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COVALENTLY BOUND CONJUGATES OF ALBUMIN AND HEPARIN: SYNTHESIS, FRACTIONATION AND CHARACTERIZATION

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ABSTRACT

Covalently bound conjugates of human serum albumin and heparin were prepared as compounds which could improve the blood-compatibility of polymer surfaces either by preadsorption or by covalent coupling of the conjugates onto blood contacting surfaces. The conjugates (10-16 weight % of heparin) were obtained by a condensation reaction between albumin and heparin using 1-ethyl-3-(dimethylaminopropyl)-carbodiimide. Unreacted albumin and heparin were removed by diethylaminoethyl (DEAE)-cellulose and Cibacron Blue Sepharose chromatography respectively. The activity of the heparin component incorporated in the albumin-heparin conjugates (Ac) was compared with that of the heparin used for the synthesis of the conjugates (Anat) by thrombin time, inhibition of Factor Xa and the activated partial thromboplastin time (APTT) assays. The Ac/Anat ratio for the above assays was as follows: Thrombin time 1.25, Factor Xa inhibition 0.5. and APTT 0.5. Gel filtration chromatography showed broad-molecular weight distributions. The conjugates were fractionated using immobilized antithrombin III (ATIII). High ATIII and low ATIII affinity conjugate fractions showed the same behavior as ATIII fractionated heparin with respect to thrombin times and Factor Xa inhibition.

INTRODUCTION

Much effort has been made to improve the blood compatibility of materials by treatment of surfaces using either heparin or human serum albumin. Human serum albumin reduces both the adhesion of blood platelets and the activation

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Keywords: albumin-heparin conjugates, heparin, anticoagulant activity, antithrombin III.

of the intrinsic blood coagulation (for reviews, see 1, 2, 3). Heparin has been covalently or ionically bound to material surfaces. In general, surfaces prepared by the first method are less blood compatible than those prepared by the latter method, where leakage of heparin from the surface might play an important role. Alternatively, controlled release of heparin from material surfaces might also be a good approach to improve blood compatibility (4).

Albumin-heparin conjugates might be useful to obtain material surfaces with improved blood compatibility either by the adsorption of the conjugate on hydrophobic materials through the albumin moiety or by covalent coupling of the conjugate to surfaces using albumin as a spacer. Presumably after contact with blood, the preadsorbed surfaces will show a gradual desorption of the conjugate, thus preventing thrombosis at the surface.

In this paper we describe the synthesis, fractionation and characterization of albumin-heparin conjugates, which will later be investigated as possible compounds for the improvement of the blood compatibility of polymeric surfaces.

MATERIALS AND METHODS

Reagents

Heparin from porcine mucosa was obtained from Diosynth, Oss, The Netherlands. The specific activity as indicated by the manufacturer was 161.5 IU/mg. Human serum albumin (No. A9511) was obtained from Sigma, St. Louis, USA and was used without further purification. Cibacron Blue Sepharose CL-6B, CNBr-activated Sepharose and Sephacryl S-300 were all purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Purified human antithrombin III was a generous gift of the American Red Cross. 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (EDC) and N-ethyl-5-phenylisoxazolium-3'-sulfonate (Woodward's reagent K) were products of Aldrich, Beerse, Belgium. Coatest Heparin was obtained from Kabi Vitrum, Stockholm, Sweden. PTT-reagent was obtained from Boehringer, Mannheim, F.R.G. Thrombin (75 NIH U/mg) was obtained from Hoffman La Roche, Switzerland. CPD human plasma was obtained from the Red Cross Bloodbank Groningen-Drenthe, Groningen, The Netherlands.

