IN VIVO BEHAVIOUR OF SOME ANTITHROMBIN III-PROTEASE AND α_1-ANTITRYPSIN-PROTEASE COMPLEXES

By

LAURENCE SHEUNG-LUN LAM, B.Sc.

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Author: Laurence Sheung-Lun Lam, B.Sc. (Calif. State University, Los Angeles)

Supervisor: Doctor E. Regoeczi, Professor of Pathology

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ABSTRACT

Proteases participate in various aspects of different biological processes to serve purposes as diverse as nutrition and biological controls, all of which are effected by the cleavage of peptide bonds. Nevertheless, proteolysis is an irreversible process because there is no known biological repair mechanism for a broken peptide bond. To ensure that proteases act only beneficially, proteolytic activities are regulated by different control mechanisms, one of which is composed of the natural protease inhibitors. The inhibitors neutralize the proteases by complex formation. The interactions between inhibitors and proteases *in vitro* have been well documented whereas information on the *in vivo* behaviours of protease-inhibitor complexes is limited at present. α_2M-protease complexes have been shown to disappear from the circulation extremely rapidly while the clearance of α_1AT-protease complexes is comparatively slow. Thus, it seems probable that different elimination pathways may exist for different types of protease-inhibitor complexes. The purpose of this study is to examine the *in vivo* behaviours of some AT III-protease complexes, with an attempt to elucidate a probable elimination pathway for such complexes.

Complexes of rabbit, human and rat AT III with rabbit plasmin and of rabbit AT III with rabbit or bovine thrombin were formed from radioactively-labelled proteins and separated from the uncomplexed reactants by preparative electrophoresis. In certain cases, unlabelled proteins were complexed and the complexes were labelled after they had formed. The *in vivo* behaviour of the complexes was studied in rabbits by the

measurement of plasma radioactivity. The integrity of the complexes after injection into the rabbits was monitored by gel filtration of selected plasma samples on Sephadex G-200. Affinity chromatographic columns, such as Sepharose-AT III, -trypsin, -heparin and -lysine were used for the isolation and study of the various components. The stability of AT III-protease complexes under different in vitro conditions was examined by gel filtration and polyacrylamide gel electrophoresis. As a comparison, the complex of rabbit α_1AT with rabbit plasmin was also studied. α_1AT-plasmin complex was separated from uncomplexed materials by affinity chromatography on a Sepharose-lysine column.

The AT III-protease complexes tested were eliminated considerably more slowly than α_2M-protease complexes. The speed of elimination of AT III-protease complexes resembled that of α_1AT-protease complexes. All five combinations of AT III-protease complexes dissociated soon after injection into rabbits, though to variable extents. However, rabbit AT III-plasmin complexes were stable for at least 6 h at 37°C in vitro. Post-complex AT III and post-complex plasmin appeared to have been altered as compared to the corresponding native proteins. Thus post-complex AT III lost more than 80% of its affinity for Sepharose-heparin and this form of the inhibitor no longer bound to Sepharose-trypsin (80% of the control AT III bound to conjugated trypsin). However, post-complex AT III was eliminated only marginally faster than the native AT III and it retained its reactivity with antibodies raised against normal AT III. Post-complex plasmin did not bind to Sepharose-AT III or -Trasylol but about 86% of its ability to bind to Sepharose-lysine was preserved. Whether the above observations for AT III-protease

complexes in rabbits hold true in other species, remains to be established.

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INTRODUCTION

In the mammalian circulation proteolytic enzymes are produced during the activation of several plasma systems such as the clotting system (1), the kallikreinkinin system (2), the complement system (3), and the fibrinolytic system (4). These systems are interrelated; activation of one system can lead to activation of one or more of the other systems as a consequence of the proteases produced. Proteases exist as inactive proenzymes until they are activated. However, proteolytic activities in excess of biological requirements are usually neutralized by various protease inhibitors, thus maintaining a delicate biological equilibrium (5,8). Overactivation or inadequate inhibition of these proteases can result in pathological states such as pulmonary emphysema (5,8), disseminated intravascular clotting (6) and haemorrhagic pancreatitis (7), just to name a few.

Mammalian plasmas contain a variety of protease inhibitors which counteract the actions of the protease by the formation of enzyme-inhibitor complexes (8). At least six of these protease inhibitors (PIs) have been isolated in pure forms and characterized with regard to their interactions with the proteases in vitro. These include alpha 1-antitrypsin (α_1-AT: 9-11); alpha 2-macroglobulin (α_2M: 12-16); alpha 1-antichymotrypsin (α_1X: 17,18); inter-alpha-inhibitor (IαI: 19,20); C1 esterase inactivator ($\overline{C1}$ INA: 21,22) and antithrombin III (AT III: 23-27). Recently, a fast reacting α_2-antiplasmin has also been described (28-33).

Except for α_1X and possibly α_2-antiplasmin, all the protease

inhibitors mentioned above have broad spectra of action. Knowledge concerning the interactions of these PIs with various proteases has been obtained mainly from studies on in vitro systems in which purified components were used. Only in a few cases has the fate in vivo of the protease-inhibitor complexes been examined. Hence, very little information on the in vivo behaviours of the protease-inhibitor complexes is available at present.

It has been established that α_2M binds all four classes of endopeptidase by a "trapping" mechanism (16). The enzymes cleave a peptide bond in a sensitive region of the macroglobulin, leading to a confomational change in the α_2M which then "traps" the enzymes irreversibly. Access of substrates to the active site of the enzymes becomes sterically hindered such that the "trapped" enzymes remain active only towards low molecular weight substrates but not to higher molecular weight natural protein substrates (14). Different binding ratios of α_2M to enzymes ranging from 1:1, (16,34,35,40), to 1:2 (36-38, 40) and 1:3 (39) have been reported. However, the results obtained by Barrett and Starkey (16) by using labelled proteases and reliable active site titration, in general, favour a binding ratio of 1:1 of α_2M to all four classes of endopeptidase.

In vivo data show that α_2M-protease complexes disappear from the circulation rapidly, usually in the order of minutes (40-45). When labelled, preformed complexes of proteases with α_2M and α_1M are injected into animals, the radioactivity is quickly accumulated by organs rich in macrophages (45-47). In view of the broad specificity of α_2M, the fast removal of such complexes seems to offer an effective defence mechanism against

excessive proteolytic activities arising *in vivo*.

Alpha 1-antitrypsin has been shown to inhibit trypsin, chymotrypsin, collagenase and elastase instantaneously at a 1:1 molar ratio but to react with plasmin and thrombin only "progressively" (40-42, 48,49). Contradictory observations concerning the stability of α_1AT-protease complexes have been reported. Complexes of human α_1AT with porcine or bovine trypsin are found to be stable to reducing and denaturing agents (50,51). However, strong nucleophilic agents (50), high pH (52,53) sodium dodecyl sulphate (54) and long periods of incubation (55) have all been found to cause dissociation of α_1AT-trypsin complexes *in vitro*. Complexes of α_1AT-trypsin, α_1AT-collagenase and α_1AT-elastase also tend to dissociate and transfer the enzymes to α_2M both *in vitro* and *in vivo* (40-44). Enzymic activities have been recovered *in vitro* to variable extents after the dissociation of proteases from α_1AT (43,44,51-55). However, destruction of the inhibitor by acidification of α_1AT-trypsin complexes did not lead to recovery of tryptic activity (48). α_1AT is thought to be altered by limited proteolysis during complex formation with proteases so that it is inactive after dissociation from the complexes (50-53).

In contrast to α_2M-protease complexes, complexes of α_1AT with proteases disappear from the circulation comparatively more slowly and there are indications of the enzymes being transferred from such complexes to α_2M (40-44). This raises the possibility that there may be different elimination pathways for different types of protease-inhibitor complexes.

Nothing is known at present about the fate of complexes of AT III with proteases in vivo. However, in vitro observations suggest that AT III may regulate different plasma enzyme systems at key positions. Heparin dramatically accelerates the otherwise "progressive" inhibition by AT III of the various proteases (23,26,56-66) although the precise mode of action of heparin is still a controversial matter. AT III inhibits factor XIIa (59,65,66) as well as its active fragments (59), and in the presence of heparin, it blocks CIs (67). It is known that active fragments of factor XIIa activate prekallikrein to kallikrein (68) and generate plasminogen activator from proactivator (69), AT III thus seems able to influence the initiation of the intrinsic clotting pathway, the kallikrein-kinin, the fibrinolytic and the complement systems.

AT III may also control the converging point of both the intrinsic and extrinsic coagulant pathways by being the major inhibitor of factor Xa (61-63, 70-72). Thrombin, the primary enzyme for fibrin-clot formation, is inhibited by AT III which accounts for approximately 75% of the "progressive" antithrombin activity of normal plasma (23,24). AT III may be an important inhibitor for maintaining a hypothetical haemostatic balance between intravascular coagulation and fibrinolysis because it also inhibits the fibrinolytic enzyme, plasmin (73). The extent of amplification achieved by the cascade mechanism for blood coagulation (74) could be further moderated by AT III via inhibition of factor IXa (57,62,72) and factor XIa (58). AT III has been shown to form stoichiometric complexes (1:1) with the various proteases and such complexes are stable in vitro to reducing and denaturing agents (26,57-59, 72,73).

Because of the difficulty in simulating in vivo situations by in vitro tests, the relative significance and contribution of the protease inhibitors to biological control can only be assessed in conjunction with information obtained from studies on the in vivo behaviours of these inhibitors and their complexes with the various proteases. The elimination pathway for AT III-protease complexes is hitherto unknown. It is, therefore, the main objective of this study to investigate the in vivo behaviours of some AT III-protease complexes, with an attempt to elucidate a probable elimination pathway for such complexes. As a comparison, the complex of rabbit α_1AT with rabbit plasmin was also studied.

MATERIALS AND METHODS

CHEMICALS

Sepharose-6B, DEAE-Sephadex, Sepharose-concanavalin A and Sephadex G-200 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Cyanogen bromide N'N'-methylene-bisacrylamide and N,N,N'N'-tetramethylethylenediamine were from Eastman Kodak Co., Rochester, N.Y., U.S.A. Potassium phosphate (tri-and di-basic), sodium hydroxide, sucrose and urea were from J.T. Baker Chemicals Co., Philipsburg, N.J., U.S.A. Sodium hydrogen carbonate, L-lysine monohydrochloride, ammonium acetate, sodium chloride, glycine, ammonium persulphate, acetic acid, ethylenediamine tetraacetic acid (disodium salt) were from BDH Chemicals Ltd.,

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Abbreviations

AmAc, ammonium acetate. AT III, antithrombin III, α_1-AT, α_1-antitrypsin, BAEE, α-N-benzoyl-L-arginine ethyl ester. CNBr, cyanogen bromide. DFP, diisopropylphosphofloridate. DFP-Pln, diisopropylphosphofloridate-inactivated plasmin. εACA, ε-aminocaproic acid. EDTA-Na_2, disodium ethylenediamine tetraacetic acid. Pln, plasmin. Pln-AT III, plasmin-antithrombin III complex. Pln-α_1AT, plasmin-α_1-antitrypsin complex, S-6B, Sepharose 6B. S-AT III, Sepharose-antithrombin III. S-T, Sepharose-Trasylol. TIU, trypsin inhibitory unit.

Poole, England. Heparin (sodium salt), ε-aminocaproic acid, α-N-benzoyl-L-arginine ethyl ester (BAEE), diisopropylphosphofluoridate (DFP) were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Ammonium sulphate, Tris (hydroxymethyl) aminomethane, sodium azide, sodium iodide, potassium hydroxide and trichloracetic acid were from Fisher Scientific Co., Toronto. DEAE-cellulose was from Whatman, Maidstone, Kent, U.K. Acrylamide was from BIO-RAD Laboratories, Richmond, Ca., U.S.A. $Na^{125}I$ and $Na^{131}I$ were from New England Nuclear, Boston, Ma., U.S.A. Zerolit FF-IP (Cl^--form) was from Zerolit Ltd., Pemberton House, Middlesex, England. Cibacron blue 3G-A was from CIBA-Geigy, Toronto, Canada.

All chemicals used were reagent grade.

AFFINITY CHROMATOGRAPHIES

a. Preparation of the Gel Matrix

Sepharose 6B was used throughout. Activation was done by the CNBr method as described by Porath et al. (75). Routinely, 60 g of washed gel was suspended in 100 ml of cold 5M potassium phosphate buffer (pH 13; 3.33 moles of K_3PO_4 + 1.67 moles of K_2HPO_4/litre) in an ice bath. Six g of CNBr dissolved in 30 ml of distilled water was added to the gel (with stirring) within a period of 2 min and the reaction allowed to go on for another 8-10 min. The activated gel was washed in a Buchner funnel under suction with 2 litres of distilled water followed by 2 litres of 0.25M $NaHCO_3$, pH 9.0. Alternatively, Sepharose-6B was activated with CNBr as described by Cuatrecasas (76). Ten g of well-washed S-6B was suspended in 5 ml distilled water and 1.1 g CNBr in 15 ml distilled water was added to it, the pH of the reaction was kept at 11 by addition

of 4M NaOH, reaction time was 10 min.

b. Coupling Lysine

Activated gel (60 g) was added to 6-7 g of lysine dissolved in 100 ml of 0.25M $NaHCO_3$, pH 9.0 and stirred gently for 20 h at 2°C. The coupled gel was washed with 2 litres af 0.5M $NaHCO_3$, pH 9.0, 2 litres of distilled water and 2 litres of the appropriate buffer.

c. Coupling Trypsin

0.3 g of trypsin in 100 ml of 0.25M $NaHCO_3$, pH 9.0 was coupled as in (b) using 50 g activated gel.

d. Coupling Heparin

The method described by Miller-Andersson et al. was followed (26). Four g of CNBr was dissolved in 30 ml distilled water. To this were added 500,000 units of heparin, then 30 g of washed Sepharose-6B was stirred into this solution and the pH kept between 10-11 by dropwise addition of 4M NaOH. The reaction was allowed to go on for 10-12 min at room temperature, then transferred to 2°C and stirred gently for 20 h. Then the coupled gel was washed with 2 litres of 1M NaCl, 2 litres of distilled water and 2 litres of the appropriate buffer.

e. Coupling AT III or Trasylol

The gel was activated by the method of Cuatrecasas and washed extensively with 0.1M $NaHCO_3$ pH 9.0, then was added to approximately 6 mg of AT III or 218 TIU of Trasylol in 0.1M $NaHCO_3$ pH 9.0 and stirred

gently for 20 h at 2°C. Subsequently the gel was washed as described above.

All affinity chromatography columns were operated at room temperature.

PROTEINS*

Trypsin (2 x crystallized) was from Worthington Biochemical Coop., Freehold, N.J., U.S.A. Trasylol and bovine serum albumin were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Urokinase was from Leo Pharmaceuticals, Copenhagen, Denmark. Bovine thrombin was from Parke-Davis Co., Detroit, Mi., U.S.A. Arvin was from Twyford Laboratories Ltd., High Wycombe, Bucks., U.K.

The thrombin preparations were kindly provided by Dr. M.W.C. Hatton. Bovine thrombin was purified from Parke-Davis commercial thrombin by chromatography on Sepharose-heparin (77) and rabbit thrombin was prepared from ACD plasma by an Al $(OH)_3$ adsorption technique followed by Sepharose-lysine affinity chromatography (78).

Isolation of Rabbit Plasmin by Sepharose-Lysine Chromatography

Plasmin was isolated using a method developed in this laboratory (77). Rabbit blood was collected into ACD anticoagulant (0.8 g citric acid, 2.2 g sodium citrate, 2.45 g glucose in 100 ml distilled water; 6 vol. blood: 1 vol. ACD). The plasma obtained was difibrinated with Arvin (0.15 U Arvin/ml plasma at 37°C for 4 h) and the clot removed by

* Unless otherwise stated, Pln, AT III, α_1AT and thrombin used in the experiments were of rabbit origin.

winding on to applicator sticks. After centrifugation at 3000 g for 10 min, 200–300 ml of the defibrinated plasma was passed through a Sepharose-lysine column (2.3 cm x 12 cm) equilibrated with 0.1M ammonium acetate pH 7.2 (AmAc) at room temperature. The column was then washed with 500 ml of 0.5M NaCl followed by 200 ml of 0.1M AmAc pH 7.2. One ampoule of urokinase (2330 Ploug U), dissolved in 4 ml of 0.1M AmAc, was loaded on to the column, the flow stopped for 15 min and 250 ml of 0.1M AmAc, pH 7.2 was passed through the column. The plasmin was step-eluted with 0.05M ε-aminocaproic acid (εACA) in 0.1M AmAc, pH 7.2. Five-ml fractions were collected using an LKB fraction collector and the protein content monitored by their absorbancy at 280 nm. The protein peak was pooled and concentrated by pressure dialysis at 2°C against the eluting buffer. Concentrated plasmin was subdivided into 0.5 ml portions and frozen at -40°C until use. The specific esterase activity of the preparations at room temperature ranged from 2 to 3 μmoles BAEE hydrolyzed/min/mg protein.

Inactivation of Plasmin by Diisopropylphosphofloridate (DFP-Pln)

Plasmin (2 mg in 0.5 ml 0.05M εACA in 0.1M AmAc pH 7.2) was inactivated by incubating with 0.1 ml DFP (0.5M in propylene glycol) for 20 h at 37°C. DFP-Pln was dialysed against the appropriate buffer before use. Inhibition was > 98% as determined by BAEE assays.

Preparations of Antithrombin III (AT III)

Rabbit plasma was made 40% saturated by adding 24 g of solid ammonium sulphate to 100 ml of plasma and stirred for 30 min at room

temperature. The precipitate was removed by centrifugation at 9000 g for 20 min at 4°C and the supernatant was dialysed against several changes of 0.02M phosphate pH 7.3 containing 0.15M NaCl, at 2°C for 24 h. 100-150 ml of the dialysed supernatant was passed through a Sepharose-heparin column (1.3 x 12 cm) equilibrated with the dialyzing buffer at room temperature. The column was then washed with 0.02M Na phosphate pH 7.3 containing 0.3M NaCl, until the absorbance of the effluent at 280 nm was < 0.01. AT III was eluted from the column by an NaCl gradient, (the mixing chamber contained 150 ml of 0.05M Tris-HCl pH 8.0 containing 0.15M NaCl, the limit solvent was 150 ml 0.05M Tris-HCl pH 8.0 containing 2M NaCl). Fractions of 5 ml were collected, the protein peaks (see result) were separately pooled and concentrated by pressure dialysis against 0.05M Tris-HCl pH 8.0 containing 0.15M NaCl at 2°C. The concentrated samples were analyzed by alkaline gels (7.5% polyacrylamide, pH 8.3) and their inhibitory activities towards plasmin were determined by using BAEE as a substrate (see below). Material with the highest inhibitory activity was stored at -40°C until use. Human and rat AT III were prepared in the same manner.

Isolation of Rabbit α_1-Antitrypsin (α_1-AT)

Rabbit α_1-AT was kindly prepared by Dr. A. Koj as described elsewhere (80). Briefly, α_1-AT was isolated from rabbit plasma by salting out with $(NH_4)_2SO_4$ followed by ion-exchange chromatography, either on DEAE-Sephadex or DEAE-cellulose (each at pH 8.7 and 6.5), and affinity chromatography on Sepharose-Cibacron Blue and Sepharose-concanavalin A. The protein thus obtained was homogenous during crossed immunoelectrophoresis

by using an antiserum to whole rabbit plasma prepared by Dr. J. Gauldie. It consisted of a fast and a slow component when electrophoresed in alkaline polyacrylamide gel. Each component possessed antitryptic activity, therefore preparations containing both components were used to form complex with plasmin without further fractionation by preparative electrophoresis.

Analytical Polyacrylamide Gel Electrophoresis

The method was modified from that of Clarke (81). Eight polyacrylamide gels (7.5%) were prepared from the following solutions: 6 ml of 40% acrylamide with 0.4% N,N'-methylene-bisacrylamide, 6 ml of 0.05M Tris-0.39M glycine buffer, pH 8.3, 4 ml of 0.1% N,N,N',N'-tetramethylethylenediamine and 16 ml of 1% ammonium persulphate. Distilled water (2-3 mm) was layered on top of the gels which were allowed to set at room temperature for an hour. The gels were pre-electrophoresed at a constant current of 4mA per gel in 0.005M Tris-0.039M glycine buffer, pH 8.3 for 1.5 h before loading. Protein solutions of less than 0.1 ml were loaded and run for 1 h under the above conditions. The gels were stained for 30 min with 0.1% amido black in 7% acetic acid and then destained with 7% acetic acid.

Preparative Polyacrylamide-gel Electrophoresis

Isolation of plasmin-antithrombin III complexes was carried out in an apparatus described by Pajdak (82). A polyacrylamide-gel column (7.5%, 3 cm x 3 cm) was prepared. The running buffer was 0.005M Tris-0.039M glycine, pH 8.3. Samples of proteins to be separated (1.0-1.5 ml)

were made dense by the addition of sucrose. A trace amount of Bromophenol Blue was added to the sample before layering it on top of the gel column. Electrophoresis was carried out at a constant current of 20 mA first at 2°C. Sampling was started when the tracking dye reached the anode chamber by collecting fractions at 10-min intervals. After the first 5 fractions had been collected the rest of the run was carried out at room temperature. The fractions were monitored for their radioactivity (see below) and selected fractions by analytical polyacrylamide-gel electrophoresis.

Determination of Esterase Activity Using Benzoyl-L-Arginine Ethyl Ester (BAEE)

The esterase activity of plasmin and its inhibition by α_1AT and AT III was assayed using BAEE as a substrate following the method of Rick (83). BAEE solution (0.17 mg per ml in 0.05M Tris-HCl pH 8.0) was freshly prepared for each assay. Plasmin (30-40 µg) was added to a test tube and the volume brought to 0.6 ml with buffer. Reaction was started by adding 2.6 ml of BAEE solution to the tube, mixed and read after transfer to quartz cuvettes of 1 cm path at 254 nm. Readings were taken every 30 sec. for 5 min.

In the inhibition studies, the molar ratio of inhibitors (α_1AT or AT III) to plasmin was approximately 1.5 in a total volume of 0.6 ml. They were preincubated for 5 min at room temperature before adding 2.6 ml of BAEE. The residual esterase activity was measured. Esterolytic activities were calculated by using the following conversion factor: 1 µmole BAEE hydrolyzed/min = Δ 254/min of 0.359 (83).

Iodination of Proteins

Samples of proteins (plasmin, thrombin, α_1AT or AT III, 2-3 mg each) were labelled with either ^{125}I or ^{131}I using the iodine monochloride method (84). Iodination was carried out at pH 8-9 in a vol. of 0.5-0.75 ml. The number of iodine atoms substituted per mole of protein was 1.5 or less. After iodination of plasmin, thrombin or α_1AT, the non-protein radioactivity was removed from the reaction mixture by passing it through a 2-ml column of Zerolit, a strong anion exchange resin. For plasmin, the column was equilibrated with 0.05M εACA in 0.15M NaCl and for α_1AT and thrombin the columns were equilibrated with 0.15M NaCl.

In the case of AT III, the free radioactivity was removed by dialyzing against three changes of 2 litres of 0.005M Tris-0.039M glycine pH 8.3 at 2°C for 30 h.

