and Favorev, V. V. (1966), *Biokhimiya 31*, 334.
Litt, M., and Ingram, V. M. (1964), *Biochemistry 3*, 560.
Marciello, R., and Zubay, G. (1964), *Biochem. Biophys. Res. Commun. 14*, 272.
Penniston, J. T., and Doty, P. M. (1963), *Biopolymers 1*, 145.
Rushizky, G. W., and Knight, C. A. (1960), *Virology 11*, 236.
Scott, J. F. (1967), *Methods Nucleic Acid Res.* (in press).
Shapiro, R., and Hachmann, J. (1966), *Biochemistry 5*, 2799.
Staehelin, M. (1959), *Biochim. Biophys. Acta 31*, 448.
Stanley, W. M., Jr., and Bock, R. M. (1965), *Anal. Biochem. 13*, 43.
Tiffany, B. D., Wright, J. B., Moffett, R. B., Heinzelman, R. V., Strube, R. E., Aspergren, B. D., Lincoln, E. H., and White, J. L. (1957), *J. Am. Chem. Soc. 79*, 1682.

Interaction of Heparin with the Plasma Proteins in Relation to Its Antithrombin Activity*

P. Porter,† M. C. Porter, and J. N. Shanberge‡

ABSTRACT: The distribution of heparin among the plasma proteins has been studied by gel filtration, anion-exchange chromatography, and electrophoresis. Protein-bound heparin appears predominantly among components in the low molecular weight group of proteins isolated from Sephadex G-200. The plasma cofactor responsible for the antithrombin activity of heparin is identified in these fractions. Electrophoretic studies indicated that albumin and fibrinogen do not form stable complexes with heparin at the normal physiological level of pH. γ-Globulin and thrombin form complexes with heparin, the latter with a reduction in its activity. The antithrombin action of heparin, however, is weak in comparison with the activity of heparin with cofactor. Cofactor combines with heparin to form an active complex identified in gel filtration eluates of heparinized defibrinated plasma. The antithrombin activity of this complex is eliminated by the addition of protamine. Chromatographically the plasma cofactor is characterized by a molecular size similar to albumin and an isoelectric point similar to γ-globulin. In electrophoretic studies the peak of cofactor activity appeared in the α-2-globulins, although the activity was distributed through the α- and β-globulins. Evidence from the staining of electrophoretograms of plasma fractions suggests that cofactor may be a glycoprotein.

A number of authors have conducted investigations into the reaction of heparin with proteins and complex bases (Fischer, 1931; Fischer and Astrup, 1935; Jacques, 1943; Gorter and Nanninga, 1952a,b). The important conclusion to be drawn from these studies is that the mode of action is one of salt formation, that the reactions are reversible, dissociation taking place in accordance with the laws of mass action. While this in itself is valuable information, the studies have been conducted largely with components which are not found in the blood plasma. Therefore the information is relevant to the anticoagulant action only in so far as these physicochemical principles may have any implication.

It has long been realized that a component of the plasma is a necessary requirement for the inhibitory action of heparin in blood coagulation. The early studies of Howell and Holt (1918), Quick (1938), and Ziff and Chargaff (1940) indicated that the required "cofactor" was either albumin or a component closely related to albumin. Ferguson (1940) demonstrated that crystalline albumin had no antithrombin activity with heparin, and Chargaff *et al.* (1941) conducting electrophoretic studies found a component in globulin fractions to have such activity.

On the basis of the physicochemical principles established by Fischer and Astrup (1935) it might be expected that heparin would combine with a number of plasma proteins. However, in the reports available on this subject discrepancies exist and the relationship of the findings to the anticoagulant action of heparin is not indicated. Chargaff *et al.* (1941) studied the effect of heparin on the plasma proteins by electrophoresis and

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indicated that albumin and the globulins were effected with the production of a new electrophoretic component. Mohlo and Mohlo-Lacroix (1952), using a paper chromatographic technique, indicated that heparin was bound mainly by albumin, only one-fifth of the bound heparin being with the globulins. However, Eiber and Danishefsky (1959) studying the *in vivo* distribution of heparin among the plasma proteins demonstrated that albumin was not involved in the binding of heparin. The bound heparin was found in Cohn fractions I–III suggesting the proteins may be prothrombin, γ-globulins, and β-1-lipoprotein.

For a closer understanding of the mechanisms of the inhibition of coagulation by heparin it is evident that at least one step in the process which needs clarification is the combination of heparin with the plasma proteins. This paper presents techniques for the study of the distribution of heparin among plasma proteins. Components involved in the binding of heparin are identified and studied in relation to the antithrombin activity of heparin in plasma.

Methods

Microelectrophoresis. Plasma samples and fractions were examined by cellulose acetate electrophoresis (pH 8.6) using a Veronal–borate buffer (Porter and Gooderham, 1966). The strips (10 × 2.5 cm) were developed for 2 hr at a potential of 150 v and the proteins were stained with ponceau S (Harleco, Philadelphia, Pa.) (0.2% in 5% acetic acid). The strips were cleared using mineral oil and scanned in a Gelman scanner.

Investigations of the mucoproteins were made by electrophoresis at pH 4.5 using a buffer made up from 0.05 M citric acid and 0.05 M disodium phosphate. The strips (10 × 2.5 cm) were developed at a potential of 150 v for 2 hr. The glycoproteins and mucoproteins were stained using Schiff's PAS reagent. Lipoproteins were identified in the electrophoretic strips developed at pH 8.6 by staining with 7B-oil red O stain (Beckman Instruments Inc., Palo Alto, Calif.) in 70% methanol.

Heparin was identified in electrophoretic strips by staining with a 2% aqueous solution of toluidine blue or 1% alcian blue (Harleco, Philadelphia, Pa.) in 0.01 N HCl. We considered the latter to be more preferable because the background staining could be removed by washing the electrophoretic strips with 0.01 N HCl, whereas this tended to elute the stained heparin band from strips stained with toluidine blue.

Immunoelectrophoresis. Analysis of proteins by immunoelectrophoresis was carried out on 1% agar buffered with 0.07 M barbiturate buffer (pH 8.6). The slides were developed at 120 v for 3 hr and precipitin lines were developed by introducing rabbit antiserum into a central trough. The rabbit antisera were prepared by injection of emulsions of plasma or plasma fractions with Freunds Bacto-Adjuvant, Incomplete, into New Zealand white rabbits as described by Porter (1964).