Synthesis and Fractionation of Conjugates

In a typical experiment, heparin (770 mg)and albumin (2590 mg) were dissolved in water (39 ml) and this solution was adjusted to pH 5.1-5.2 with HCl (1N). During the addition of 1 N HCl, a white precipitate was formed, which contained both heparin and albumin (5). The precipitate dissolved almost completely within 1 hour. Eight 1 ml portions of EDC (c = 32.5 mg/ml) solution were added to the resulting solution at 30 minute intervals. The pH was maintained at 5.1-5.2 by the addition of HCl (1 N) or NaOH (1N). Thirty minutes after the last addition of EDC, the pH was adjusted to pH 7.5 with NaOH (1 N) and the solution was gently stirred for 20 hours. Finally the solution was dialyzed for 2 hours against 0.025 M Tris-HCl, pH 7.5. The total experiment was performed at room temperature.

Separation of the albumin-heparin conjugate from free albumin and heparin was achieved by the following chromatographic procedures. The dialyzed

solution containing the conjugate was applied to a column of DEAE-cellulose (Whatman DE 32) which was previously equilibrated with 0.025 M Tris-HCl, pH 7.5. In order to remove unbound material, the column was washed with 1 column volume of 0.025 M Tris-HCl, pH 7.5. It was shown that 5-10 mg of reactants and products could be bound per ml packed gel. Free albumin was eluted with approximately 2 column volumes of 0.15 M NaCl-0.025 M Tris-HCl, pH 7.5. This eluate was discarded. To elute both the albumin-heparin conjugate as well as free heparin, a 0.50 M NaCl-0.025 M Tris-HCl, pH 7.5, buffer solution was used. Fractions with an optical density exceeding 0.2 at 280 nm were pooled and used for further purification with a Cibacron Blue Sepharose column. This material binds albumin and the interaction can be broken with potassium thiocyanate (KSCN) (6,7). The pooled fractions obtained with the DEAE-cellulose column were applied to a column of Cibacron Blue Sepharose (binding capacity ca 15 mg conjugate per ml packed gel). This column was equilibrated with 0.17 M NaCl-0.025 M Tris-HCl, pH 7.5. Free heparin was removed by eluting the column with 1 column volume of 0.17 M NaCl-0.025 M Tris-HCl, pH 7.5, buffer. Then the albumin-heparin conjugate was eluted with 0.17 M NaCl-0.25 M KSCN-0.025 M Tris-HCl, pH 7.5. Fractions with an optical density exceeding 0.2 at 280 nm were pooled and extensively dialyzed against water. The conjugate was collected by freeze drying and the product was stored at 4^{0}C. This product was used for further studies. All chromatographic procedures were performed at 4^{0}C.

Determination of Heparin Concentrations

Heparin concentrations of the different column eluates were routinely determined according to a slightly modified metachromic assay described by Jacques et al. (8). To a solution of Azure A (3 ml, OD 505 nm ± 0.8) in barbital buffer (pH 8.6, ionic strength 0.04) an aqueous solution of heparin (5-100 μl, maximal concentration 5 mg/ml) was added. After mixing, the adsorption at 505 nm was measured against a similar solution of Azure A containing no heparin. The weight percentage of heparin in the albumin-heparin conjugates obtained after freeze drying was determined by 2 independent methods, the metachromic assay described above and the uronic acid assay described by Blumenkranz (9).

Determination of Albumin Concentrations

The albumin concentrations of the different column eluates were determined spectrophotometrically (10). The weight percentages of albumin in the albumin-heparin conjugates obtained were determined by the same method.

Biological Assays of the Albumin-Heparin Conjugate

Thrombin times of citrated plasma were performed with a Mechrolab Clottimer (Heller Laboratories, Santa Rosa, U.S.A.). A solution of heparin or albumin-heparin conjugate dissolved in saline (50 μl) was added to the plasma (950 μl). After 5 minutes incubation at 37^{0}C, the plasma solution (100 μl) was added to a thrombin solution (200 μl, 3.0 NIH U) at 37^{0}C and clotting times were registered.

Activated parital thromboplastin time (APTT) was also determined with a Mechrolab Clottimer. A solution of heparin or albumin-heparin conjugate in

saline (50 μl) was added to plasma (950 μl). After 5 minutes incubation at 37°C, the plasma solution (100 μl) was mixed with a suspension of Kaolin and Cephalin (PTT-reagent from Boehringer, 100 μl). After an incubation time of exactly 3 minutes, $CaCl_2$ (100 μl, 0.025 M) was added and the clotting time was registered.