Sephadex Gel Filtration

Selected plasma samples from rabbits injected with labelled proteins were chromatographed on a Sephadex G-200 column (2.3 cm x 50 cm) equilibrated with 0.02M Na phosphate pH 7.4 containing 0.3M NaCl at room temperature. Azide (0.1% w/v) was added to the buffer as bacteriocidic agent. Rabbit blood was collected into test tubes containing disodium ethylenediamine tetraacetic acid (EDTA-Na_2) as an anticoagulant (5 mg/ml of blood). Plasma was obtained by centrifugation at 3000 g for 10 min. Plasma, (1 ml, made denser by the additon of sucrose), was layered on to the top of the column which was developed with the equilibrating buffer at a flow rate of approximately 40 ml/h. Fractions of approximately

5 ml were collected with an LKB fraction collector and monitored by measuring both their absorbance at 280 nm and their radioactivity.

Preparation of Plasmin-α_1AT Complex

Plasmin was dialyzed against 0.1M AmAc pH 7.2 to remove εACA from the preparation. A complex between labelled α_1AT and unlabelled plasmin was formed by incubating the two proteins together (1 mg plasmin + 1 mg α_1AT) at 37°C for 1-1.5 h. Because preliminary experiments showed that α_1At had no affinity for Sepharose-lysine, uncomplexed α_1AT was removed by passing the incubation mixture through a Sepharose-lysine column (1.3 cm x 11 cm) equilibrated with 0.1M AmAc, pH 7.2 followed by washing with the same buffer.

The bound proteins were step-eluted with 0.1M AmAc, pH 7.2 containing 0.1M εACA. Fractions with the highest radioactivity were pooled and concentrated by pressure dialysis against 0.005M Tris pH 8.0 containing 0.15M NaCl at 2°C. The identity of the eluted protein as a complex was confirmed by performing a parallel experiment with unlabelled proteins. The protein not retained by Sepharose-lysine showed strong antitryptic activity, whereas the bound protein had neither antitryptic activity nor BAEE esterase activity. Furthermore, a molecular species with intermediate electrophoretic mobility (as compared to Pln and α_1AT) was detected when the eluted fractions were analysed with disc gel electrophoresis.

Preparations of Thrombin-AT III Complex

Labelled bovine or rabbit thrombin (in 0.15M NaCl) and rabbit

AT III (in 0.005M Tris-HCl pH 8.0) were incubated at 37°C for 1 h and the complex thus formed was isolated by preparative electrophoresis and pressure dialysed against 0.15M NaCl.

Preparation of Plasmin-Antithrombin III (Pln-AT III) Complex

Depending on the experimental design, different kinds of labelled Pln-AT III were prepared.

(a) A dual-labelled complex was prepared by incubating differently labelled plasmin and AT III together.

(b) A single-labelled complex was formed by incubating unlabelled Pln with labelled AT III.

(c) A Pln-AT III complex was also formed using unlabelled proteins and after purification by preparative electrophoresis, it was labelled with iodine.

In each case, the incubation mixture contained approximately 1 mg Pln in 0.1M AmAc pH 7.2 containing 0.05M εACA and 1 mg AT III in 0.005M Tris-HCl pH 8.0. After incubating for 1 h at 37°C, the incubation was continued at 4°C for 20 h.

Uncomplexed proteins were separated from the complex by preparative electrophoresis as already described (p.12). The fractions were monitored by their radioactivity and selected fractions were analyzed by disc gel electrophoresis. The fractions containing the complex were pooled and concentrated by pressure dialysis against 0.05M Tris-HCl pH 8.0 containing 0.15M NaCl at 2°C.

Complexes between rabbit plasmin and human or rat AT III were formed and isolated by essentially the same technique.

In vivo Experiments

(a) Animals

New Zealand white rabbits with body weights ranging from 2.5-5.5 kg were used. They were fed a standard pelleted diet and the drinking water contained 0.005% (w/v) NaI.

(b) Measurement of the Radioactive Dose

Standards for each injection were prepared as follows: 1-2 drops of the material to be injected were transferred to a test tube, the amount was determined by the difference in weight of the test tube before and after the transfer. The drops were then immediately washed over into a 50-ml volumetric flask using 40% urea in 0.2M KOH (this disrupted hydrogen bonding, thus minimizing attachment of labelled proteins to the test tube wall). Duplicates of 2 ml of this preparation were pipetted into test tubes and counted as standards. The amount of radioactive proteins injected was determined by weighing the syringe before and after delivering its contents. The dose received by the animals was calculated from the above data (85).

The amount of proteins injected into the rabbits was in the range of 0.3 mg-1 mg.

(c) Preparation of Samples for Counting

Labelled preparations were injected into the rabbits via a

marginal ear vein and blood samples collected from the other ear at appropriate time intervals. Plasma was obtained by centrifuging blood samples collected into heparinized tubes at 3000 g for 10 min. Duplicates of 0.5 ml plasma were pipetted into test tubes and the volumes made up to 2 ml by adding 1.5 ml of 0.15M NaCl for total plasma radioactivity countings.

Non-protein radioactivity was measured by deproteinizing the plasma samples with 2 ml of 20% trichloroacetic acid and counting 2 ml of the supernatant. Protein bound radioactivity was then calculated from the difference between total and non-protein radioactivities.

The geometry of the samples for counting was kept constant by using 15 mm x 85 mm test tubes with a 2 ml sample volume. Radioactivities were measured in a Packard model 5986 multichannel analyzer with approximately 60% photopeak efficiency for ^{125}I and 29% for ^{131}I.

Isotopic decays were corrected by reference to the standards which were counted along with all the samples.

In all experiments the plasma sample taken 5 min after injection was used as 100% activity and subsequent samples were expressed as a percentage of this value. Protein bound radioactivity was plotted against time on 2-cycle semilogarithmic paper.

The following sampling schedules were followed whenever possible:

1. For DFP-Pln

DFP-Pln injected was either in 0.15M NaCl or 0.15M NaCl containing 0.05M εACA depending on the experimental design. The first

blood sample was collected 5 min after injection and subsequent samples were collected at 30 min-intervals for 5-6 h.

2. For Pln-α_1AT Complex and Uncomplexed α_1AT

Uncomplexed labelled α_1AT in 0.15M NaCl was injected three days before the Pln-α_1AT complex (in 0.005M Tris-HCl pH 8.0 containing 0.15M NaCl, with the label in the α_1AT moiety). Three samples were collected on the first day, that is, at 5 min, 1.5 h and 6.5 h after injection. Two samples (one in the morning and one in the afternoon) were taken daily on the second and third days. Pln-α_1AT complex was injected on the fourth day. Further samples were obtained at 5 min and then at 30 min intervals for 6 h.

3. For Pln-AT III Complex and Uncomplexed AT III

After injecting the dual-labelled complex in 0.05M Tris-HCl pH 8.0 containing 0.15M NaCl, samples were taken at 5 min, 0.6 h, 1.5 h, 2.5 h, 3.5, 6.5 h, 11 h, 23.5 h and 30 h.

When single-labelled complex (labelled in the AT III moiety) was injected together with a differently labelled uncomplexed AT III, the same time table as that for the dual-labelled complex was used for the first two days. Then 2 samples were taken daily until the termination of the experiment at approximately 100 h.

4. Reisolation of Dissociated AT III and Uncomplexed AT III

The complex formed between labelled AT III and unlabelled Pln was injected simultaneously into a rabbit with a differently labelled uncomplexed AT III. Approximately 30 h after injection, 30 ml of blood

was collected into 5 ml of ACD anticoagulant. Reisolation of the injected AT III preparations by Sepharose-heparin column was attempted as described (p.12).

The protease-binding activity of the reisolated labelled AT III forms was determined by loading the proteins on to Sepharose-trypsin columns (1.3 cm x 6 cm) equilibrated with 0.05M Tris-HCl, pH 8.0 containing 0.15M NaCl at room temperature. Samples were dialyzed against the same buffer before loading. The radioactivity that passed through the column was rechromatographed on a second column of Sepharose-trypsin to ensure that overload conditions did not influence the results. The binding capacity of the Sepharose-trypsin batch with regard to AT III was tested on a separate column using labelled AT III which had not been injected into an animal.

5. Examination of the Plasmin after its Dissociation from the Pln-AT III Complex In vivo

A dual-labelled complex between rabbit Pln and rat AT III was formed and isolated as described. The sample was injected into a rabbit. Eleven h later a blood sample (10 ml) was collected into EDTA-Na_2 and the cells were removed by centrifugation at 3000 g for 10 min. The plasma, containing the complex, dissociated Pln and AT III, was utilized as follows: 1 ml was chromatographed on Sephadex G-200; another one ml of plasma was subjected to affinity chromatography on a column of Sepharose-lysine (1.5 ml volume equilibrated with 0.15M NaCl). The column was washed with approximately 20 ml of 0.15M NaCl and eluted first with 0.5M NaCl followed by 0.1M εACA. The fractions eluted by εACA were

pooled and dialyzed against 0.1M NaCl (3 x 500 ml) at 2°C. Following concentration in an Amicon ultrafiltration chamber, one portion of the protein solution was loaded on a column of S-AT III (1 ml volume equilibrated with 0.1M NaCl), washed with approximately 30 ml of 0.1M NaCl and then eluted with 1M NaCl.

The other portion was loaded on a column of S-T (1 ml volume; equilibrated with Ringer's lactate), washed with approximately 30 ml Ringer's lactate and eluted with 1M NaCl.

Effluent fractions from the Sephadex G-200 column, containing dissociated Pln, were pooled and concentrated by pressure dialysis against 0.1M NaCl. The protein solution thus obtained was analyzed by chromatographies on Sepharose-lysine and Sepharose-AT III as already described above.

RESULTS

Isolation of Rabbit Plasmin

The plasmin was obtained by activating plasminogen adsorbed to Sepharose-lysine by passing urokinase through the column, and was eluted in a sharp peak with 0.1M AmAc pH 7.2 containing 0.05M εACA (Fig. 1). The quality and characteristics of plasmin prepared this way have been described (77).

The esterolytic activity of 32 preparations of plasmin ranged from 2 to 3 μmoles BAEE hydrolyzed/min/mg at room temperature. Approximately 2-3 mg of plasmin was usually obtained from 100 ml of plasma. The plasmin did not migrate too far from the origin of the gel column when electrophoresed in alkaline gel (7.5% polyacrylamide, pH 8.3), and it showed a broad and diffused band(s) after staining (refer to gel no. 4 in Fig. 6). The presence of εACA allowed the plasmin preparations to be concentrated without causing precipitation of the protein. Removal of εACA from the preparation by dialysis resulted in a cloudy solution.

Preparation of Rabbit, Human and Rat Antithrombin III (AT III)

After passing the supernatant from 40% saturated plasma through Sepharose-heparin, the NaCl gradient resolved the adsorbed proteins into three effluent peaks. Fig. 2 shows a typical elution pattern for a preparation of rabbit AT III. The peaks in the shaded areas were separately pooled and concentrated. Peak II contained over 80% of the AT III

activity and was, in most cases, immunologically pure as judged by immunoelectrophoresis using a polyvalent guinea pig antiserum to rabbit serum (kindly provided by Dr. J. Gauldie). When electrophoresed in 7.5% alkaline gel, the peak II protein resolved into two bands. Occasionally one or two additional very faint bands could also be observed. Peak II AT III was used for all experimental purposes without further treatment. The remaining antithrombin activity (< 20%) could be found in peaks I and III but both contained some other protein contaminants at variable proportions as judged by electrophoresis in 7.5% alkaline polyacrylamide gels.

Qualitatively similar results were obtained from eight preparations of rabbit AT III and one preparation each of human and rat AT III isolated by the above method. The possible presence of trace amounts of heparin in these AT III preparations had not been excluded.

Inhibition of Plasmin Esterase Activity by Rabbit α_1AT and Rabbit, Human and Rat AT III

Both α_1AT and AT III showed 'progressive' inhibition of plasmin esterolytic activity. Fig. 3 depicts the time course of inhibition of plasmin by α_1AT and AT III. α_1AT inhibited over 80% of the Pln BAEE activity over a period of 30 min. When rabbit AT III and plasmin were incubated at a molar ratio of 0.7 the inhibition levelled off at 40% of original activity. When the AT III/Pln ratio was increased to 1.5, over 90% inhibition was reached in 5 min. Human or rat AT III (ratio of AT III/Pln = 1.5) appeared to inhibit rabbit Pln more slowly than rabbit AT III, thus only 30% and 37% Pln inhibition, respectively, was reached at 5 min at room

temperature (see Table 1). However, over 90% inhibition of Pln by both human and rat AT III (AT III/Pln = 1.5) could be achieved over an incubation period of one hour.

The rates of inhibition of Pln by the different AT III preparations stated above should not be taken as representative values for the reaction rates, because heparin might have been present in varying (trace) amounts in these AT III preparations and thus it could have influenced the speed of the reaction. It was, however, for the purpose of the present studies, sufficient to demonstrate that the α_1AT and AT III preparations were capable of inhibiting plasmin and that Pln-α_1AT and Pln-AT III complexes could be isolated for studies in vivo.

Isolation of Plasmin-α_1-Antitrypsin Complex (Pln-α_1AT) by Sepharose-lysine Chromatography

Because α_1AT had no affinity for Sepharose-lysine, the uncomplexed α_1AT could be separated from the Pln-α_1AT by chromatographing the incubation mixture on Sepharose-lysine. Fig. 4 is a typical chromatogram obtained from a Sepharose-lysine column.

The material in peak I came through the column by washing with 0.1M AmAc pH 7.2 and had strong antitryptic activity and a mobility similar to that of native α_1AT in alkaline polyacrylamide gel. The material in peak II was eluted by 0.1M εACA in 0.1M AmAc pH 7.2. It had no antitryptic activity and contained a molecular species the electrophoretic mobility of which was different from that of free Pln and α_1AT. Fig. 5 shows the 7.5% alkaline polyacrylamide gel pattern of the proteins

in peaks I and II as well as Pln and native α_1AT.

The chromatographic behaviour on Sepharose-lysine and the electrophoretic mobility of the complex formed between Pln and labelled α_1AT were the same regardless whether labelled or unlabelled α_1AT was used to make the complex. Thus, the excess labelled α_1AT passed through the Sepharose-lysine column, just as the unlabelled did, while the Pln-α_1AT complex was retained. The εACA-eluted peak had negligible BAEE esterase activity. From its electrophoretic pattern this peak was concluded to contain the Pln-α_1AT complex and was used therefore for in vivo experiments.

Isolation of Plasmin-antithrombin III Complex (Pln-AT III) by Preparative Electrophoresis

A complex was formed when Pln and AT III were incubated together but some unreacted proteins were always present. The complex was isolated by preparative electrophoresis. Fig. 6 shows the 7.5% polyacrylamide gel patterns of the proteins from each isolation step.

The complex before and after isolation by preparative electrophoresis was also analyzed by gel filtration on Sephadex G-200 as shown in Figs. 7 and 8. Over 80% of both radioactivities were in the complex peak after preparative electrophoresis as compared to approximately 70% before. However, the extent of improvement was not 100%. The preparative electrophoretic step served only to remove as much of the uncomplexed radioactivities as possible so that the data obtained from the animal experiments could reflect more closely the behaviour of the complexes

in vivo.

In vivo Experiments

To investigate the fates of the complexes formed between rabbit plasmin on the one hand, and rabbit AT III, rat AT III, human AT III, and rabbit α_1AT, on the other, such complexes were prepared and isolated as described and injected into rabbits. In addition, complexes formed between rabbit thrombin and rabbit AT III, and bovine thrombin and rabbit AT III were also examined. The disappearance of protein bound radioactivity from the circulation was followed. For comparison, the disappearance of labelled rabbit AT III, rabbit α_1AT and diisopropylphosphofluoridate-inactivated rabbit plasmin (DFP-Pln) were also studied. DFP-Pln was used instead of active Pln to avoid complex formation in vivo so that unambiguous interpretations of the data could be made.

Fig. 9 shows the disappearance of labelled DFP-Pln from the circulation studied in 10 rabbits. Plasma curve of DFP-Pln followed a single exponential decay with a half-life of 2.3 h.

Different preparations of rabbit α_1AT and rabbit AT III were labelled and studied in 5 and 10 rabbits, respectively. The representative plasma curves are shown in Fig. 10. Both labelled α_1AT and labelled AT III disappeared from the circulation according to higher-order kinetics during the initial 40 h after which the disappearance rates appeared to follow first-order kinetics. The half-lives of this latter portion of the curves for α_1AT and AT III were found to be 42.5 h and 41 h, respectively.

(A) Pln-^{131}I-α_1AT Complex

Complexes formed between ^{131}I-α_1AT and unlabelled Pln were isolated and studied in 12 rabbits. The plasma curve of the protein bound radioactivity appeared to be single exponential until approximately 70% of the injected radioactivity had disappeared (Fig. 11). The half-life of this portion of the curve was approximately 0.8 h. The remaining radioactivity disappeared in a more complex manner and clearance seemed to become slower with increasing time.

(B) The Fate of Different Plasmin-AT III Complexes In vivo

1. Complex Formed from Unlabelled Pln and Labelled AT III

Such complexes were produced and injected simultaneously with differently labelled native AT III into two rabbits to comparatively study their behaviours.

Plasma curves of the two labelled proteins followed each other closely, especially during the first 30 hours of injection. Thereafter they became slightly divergent in as much as the radioactivity representing the AT III in the complex disappeared somewhat more rapidly than that representing the native AT III. The mean half-lives of the plasma slopes were 38 h for the complexed AT III and 47 h for the native AT III. One of these experiments is shown in Fig. 12.

Results of studies by Sephadex G-200 chromatography on selected plasma samples are shown in Fig. 13. Dissociation of the complex could already be detected by gel filtration of the plasma samples taken as early as 5 min after injection (Fig. 13a). At 6.23 h approximately

84% of the radioactivity representing the complexed AT III eluted with the 4 S peak, that is, in coincidence with the native AT III marker (Fig. 13b). No more complex could be detected in the plasma taken around 29 h after injection, as the label originally associated with the complex now was in the chromatographic position of the native AT III (Fig. 13c).

2. Complex Formed from Unlabelled Pln and Unlabelled AT III and Labelled with ^{125}I after it was Isolated by Preparative Electrophoresis

It is conceivable that iodination could change the biological behaviour of a protein. To exclude the possibility that the observed dissociation of the Pln-AT III complex was an artifact due to iodination, complex was formed between unlabelled Pln and unlabelled AT III, isolated and then iodinated with ^{125}I. Iodination at this stage would unlikely alter the tendency of a preformed complex to dissociate. Such complexes were prepared at three separate occasions and each was studied in a rabbit together with ^{131}I-native AT III as a marker. Plasma curves from one of these experiments is shown in Fig. 14. It is seen that the radioactivity representing the complex disappeared more rapidly during the initial 50 h as compared to that representing the native AT III. Thereafter the plasma slope of the complex appeared to decay in a single-exponential manner. Similar observations were made also in the other

animals. The half-life of the label representing the complex at this later stage was 35.3 h (± 2.4 S.E.M.), whereas that of the native AT III was 45.7 (± 3.5 S.E.M.).

Gel filtration through Sephadex G-200 of three plasma samples from the animal depicted in Fig. 14 showed that dissociation of the complex indeed took place (Fig. 15). Whereas in the 5-min sample most of the ^{125}I was in the complex position (Fig. 15a), a gradual shift of the radioactive protein peak to a lower-molecular-weight position with increasing time is apparent, indicating dissociation of the complex (Fig. 15b & c).

Thus the above results showed that Pln-AT III complex made from unlabelled components also dissociated in vivo and, consequently, that dissociation was not an artifact due to iodination.

3. Complex Formed from Labelled Pln and Labelled AT III

To further investigate the fate of Pln-AT III complexes, dual-labelled complexes were formed from ^{125}I-Pln and ^{131}I-AT III and purified by preparative electrophoresis. Five preparations of such complexes were studied in six rabbits. Results concerning the elimination of this kind of complexes from the circulation are summarized in Fig. 16. It is seen that the labelled proteins were eliminated at markedly different rates indicating dissociation of the complex soon after injection. ^{125}I-Pln of the complex disappeared much more rapidly than the corresponding ^{131}I-AT III. Data obtained by Sephadex G-200 chromatography of selected plasma samples from one of these animals are shown in Fig. 17.

They are consistent with the interpretation of the plasma curves in terms of dissociation.

4. Complexes Formed from Labelled Rabbit Plasmin and Labelled Rat or Labelled Human AT III

The complexes studied so far were formed from homologous proteins thus raising the possibility that dissociation was a feature unique to the rabbit proteins. To expand the scope of investigation, dual-labelled complexes were formed between rabbit Pln and human or rat AT III and their behaviours studied in vivo. Dissociation was observed with both of these complexes although the differences in the rates of disappearance of the labelled proteins were not as marked as those of the rabbit Pln-rabbit AT III complex. Plasma curves of these complexes are shown in Fig. 18 and 19. During the first 1-1.5 h the labelled proteins disappeared closely together, but thereafter their plasma radioactivity curves began to diverge. Hence, it appeared that the complexes of rabbit Pln with human or rat AT III dissociated more slowly than rabbit Pln-rabbit AT III complexes.

Behaviour of Thrombin-Antithrombin III Complexes In Vivo

To see if the tendency to dissociate was specific to complexes of plasmin, thrombin was used for complex formation with AT III and its behaviour investigated.

One preparation of complexes was made from labelled rabbit AT III and labelled rabbit thrombin (ratio of AT III/thrombin = 2) and one from labelled bovine thrombin and labelled rabbit AT III (ratio of AT III/

thrombin = 1.2) and both preparations were studied in one rabbit each. Fig. 20 shows the plasma curves of the rabbit thrombin - rabbit AT III complex. As judged by the difference between the disappearance rates of the labelled proteins, rabbit thrombin-AT III complexes seem to dissociate in vivo more rapidly than Pln-AT III complexes. Similar plasma curves were obtained for the bovine thrombin-rabbit AT III complex. The label representing either thrombin disappeared rapidly, especially at the beginning: by 5 min after injection approximately 30% of the initial thrombin radioactivity had left the plasma. By comparison to the situation with plasmin-AT III complexes, AT III consumption from thrombin-AT III complexes was enhanced and from superimposition of the AT III curves in Figs. 16 and 20, it appears that the excess loss in AT III occurred during the early phase of the experiment (< 10 h).

Gel filtration through Sephadex G-200 of the dual-labelled rabbit thrombin-rabbit AT III preparation in the presence of 1 ml carrier plasma showed the presence of two complex peaks, one of which eluted between the 19 S and 7 S plasma protein peaks and the other one between the 7 S and the 4 S peaks (Fig. 21a). The preparation also contained a small amount (approx. 9%) of uncomplexed thrombin as well as some free AT III, the precise amount of which could not be determined from the chromatogram. Analysis of selected plasma samples from the rabbit that received this preparation on Sephadex G-200 (one chromatogram shown in Fig. 21b) showed the disappearance of the complex peaks with time. This was accompanied by a shift of the label representing the complexed AT III to the position of the 4 S peak. Similar observations were also made with the dual-labelled bovine thrombin-rabbit AT III complex.

Some Properties of Post-Complex ^{131}I-AT III

From the foregoing sections it is apparent that complexes of rabbit, human and rat AT III with rabbit plasmin or rabbit or bovine thrombin are all unstable *in vivo* to variable extents. It is also apparent that AT III which had dissociated from a complex with a protease ('post-complex' AT III) is cleared from the circulation only marginally faster than native AT III. In view of these facts it seemed interesting to investigate some of the properties of post-complex AT III relative to the native protein.