Thin Layer Gel Filtration and Electrophoresis. Thin layer studies were carried out using Sephadex G-150 superfine made up to gel consistency by adding 6.5 g to 100 ml of 0.85% NaCl in 0.1 M Tris-HCl buffer (pH 7.2). Glass plates cut to required size were cleaned thoroughly with Haemo-Sol and spread with a 0.5-mm layer of the gel. The plate was placed in an enclosed chamber and set at an inclination of between 10 and 20° from the horizontal. The gel was connected to a reservoir of the Tris-buffered saline by a Whatman No. 1 filter paper strip. A flow of solvent was allowed for 15–18 hr in order to establish an equilibrium within the gel before applying the samples (3 μl) by micropipet. The plate was developed for a further 10 hr at 5°.

In order to identify the position of the components a filter paper cut to size was placed on the surface of the gel and the slide was dried in an air oven at 50°. The proteins and polysaccharides were then detected in the paper as described for the electrophoretic technique.

Thin layer separation was combined with electrophoresis to provide additional resolution of the components. After development by gel filtration, the thin layer plate was placed in an electrophoresis bath and connected with the Veronal–borate buffer (pH 8.6) by filter paper strips. A potential of 120 v was applied at right angles to the direction of the original chromatographic flow. After electrophoresis for 3 hr at 4° the components were detected as previously described.

Gel filtration chromatography was carried out on Sephadex G-200 in glass columns (45 × 2.5 cm) at 4°. Elution was carried out with 0.85% NaCl in 0.1 M Tris-HCl buffer (pH 7.2). Volumes of 10–15 ml of plasma were applied to the columns and a flow rate of approximately 5 ml/hr was maintained. The eluate was collected using an LKB RadiRac fraction collector and drop counter (64 drops/tube). The protein pattern of elution was obtained by reading the eluates suitably diluted, at 280 mμ in quartz cells, 10-mm light path, against a solvent blank using a Beckman DU spectrophotometer. Fractions were bulked by reference to the elution pattern.

Anion-exchange chromatography on DEAE-cellulose was carried out with the recovery of fractions as described by Porter (1966). Components were eluted with 0.02 M phosphate buffer (pH 7.2) with a stepwise increase in NaCl concentration. Alternatively, elution was carried out with a simple decreasing pH gradient obtained by the continuous addition of 0.05 M NaH_2PO_4 to 0.02 M phosphate buffer (pH 7.2).

Fibrinogen, Thrombin, and Antithrombin Activity. The estimation of antithrombin activity depends on the measurement of the inactivation of thrombin. The strength of thrombin was measured in terms of the coagulation of fibrinogen.

For the preparation of fibrinogen, citrated human plasma was first absorbed at room temperature with Filter-Cel (Johns Manville) (30 mg/ml for 15 min); after centrifuging at 3000 rpm for 30 min, the supernatant was incubated at 37° for 5 hr. The inclusion of this preliminary step resulted in a preparation having improved clotting properties and a more easily identified end point in thrombin assays.

The supernatant was dialyzed against 0.85% NaCl in 0.015 M sodium oxalate for 30 hr with several changes

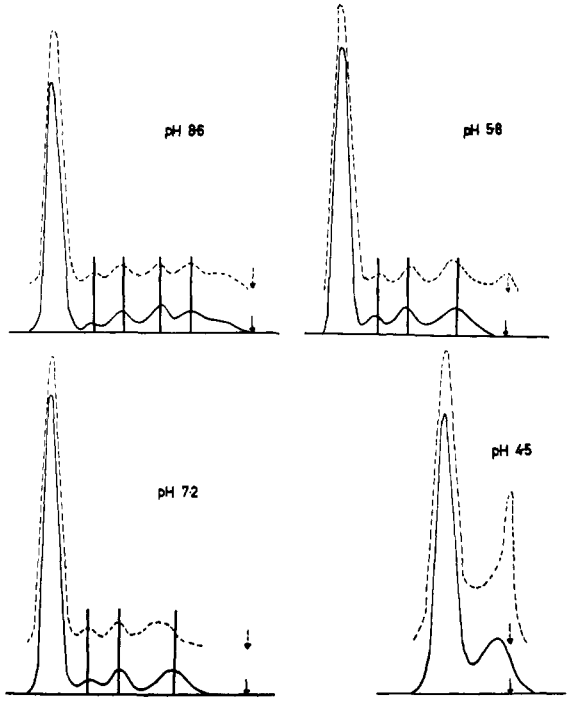

FIGURE 1: Studies of the interaction of heparin with the plasma proteins by thin layer gel filtration using Sephadex G-150 superfine. The protein pattern was obtained using a two-dimensional technique combining gel filtration with electrophoresis and staining with ponceau S. Protein-bound heparin was separated from its complexes during electrophoresis and its region of distribution was indicated by staining with alcian blue.

of solution. The oxalated, Filter-Cel-absorbed plasma was absorbed with 10% (w/v) $BaSO_4$ for 10 min with continuous stirring followed by centrifuging at 3000 rpm for 30 min.

Throughout the remainder of the preparation, the temperature was maintained below −3°. Cohn buffer (pH 6.0) was added to the supernatant plasma (1.5 ml/l. of plasma) and 53% alcohol at −20° was added slowly with stirring (177 ml/l. of plasma). The buffer is composed of 4 M sodium acetate buffered to pH 6.0 with 10 M acetic acid (Cohn *et al.*, 1946). The precipitated fibrinogen was centrifuged at 2000 rpm at −3° for 20 min. The precipitate was washed two times in the cold with glycine–citrate–alcohol solution following which it was dissolved in one-quarter the original plasma volume of 0.85% NaCl in 0.1 M Tris-HCl buffer (pH 7.2).

The glycine–citrate–alcohol solution was prepared as follows. A citrate–alcohol solution was prepared by adding 65 ml of absolute alcohol to 935 ml of 0.055 M sodium citrate–citric acid buffer (pH 7.5). Glycine (75 g) was made up to 1 l. with this solution.