Anti-Factor Xa activity of heparin and the albumin-heparin conjugate was determined according to the method described by Teien (11).

Gel Filtration Study of the Conjugate of Albumin and Heparin

Albumin-heparin conjugate (75 mg) was dissolved in 5 ml 0.17 M NaCl-0.025 M Tris-HCl, pH 7.5. This solution was applied to a column (2.6 cm x 95 cm) of Sephacryl S-300, which was equilibrated with 0.17 M NaCl-0.025 M Tris-HCl, pH 7.5. Elution was performed with the same buffer at a flow rate of 20 ml/hr and 4.5 ml fractions were collected. A solution of albumin (60 mg) and heparin (15 mg) in 5 ml 0.17 M NaCl-0.025 M Tris-HCl, pH 7.5, was eluted in the same way.

Fractionation of the Albumin-Heparin Conjugate with Immobilized Antithrombin III

Purified antithrombin III was coupled via amino groups to CNBr-activated Sepharose in the presence of heparin (12). Antithrombin III (46 mg) as received, was desalted with Sephadex G 25 and mixed with CNBr-activated Sepharose (20 ml) in 50 ml of 0.15 M NaCl-0.10 M $NaHCO_3$, pH 8.0. In order to protect the heparin binding site of the antithrombin molecule, heparin (150 mg) was also added. The mixture was gently rotated in a round bottomed flask for 20 hours at 0°C. Excess CNBr-activated groups were blocked by the addition of 5 ml of 1.0 M ethanol-amine solution, pH 8.0 (13). The anti-thrombin III immobilized gel was sequentially washed with 0.17 M NaCl and 1.5 M NaCl, collecting all washings. Quantitation of antithrombin III levels in the collected eluate revealed that 70% of the total antithrombin III was covalently bound to the gel. Affinity chromatography of both the albumin-heparin conjugate and heparin was performed as follows. A sample of albumin-heparin conjugate (100 mg in 10 ml 0.17 M NaCl-0.025 M Tris-HCl, pH 7.5) or heparin (5 mg in 5 ml 0.17 M NaCl-0.025 M Tris-HCl, pH 7.5) was applied to a column containing 20 ml of the prepared antithrombin III Sepharose gel, which was previously equilibrated with 0.17 M NaCl-0.025 M Tris-HCl, pH 7.5. Non-adsorbed materials (further indicated as low affinity (LA) heparin or albumin-heparin) were collected by washing the column with 2 column volumes of 0.17 M NaCl-0.025 M Tris-HCl, pH 7.5. The adsorbed materials (further indicated as high affinity (HA) heparin or albumin-heparin) were collected by washing the column with 2.0 M NaCl-0.025 M Tris-HCl, pH 7.5. The different fractions obtained were characterized by measuring the antithrombin and anti-factor Xa activities.

RESULTS

Preparation and Fractionation of Covalently Bound Albumin-Heparin Conjugates

The method developed for coupling albumin and heparin with EDC was based

on the procedure described by Danishefsky for the coupling of aminoethyl agarose and heparin (14). Woodward's reagent K is also often used for the formation of amide bonds (e.g. 15). The coupling of albumin and heparin was also tried with this reagent, however, no detectable amounts of coupling product were formed although temperature, pH, reaction time and the weight ratio of heparin and Woodward's reagent K were varied. The yield obtained with the EDC coupling method was 35-40% based on the original amount of albumin used and this was not dependent on the scale used for the coupling reaction (50 mg-10 g albumin). Figure 1 shows the elution profile of the dialyzed reaction mixture using a DEAE-cellulose column.