Complexes were formed from unlabelled rabbit Pln and labelled rabbit AT III, isolated and injected together with differently labelled native AT III as the control into two rabbits. Approximately 30 h later, i.e. when according to plasma curve measurements essentially all the complex injected had dissociated, (residual Pln level: < 3%), 30 ml of blood were collected into 5 ml of ACD. The plasma obtained by centrifugation was made 40% saturated with solid $(NH_4)_2SO_4$ and the dialysed supernatant chromatographed on a column of Sepharose-heparin as described in Methods.

Table II summarizes the results of this experiment from which it is apparent that 81% of the label representing the post-complex AT III passed through the Sepharose-heparin column, whereas 70% of the radioactivity representing the native AT III was bound by the column. This indicated that the heparin-binding site of the post-complex AT III was no longer functional.

It has been shown that the heparin-binding site of the AT III molecule is different from the enzyme-inhibiting site (27). The next experiment was designed to test the inhibitory activity of the post-complex AT III relative to that of native AT III using Sepharose-trypsin affinity chromatography. Control experiment done with labelled AT III which had not been injected into an animal showed that 80% of the AT III would bind to Sepharose-trypsin. Hence, the Sepharose-trypsin affinity column provided a means for checking the activity of AT III.

As shown in Table II, almost all the label representing post-complex AT III (> 94%) passed through the column. In contrast, 80% of the injected labelled native AT III was bound by the column, which was comparable to value obtained with not-injected control AT III.

These observations show that after dissociation from the complex, the affinity for heparin of AT III is reduced or abolished and that post-complex AT III no longer reacts with immobilized trypsin.

Some Properties of Post-Complex ^{125}I-Pln

A dual-labelled rabbit ^{125}I-Pln/rat ^{131}I-AT III complex was formed, isolated and injected into a rabbit. At 7 min and at approximately 11 h after injection, plasma samples were taken for the following analyses:

1. Sephadex G-200 Gel Filtration

One ml of the plasma taken 7 min after injection was chromatographed on Sephadex G-200 and the chromatogram is shown in Fig. 22. Almost all of the labels representing the rat AT III and rabbit Pln eluted together at the 7 S peak indicating the presence of a complex.

One ml of the plasma sample taken approximately 11 h after injection was chromatographed in the same way. Fig. 23 shows the chromatogram of this sample. Two new peaks appeared in addition to the complex peak, one of them eluted just prior to the 4 S peak and the other one eluted in the salt volume of the column. The radioactivity in the salt volume is likely to be the iodide which has been released from the proteins during catabolism. The radioactivities eluting with the ascending limb of the 4 S peak were assumed to represent the Pln and AT III dissociated from the complex. The fractions containing this material (# 19-21) were therefore pooled for analyses which are described in the following sections.

2. Affinity Chromatography of Fractions 19, 20 and 21 of 11-hour Sample from Sephadex G-200 on Sepharose-lysine and on Sepharose-AT III

After pooling, the fractions were concentrated in an Amicon ultrafiltration chamber and dialyzed against 0.1M NaCl. When chromatographed on Sepharose-lysine, more than 98% of the ^{131}I-AT III passed through the column by washing with 0.1M NaCl and only about 9% ^{125}I-Pln was in this portion. Elution with 0.1M εACA recovered approximately 86% of the ^{125}I-Pln as shown in Fig. 24. This result suggests that Pln derived from an AT III complex retains its ability to bind to, and be removed from Sepharose-lysine.

When a sample of this Sephadex G-200-derived starting material was chromatographed on Sepharose-AT III, 96% of the ^{125}I-Pln failed to bind to the inhibitor.

3. Affinity Chromatography of the 11-hour Plasma Sample on Sepharose-lysine

One ml of the sample was loaded on to a Sepharose-lysine column and washed with 0.1M NaCl followed by 0.5M NaCl. Then the bound radioactivities were eluted with 0.1M εACA as shown in Fig. 25. It can be seen that 95% of the ^{131}I-AT III passed through the column by washing with 0.1M NaCl. On the other hand, only 48% of the ^{125} I-Pln, presumed still to be in the complex form, came through. Washing with 0.5M NaCl did not displace any more radioactivities from the column. Elution with 0.1M εACA recovered 36% of the ^{125}I-Pln. Because this ^{125}I-Pln peak was associated with negligible quantities of ^{131}I-AT III (≤ 2%) it was inferable that this material represented ^{125}I-Pln which had dissociated from the complex. These results further strengthen the view expressed above that Pln retains its ability to bind to Sepharose-lysine after its dissociation from the Pln-AT III complex.

4. Affinity Chromatography of Post-Complex ^{125}I-Pln on Sepharose-AT III (S-AT III)

In a control experiment, labelled Pln in 1 ml of 0.1M NaCl with or without 0.46 mg added heparin was passed through separate columns of S-AT III. It was found that approximately 33% and 46% of the radioactivity became irreversibly bound to the affinity medium in the absence and presence of heparin, respectively. The unretained Pln could have been due to either inactive protein in the preparation or unfavourable conditions prevailing in the flow system for maximal interaction between Pln and AT III, the reaction being time-dependent. Therefore, when analyzing the test sample, time was allowed for inter-

action by stopping the flow for 5 min after loading.

The test sample was ^{125}I-Pln adsorbed from the 11-hour plasma sample on Sepharose-lysine and subsequently recovered by elution with 0.1M εACA. Before application to the S-AT III column, the protein was dialyzed against 0.1M NaCl. Approximately 84% of the loaded radioactivity came through the S-AT III column when washing with 0.1M NaCl and an additional 12% when washing with 1M NaCl (see Fig. 26). This means that approximately 96% of the ^{125}I-Pln in this sample failed to react with S-AT III. This is in keeping with the behaviour of the fractionated material from the Sephadex G-200 column as already described on p. 34.

5. Affinity Chromatography of Post-Complex ^{125}I-Pln on Sepharose-Trasylol (S-T)

Preliminary experiments showed that 68% of control ^{125}I-Pln was bound irreversibly by this batch of S-T, but also 25% of DFP-Pln.

On chromatography of a sample of ^{125}I-Pln eluted by εACA from Sepharose-lysine, 57% came through by washing the S-T column with Ringer's lactate and an additional 19% by washing it with 1M NaCl (see Fig. 27), the rest being irreversibly bound by the medium. As S-T bound a similar fraction of DFP-Pln, it seems likely that the irreversibly bound portion of the test sample represented non-specific binding.

To summarize the results from the above series of experiments, it seems justifiable to conclude that Pln dissociated from Pln-AT III complex retains its affinity for Sepharose-lysine but it is inactive towards S-AT III and S-Trasylol. On the other hand, post-complex

AT III lacks affinity for Sepharose-trypsin and exhibits little or no affinity for Sepharose-heparin. In brief, both the post-complex Pln and AT III appear to be grossly altered.

Stability of Pln-AT III Complexes In vitro Under Different Incubating Conditions

Unlabelled rabbit Pln (in 0.05M εACA in 0.1M AmAc pH 7.2) and unlabelled rabbit AT III (in 0.05M Tris-HCl pH 8.0, ratio of AT III/Pln ≈ 1.3) were incubated at 37°C in the presence of azide (0.1% w/v), for approximately 29 h. Small subsamples were taken at 2, 3.5, 6, 11, 23.5 and 29 h and immediately frozen until they were analyzed by disc gel electrophoresis (7.5% polyacrylamide, pH 8.3). Fig. 28 shows the gel patterns. The complex appeared to be stable under these experimental conditions in all the samples up to 6 h of incubation but in the 11-h sample dissociation of the complex was already evident from a broadening of the complex band towards the anode. In later samples, decreases in the intensity of the band representing the complex were parallelled by the appearance of additional bands on the cathodic side of the complex. At the same time, signs of proteolysis of the unreacted AT III became detectable..

Fig. 29 shows the gel patterns of the complex treated in the same way as the above-mentioned samples, but with the addition of 163 units of Trasylol after the first hour of incubating the Pln and AT III together at 37°C. Addition of Trasylol at this stage would unlikely interfere with complex formation which would have already been completed. It is evident from these gels that proteolysis in these samples was

reduced. However, dissociation of the complex could still be observed as shown by the decrease in the intensity of the complex band which became noticeable in samples taken after 6 h of incubation. The intensity of the AT III bands increased parellel to the decrease in the complex band. This suggests that after its dissociation from the complex, AT III possessed similar electrophoretic mobility to that of the native AT III. Addition of Trasylol to the incubation mixture also resulted in the appearance of a band near the very top of the gels when the samples were electrophoresed. The material in this band could represent a complex of Pln and Trasylol. However, this assumption has to be confirmed experimentally. For comparison, Fig. 30 shows the gel patterns of the same complex which was stored at 4°C for 29 h, both in the presence and absence of Trasylol. Dissociation could not be observed in either sample.

A small quantity of a complex formed from ^{125}I-rabbit Pln and ^{131}I-rat AT III (the same preparation as the one used for the experiment shown in Fig. 19) was incubated with 2 ml of whole rabbit blood, anticoagulated with sodium citrate, in the presence of azide (0.1% w/v) for approximately 30 h at 37°C. Then the cells were removed by centrifugation at 3000 g for 10 min and the plasma thus obtained was filtered through Sephadex G-200. Results from a control sample from a rabbit 7 min after injecting not-incubated complexes from this preparation, has already been introduced in Fig. 22. Fig. 31 shows the chromatogram of the incubate. It is seen that very little complex remained and the radioactivities shifted to two new positions, one of which corresponded to the 19 S peak and the other one to the ascending limb of the 4 S peak.

A portion of the same complex preparation was also incubated with 1 ml of rabbit plasma under identical conditions and then filtered through Sephadex G-200. The chromatogram from this run in Fig. 32 shows that although some of the complex had dissociated, nevertheless the extent of complex lysis was considerably less than when incubation was in whole blood.

DISCUSSION

Knowledge concerning the fate *in vivo* of complexes formed between natural inhibitors and proteases is fragmentary. The available information in the literature, although scanty, seems to suggest that there is more than one pathway for the clearance of these complexes. Thus the complexes of α_2M-protease are eliminated rapidly by the reticulo-endothelial system (40-47) while that of α_1AT-protease are eliminated comparatively slowly (40-44). Complexes of α_2M-protease have been studied in dogs (42,43,45), in man (44), and in rabbits (46), and their half-lives have been found to be in the order of minutes in all the hosts studied.

The rate of elimination of α_1AT-protease complexes seems to vary depending on the types of proteases in the complex and the hosts in which they are studied. Thus complexes of α_1AT with trypsin have half-lives of 45 min in dogs (42,43,45), and 3.5 h in man (44) while the half-lives of α_1AT-elastase are about 30 min and 1-1.5 h, respectively. The results in this study showed that α_1AT-plasmin complex had a half-life of 0.8 h in rabbits. Dissociation and transfer of proteases from α_1AT-protease complexes to α_2M have been observed (40-45). This led to the proposal that α_1AT served as a protease carrier, particularly in the extracellular space, where the concentration of α_2M is low (44). The importance of this proposed role for α_1AT, however, will depend on the rate of dissociation of the α_1AT-protease complexes as compared to their rates of elimination. When complex of labelled rabbit α_1AT with unlabelled plasmin was injected in rabbits in the present study, approximately 70% of the

radioactivity disappeared in a single-exponential manner with an average half-life of 0.8 h. The remaining radioactivity was cleared in a more complicated manner and clearance became slower with increasing time. This could probably be explained by slow dissociation of the α_1AT-plasmin complex although no direct evidence can be given. The rates of elimination of labelled native α_1AT and α_1AT-plasmin complex differed greatly as shown in fig. 10 and 11. The faster disappearance of radioactivity in fig. 11 could be assumed to reflect the clearance of α_1AT in a complexed form. The above observations seem to suggest that the elimination of α_1AT-plasmin complex in rabbits does not rely heavily on α_1AT to act as a protease transfer protein, as has been observed in the clearance of α_1AT-trypsin and α_1AT-elastase complexes (40-45). Not enough studies have been done at present to allow any conclusion to be drawn concerning the functional states of α_1AT and the proteases after dissociation. The observed transfer of proteases to α_2M from α_1AT-protease complexes (40-45) does not necessarily indicate that the proteases are enzymatically active after dissociation because α_2M has been found to be able to form primary complexes with inactivated enzymes (87).

The present studies focus mainly on the protease clearance pathway mediated by AT III. Complexes of rabbit, human and rat AT III with rabbit plasmin and of rabbit AT III with rabbit and bovine thrombins have been tested. It appears that the stability of these complexes <u>in vivo</u> depends on the origin of AT III as well as on the type and source of the proteases. Even in the case of the relatively stable complexes of human and rat AT III with rabbit plasmin, the half-lives of the plasma radioactivity curves were in the order of hours (cf. fig. 18 and 19).

Thus from a kinetic point of view, protease clearance through AT III is similar to that by the α_1AT system. However, as only plasmin and thrombin have been tested, this conclusion should not be generalized before the other proteases, which are inhibited by AT III, have also been studied. Nevertheless, the present results seem to be in agreement with Ohlsson's concept (7,44) that α_2M occupies a key position in the defence mechanisms against endogenous proteases because it is the only plasma inhibitor, thus far, whose complexes with proteases are eliminated immediately. Furthermore, saturation of α_2M in the circulation with protease is always followed by irreversible shock (43).

Dissociation of all five combinations of AT III-protease complexes was detectable in vivo, although to variable extents. The data suggests that thrombin-AT III complexes are less stable in vivo than plasmin-AT III complexes and complexes formed by human or rat AT III with rabbit plasmin dissociate less readily than those formed by rabbit AT III. The variations in the stability of AT III-protease complexes could be due to the difference in affinities of AT III molecules from different species for different proteases. In fact, human AT III has been observed to show less affinity for human Xa than for bovine Xa (96,97). It is also possible that different AT III-protease complexes interact differently with the plasma membrane of the cells which are involved in the elimination of the complexes. It seems, therefore, that the biological fate of AT III-protease complexes may vary considerably depending on the origin and type of both AT III and proteases.

Stable complex of human AT III with human plasmin has been observed

in vitro (73). However, the stability of the complex has not been followed beyond the first hour after it has been formed. In the present study, the stability of rabbit AT III-plasmin complex was followed for 30 h at 37°C. The results showed that the complex was stable *in vitro* for at least 6 h (cf. figs. 28 and 29) although dissociation of the same became apparent *in vivo* much earlier. This difference in the *in vivo* and *in vitro* behaviours of AT III-plasmin complex raises the possibility of the existence of a biological pathway which expedites the breakdown of such complex. The exact location and mechanism of such pathway cannot be given at present, but the observation that less intact complex remained detectable after prolonged incubation with whole blood than after incubation with plasma (cf. figs. 31 and 32) suggests that cells might play a role. It is conceivable that interaction of a complex with the plasma membrane of certain cells effects the release of some factors which promote dissociation, though this is pure speculation at present.

Incubation of an inhibitor with excess enzyme may result in proteolytic breakdown of the complex formed as has been observed for human α_1AT and porcine trypsin (88) and for human AT III and human thrombin (89). In the present case, the AT III-plasmin complex was always formed in the presence of molar excess of AT III and polyacrylamide-gel data showed that dissociation and proteolysis of the AT III-plasmin complex were independent phenomena. In the absence of Trasylol (fig. 28) preformed AT III-plasmin complex showed signs of secondary proteolysis after 6 h of incubation at 37°C *in vitro*. However, when Trasylol was present (fig. 29) the preformed AT III-plasmin complex disappeared gradually without secondary proteolysis, suggesting that the association between

rabbit plasmin and rabbit AT III may be of a reversible nature.

Affinity chromatographic studies indicate that the properties of plasmin and AT III released from the complex differ from those of the corresponding native molecules. Post-complex plasmin no longer binds to Sepharose-AT III or Sepharose-Trasylol. However, the observations presented in fig. 29 imply that it still can react with the stronger inhibitor, Trasylol, in solution. The apparently contradictory reactivity of post-complex plasmin towards immobilized Trasylol and Trasylol in solution could probably be the result of limited accessibility of the former to the plasmin due to steric hinderance presented by the gel matrix or that the ability of Trasylol to react with plasmin might have been altered during the coupling procedure. After prolonged incubation of the complex with whole blood or plasma (cf. fig. 31 and 32), a large portion of post-complex plasmin was eluted in the void volume of the Sephadex G-200 column. This suggests that possible interaction of post-complex plasmin with α_2M had occurred. As α_2M has been found to be able to form primary complexes with inactivated enzymes (87), the gel filtration finding alone would not necessarily mean that post-complex plasmin is enzymatically active. Therefore, the functional state of post-complex plasmin requires further studies before any conclusion concerning details can be made.

Binding of AT III to heparin is probably a function of lysyl residues (27). Reduced binding of post-complex AT III to heparin therefore signifies a change in the availability of such residues perhaps as the result of a conformational change in the inhibitor. Decreased heparin binding by AT III complexed with thrombin has been reported (90,91). Parallel to the altered affinity for heparin, post-complex AT III no

longer binds to Sepharose-trypsin. All this suggests that post-complex AT III is profoundly different from the native inhibitor. Considering the prevailing physiological tendency regarding defunct plasma proteins is their fast removal from the circulation (92), the long survival of post-complex AT III in the plasma is a startling finding. Post-complex AT III preserves its reactivity with antibodies raised against normal AT III (93). This, in conjunction with the long survival of post-complex AT III in the circulation, could give rise, in certain circumstances, to overestimates of functional plasma AT III concentration if the inhibitor is only measured immunologically. The interesting and hitherto unexplained observation (94) that large doses of human AT III infused into anaesthetized dogs remained immunologically detectable long after the excess thrombin inhibitory activity had been lost, could now be understood on this basis. Whether post-complex AT III occurs in human plasma under pathological conditions and if it also has a long life, comparable to its counterpart in the rabbit, remains to be elucidated.

In summary, the present studies have shown that protease clearance mediated by AT III is comparatively slower than that of α_2M-protease complexes. Dissociation of AT III complexes in vivo, though to variable extents, seems to be a common phenomenon. The post-complex AT III and proteases appear to be altered and they have different properties as compared to the corresponding native molecules. The possibility that these observations are particular to the species studied has not been ruled out. Further studies of AT III-protease complexes in other hosts are clearly needed before any generalization can be made.

ADDENDUM: Since the completion of this work, a brief report by Jesty (98) appeared in which he studied the reversal of the reaction of bovine thrombin with antithrombin III by methods of equilibrium perturbation. He observed that the addition of the thrombin substrate Phe-Pip-Arg-p-nitroanilide (S-2238) at a level 20 times the K_m value of thrombin, caused dissociation of the thrombin-AT III complex. This further supports the views gained in the present investigations that complexes of AT III-protease are not as stable as hitherto widely assumed in the literature.

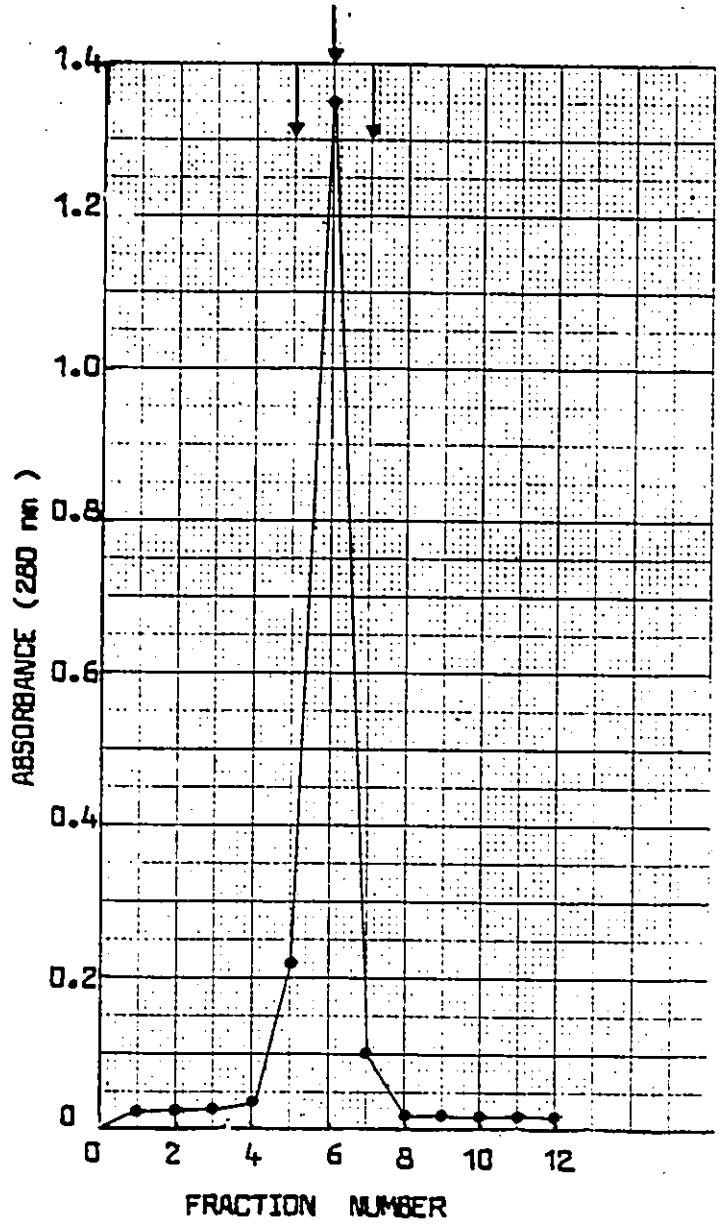


Figure 1. Elution of plasmin from Sepharose-lysine column by 0.1M AmAc pH 7.2 containing 0.05M EACA. The fraction volume was 8 ml. Those fractions marked by arrows were pooled and concentrated by pressure dialysis against the running buffer. (column size 2.5 x 13 cm)
$E_{280}^{1\%}$=20 was used for calculations of plasmin concentrations. (A. Koy, personal communication).