Bovine thrombin (Parke Davis and Co., Detroit) was used for the antithrombin studies. The thrombin was purified before use in the thrombin inactivation studies by chromatography on DEAE-cellulose. The freeze-dried thrombin was dialyzed against 0.02 M phosphate buffer (pH 7.7) at 4° overnight and applied to the column equilibrated with the same buffer. Elution was continued with this buffer till the "fall-through" fraction was cleared from the column. Elution of the thrombin considered most satisfactory for the studies was carried out with 0.02 M phosphate buffer containing 0.15 M NaCl. Solutions were stored at −40° until required for use.

Thrombin activity was measured by coagulation of human fibrinogen under standardized conditions of temperature, pH, and ionic strength. Coagulation times were measured in (10 × 1 cm) thin-walled glass tubes placed in a rack in a glass-walled water bath at 37°. Into each tube was pipetted 0.2 ml of human fibrinogen solution and after 3–5-min temperature equilibration 0.2 ml of thrombin solution was added. Coagulation was timed by stop watch and the end point was determined by the wire hook method. For calibration before use in assaying thrombin activity the fibrinogen solution was diluted with 0.85% NaCl in 0.1 M Tris-HCl buffer (pH 7.2) until a thrombin solution of 10 units/ml (prepared from Parke Davis standard) produced a coagulation time of the order of 15 sec. Standard thrombin solution having a range of thrombin activities 1–10 units/ml were prepared from the Parke Davis strandard and the coagulation times were assessed. A plot of log coagulation time (seconds) against log (thrombin units) gave a linear relationship which was used in measuring the thrombin activity of unknown solutions. With clotting times of the order of 15 sec the standard deviation of ten measurements was 0.2 sec; for coagulation times of 50 sec (2.5 units of thrombin) the standard deviation for measurements was 1.4 sec.

The action of heparin with the component responsible for its antithrombin activity, cofactor, produces an immediate inactivation of thrombin. The heparin–cofactor activity in plasma fractions investigated and recorded in this text was identified and assayed in terms of the number of thrombin units inactivated immediately by a fraction containing heparin (0.25 unit/ml). There was no inactivation of thrombin by heparin alone at this concentration even over periods of 30 min.

Defibrinated Plasma. A 30-ml sample of blood was drawn from a normal donor into a silicone-coated syringe containing 0.2 ml of 30% EDTA. The blood was centrifuged at 2000 rpm for 30 min to obtain the plasma, and the plasma was further centrifuged at 10,000 rpm for 30 min to remove any remaining platelets. This procedure was adopted in order to minimize the disruption of platelets and the consequent release of platelet factors. The plasma sample was diluted with an equal volume of the 0.1 M Tris-HCl–0.85% NaCl buffer (pH 7.2), and the fibrinogen was removed by adding 2 units of thrombin for every 10 ml of diluted plasma. Clot formation was prevented by stirring and the fibrin was removed as strings adhering to the stirrer. The defibrinated plasma was allowed to stand at refrigerator temperature of 4° overnight when a small amount of remaining fibrinogen precipitated as fibrin and was removed by centrifugation.

Heparin. Sodium heparin with an activity of 150

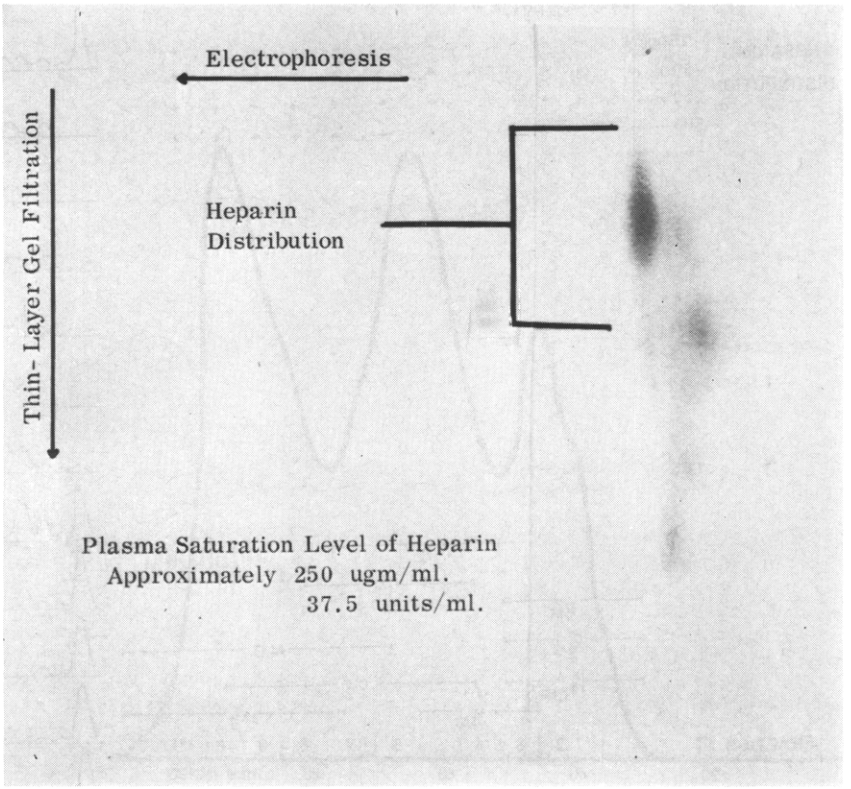

FIGURE 2: Thin layer gel filtration studies of interaction of heparin with plasma proteina. Chromatography of heparinized plasma on Sephadex G-200 giving pooling data for fractions and the distribution of heparin for four different heparin concentrations. Protein patterns obtained by cellulose acetate electrophoretic analysis of the 11 selected fractions are shown to the right of the chromatography elution pattern.

units/mg (Abbott Laboratories, North Chicago, Ill.) was used for all the studies.

Protamine. Protamine sulfate was obtained from Delta Chemical Works Inc., New York, N. Y.

Albumin. Bovine albumin powder was obtained from Armour Laboratories, Chicago, Ill.

Results

Electrophoretic Studies of the Interaction of Heparin with the Plasma Proteins. Preliminary studies of the interaction of heparin with the plasma proteins were carried out by electrophoresis on cellulose acetate. Since the formation of an ionic complex would depend to a considerable extent upon the pH of the solution, the investigations were carried out at four different pH values, 8.6, 7.2, 5.8, and 4.5. The electrophoresis in each case was carried out with a potential of 150 v. Solutions of albumin (3%), fibrinogen (1%) and thrombin (1000 units/ml) were prepared in 0.85% NaCl and for comparison the same solutions were prepared containing heparin at a concentration 0.2% (w/v). Comparative electrophoretic studies were also carried out for normal and heparinized plasma containing 0.2% heparin.