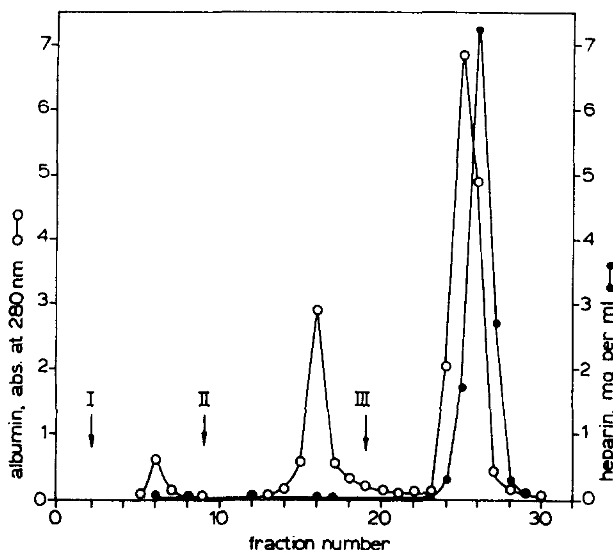

FIG. 1

Fractionation of albumin, heparin and the albumin-heparin conjugate on DEAE-cellulose. The arrows indicate: I, elution with 0.025 M Tris-HCl, pH 7.5; II, elution with 0.15 M NaCl-0.025 M Tris-HCl, pH 7.5; III, elution with 0.50 M NaCl-0.025 M Tris-HCl, pH 7.5. Fractions of about 20 ml were collected.

Figure 1 shows three separated albumin peaks. The first represents free albumin not bound on the column, the second represents free albumin bound on the column and the third represents albumin bound heparin. The fact that the third albumin peak doesn't coincide with the heparin peak demonstrates that free heparin was still present. Free heparin could be effectively removed by chromatography with Cibacron Blue Sepahrose. The elution profile is shown in Figure 2.

From Figure 2 it appears that the fractions obtained by eluting the column with KSCN, contained albumin as well as heparin. It may be concluded that albumin coupling with heparin has occurred, free heparin doesn't interact with Cibacron Blue Sepharose, and heparin doesn't bind to albumin at physiological pH (5).

Rechromatography of the freeze dried albumin-heparin conjugate obtained on DEAE-cellulose and Cibacron Blue Sepharose showed that the preparation still contained about 5% free albumin, and no free heparin could be detected.

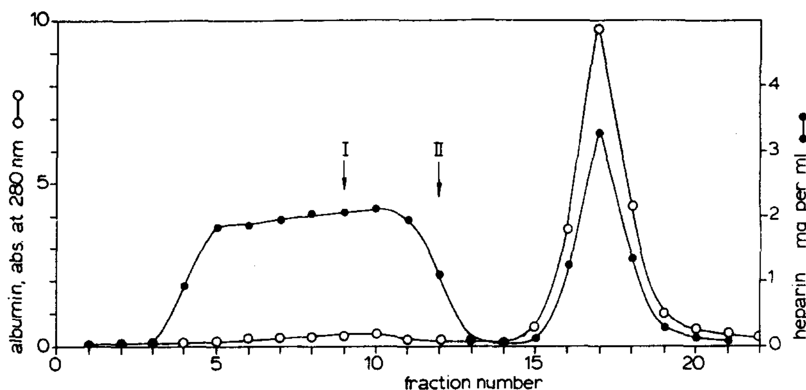

FIG 2

Fractionation of heparin and albumin-heparin conjugate on Cibacron Blue Sepharose. The arrows indicate: I, elution with 0.17 M NaCl-0.025 M Tris-HCl, pH 7.5; II, elution of the column with 0.17 M NaCl-0.25 M KSCN-0.025 M Tris-HCl, pH 7.5. Fractions of 20 ml were collected.

Characterization of the Albumin-Heparin Conjugates

The weight % of heparin in the conjugates obtained using identical procedures, varied between 10 and 12% as measured by the 2 colorimetric methods for the determination of heparin as previously described. Non systematic differences in the weight % of heparin up to 0.5% were observed when the results obtained with both methods were compared. When the coupling procedure was performed with a higher ratio of heparin to albumin, purified conjugates contained only a slightly higher weight % of heparin. Representative data are given in Table 1.