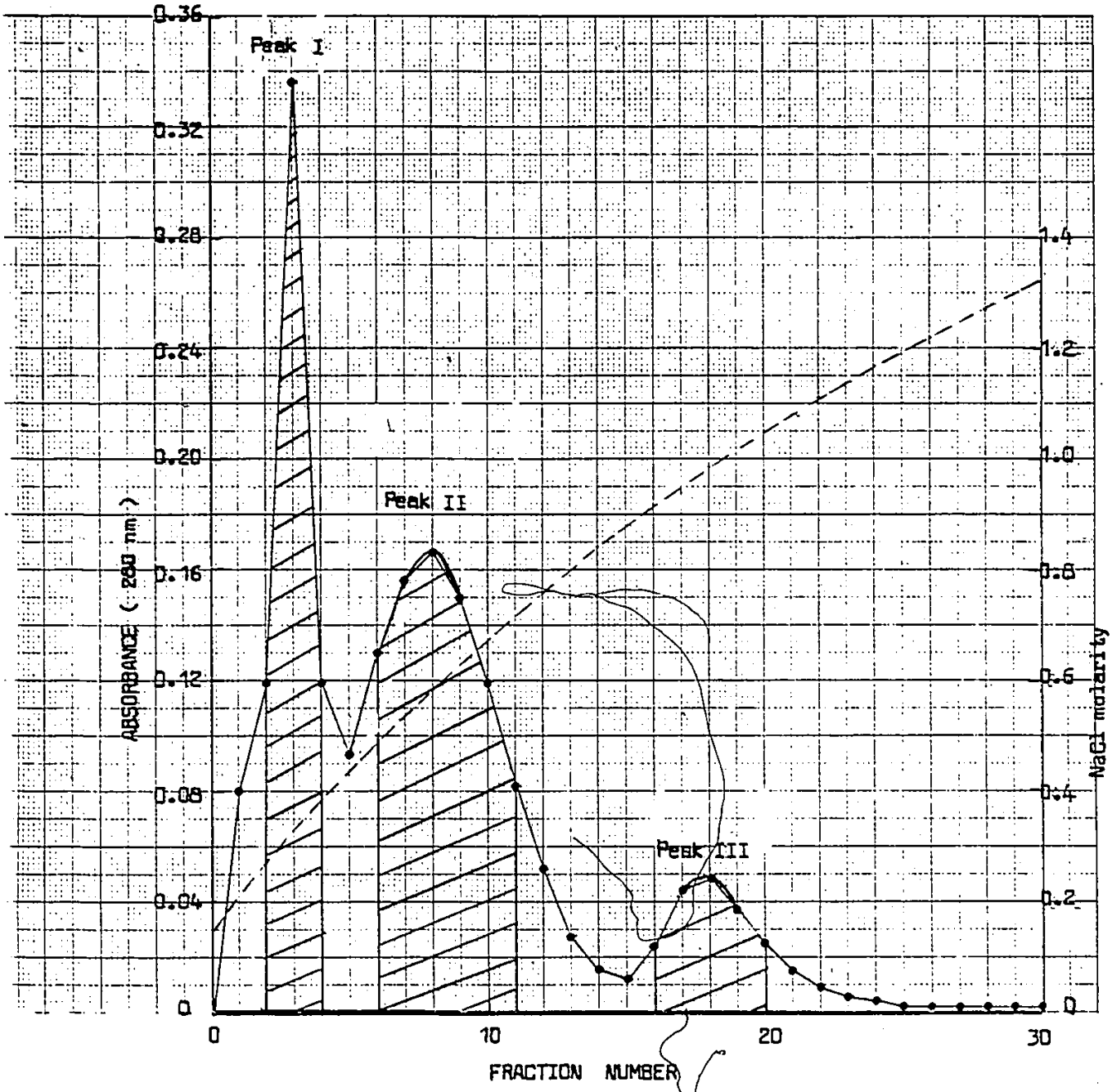


Figure 2. NaCl gradient elution of rabbit antithrombin III from Sepharose-heparin column.

The fraction volume was 5 ml. The mixing chamber contained 150 ml of 0.05M Tris-HCl pH 8.0 containing 0.15M NaCl and the limit solvent was 150 ml of 0.05M Tris-HCl pH 8.0 containing 2M NaCl. Column size was 1.5 x 12.5 cm. The fractions in the shaded areas were pooled and concentrated separately. $E_{280}^{1\%}$=10.5 (27) was used for calculating the AT III concentrations. The broken line represents the NaCl gradient.

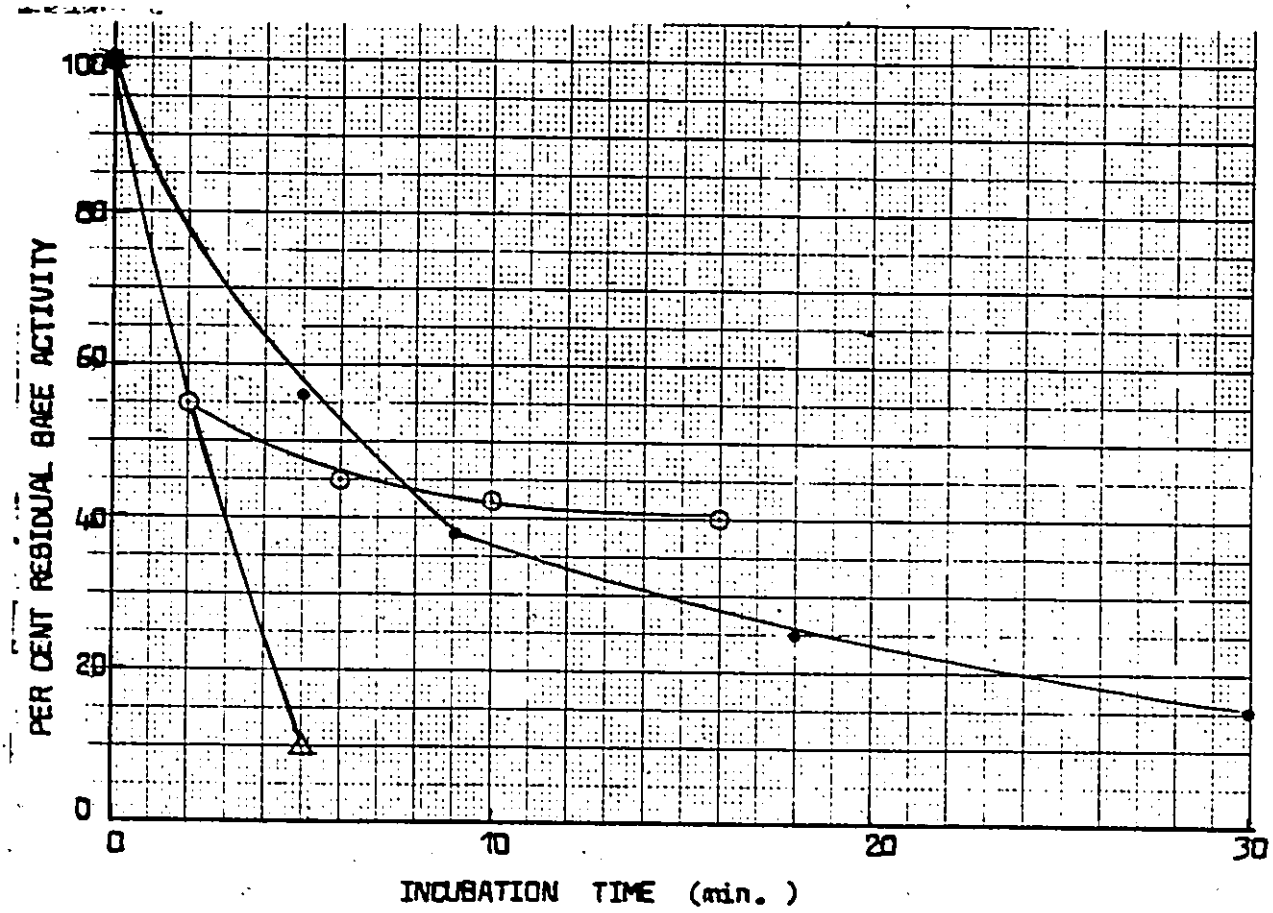


Figure 3. Inhibition of plasmin esterolytic activity (BAEE) by rabbit α_1AT or AT III. Test conditions are given in the text. Closed circles represent inhibition by α_1AT at a molar ratio of α_1AT:Pln=3:1. Open circles represent inhibition by AT III at a molar ratio of AT III:Pln=0.7:1. Open triangles represent AT III:Pln=1.5:1. $E^{1\%}_{280}$=5.1 (80), 10.5 (27), and 20 were used for calculating the protein concentrations of α_1AT, AT III and Pln, respectively. The molecular weights of α_1AT, AT III, and Pln were assumed to be 58000 (80), 67000 (26), and 86000 (95) daltons, respectively.

TABLE 1 Inhibition of rabbit plasmin esterolytic activity by rabbit α_1AT, rabbit AT III, rat AT III and human AT III.

Inhibitor	Molar ratio (inhibitor: enzyme)	% Inhibition
rabbit α_1AT	3	55
rabbit AT III	1.5	92
rat AT III	1.5	37
human AT III	1.5	30

The inhibitors and plasmin were preincubated for 5 min. at room temperature before assaying with BAEE. Assay conditions are given in the text. $E_{280}^{1\%}$=20,* 5.1 (80), and 10.5 (27) were used for the calculation of protein concentrations of plasmin, α_1AT and AT III, respectively.

* A. Koj, personal communication.

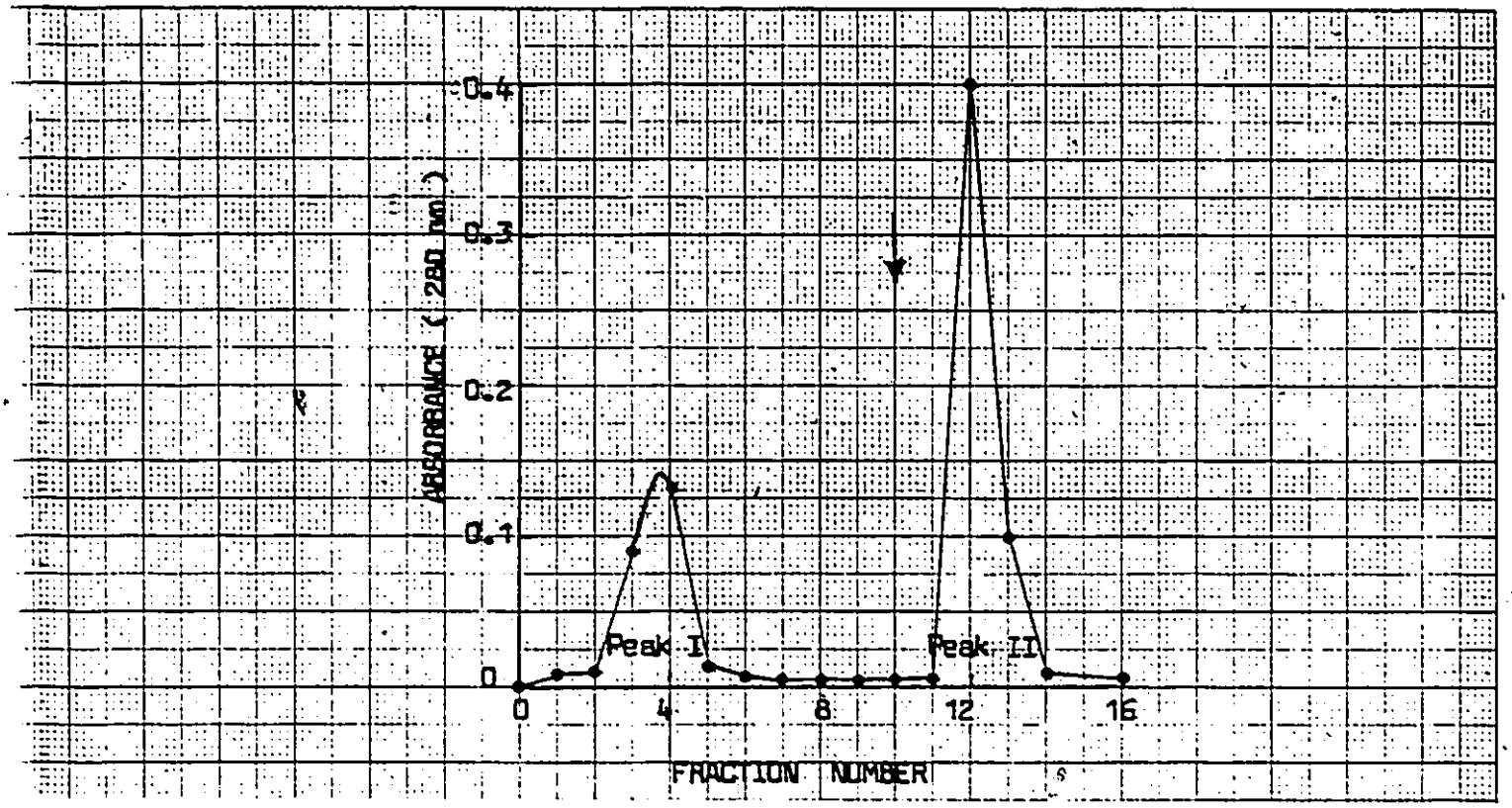


Figure 4. Isolation of Pln-αAT complex by Sepharose-lysine chromatography. The fraction volume was 4 ml. The column size was 1.5 × 11 cm. The running buffer was 0.1M AmAc pH 7.2. Peak I passed through the column by washing with 0.1M AmAc pH 7.2. Elution of the adsorbed proteins with 0.1M AmAc pH 7.2 containing 0.1M EACA was started as indicated by the arrow.

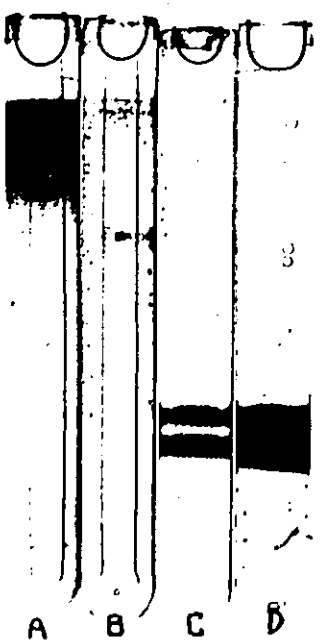


Figure 5. Polyacrylamide-gel electrophoresis (7.5%, pH 8.3) of: (A) rabbit plasmin, (B) pln-α_1AT complex eluted from Sepharose-lysine; peak II, (C) uncomplexed α_1AT; peak I, and (D) control native α_1AT.

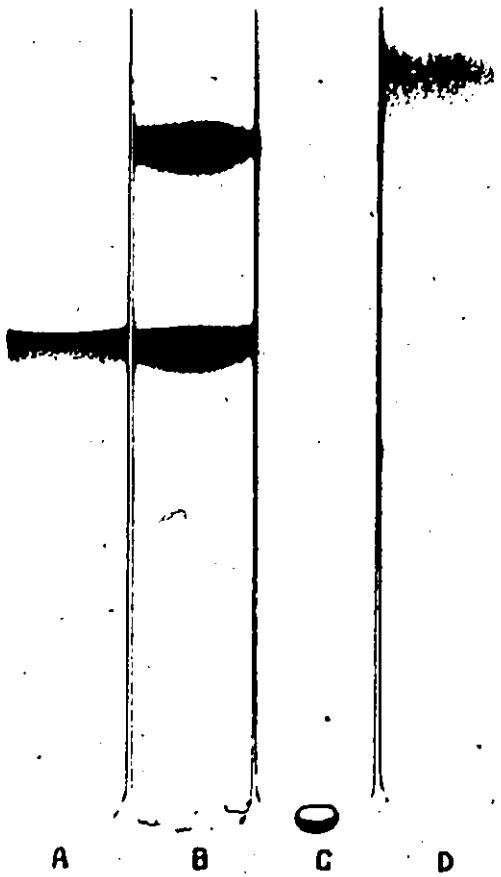


Figure 6. Polyacrylamide-gel electrophoresis of: (A) native rabbit AT III, (B) incubation mixture of rabbit AT III and pln (1.5:1) before preparative electrophoresis, (C) rabbit pln-AT III complex separated by preparative electrophoresis and (D) rabbit plasmin.

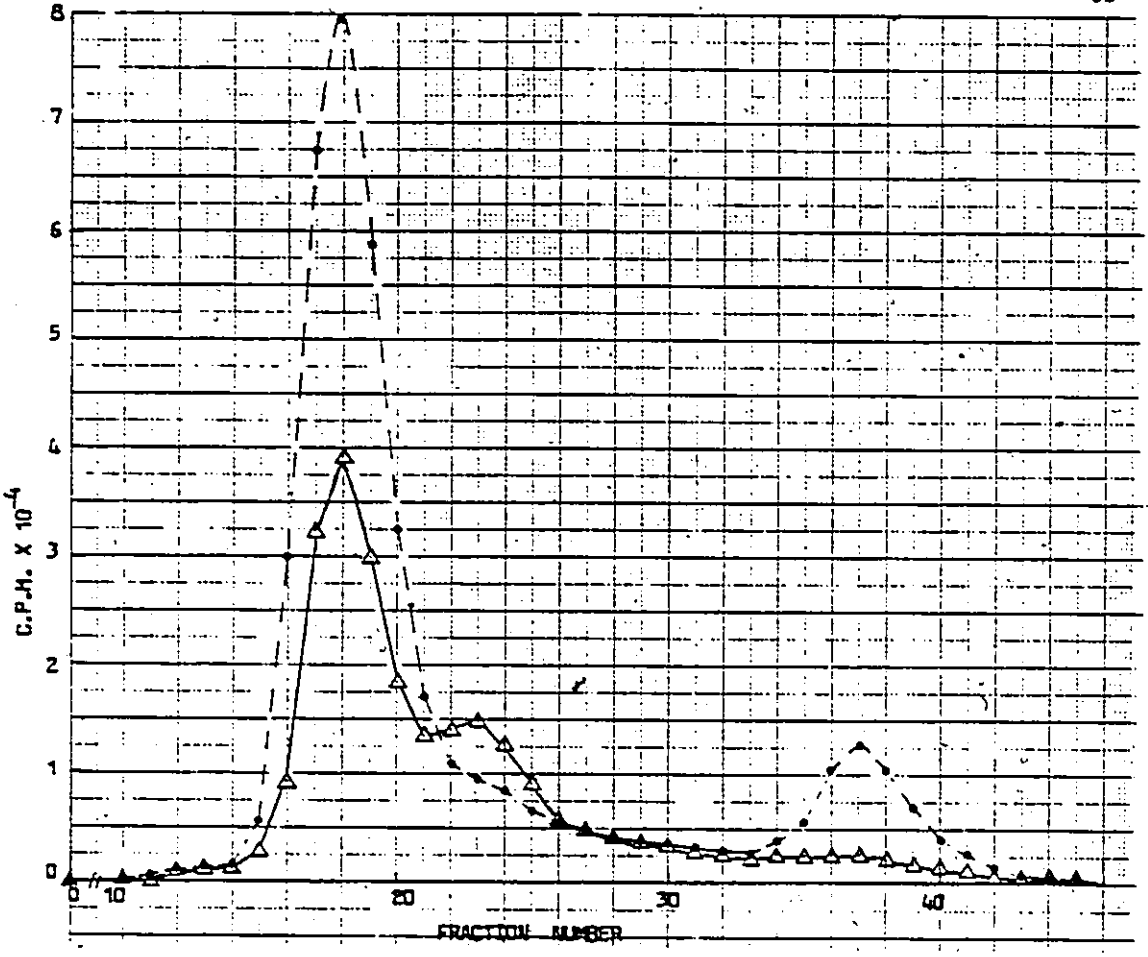


Figure 7. Sepnadex G-200 gel filtration of the complex of ^{125}I Pln (●— — —●) with ^{131}I AT III (Δ———Δ) before preparative electrophoresis. Fraction volume was approx. 5 ml, collected at a flow rate of approx. 40 ml/h. The running buffer was 0.02M Na-phosphate pH 7.3 containing 0.3M NaCl. The column size was 2.3 x 50 cm.

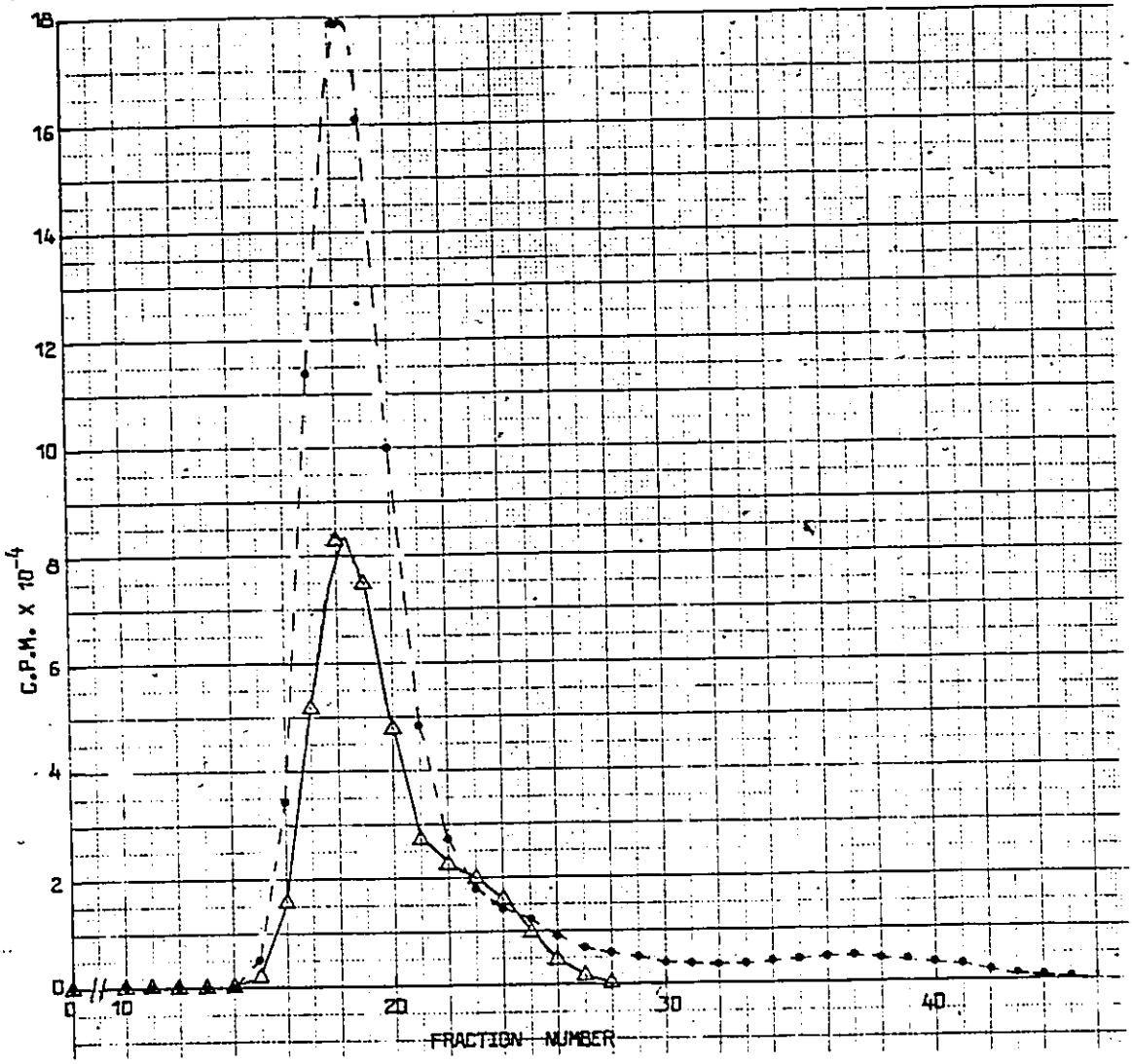


Figure 8. Sephadex G-200 gel filtration of the complex of ^{125}I PIn (●— – —●) with ^{131}I AT III (△———△) after preparative electrophoresis. Running conditions were the same as those given in fig. 7.