Owing to the acidic character of heparin the isoelectric point of the complex is less than that of the original component. The binding of heparin by a component may, therefore, be identified by a change in electrophoretic mobility, the complex migrating more quickly toward the anode than the uncombined component. The change in isoelectric point also produces solubility differences and the binding of heparin could often be identified by the precipitation of protein at the origin in the electrophoretic studies at the lower pH values.

Electrophoretic studies at pH 8.6 revealed no evidence for the complexing of heparin by any of the plasma components. Heparin migrated freely toward the anode at approximately twice the rate of albumin.

In the electrophoretic studies at pH 7.2 thrombin showed mobility changes indicative of heparin complexing, but fibrinogen and albumin gave no evidence of complexing. The comparative study of normal and heparinized plasma at pH 7.2 revealed a change in mobility of the γ-globulins toward the anode, indicating heparin complexing.

The binding of heparin by thrombin and albumin was evident from mobility changes in electrophoretic studies at pH 5.8, whereas the interaction of heparin with fibrinogen resulted in an insoluble complex at this pH. In the electrophoretic studies of heparinized plasma at pH 5.8 there was precipitation at the origin, presumably due to the fibrinogen–heparin complex and a slight change in mobility of the γ-globulins.

Thrombin and fibrinogen were insoluble at pH 4.5

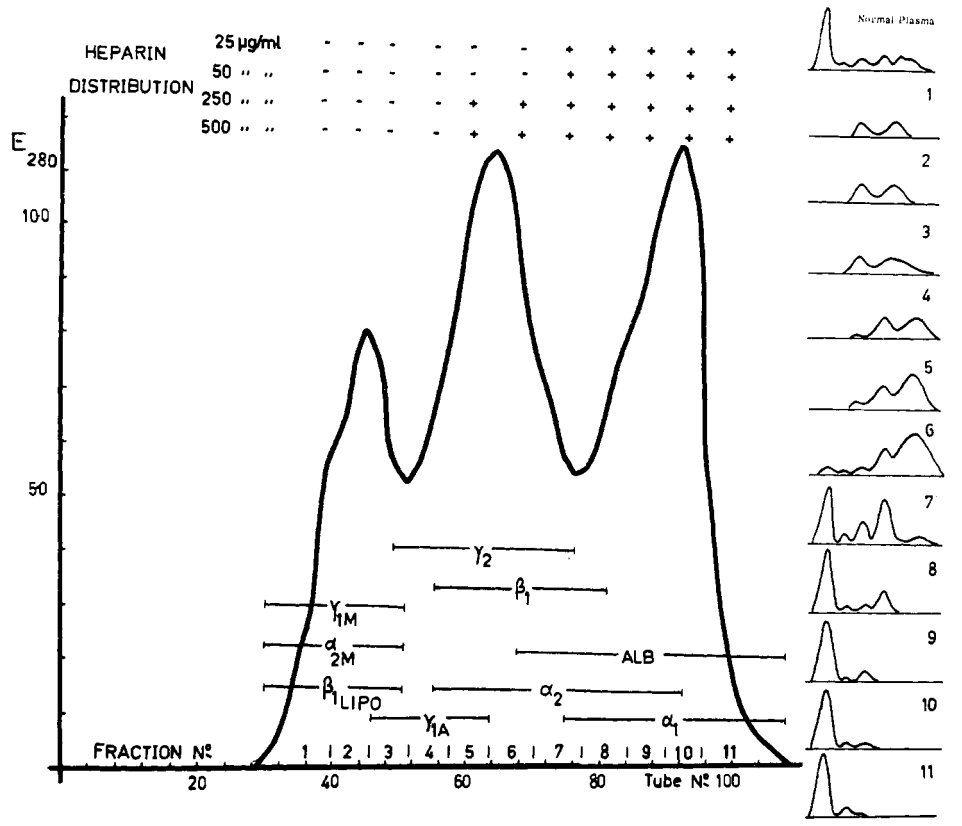

FIGURE 3: Chromatography of heparinized and nonheparinized defibrinated plasma on Sephadex G-200 with assay of heparin–cofactor activity. (upper figure) Nonheparinized defibrinated plasma. (lower figure) Heparinized defibrinated plasma. The assays of cofactor activity are presented in terms of the number of thrombin units which are immediately inactivated by the presence of each fraction. The assays for nonheparinized plasma required the addition of heparin (0–25 units/ml) to each fraction.

and so were their heparin complexes; hence, there was no further information to be gained by electrophoretic studies at this pH. Albumin, however, was soluble and migrated slowly toward the anode, but the binding of heparin by albumin resulted in an insoluble complex which was immobile on electrophoresis. In the electrophoretic study of heparinized plasma at pH 4.5 a considerable precipitation of protein was apparent at the origin. We were unable to identify any free heparin in the electrophoretogram, indicating that all the available heparin (200 mg %) was complexed with the plasma proteins at this pH. Since the greatest part of the albumin in the heparinized plasma was uncomplexed and free to migrate, it was evident that there was remaining a capacity in the plasma to bind further heparin at pH 4.5.

Thin Layer Gel Filtration Studies. We considered the information yielded by electrophoresis studies to be of limited value since the method would only indicate the ability of a certain component to yield a stable complex at a given pH, ionic strength, and potential difference. The applied potential is obviously an undesirable factor leading to dissociation of the complex, and information on less stable complexes under normal physiological conditions is inevitably lost.

Gel filtration is the method of choice for studying the binding of heparin by the plasma proteins. Fractionation may be carried out without change of pH or ionic strength, or introducing any factor liable to dissociate the complexes.

Heparin is a relatively small molecule and is probably completely included within the gel structure of Sephadex G-200 and G-150, whereas the plasma proteins are partially or completely excluded. Using Sephadex G-150 superfine in thin layer studies, free heparin could be identified migrating through the gel at a slower rate than the proteins. In a number of studies in which various heparin concentrations were added to plasma, the heparin was completely bound by the proteins at concentrations less than 250 µg/ml, a level of 37.5 units/ml. In excess of this level, free heparin could be identified.