TABLE 1

Percentage of Heparin Present in the Albumin-Heparin Conjugates Prepared by Using Different Heparin Albumin Ratios.

grams heparin used	grams albumin used	ratio heparin/ albumin	mg EDC used	weight % of heparin in conjugate	yield (albumin used for coupling/ albumin present in conjugate)
0.15*	0.50*	0.3	50	10.2	52%
0.50	0.50	1.0	50	13.5	56%
1.50	0.50	3.0	50	15.6	61%

*Standard concentration of reactants.

The elution patterns of the albumin-heparin conjugate and a mixture of albumin and heparin on Sephacryl S-300 are shown in Figure 3.

The elution volume of the conjugate on Sephacryl S-300 (exclusion limit 1 x 10^6 D for globular proteins) is smaller than the elution volume of both heparin and albumin. The fact that the elution profiles coincide shows that the coupling reaction was successful and that the albumin-heparin conjugate is stable at physiological pH. From the results shown in Figure 3 it can be concluded that the molecular weight distribution of the conjugate is rather broad.

The biological activity of the albumin-heparin conjugates was compared with the activity of native heparin. Antithrombin activity (Fig. 4), anti-Factor Xa activity (Fig. 5) and activity against activated coagulation factors of the intrinsic pathway (APTT test, Fig. 6) were measured.

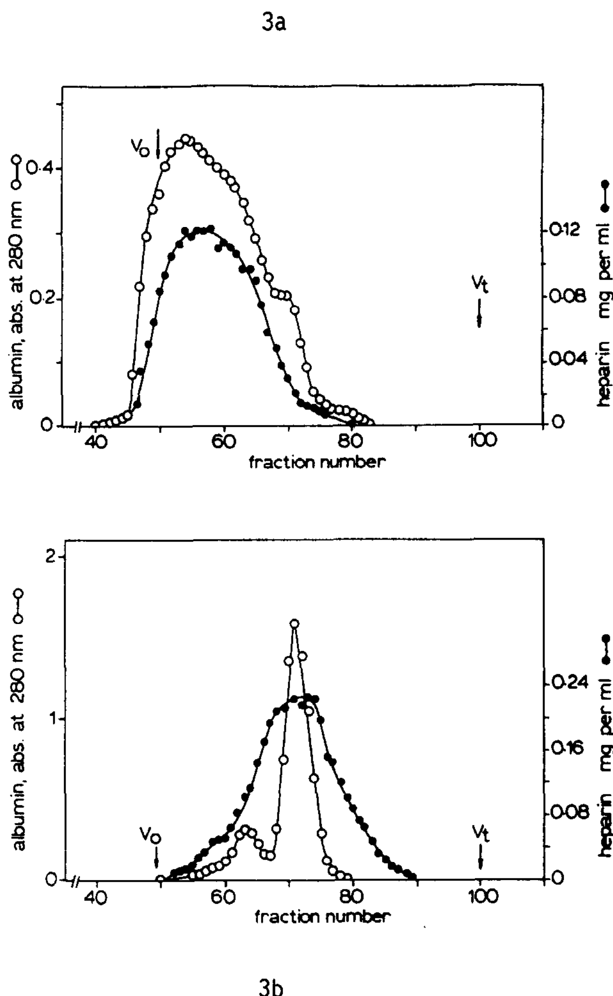

FIG. 3

Gel filtration of the albumin-heparin conjugate and of a mixture of albumin and heparin.

3a. Conjugate on Sephacryl S-300.

3b. A mixture of albumin and heparin on Sephacryl S-300.

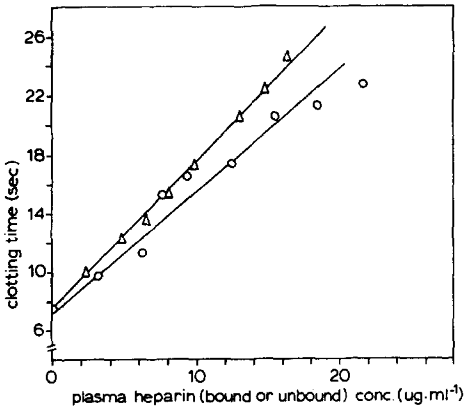

FIG. 4

Thrombin clotting times as a function of the heparin [bound (Δ) or unbound (o)] concentration.