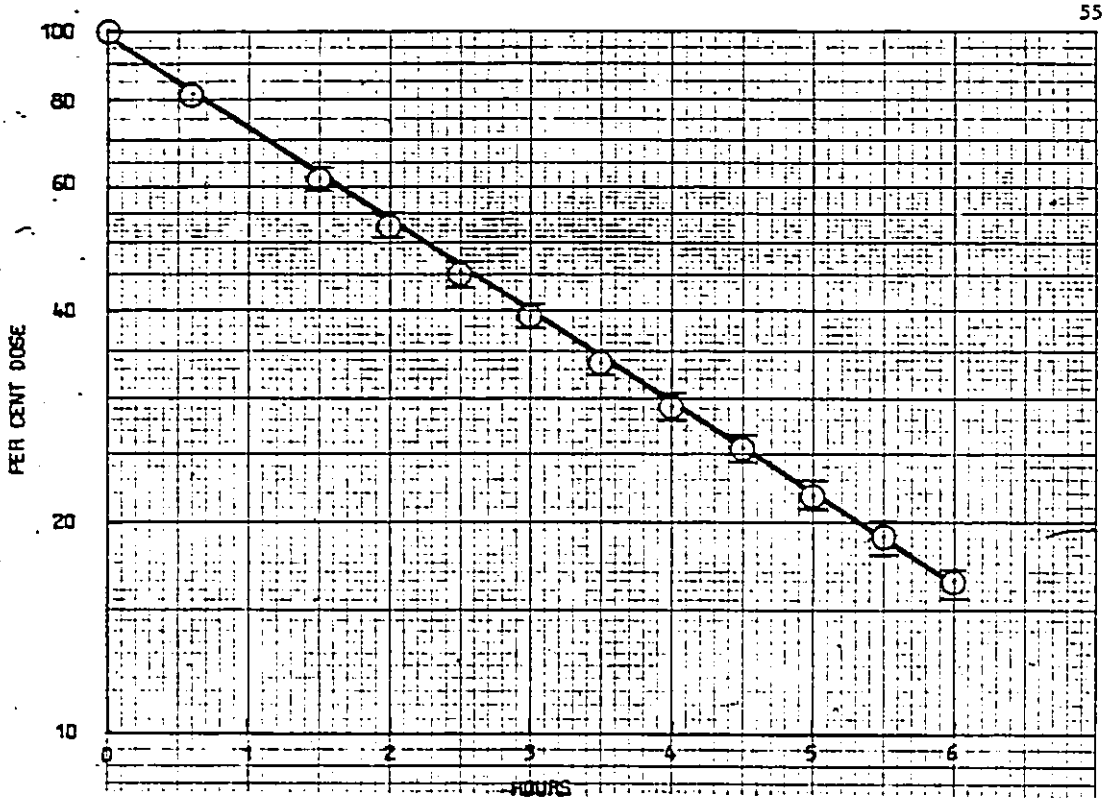


Figure 9. Protein-bound radioactivity in the plasma of rabbits following the injection of labelled diisopropylphosphofluoride-inactivated rabbit plasmin. Each point is the mean of ten experiments. Horizontal bars are standard errors of the means. (Only shown where the errors exceeds the size of the symbol).

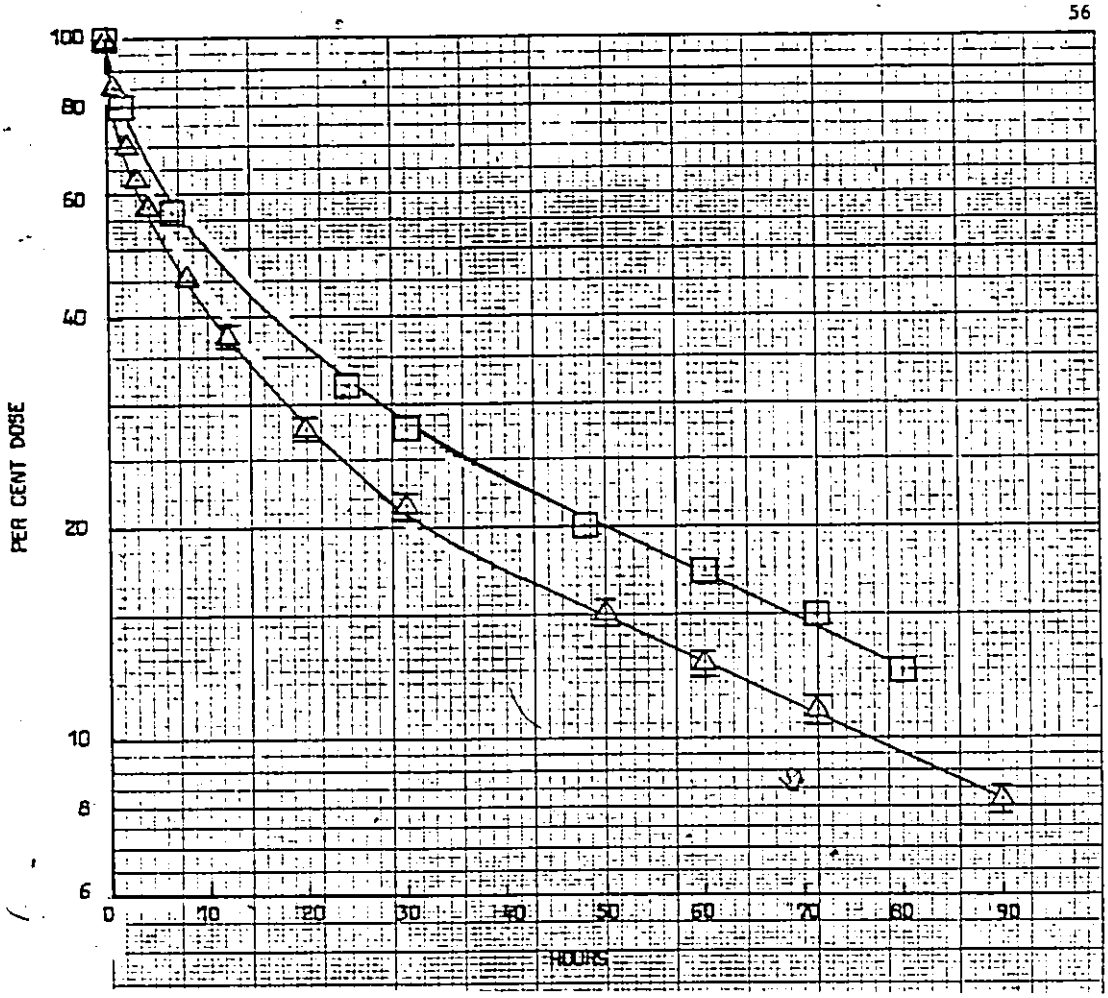


Figure 10. Protein-bound radioactivity in the plasma of rabbits following the injection of labelled rabbit α_1AT (□) or rabbit AT III (△). Each point is the mean of five experiments in the case of α_1AT and ten in the case of AT III. Horizontal bars are the standard errors of the means (only shown where the error exceeds the size of the symbols).

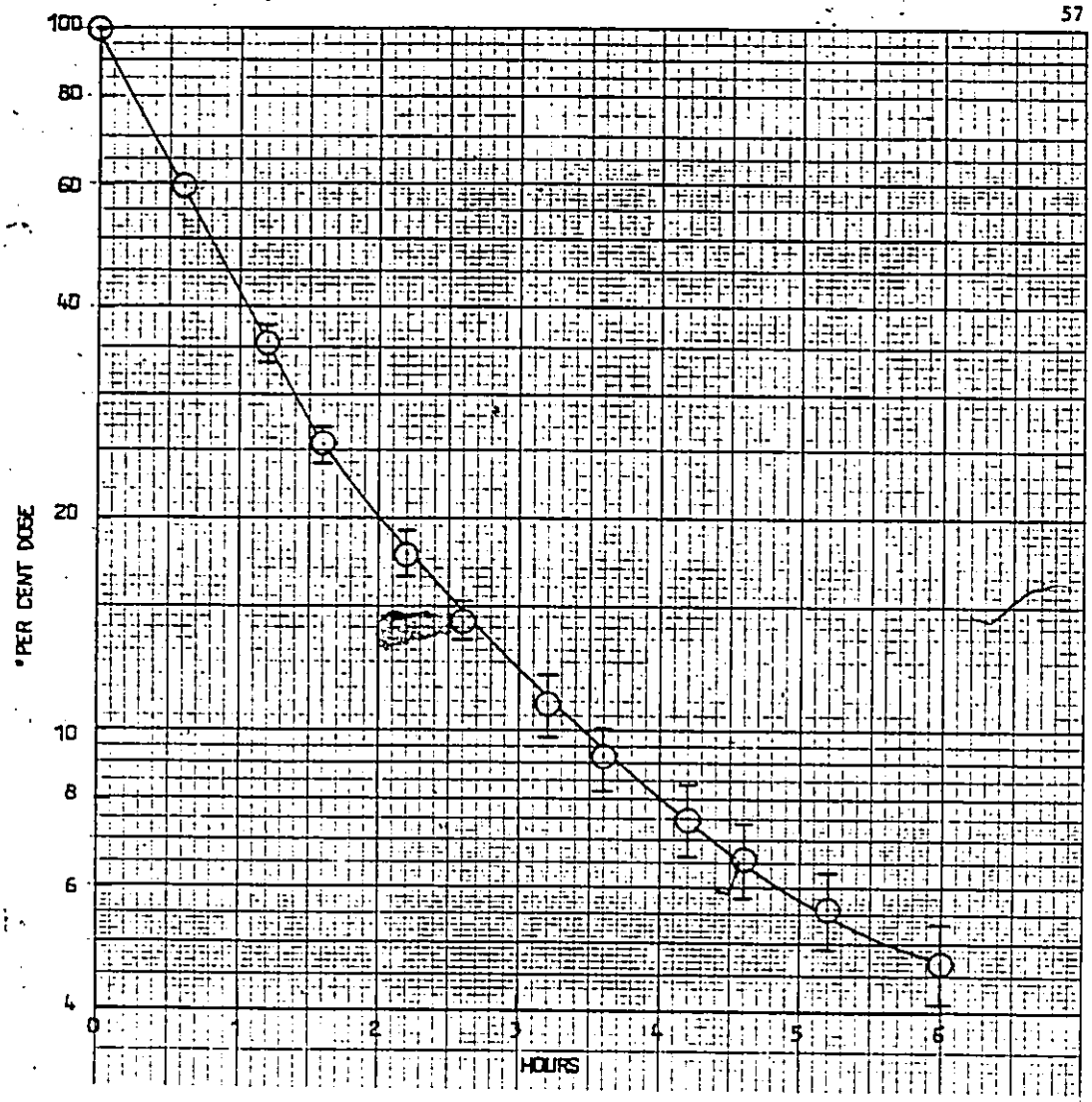


Figure 11. Protein-bound radioactivity in the plasma of rabbits following the injection of the complex of ^{131}I-α_1AT with plasmin. Each point is the mean of twelve experiments. Horizontal bars are the standard errors of the means (only shown where the error exceeds the size of the symbol).

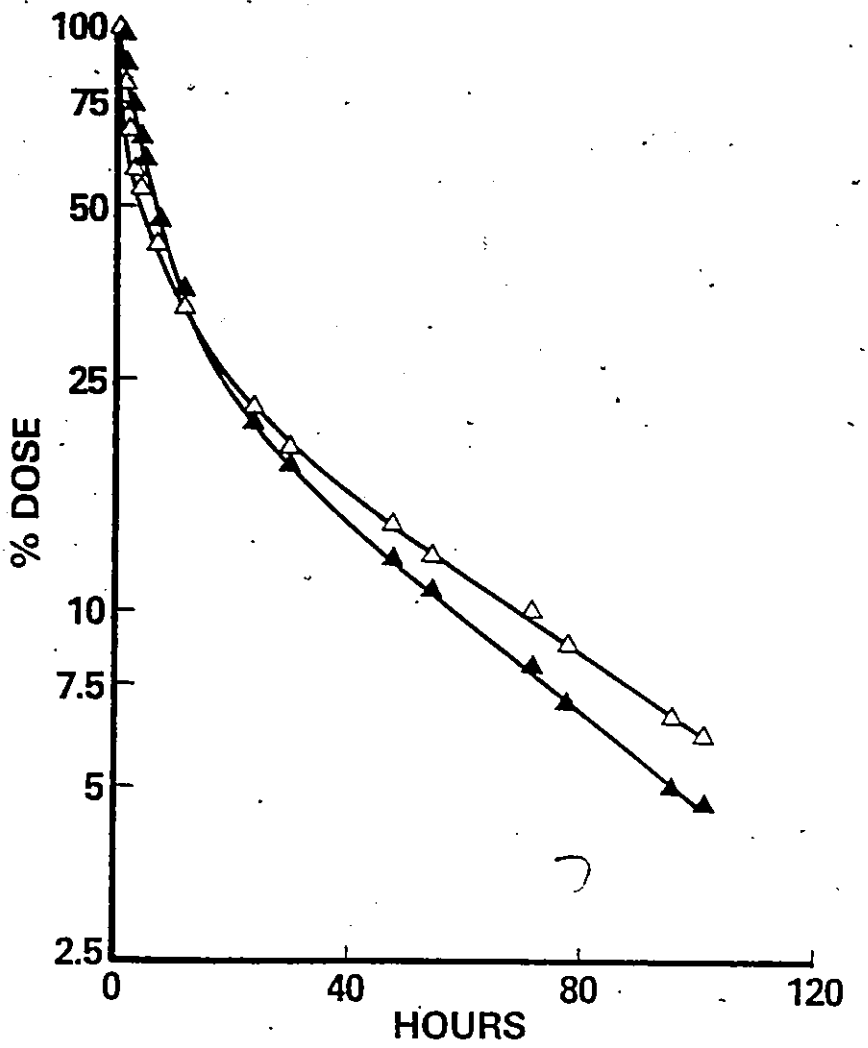


Figure 12. Protein-bound radioactivities in the plasma of a rabbit following the injection of homologous, control ^{131}I-AT III (△) and homologous ^{125}I-AT III (▲) that had been complexed with homologous plasmin.

Figure 13a.

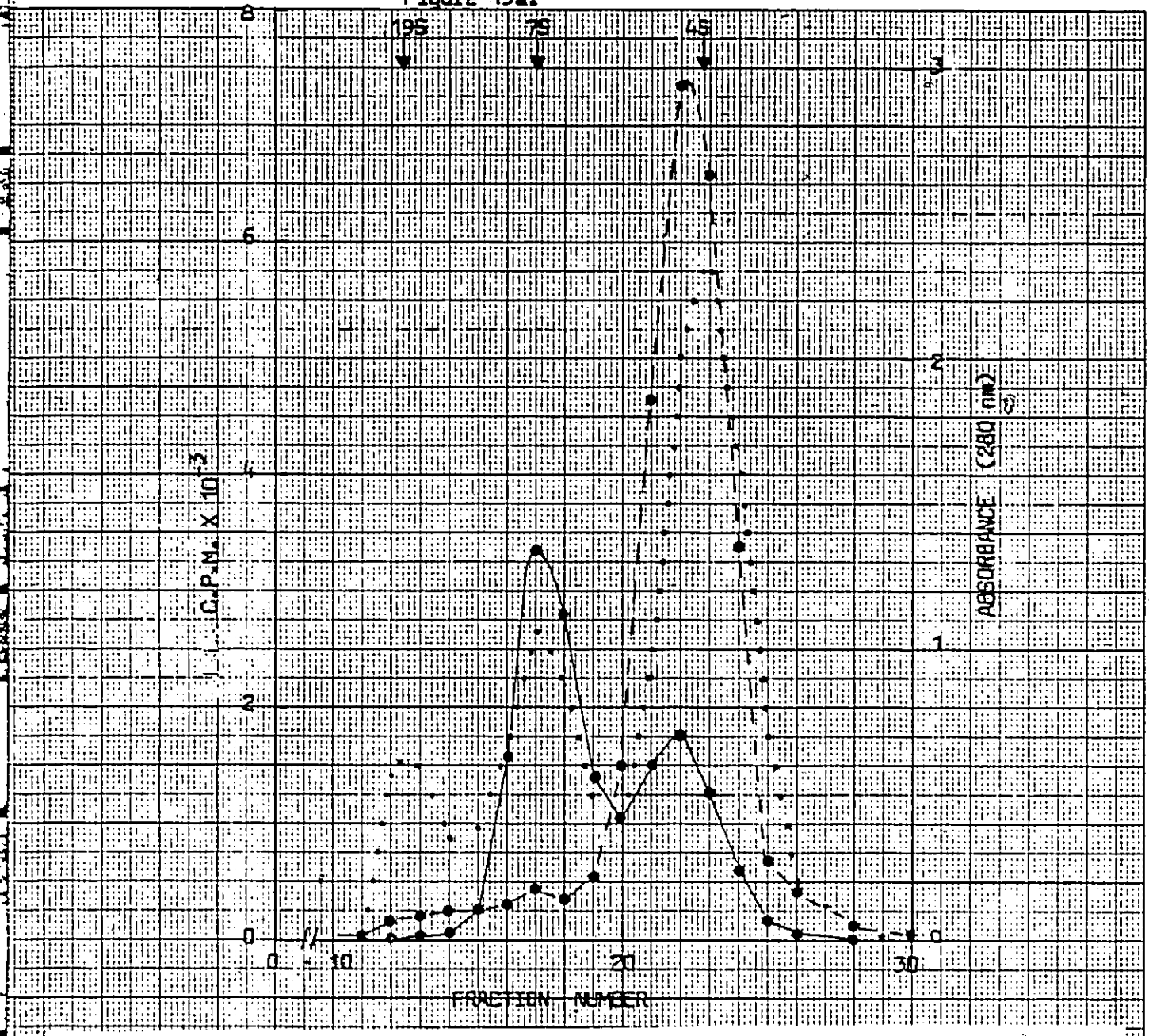


Figure 13. Sephadex G-200 chromatograms of three plasma samples obtained from a rabbit after 5 min (13a), 6.23 h (13b) and 29 h (13c) following the injection of a mixture of homologous ^{131}I-AT III not exposed to protease (●– – – –●) and homologous ^{125}I-AT III (●————●) that had been complexed with unlabelled homologous plasmin. Absorbance of plasma proteins at 280 nm in the effluent is shown by the dotted line.

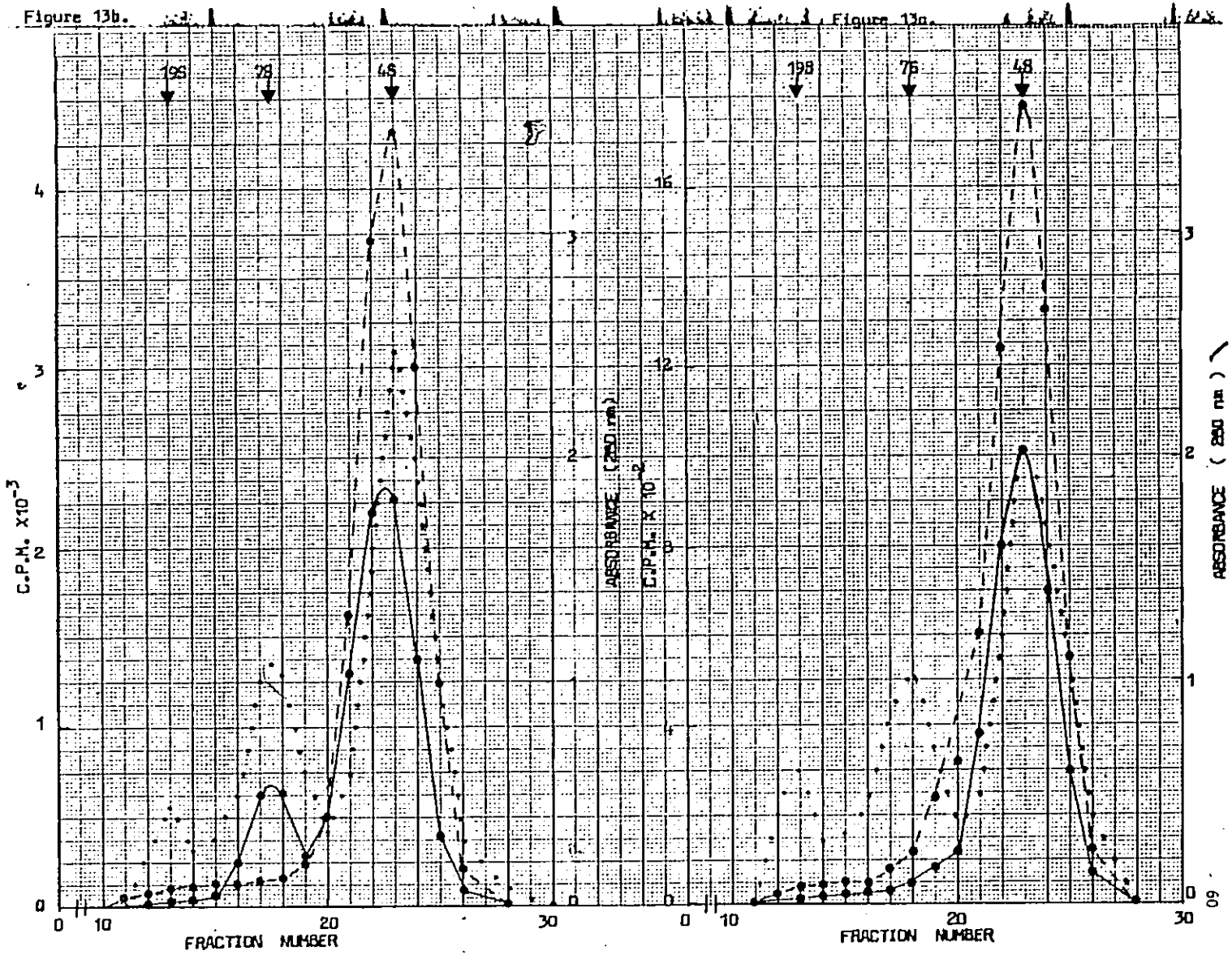
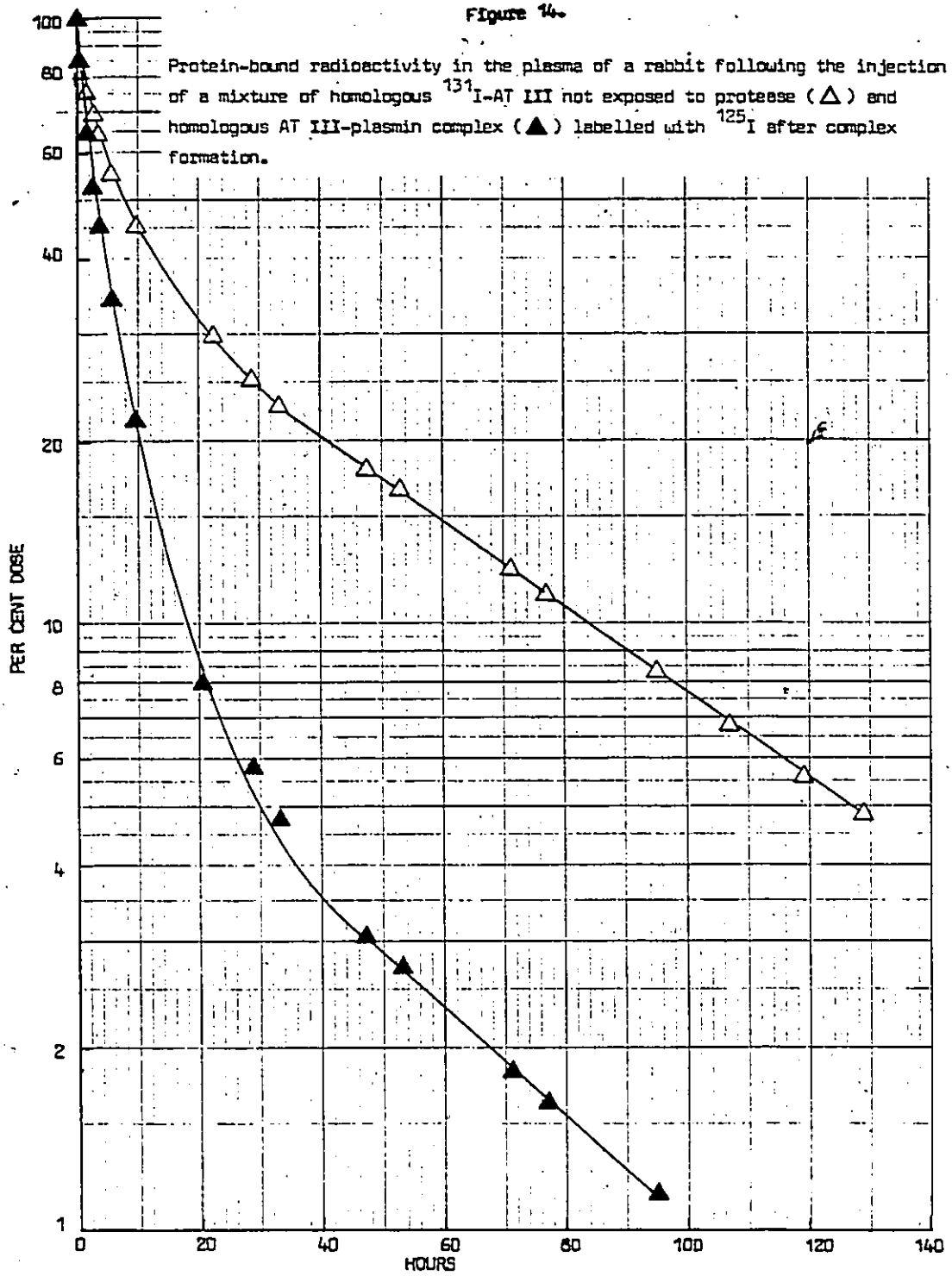


Figure 13b.
Figure 13a.
196
28
46
198
76
48
C.P.M. X10⁻³
ABSORBANCE (280 nm)
FRACTION NUMBER
FRACTION NUMBER

Figure 14.

