46), we found that heparin affinity for fibrinogen is weak. On the other hand, heparin-fibrinogen complexes can be formed under appropriate circumstances (42, 43, 47–51, inter alia), related for the most part to ionic strength and pH conditions, as is typical of electrostatic interactions between polyelectrolytes like heparin and proteins (52, 53). However, fibrinogen preparations are usually heavily contaminated with Clg (9, 11, and this study), and this fact has not been taken into account in certain experiments cited above whose proper interpretation would require differentiating effects due to indirect heparin binding of fibrinogen via Clg, from those due to direct binding. In any case, heparin-fibrinogen complexes are probably not stable under ionic strength and pH conditions in the physiologic range (47).

Heparin is known to be capable of binding several plasma proteins (51, 54, 55), including thrombin (46, 51, 56, 57) and certain other coagulation factors (46, 48), and an inhibitor known as antithrombin-heparin cofactor (46, 48, 57, 58). The results of our electrophoretic analyses of material from plasma that had been retained on a heparin-Sepharose column are consistent with those observations, in that several electrophoretic bands were found, including the minor one corresponding to Clg (Fig. 9). The numerous proteins binding to the heparin affinity column serve to underscore one of the unique features of the heparin–C1g interaction, namely, that of all the heparin-protein complexes formed in plasma, only that between Clg and heparin appears to be cold-insoluble to any appreciable extent. That is, the relatively intense staining of the Clg band in the heparin-induced cryoprecipitate of normal or afibrinogenemic plasma is quite impressive when compared with its relatively weak intensity in the eluate fraction of the heparin affinity column (cf. Figs. 8 and 9). This fact, plus the discovery that plasma Clg can be precipitated in high yield in the presence of optimal amounts of heparin, offers a promising new first step in the purification of Clg which should result in a much higher recovery of material than can be obtained by application of presently existing preparative methods (7, 11, 19). Furthermore, although a relatively simple ammonium sulfate precipitation method has been described (59) that can be used to prepare band I fibrinogen essentially free of fibrinogen catabolites, the use of heparin for this purpose introduces a new and simple way to obtain such molecules directly from plasma.

The antithrombin-heparin cofactor has an affinity for those heparin molecules possessing high anticoagulant activity (28, 40, 41). This discovery provides a convenient means for separating heparin molecules with low anticoagulant potential from those with high potential. In our analysis of the heparin in the heparin-precipitable fraction, we observed that the specific anticoagulant activity of heparin recovered in this fraction was almost the same as that of the starting heparin. Failure to recover heparin of substantially higher or lower specific activity under precipitation conditions in which heparin was in molar excess with respect to Clg (heparin/Clg ratio = 13–14:1) indicates that, unlike the plasma antithrombin-heparin cofactor, the heparin binding components of the heparin-induced precipitate are not selective for heparin molecules possessing high or low anticoagulant potential. This result is not surprising inasmuch as Smith and von Korff (2) were able to induce cryoprecipitation with a number of different sulfated polysaccharides that had little or no anticoagulant potential. Furthermore, they observed optimal precipitation in plasma at a heparin level in the same concentration range as we found in this study, although the specific anticoagulant activity of their preparation was not more than 1/10 that of the preparations we used.

It is widely appreciated that fibrinogen is a major and constant constituent of the heparin precipitate of normal as well as of pathological plasma (2, 3, 13, 15). Certain early observations led to the inference that the fibrinogen in the fraction might have been altered in some way (2), perhaps by having been converted to a form of fibrin. Inasmuch as fibrin can circulate in a soluble form in blood under pathological conditions (14), and perhaps, to a limited extent, even in normal subjects (60), it might be included as a significant component of the heparin-precipitable fraction under proper circumstances. Nevertheless, our present studies support those of Fyrand et al. (15) in indicating that fibrin is not a usual or necessary component of the fraction from normal plasma. In fact, the fibrinogen in the heparin precipitate appears to be as close to the “native” form of the molecule as can be found in blood. That is, electrophoretic analyses indicate that fibrinogen in this fraction is characterized by the presence of intact Aα chains (i.e., band I).

3 Partially degraded circulating catabolites lacking portions of the molecule containing the carboxy-terminal portion of the Aα chain, even though present in plasma (35, 59, 61) or in mixtures of Clg and heparin, failed to participate in formation of such cold-precipitable complexes (Figs. 6 and 8). The failure of fibrinogen catabolites migrating in electrophoretic band II or

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beyond to participate in heparin-induced precipitation implies that the region of the fibrinogen Aα chain accounting for precipitation with CIg is contained within a segment representing the difference in size (∼13,200) between the intact Aα chain and the principal Aα chain remnant characterizing band II fibrinogen. The carboxy-terminal region of the Aα chain also contains a site(s) that is utilized during factor XIIIa-catalyzed cross-linking of fibrin α chains (62–64), and possibly during cross-linking with CIg (11) as well.

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