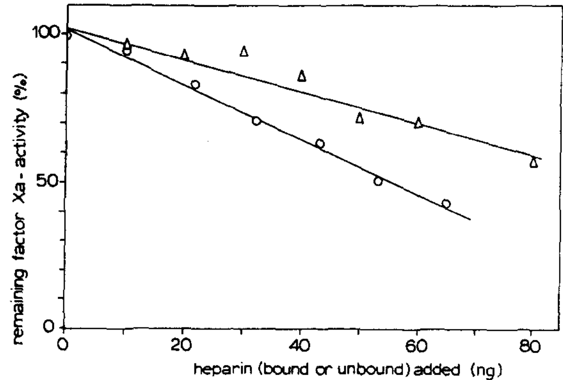

FIG. 5

Factor Xa inhibition as a function of the amount of heparin [bound (Δ) or unbound (o)] added in the presence of antithrombin.

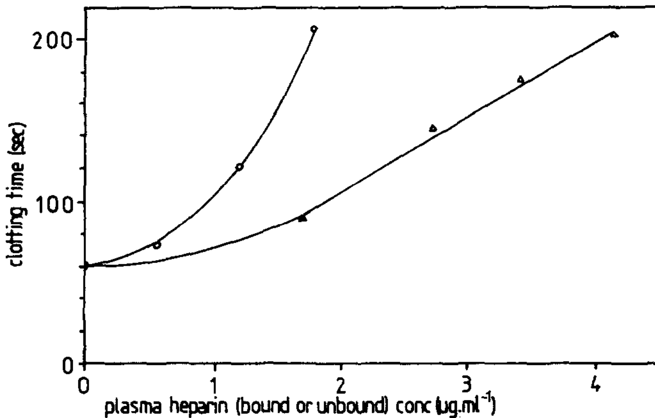

FIG. 6

APTT as a function of the heparin [bound (Δ) or unbound (o)] concentration.

The results show that heparin bound to albumin still possesses a substantial heparin activity. Heparin in the albumin-heparin conjugate is more active than unbound heparin when tested with thrombin (ratio 1.25). An activity ratio of 0.5 was measured using a test for Factor Xa inhibition and the APTT test.

Heparin and the albumin-heparin conjugates were fractionated with immobilized antithrombin III to yield portions with low affinity (LA) and high affinity (HA) for antithrombin III. Figure 7 shows the elution profile of the albumin-heparin conjugate on antithrombin III Sepharose. The LA and HA albumin-heparin conjugates obtained as well as LA and HA heparin were characterized by determining the antithrombin activity and the anti-Factor Xa activity (Fig. 8).

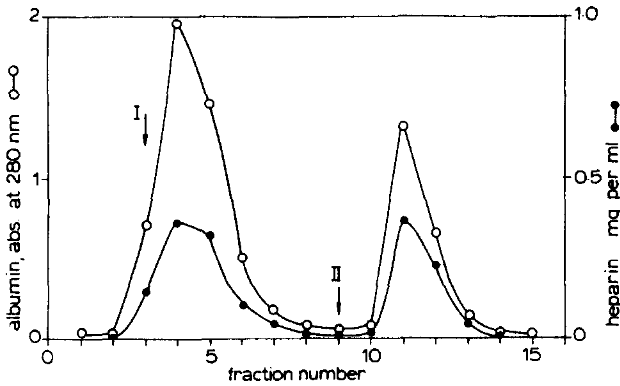

FIG. 7

Elution profile of albumin-heparin conjugate on anti-thrombin III Sepharose. Arrows indicate: I, elution with 0.17 M NaCl-0.025 M Tris-HCl, pH 7.5; II, elution with 2.0 M NaCl-0.025 M Tris-HCl, pH 7.5. Fractions of 5 ml were collected.