Protein-bound radioactivity in the plasma of a rabbit following the injection of a mixture of homologous ^{131}I-AT III not exposed to protease (△) and homologous AT III-plasmin complex (▲) labelled with ^{125}I after complex formation.



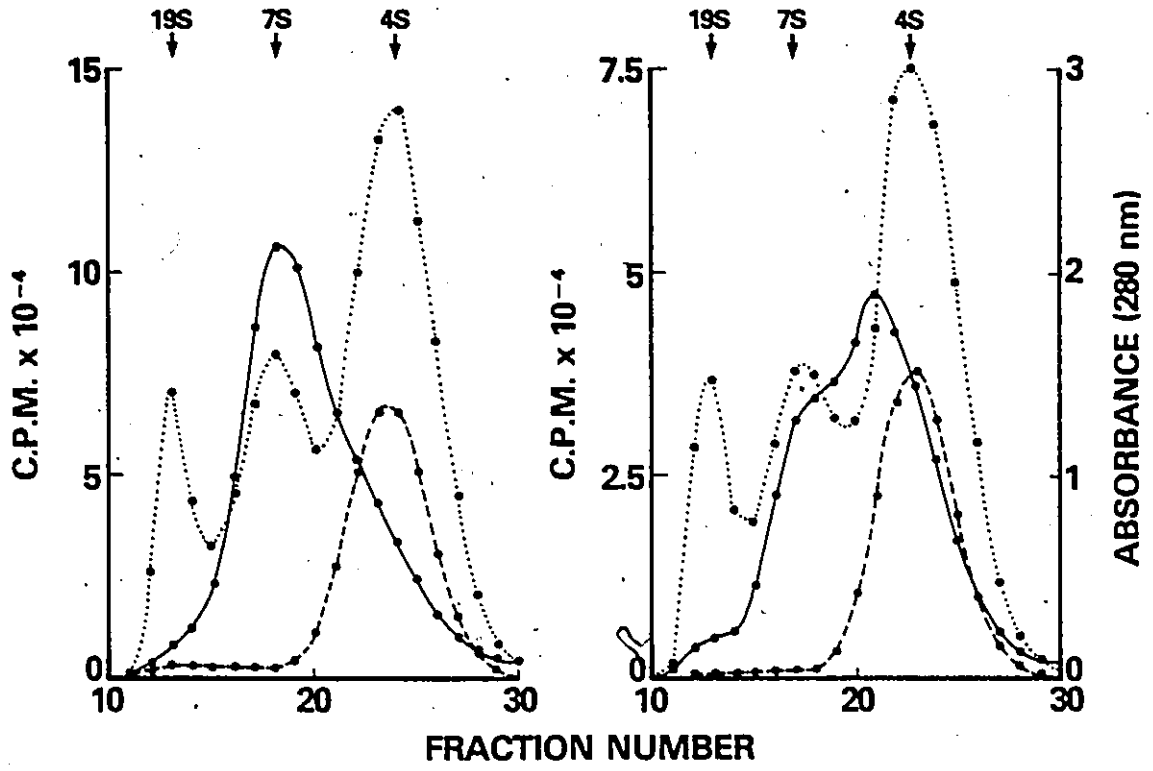
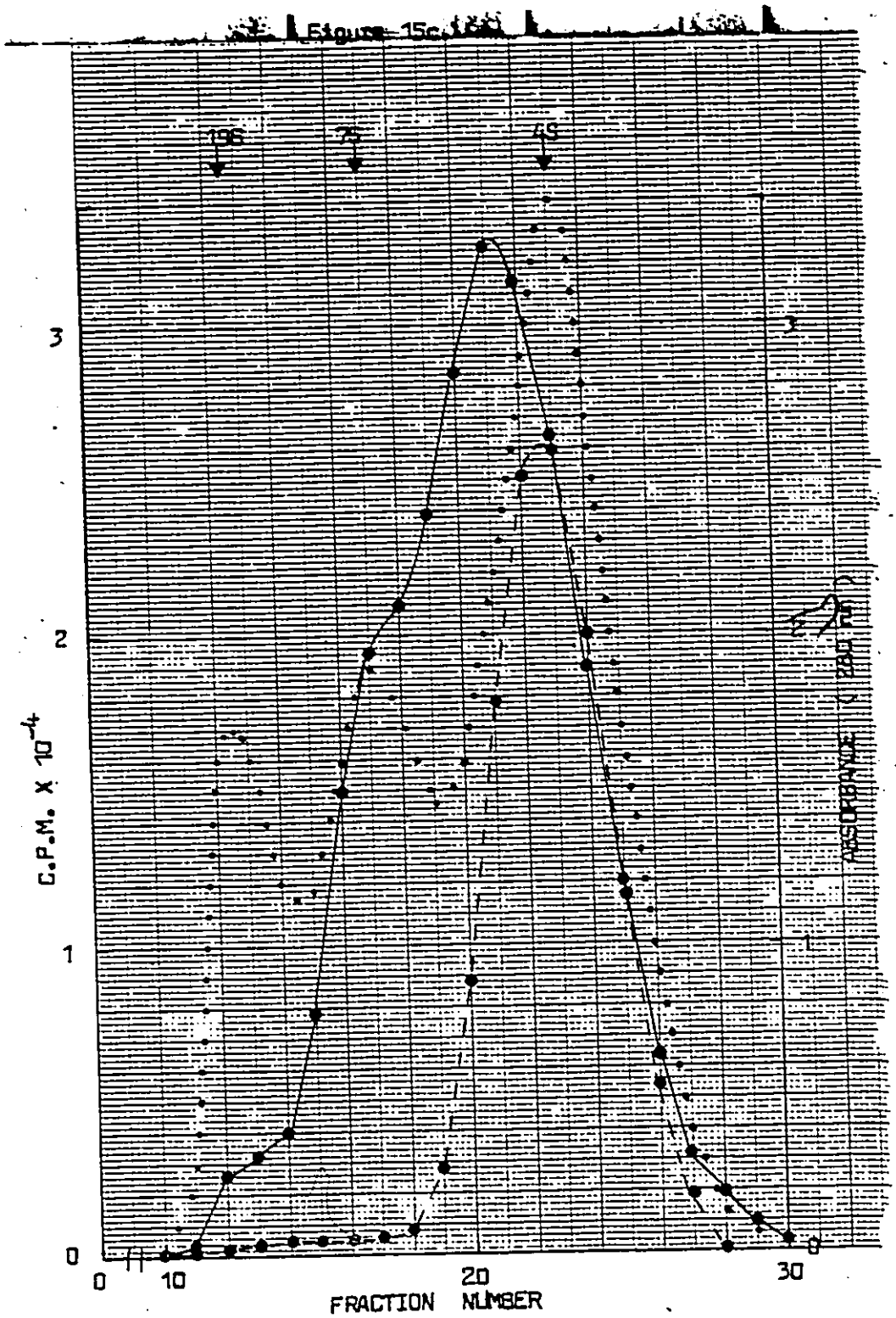


Figure 15a.

Figure 15b.

Sephadex G-200 chromatograms of three plasma samples obtained from a rabbit after 5 min. (15a), 3.5 h (15b), and 6 h (15c) following the injection of a mixture of homologous ^{131}I-AT III not exposed to protease (●– – – –●) and homologous AT III-plasmin complex (●————●) labelled with ^{125}I after complex formation. Absorbance of plasma proteins at 280 nm in the effluent is shown by the dotted line.


196
75
49
C.P.M. X 10^{-4}
ABSORBANCE (280 nM)
FRACTION NUMBER
0
10
20
30
1
2
3

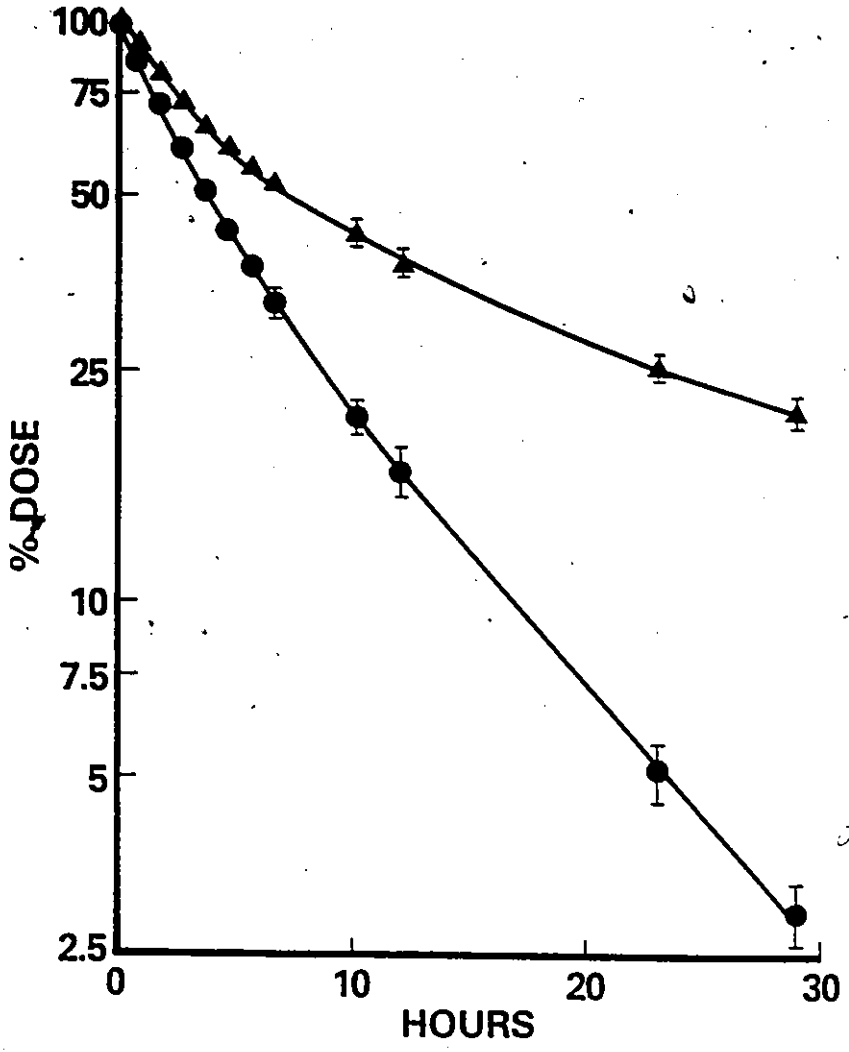


Figure 16. Protein-bound radioactivity in the plasma of rabbits following the injection of homologous complex of ^{131}I-AT III (▲) with ^{125}I-plasmin (●). Each point is the mean of six experiments. Horizontal bars are standard errors of the means. (Only shown where the error exceeds the size of the symbol).

Figure 17a.

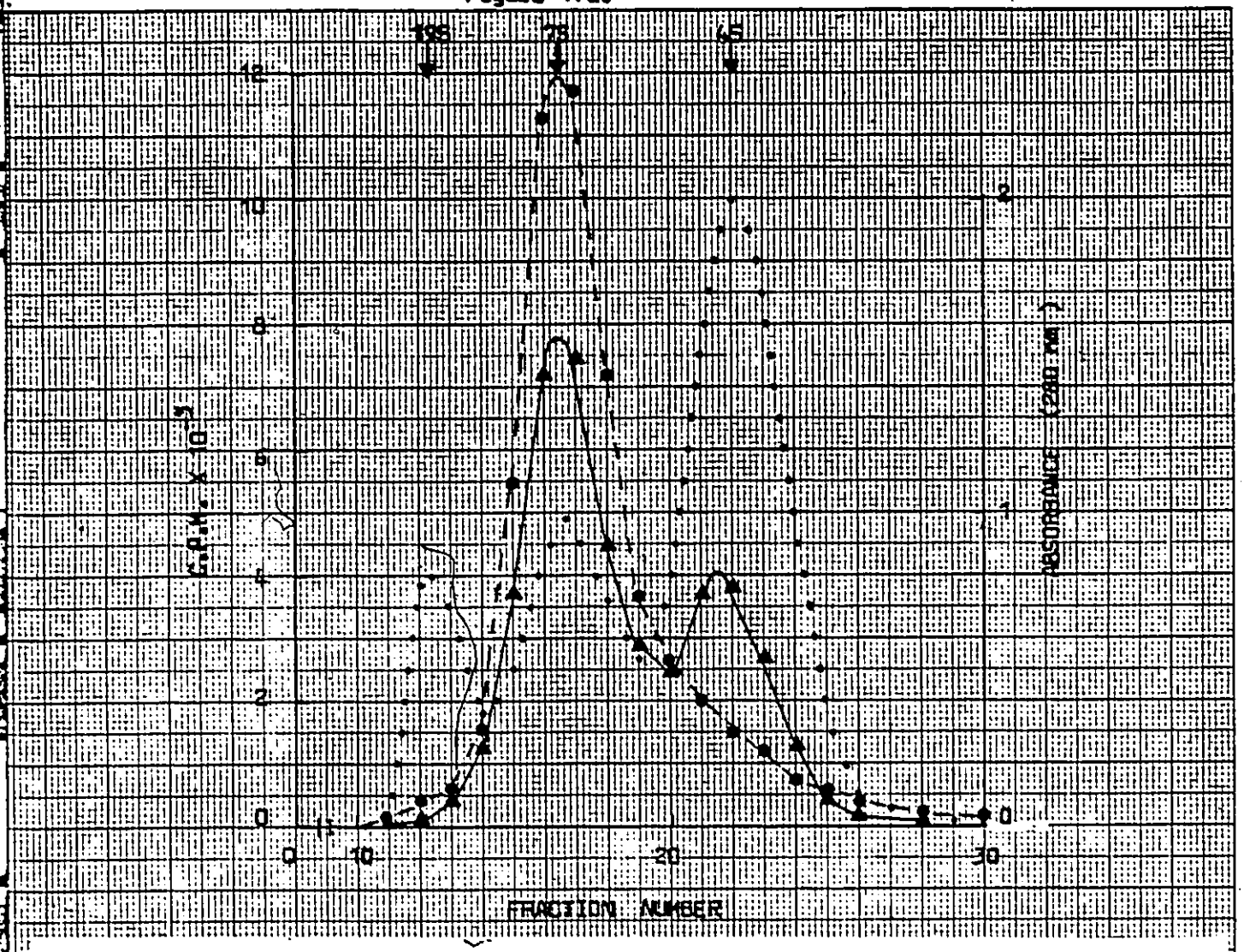


Figure 17. Sephadex G-200 chromatograms of three plasma samples obtained from a rabbit after 5 min (17a), 6.4 h (17b) and 24 h (17c) following the injection of a homologous complex of ^{131}I-AT III (▲——▲) with ^{125}I-plasmin (●– – – –●). Absorbance of plasma proteins at 280 nm in the effluent is shown by the dotted line.

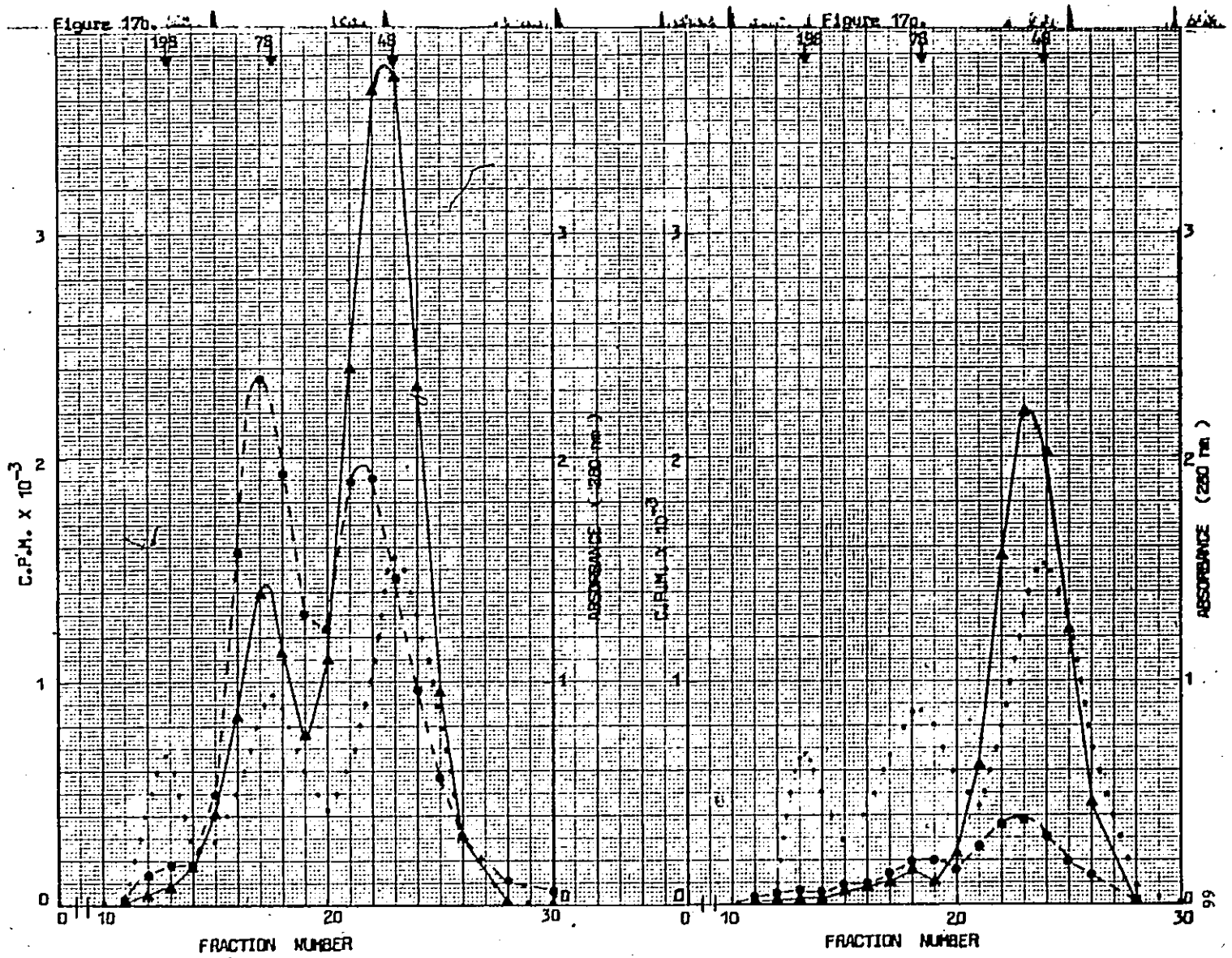


Figure 17b.
198
78
48
C.P.M. X 10^{-3}
ABSORBANCE (280 nm)
FRACTION NUMBER
0
10
20
30
1
2
3

Figure 17a.
198
78
48
C.P.M. X 10^{-3}
ABSORBANCE (280 nm)
FRACTION NUMBER
0
10
20
30
1
2
3

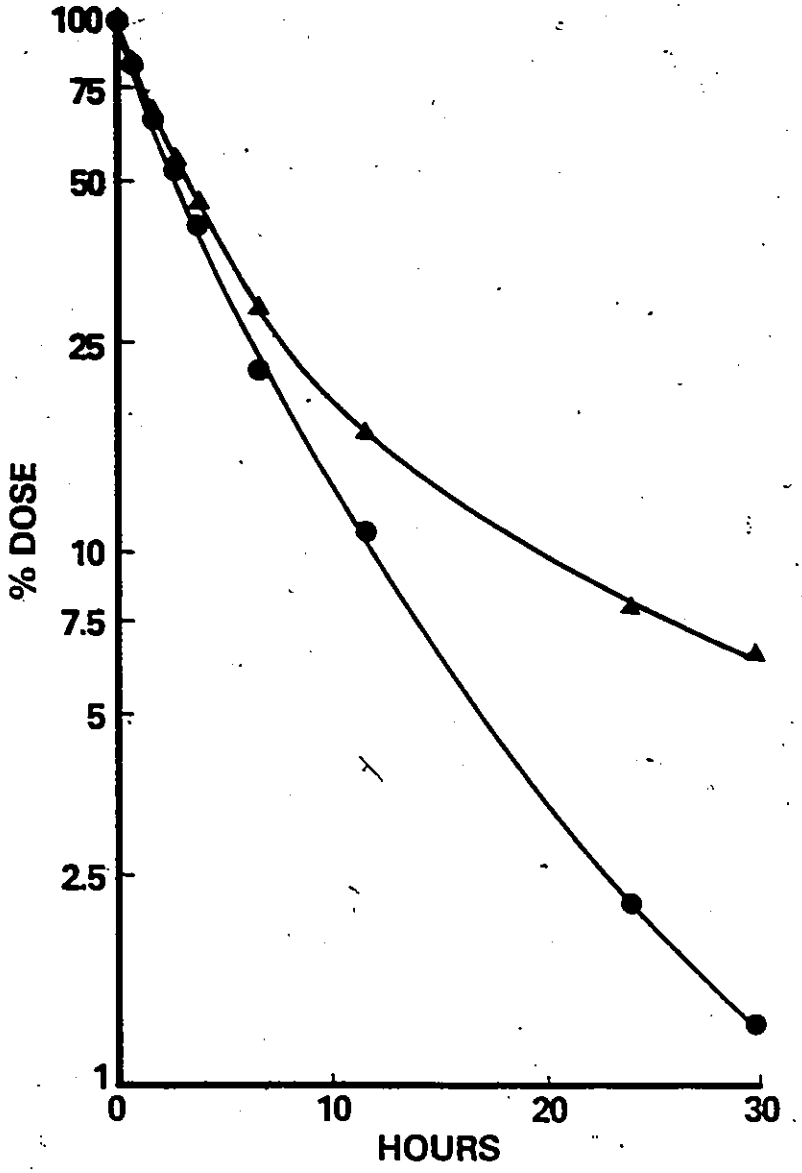


Figure 18. Protein-bound radioactivities in the plasma of a rabbit following the injection of a complex of humman ^{131}I-AT III (▲) with rabbit ^{125}I-plasmin (●).