In order to identify the proteins to which the heparin was bound, the two-dimensional technique combining thin layer gel filtration with electrophoresis was used. After the proteins and bound heparin had been separated by the conventional thin layer method, heparin was separated from its complexes and the proteins were further resolved by electrophoresis (Figure 1). The proteins

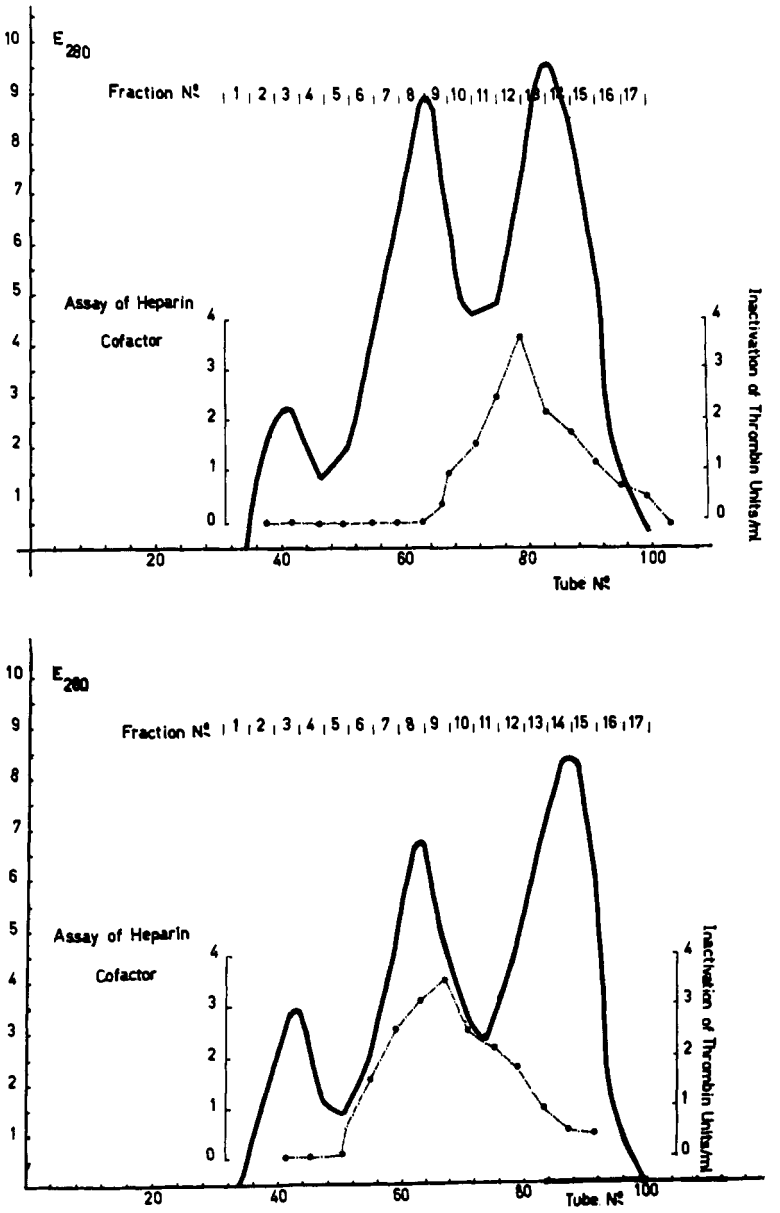

FIGURE 4: Neutralization of antithrombin action of heparin in defibrinated plasma by protamine. The ordinate gives the coagulation time for fibrinogen and thrombin (5 units/ml) immediately after the interaction of thrombin with solutions of heparin and defibrinated plasma containing various levels of protamine. The abscissa gives the concentration of protamine in the mixture (micrograms per milliliter) and the curves refer to heparin concentrations of 0, 0.25, and 0.50 unit/ml.

which bound heparin were present in the slower migrating group in which albumin was the predominant component.

Gel Filtration Chromatography. The investigations were extended to the use of column fractionation on Sephadex G-200 with a view to a closer identification of the components responsible for binding heparin. Studies were conducted with citrated pooled plasma containing four different levels of heparin. The eluates were divided into fractions by reference to the elution pattern and the tube number so that as far as was possible, identical fractions were taken in each study. The addition of heparin to the plasma produced no marked change in the pattern of protein elution from the column. Cellulose acetate electrophoresis at pH 8.6 revealed no apparent difference in the composition of the 11 selected fractions composed of normal and heparinized plasma. The electrophoretic patterns for the components in the fractions are presented alongside of the gel filtration elution pattern in Figure 2. The electrophoretic analysis facilitated the identification of bound heparin which was split from its complexes during electrophoresis at pH 8.6 and could

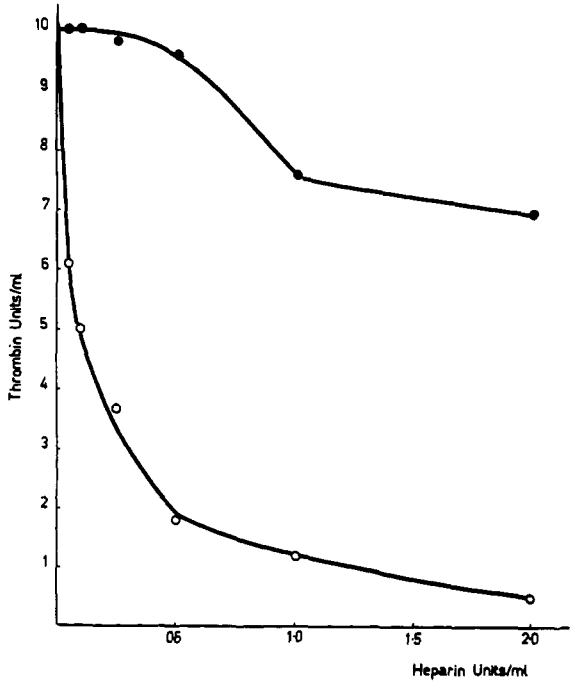

FIGURE 5: The inactivation of thrombin by heparin and by heparin with plasma cofactor. (●———●) Thrombin (10 units/ml) with increasing amounts of heparin in 0.85% NaCl. (○———○) Thrombin (10 units/ml) with increasing amounts of heparin in Sephadex G-200 eluate containing plasma cofactor.

be identified with alcian blue.