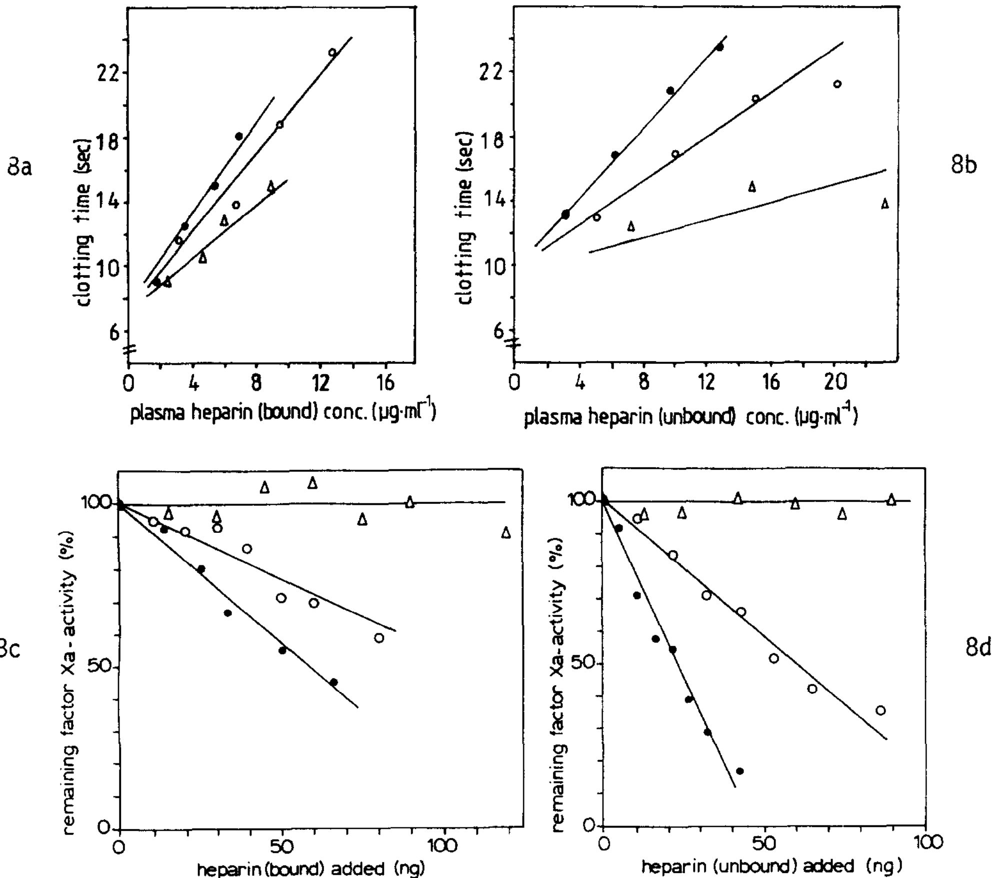

FIG. 8

Anticoagulant activities of LA and HA albumin-heparin conjugates and LA and HA heparins. 8a. Thrombin times of plasma in the presence of conjugate; 8b. Thrombin times in the presence of heparin; 8c. Factor-Xa inhibition in the presence of conjugate; 8d. Factor-Xa inhibition in the presence of heparin. (o) unfractionated, (Δ) LA material, (o) HA material.

DISCUSSION

The results presented here demonstrate that albumin-heparin conjugates can be reproducibly synthesized using EDC as a coupling agent. The conjugate contains about 40% of the albumin available for the reaction. EDC activates carboxyl groups which are present in heparin as well as in albumin (99 per molecule, (16)). The activated carboxyl groups react almost exclusively with amine groups of albumin. In principle a wide variety of coupling products like alb-hep, alb-alb, alb-alb-hep, hep-alb-hep can be expected. The results presented in Table I show that a variation of the ratio of albumin to heparin in the reaction mixture (0.3-3.0) only leads to small changes in the weight percentage of heparin in the conjugate while the yields were almost the same. This indicates that the carboxyl groups in heparin are more reactive than those in albumin, which might be due to more pronounced shielding of the carboxyl groups in albumin as compared to heparin.