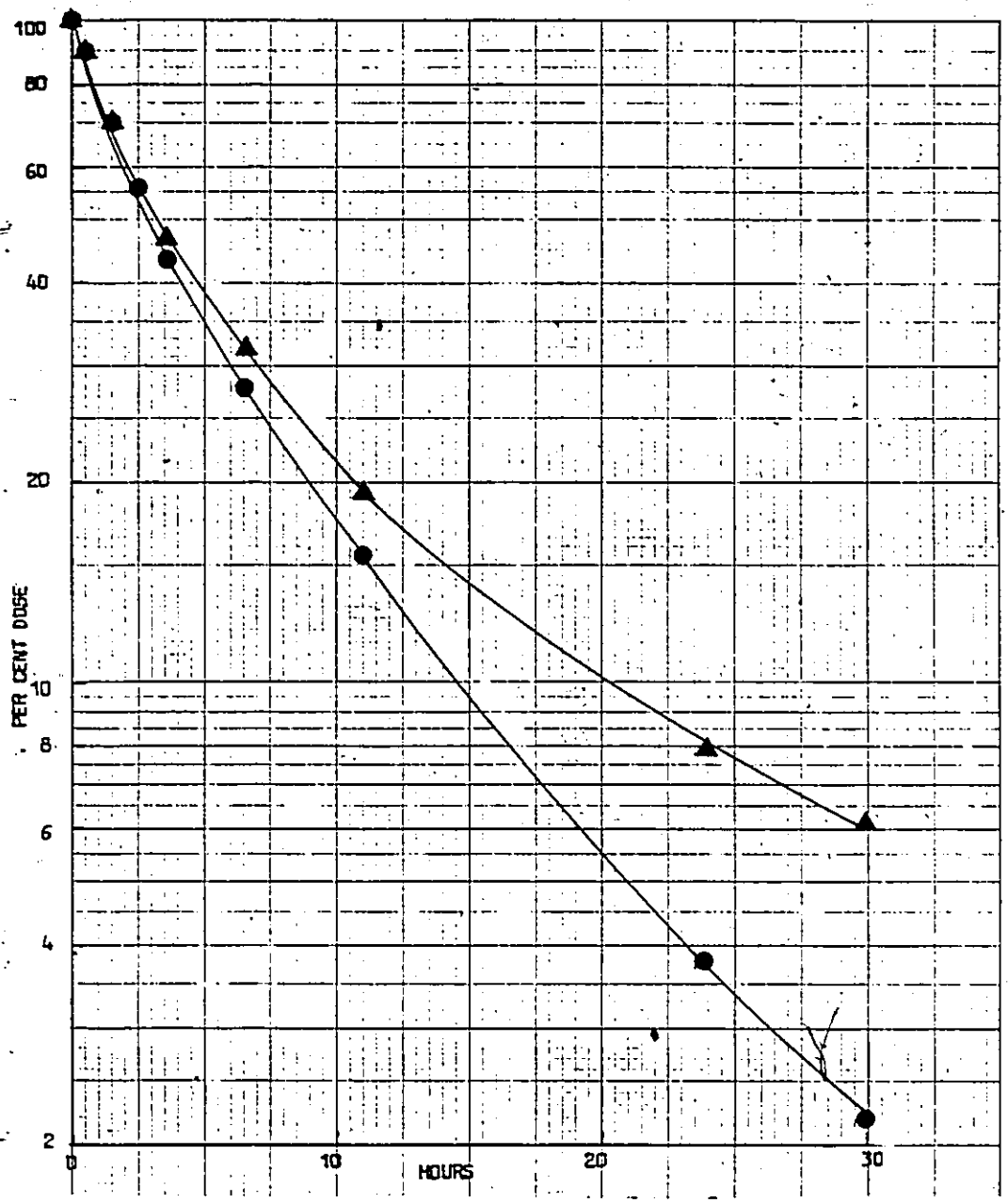


Figure 19. Protein-bound radioactivity in the plasma of a rabbit following the injection of a complex of rat ^{131}I-AT III (▲) with rabbit ^{125}I-plasmin (●).

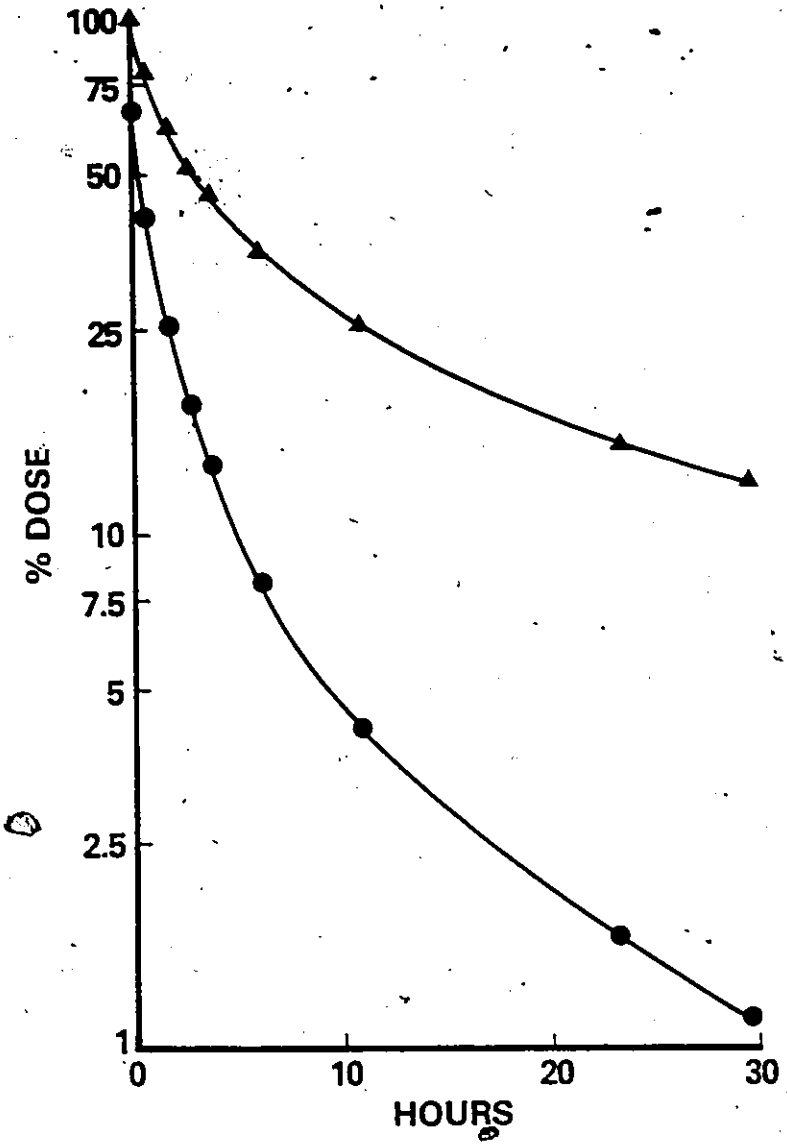


Figure 20. Protein-bound radioactivities in the plasma of a rabbit following the injection of homologous complex of ^{131}I-AT III (▲) with ^{125}I-thrombin (●). By the time the first sample was obtained (5 min.) a significant portion of the radioactivity attached to thrombin had left the plasma.

Figure 21a.

Figure 21b.

Sephadex G-200 chromatograms of two plasma samples obtained from a rabbit 5 min (21a) and 6 h (21b) following the injection of a homologous complex of ^{131}I-AT III (▲——▲) with ^{125}I-thrombin (●- - - -●). Absorbance of plasma proteins at 280 nm in the effluent is shown by the dotted line.

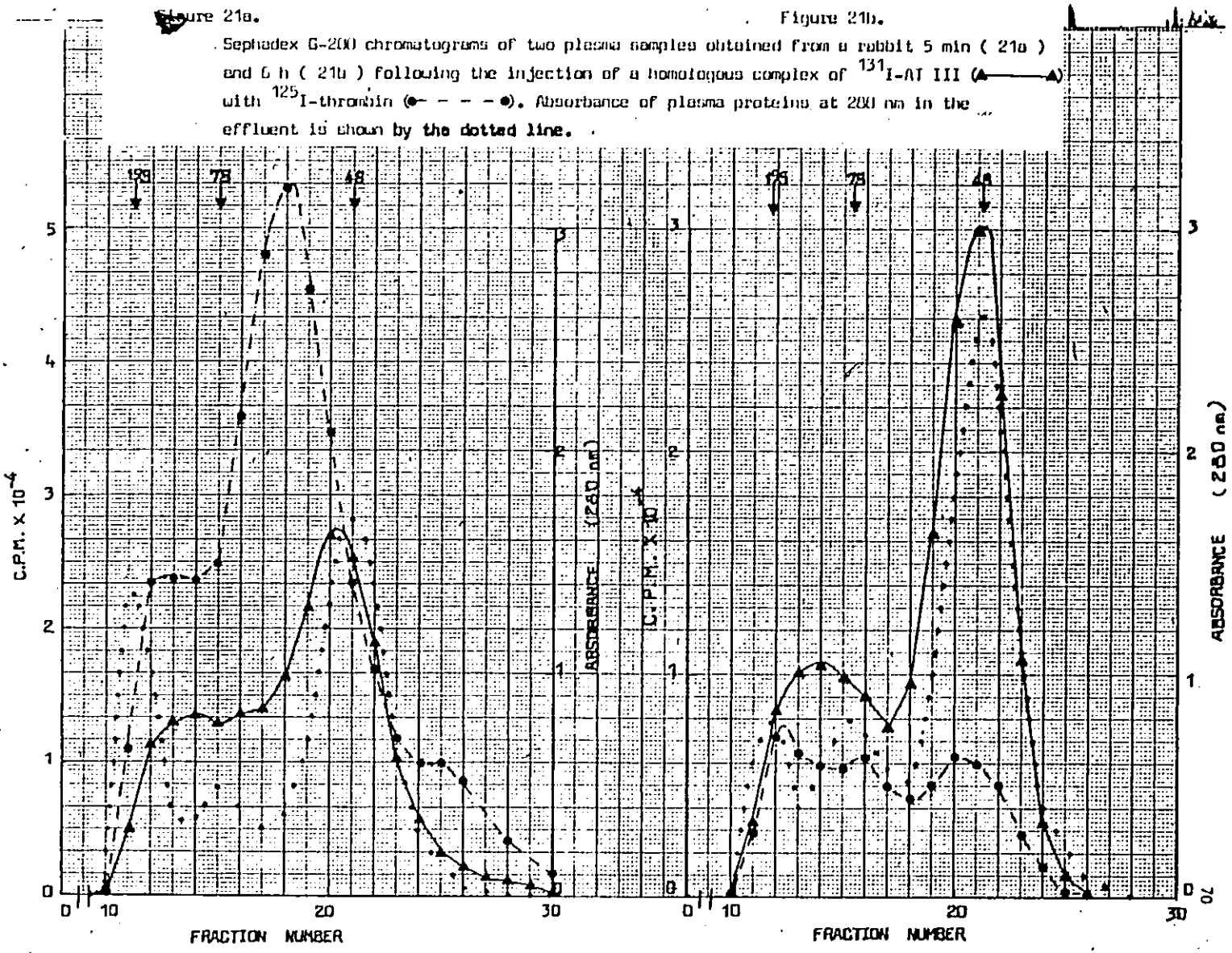


TABLE II Behaviour of different forms of rabbit AT III on Sepharose-heparin and Sepharose-trypsin

Types of AT III	% Bound by		
	Sepharose-heparin	Sepharose-trypsin no. 1	Sepharose-trypsin no. 2
AT III (post-complex)	19%	6%	< 5%
AT III (injected control)	70%	80%	0
AT III (not-injected control)	70%	80%	0

Each value is the mean of two experiments. The use of two subsequent Sepharose-trypsin columns is explained in the Methods section.

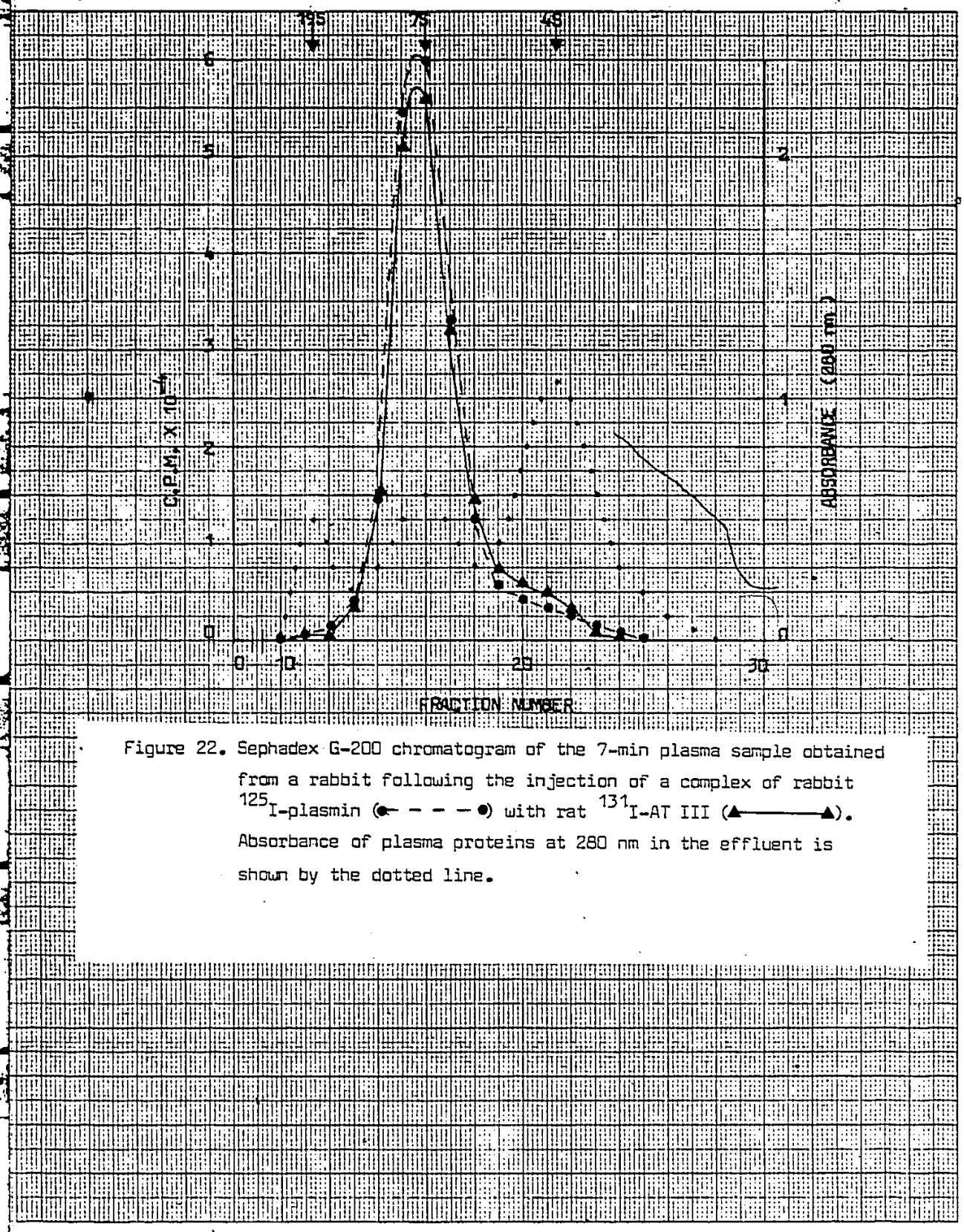


Figure 22. Sephadex G-200 chromatogram of the 7-min plasma sample obtained from a rabbit following the injection of a complex of rabbit ^{125}I-plasmin (●– – – –●) with rat ^{131}I-AT III (▲————▲). Absorbance of plasma proteins at 280 nm in the effluent is shown by the dotted line.

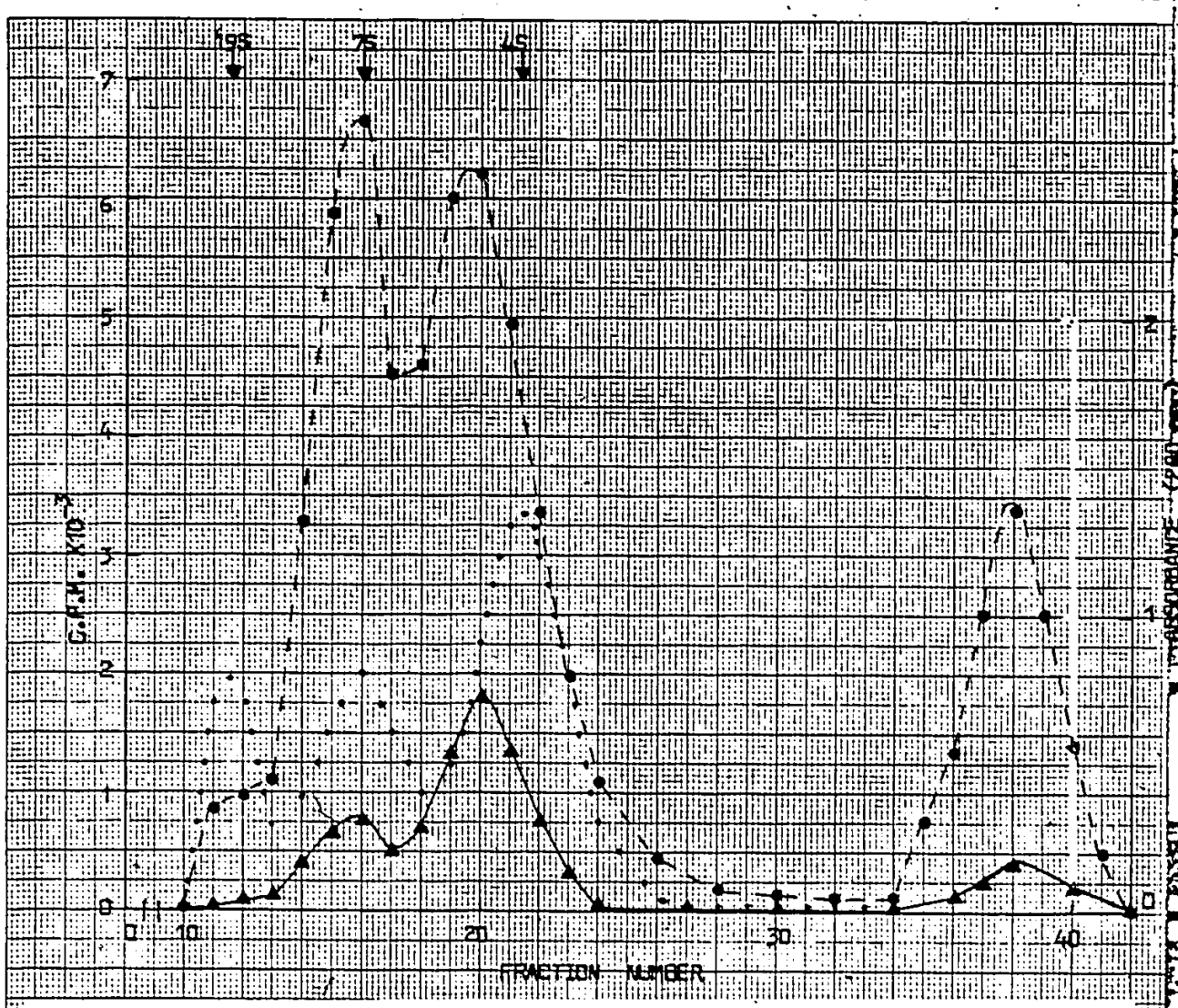


Figure 23. Sephadex G-200 chromatogram of a plasma sample obtained from a rabbit after 11 h following the injection of a complex of rabbit ^{125}I-plasmin (●– – – –●) with rat ^{131}I-AT III (▲————▲). Absorbance of plasma proteins at 280 nm in the effluent is shown by the dotted line.

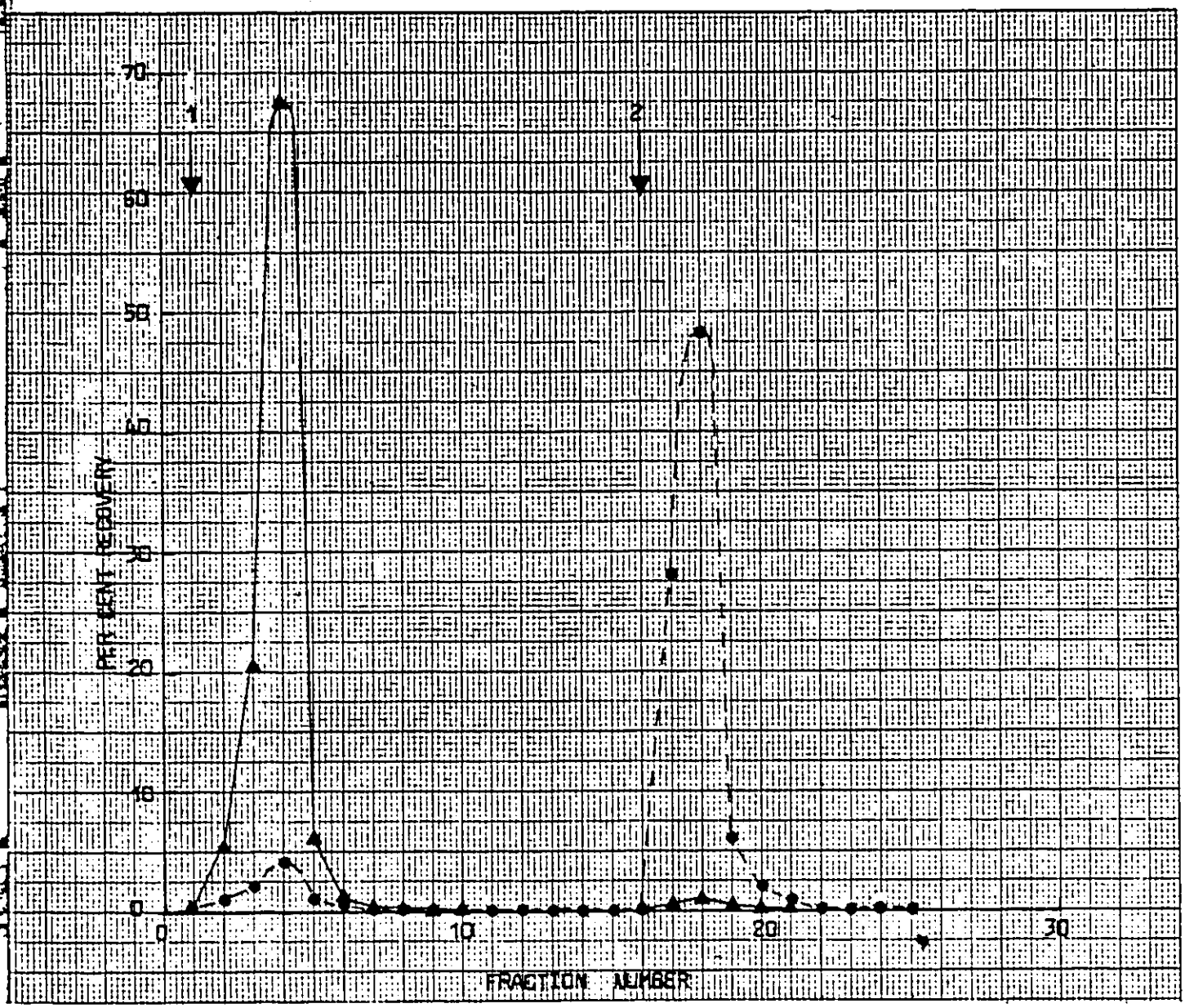


Figure 24. Sepharose-lysine affinity chromatogram of the pooled fractions 19, 20 and 21 from Sephadex G-200 gel filtration of the 11-h plasma sample obtained from a rabbit following the injection of a complex of rat ^{131}I-AT III (▲——▲) with rabbit ^{125}I-plasmin (●– – – –●). Arrow 1 indicate the start of the washing step by 0.1M NaCl and arrow 2 shows the elution of the bound material by 0.1M EACA (see text for details).