In the studies of plasma containing heparin at 25 and 50 μg/ml, bound heparin was identified in fractions 7–11. These comprise the low molecular weight components of the plasma proteins consisting mainly of albumin and the α-globulins. When the heparin concentration in the plasma was increased tenfold, bound heparin was identified in fractions 5 and 6 as well as 7–11; thus γ- and β-1-globulins become involved. Free heparin was present at the highest concentration studied (500 μg/ml) and appeared in eluates immediately following the main protein elution.

It is outside the interpretation of the findings to suggest that heparin was bound chiefly by albumin and the α-globulins. It would be quite possible that a minor component undetected by the electrophoretic technique was responsible for the complexing with heparin. To achieve greater specificity other methods needed to be brought to bear on the problem. The outstanding property of heparin which was considered to be a most useful index was its ability to inactivate thrombin. Consequently a study of the cofactor responsible for the antithrombin action of heparin was embarked upon.

Identification of Heparin–Cofactor Activity in Sephadex G-200 Eluates of Defibrinated Plasma. Owing to the fact that fibrinogen was present in many of the early fractions of plasma fractionated on Sephadex G-200, it was not possible to study the antithrombin activity of heparin with components in these fractions. To obviate this problem, investigations were conducted with defibrinated plasma. The cofactor activity was identified by assessing the antithrombin activity developed immediately in eluate samples with the addition of heparin (0.25 unit/ml). The antithrombin activity was expressed in terms of the number of units of thrombin inactivated in a thrombin solution standardized by coagulation of fibrinogen. By measuring the immediate antithrombin activity compared with a control solution containing no heparin, the activity due to heparin and "cofactor" only was assessed, and the inclusion of the action of normal plasma antithrombin was eliminated from the measurement. The cofactor activity was identified within the low molecular weight group of proteins (Figure 3).

Positive evidence that heparin combines with cofactor to produce a complex molecule, which possesses the immediate antithrombin activity, was provided by fractionation of defibrinated plasma containing heparin (10 units/ml). The immediate antithrombin action was determined in the G-200 eluates without the necessity of adding heparin as described previously. The immediate antithrombin action was detected in earlier eluates than in the defibrinated plasma control containing no heparin (Figure 3). This was taken to signify a complexing action between cofactor and heparin giving rise to a larger molecule with a higher exclusion coefficient for the Sephadex gel.

Substantiating evidence for the presence of a heparin cofactor complex in these gal filtration eluates was provided by studies of the effect of protamine on the immediate antithrombin activity. Protamine forms complexes with heparin which are ineffective in blood coagulation (Chargaff, 1938) and has been used as a heparin antagonist *in vivo* (Portman and Holden, 1949). In the present investigations the interaction of protamine and heparin was first studied in relation to the antithrombin action of heparin in defibrinated plasma. Increasing concentrations of protamine were added to solutions of heparin in defibrinated plasma. The immediate inactivation of thrombin (5 units/ml) was studied by measurement of the coagulation time for purified human fibrinogen solution. The neutralizing effect of protamine on the antithrombin action of heparin at two different levels, 0.25 and 0.5 unit/ml, is shown in Figure 4. An excess of protamine over heparin produced an apparent enhancement of the thrombin activity resulting in a shortening of the coagulation time in the thrombin–fibrinogen reaction. This fibrinoplastic effect was adequately accounted for in control solutions containing protamine concentrations up to 50 μg/ml (Ferguson, 1940).

The immediate antithrombin activity in the eluates from the Sephadex G-200 fractionation of heparinized plasma was completely eliminated by the action of protamine at concentrations of 5–10 μg/ml. From the equivalence point of protamine and heparin in defibrinated plasma these findings would suggest that the activity in these eluates may be mediated by heparin in concentrations less than 0.5 unit/ml. Heparin alone shows negligible antithrombin activity at this level. Thus, it would

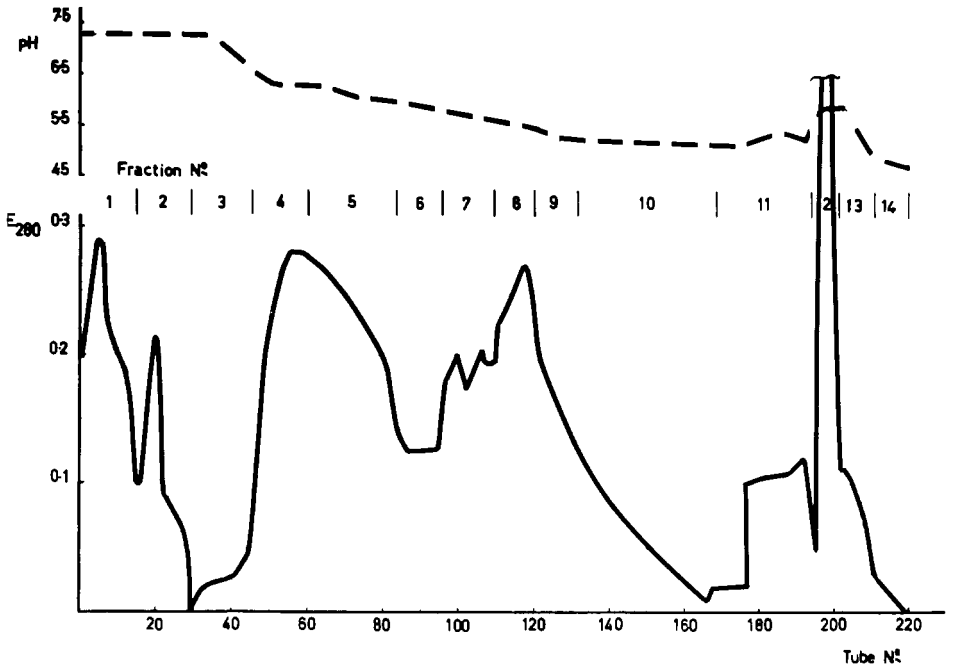

FIGURE 6: Chromatography of Sephadex G-200 low molecular weight plasma proteins on DEAE-cellulose. The decreasing pH gradient for elution and pooling data for 14 fractions described in the text are shown above the elution pattern.

appear that the immediate antithrombin activity eliminated by the action of protamine must be due to the combination of heparin with a cofactor responsible for potentiating this effect.