The elution profile of the unpurified albumin-heparin conjugate on DEAE-cellulose was measured by determining heparin concentrations and albumin concentrations in the subsequent fractions. Figure 1 shows that after the elution of the column with 0.50 M NaCl, two main peaks were observed, which indicates that albumin-heparin as well as free heparin is present. Further purification was carried out with Cibacron Blue Sepharose. With this material free heparin could be effectively removed, indicating that the heparin moiety in the conjugate doesn't prohibit the binding of the albumin moiety onto Cibacron Blue Sepharose.

The anticoagulant activity of the conjugate is different from that of heparin. As pointed out by Danishefsky et al. (17), modification of heparin carboxyl groups leads to a decreased anticoagulant activity. In the coupling reaction of albumin and heparin, heparin carboxyl groups are modified and consequently the heparin activity changed. As expected, the anti-Factor Xa activity (Fig. 5) and the activity measured with the APTT assay (Fig. 6) of the conjugate, based on the amount of heparin present in the conjugate, is lower (ratio 0.5) than native heparin; but in contrast, the antithrombin activity (Fig. 4) is slightly higher than native heparin. This might be explained by the following. Thrombin inactivation by antithrombin III in the presence of heparin proceeds via a complex of antithrombin III and thrombin with heparin. Factor Xa inactivation, however, only requires binding of heparin with antithrombin III (18). To neutralize thrombin activity, heparin molecules must contain binding regions for both antithrombin III and thrombin. Heparin is heterogeneous and contains molecules which bind either antithrombin III or thrombin. Combination of such molecules in conjugates like hep-alb-hep can lead to increased rates of thrombin inactivation by providing a single molecule with both thrombin and antithrombin III binding sites. Another possibility is that albumin enhances the activity of conjugated heparin.

Teien et al. (19) prepared heparin-dextran-albumin conjugates and claimed these to have the same activity as heparin in test systems such as APTT, Thrombotest and thrombin clotting times. These conjugates were not purified and a considerable amount of free heparin was present, which makes the interpretation of the results difficult.

As expected, the amide linkage between albumin and heparin is stable. With gel filtration chromatography and rechromatography of purified conjugates no free heparin was detected. However, rechromatography of purified conjugate with DEAE-cellulose showed that the conjugate still contained 5-10%

of free albumin. This is caused by the fact that free albumin is not removed quantitatively by eluting the DEAE-cellulose column with 0.15 M NaCl-0.025 M Tris-HCl, pH 7.5. Because no free heparin was detected in the conjugates obtained, the free albumin does not originate from decomposed conjugate.

Affinity chromatography with antithrombin III Sepahrose provides heparin fractions with high antithrombin III affinity (and high anticoagulant activity) and with low antithrombin III affinity (and low anticoagulant activity) (12,20,21). The albumin-heparin conjugates can be fractionated in a similar way into low affinity (LA) and high affinity (HA) conjugates (Fig. 7), which were compared with unfractionated conjugate by determining the respective antithrombin and anti-Factor Xa activities. Because the antithrombin III column used had a low heparin binding capacity, elution was performed step wise. The weight % of heparin for HA and LA conjugates was 14.1 and 9.5, respectively. The differences in these weight fractions can be explained by the following facts. The ratio of HA to LA increases with increasing average molecular weights of heparin (21). Therefore, the heparin components of HA conjugates may be of higher average molecular weight than the heparin components of LA conjugates. Consequently, the HA conjugate contains a higher weight % of heparin than either the LA and unfractionated conjugate. The observation that the heparin binding capacity of the antithrombin III column used for fractionation of the conjugate is slightly higher than for native heparin (3.0 mg and 2.8 mg, respectively) support these presumptions.

Current investigations deal with the efficacy of adsorbed albumin-heparin conjugates for the prevention of surface thrombosis.

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