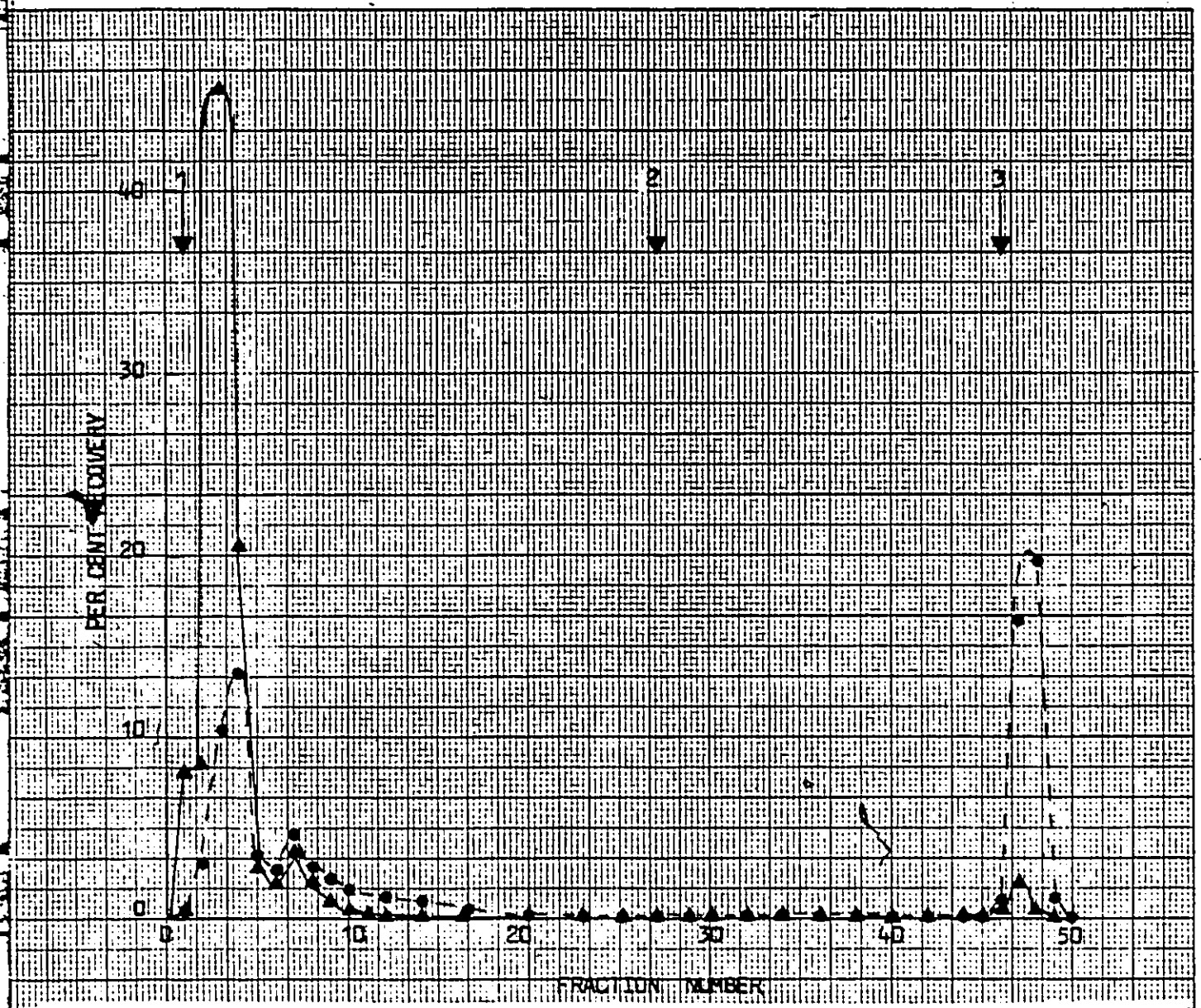


Figure 25. Sepharose-lysine affinity chromatogram of one ml of the 11-h plasma sample obtained from a rabbit following the injection of a complex of rat ^{131}I-AT III (▲——▲) with rabbit ^{125}I-plasmin (●– – – –●). Arrows 1 and 2 indicate the start of the washing steps by 0.15M NaCl and 0.5M NaCl, respectively. Arrow 3 marks the start of elution by 0.1M EACA.

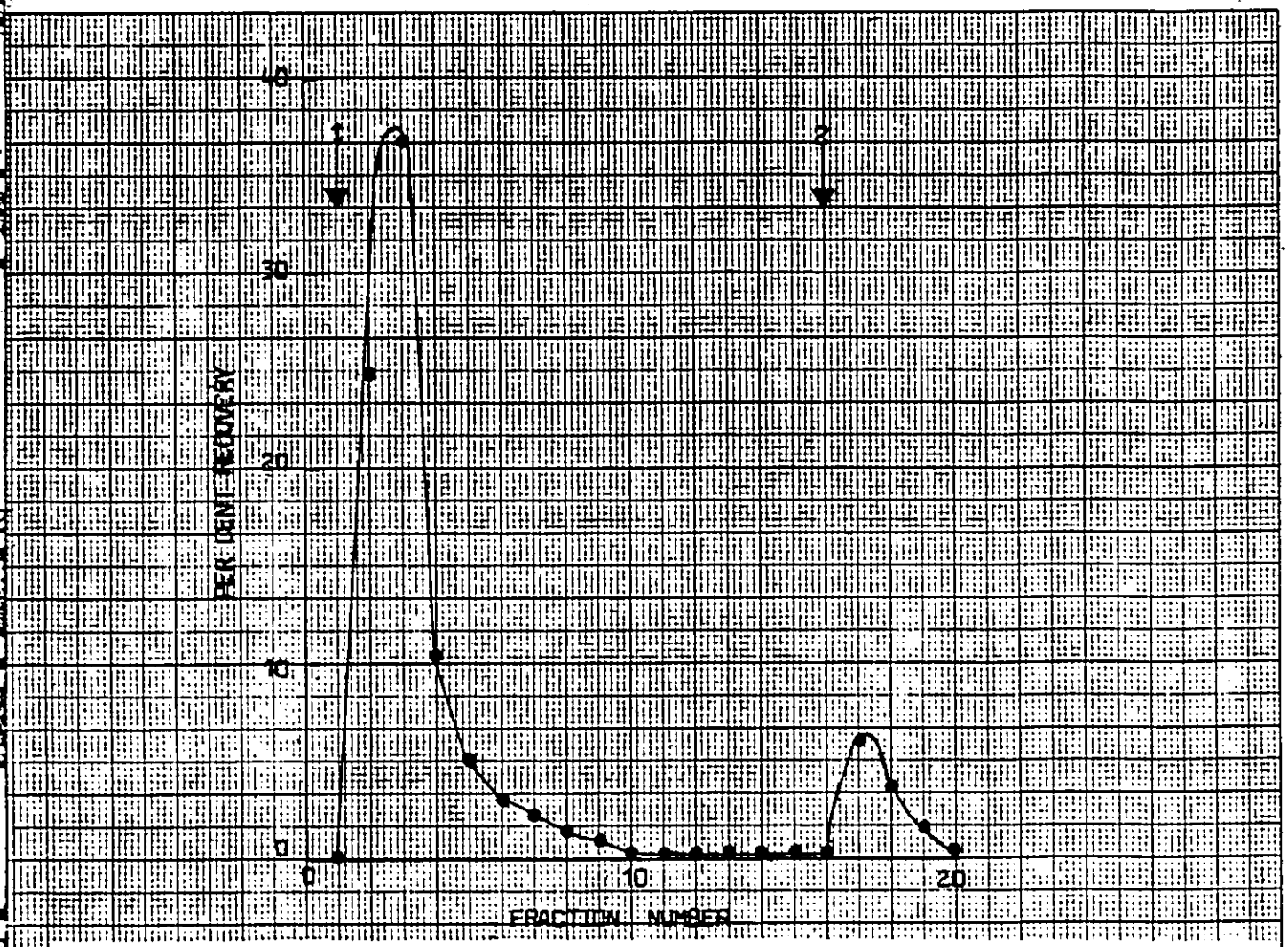


Figure 26. Sepharose-AT III affinity chromatogram of post-complex ^{125}I-rabbit plasmin. The beginning of the washing and eluting steps are indicated by arrows 1 and 2, respectively.

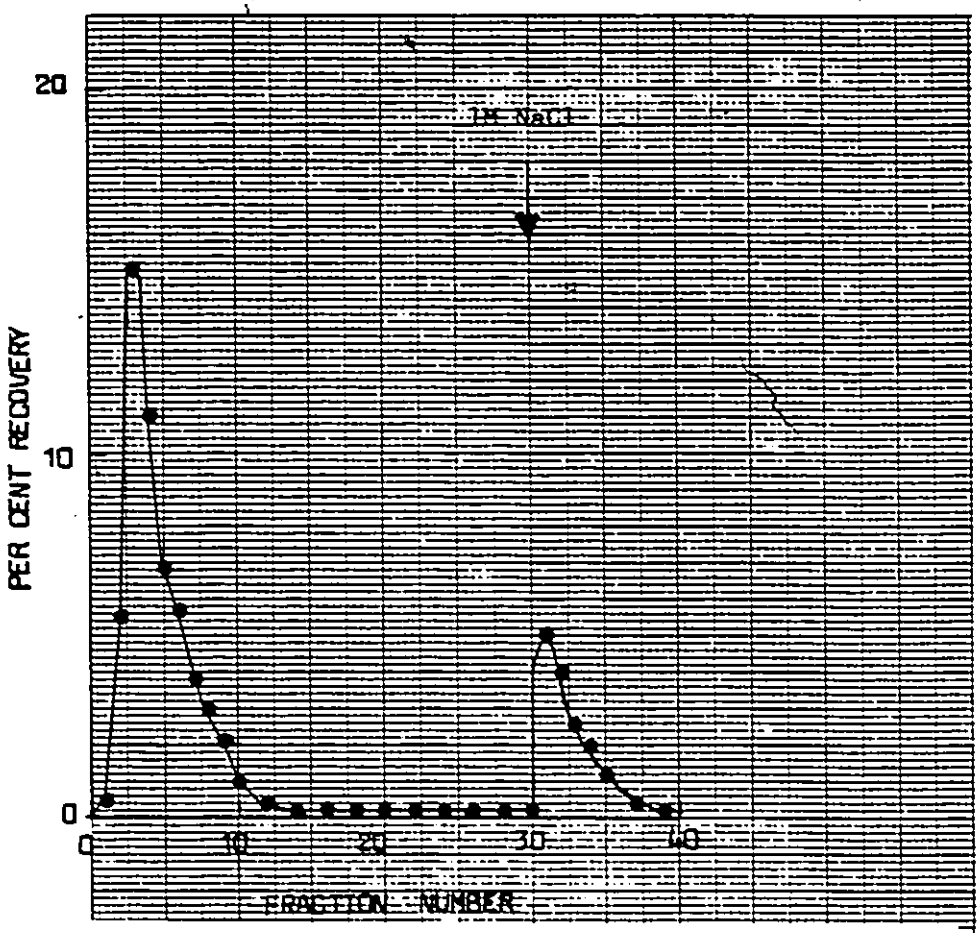


Figure 27. Sepharose-Trasylol affinity chromatogram of post-complex rabbit ^{125}I-plasmin. Elution of the bound radioactivity is indicated by the arrow.

Figure 28. Electrophoretograms of a mixture of rabbit AT III with rabbit plasmin (molar ratio 1.3:1) incubated at 37 °C in the presence of sodium azide (0.1% w/v) for increasing periods of time. Incubation was in the absence of Trasylol. Subsamples were collected for electrophoresis (from left to right) at 1, 2, 3.5, 6, 11, 23.5 and 29 h. Load: 35 ug protein per gel. Migration from top to bottom. The AT III-plasmin complex band is marked by arrow.

Figure 29. Electrophoretograms of a mixture of rabbit AT III with rabbit plasmin (molar ratio 1.3:1) incubated at 37 °C in the presence of sodium azide (0.1% w/v) for increasing periods of time. 163 units of Trasylol was added after the first hour of incubation. Conditions as in fig. 28. The AT III-plasmin band is marked by arrow.

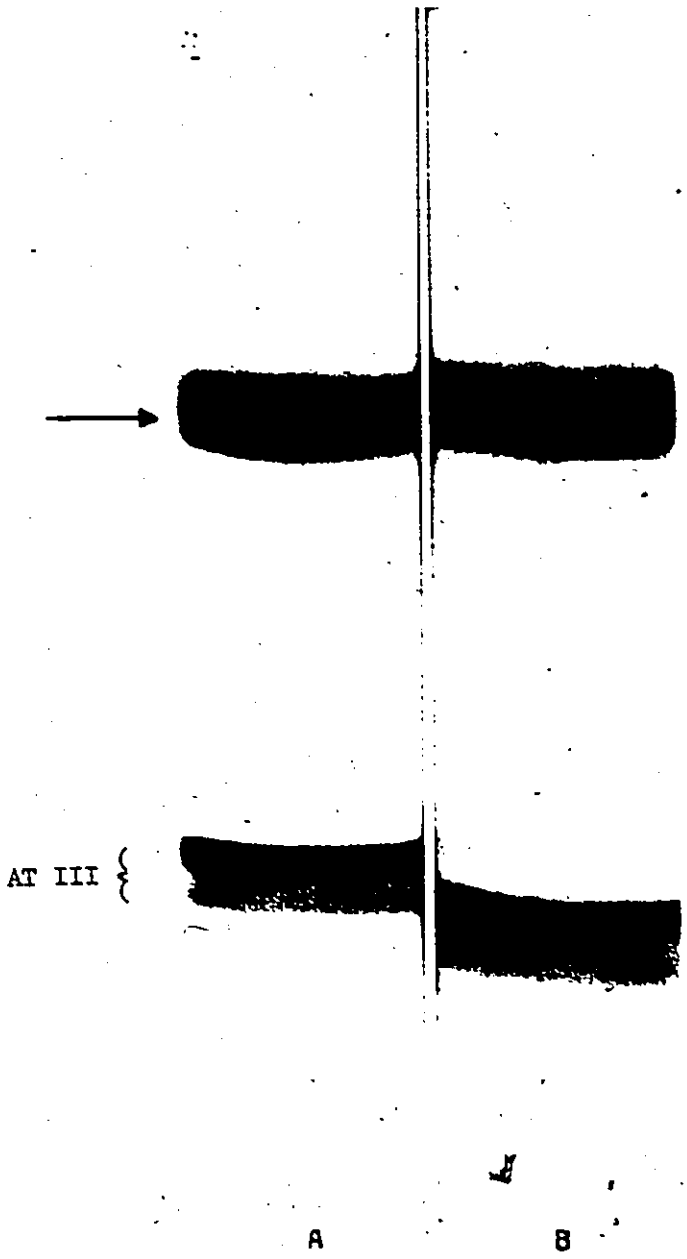


Figure 30. Electrephoretograms of a mixture of rabbit AT III with rabbit plasmin (molar ratio 1.3:1) incubated at 4 °C in the presence of sodium azide (0.1% w/v) for 29 h. (A) No Trasylol was added. (B) 25 units of Trasylol were added after the first hour of incubation. The AT III-plasmin complex band is marked by arrow.

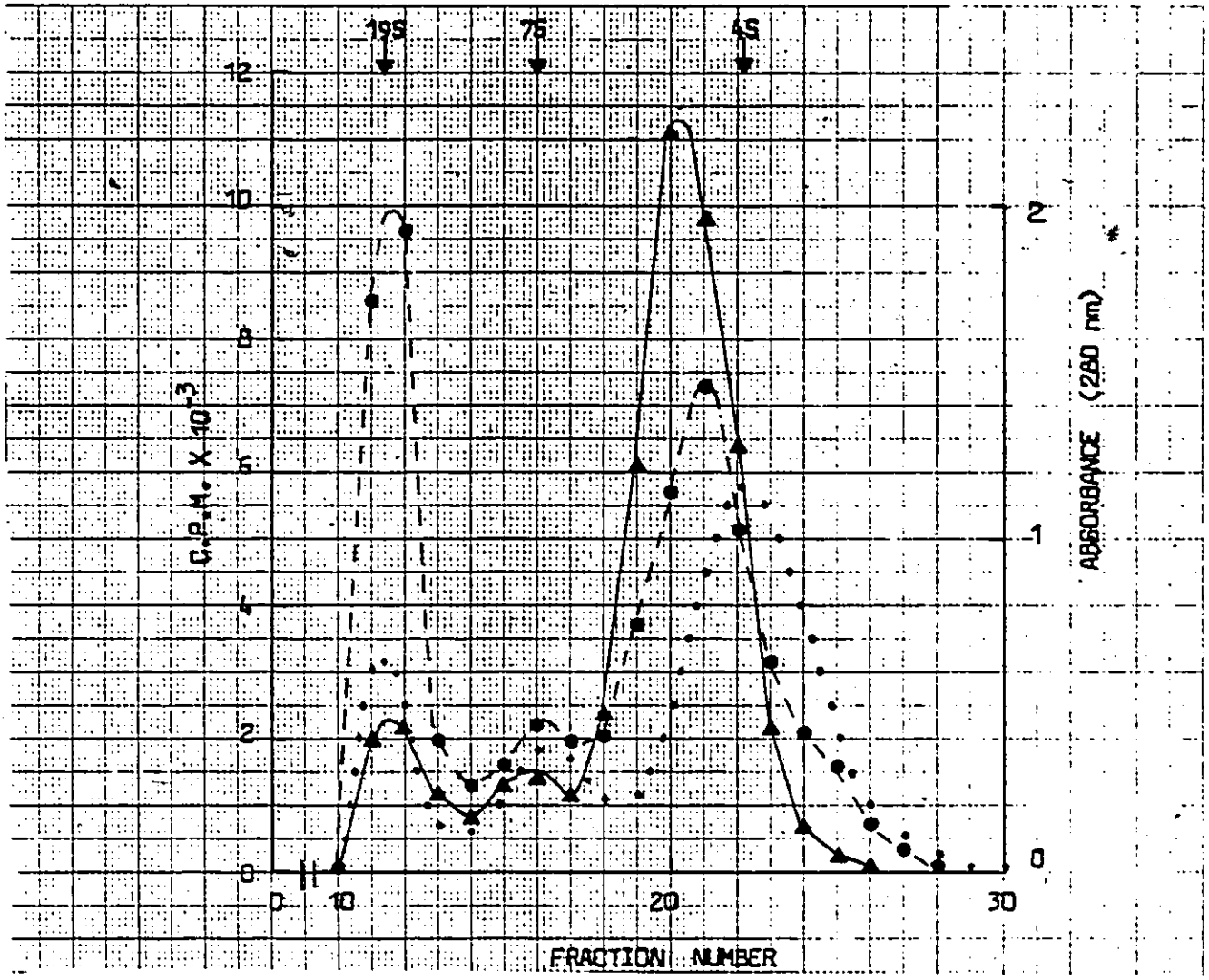


Figure 31. Sephadex G-200 chromatogram of a complex of rabbit ^{125}I-plasmin (●– – – –●) with rat ^{131}I-AT III (▲——▲) after incubating with 2 ml of citrated rabbit blood at 37 °C for 30 h, in the presence of 0.1% w/v of sodium azide. The dotted line represents the absorbance at 280 nm of plasma proteins.

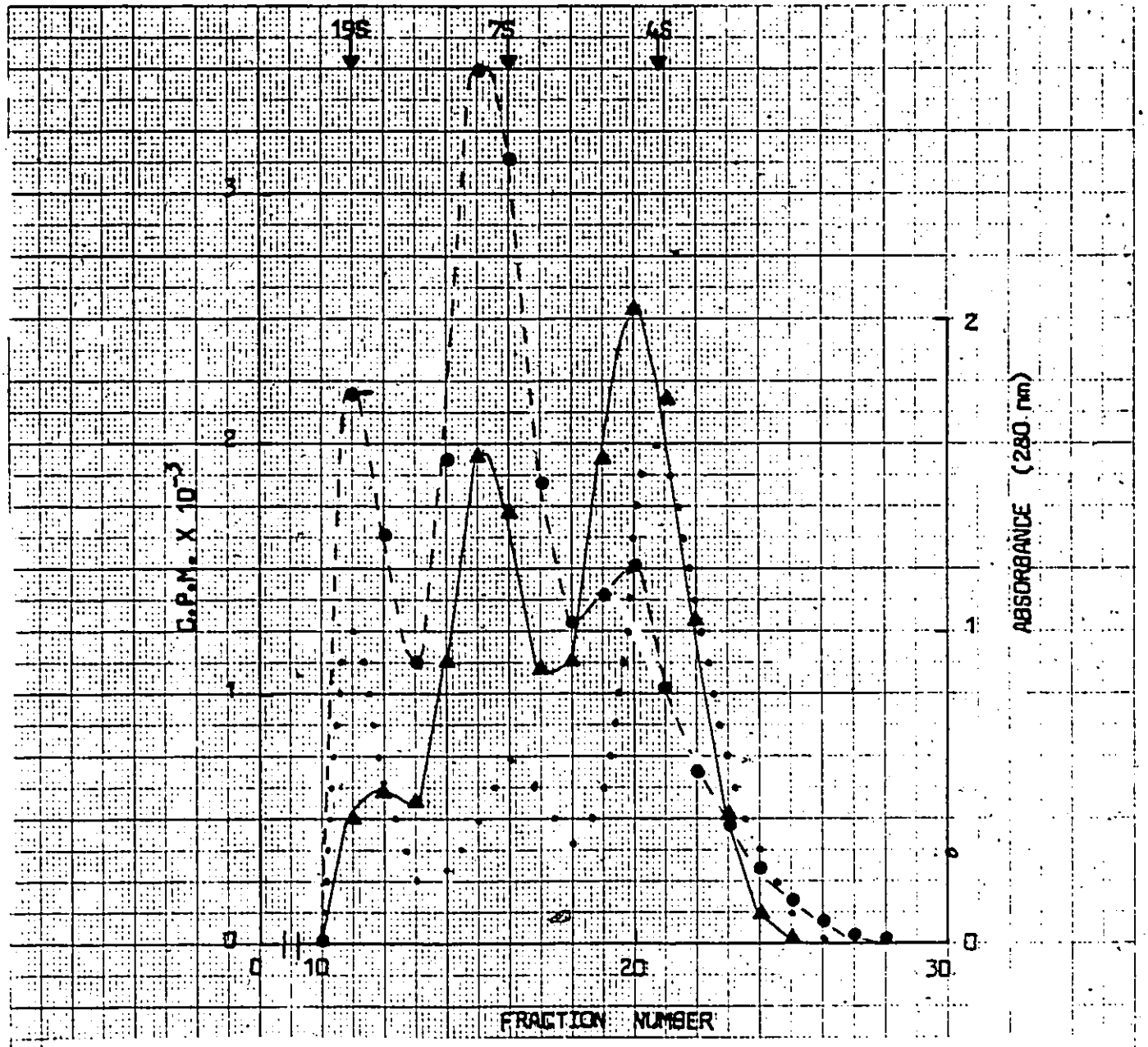


Figure 32. Sephadex G-200 chromatogram of a complex of rabbit ^{125}I-plasmin (●– – – –●) with rat ^{131}I-AT III (▲——▲) after incubating with 1 ml of citrated rabbit plasma at 37°C for 30 h, in the presence of 0.1% w/v of sodium azide. Absorbance at 280 nm of the plasma proteins is shown by the dotted line.

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