The Antithrombin Action of Heparin. The electrophoretic studies revealed that thrombin formed a complex with heparin at the physiological level of pH. Studies of the effect of heparin on thrombin revealed that it had no antithrombin activity at levels less than 0.5 unit/ml. However, in the presence of plasma protein fractions containing cofactor much smaller quantities exhibited strong activity. The immediate antithrombin activity of heparin, compared with the activity of heparin in the presence of cofactor, at various concentrations of heparin is illustrated in Figure 5.

In the electrophoresis and gel filtration studies described above, γ-globulin was demonstrated to react with heparin, but the indications were that the low molecular weight group of proteins containing the cofactor preferentially bound heparin. We felt it necessary to consider the possibility that the presence of γ-globulin in solution might result in an inactive complex with the reduction of potential antithrombin activity.

The antithrombin studies with heparin and cofactor were repeated with the introduction of γ-globulin samples from the Sephadex G-200 fraction of defibrinated plasma. There was no significant effect on the antithrombin activity suggesting that γ-globulins were unable to cause sufficient dissociation of the heparin–cofactor complex to result in significant reduction in the inactivation of thrombin.

Characterization of Cofactor by Anion-Exchange Chromatography and Cellulose Acetate Electrophoresis. Elution of proteins from the anion-exchange medium, DEAE-cellulose, progresses roughly in accordance with decreasing isoelectric point. Those components having the highest isoelectric point appear first in the elution pattern. The anionic or cationic behavior of a protein at a given pH is a determining factor in the binding of heparin. Therefore, it was considered that a study of the low molecular weight fraction from Sephadex G-200 on DEAE-cellulose would provide information relevant to the binding of heparin by components in this fraction and also the nature of cofactor. Elution was carried out with a simple pH gradient and the pH of eluates was recorded. The elution pattern obtained is illustrated in Figure 6.

The cofactor activity was identified mainly in the "fall-through fractions" 1 and 2 and some activity was present in fraction 3. It is evident, therefore, that the component had no reaction with the anion exchange cellulose at pH 7.2. Immunoelectrophoretic analysis revealed that the components appearing in fractions 1, 2, and 3 were 7S γ-globulin, a β-1-globulin, and an α-2-globulin, respectively. Electrophoresis on cellulose acetate with staining by ponceau S revealed that fractions 1–3 consisted predominantly of the β-1-globulin. Albumin was the most abundant component in the mixture appearing in fractions 4–13 and α-globulins appeared in fractions 6–14.

Further studies of cofactor were carried out by electrophoretic fractionation on cellulose acetate at pH 8.6. The Sephadex G-200 fractions containing cofactor were concentrated approximately tenfold by ultrafiltra-

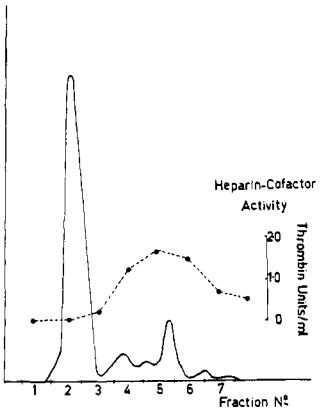

FIGURE 7: Fractionation of Sephadex G-200 low molecular weight plasma protein fraction by electrophoresis at pH 8.6 on cellulose acetate. The protein pattern was obtained by scanning a strip stained with ponceau S. The anode is on the right. The heparin cofactor activity assayed in eluates from sections of four strips run concurrently is presented in terms of thrombin units immediately inactivated by each fraction containing heparin (0.25 unit/ml).

tion through Visking membrane. Approximately 5 μl of the concentrate was applied to each of five cellulose acetate strips (10 $\times$ 2.5 cm) ruled in 0.5-cm sections and electrophoresis was carried out at pH 8.6 for 2 hr with a potential of 160 v. The center strip was stained with ponceau S and the remaining four strips were cut into 0.5-cm sections. The sections were combined according to distance from the origin and the protein eluted with 1 ml of Tris-buffered saline (pH 7.2) in stoppered tubes (5 $\times$ 1 cm). Elution was continued overnight at 4°. The heparin–cofactor activity was detected throughout the α-2- and β-globulins, the peak of activity appearing in the slow α-2-globulins (Figure 7).

Further electrophoretic studies were carried out with staining for glycoprotein and lipoprotein. The components in the globulin region where cofactor activity was identified stained predominantly for glycoprotein with Schiff's PAS, and this was also the case for the DEAE-cellulose fractions containing cofactor. There was no lipoprotein by staining or its equivalent.

Discussion

Previous investigations on the interaction of heparin with the plasma proteins have not related this property with its mechanism of antithrombin action. Whether heparin has a selective affinity for the cofactor or is distributed randomly among the plasma proteins is not known. From the laws of mass action it may be inferred that heparin should distribute itself among the proteins according to their relative concentrations and the dissociation constants of the complexes. Thus, for a minor component of the plasma to play a part comparable with one of the more abundant components it must form a heparin complex with a low dissociation constant.

On the basis that the reaction of heparin with protein is similar to simple salt formation, the most stable complex would be formed when the pH of the solution is on the acidic side of the isoelectric point of the protein. This, however, does not negate the possibility of a protein forming a complex with heparin, when the pH of solution is on the alkaline side of the isoelectric point. Jacques (1943) was able to demonstrate this point with histone and gelatin. However, on the alkaline side of the isoelectric point, the stability of the complex is considerably reduced and dissociation increases with increasing pH. Obviously such easily dissociated complexes would not be indicated by the electrophoretic technique since the application of a potential difference would facilitate the dissociation and the components would migrate separately. Thus, the preliminary electrophoretic studies provide only an indication of the ability of a given component to form a stable complex with heparin at a given pH.

In most of the literature on the subject of the binding of heparin by the plasma proteins (Howell and Holt, 1918; Quick, 1938; Chargaff *et al.*, 1941; Mohlo and Mohlo-Lacroix, 1952) albumin has been considered to be one of the main components involved. In the present experiments the preliminary electrophoretic studies with a heparinized albumin fraction indicated that albumin formed a stable complex at pH 5.6 but not at pH 7.2. However, albumin was the most abundant component identified in the gel filtration fractions containing bound heparin, and in spite of its inability to form a stable complex at the physiological level of pH, it could well play a prominent role in the binding of heparin because of its quantity.

The most important information required from the clinical point of view would be to determine whether there was any preferential binding of heparin and not which components might or might not form complexes. From this standpoint the isoelectric point of the components involved is probably the most important criterion to consider. The relevent information was provided by the fractionation studies on DEAE-cellulose using a pH-gradient elution. Cofactor activity was identified in the first fraction not taking part in the anion exchange at pH 7.2 indicating that the component responsible could have an isoelectric point greater than this value. Albumin appeared in fractions where the pH of the eluates was less than 6.1, and the majority of the α-globulins were not eluted until the pH had fallen below 5.7. It would, therefore, be expected that these components would have isoelectric points which were too low for them to form stable complexes with heparin at the physiological pH value. The anion-exchange studies indicated that three components would have suitably high isoelectric points to allow them to form stable complexes with heparin at the physiological pH value. These were an α-2-, a β-1-, and γ-globulins, and all were identified immunoelectrophoretically in the fractions containing cofactor activity. γ-Globulin had also been indicated to form a stable complex at pH 7.2 by the preliminary electrophoretic studies of heparinized plasma.

The complexing action of γ-globulin provided interesting insight into the degree of specificity required in the reaction of heparin with cofactor to produce antithrombin action. Certainly by virtue of its quantity γ-globulin could predominate in the binding of heparin in plasma. However, heparin exhibited no antithrombin activity when introduced into gel filtration fractions of plasma consisting mainly of γ-globulin. Thus, for heparin to act effectively as an antithrombin and not to have its activity reduced by ineffective combinations it must exhibit a special affinity for cofactor.

Evidence in favor of this thesis was provided by gel filtration studies of plasma containing heparin at concentrations of 25 and 50 μg/ml. The bound heparin appeared predominantly with the low molecular weight fractions which contain cofactor and exclude γ-globulin (Figure 2). Also gel filtration studies of heparinized defibrinated plasma provided positive evidence that cofactor forms a complex with heparin which is capable of inactivating thrombin (Figure 3). Further evidence for the preferential binding of heparin by cofactor was provided by the antithrombin studies in the presence of γ-globulin. If the combination between cofactor and heparin were a reversible reaction, then in accordance with the laws of mass action, it would be expected that dissociation of the complex, with consequent reduction in antithrombin activity, would take place if a second protein were introduced into the system. The dissociation would, of course, depend upon the concentration of the second protein and the dissociation constant of its complex with heparin. The introduction of a base such as protamine leads to complete dissociation of the heparin–cofactor complex and complete elimination of all antithrombin activity owing to the complex. However, the introduction of a γ-globulin fraction into the reaction of heparin and cofactor with thrombin brought about no significant decrease in the inactivation of thrombin. Thus, in spite of the greater abundance of γ-globulin, it apparently causes no effective dissociation of the heparin–cofactor complex.

The importance of cofactor in the role of heparin as an antithrombin is evident from the antithrombin studies (Figure 5). While the electrophoretic studies indicated complex formation between thrombin and heparin at pH 7.2, the inactivation of thrombin by heparin alone is a minor consideration in comparison with its action in the presence of cofactor. Gorter and Nanninga (1952a) doubted the necessity of a cofactor for the formation of heparin–thrombin complexes. This is so, but the ability of heparin to inactivate thrombin is so potentiated by cofactor that there is no doubt that the predominant action of heparin is a result of its combination with cofactor.

These investigations have indicated a number of physicochemical characteristics attributable to cofactor which will be of value in its ultimate characterization in human plasma. The gel filtration studies suggest that it may have a molecular size similar to albumin. This represents a considerable difference from the observation of Fitzgerald and Waugh (1955) who from ultracentrifuge data assessed the molecular weight of cofactor in bovine plasma to be approximately 15,000. Anion-exchange chromatography indicates that cofactor has anionic properties at pH 7.2 thus accounting for its ability to form stable complexes with heparin in plasma. Electrophoretic studies indicated that the peak of cofactor activity appeared in the region of the α-2-globulins. Loeb, quoted by Soulier (1963), considers that cofactor may be a lipoprotein, but our studies provided no evidence to substantiate this. The components migrating in the region where cofactor activity appeared stained strongly for carbohydrate, suggesting that cofactor may in fact be an α-2-glycoprotein.

References

Chargaff, E. (1938), *J. Biol. Chem. 125*, 671.

Chargaff, E., Ziff, M., Moore, D. H. (1941), *J. Biol. Chem. 139*, 383.

Cohn, E. J., Strong, L. E., Hughes, W. L., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L. (1946), *J. Am. Chem. Soc. 68*, 459.

Eiber, H. B., and Danishefsky, I. (1959), *Proc. Soc. Exptl. Biol. Med. 102*, 18.

Ferguson, J. H. (1940), *Am. J. Physiol. 130*, 759.

Fischer, A. (1931), *Biochem. Z. 240*, 357.

Fischer, A., and Astrup, T. (1935), *Biochim. Z. 278*, 326.

Fitzgerald, M. A., and Waugh, D. F. (1955), *Arch. Biochem. Biophys. 58*, 431.

Gorter, E., and Nanninga, L. (1952a), *Proc. Faraday Soc. 13*, 205.

Gorter, E., and Nanninga, L. (1952b), *Proc. Koninkl. Ned. Akad. Westenschap. 55*, 351.

Howell, W. H., and Holt, E. (1918), *Am. J. Physiol. 47*, 328.

Jacques, L. B. (1943), *Biochem. J. 37*, 189.

Mohlo, D. M., and Mohlo-Lacroix, L. (1952), *Compt. Rend. 235*, 522.

Porter, P. (1964), *J. Comp. Pathol. Therap. 74*, 108.

Porter, P. (1966), *J. Comp. Pathol. Therap. 76*, 197.

Porter, P., and Gooderham, K. R. (1966), *Res. Vet. Sci. 7*, 25.

Portman, A. F., and Holden, W. D. (1949), *J. Clin. Invest. 28*, 1451.

Quick, A. J. (1938), *Am. J. Physiol. 123*, 712.

Soulier, J. P. (1963), *Thromb. Diath. Haemorrhag., Suppl. 9*, 37.

Ziff, M., and Chargaff, E. (1940), *J. Biol. Chem. 136*, 689.