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PARTIAL PURIFICATION AND CHROMATOGRAPHIC PROPERTIES OF INACTIVE RENIN FROM HUMAN AMNIOTIC FLUID

RODNEY L. JOHNSON, ALAN M. POISNER and ROBERT D. CRIST

Department of Pharmacology (R. L. J. and A. M. P.) and Department of Gynecology and Obstetrics (R. D. C.), University of Kansas Medical Center, College of Health Sciences and Hospital, Kansas City, KS 66103, U.S.A.

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Abstract—Inactive renin from human amniotic fluid was partially purified using gel filtration and several types of affinity chromatography. The chromatographic steps employed resulted in a 62-fold purification of inactive renin with an overall yield of 11 per cent. Inactive renin was retained on an Affi-Gel Blue column and could be eluted with 1.4 M NaCl. Chromatography on a concanavalin A–agarose affinity column yielded two fractions. The non-retained fraction contained the inactive renin while the retained fraction contained cathepsin D. Acid or pepsin activation of the non-retained inactive renin, followed by concanavalin A chromatography, resulted in the retention of 33 per cent of the applied renin activity. Inactive renin was also not retained on a pepstatin affinity column. However, after the activation of the inactive renin with pepsin, the enzymatic activity was bound to the pepstatin column. Gel filtration on Sephacryl S-200 indicated that inactive renin had a molecular weight of 39,000. No change in molecular weight was observed after activation with pepsin.

Human amniotic fluid has been found to contain an inactive form of renin which can be activated by treatment with either acid [1] or proteolytic enzymes [2, 3]. Lumbers [1] demonstrated that the ability of amniotic fluid to generate angiotensin I was increased if the amniotic fluid was pretreated at a pH of 3.3. Later work [2,3] showed that the treatment of amniotic fluid with proteolytic enzymes such as pepsin, cathepsin D, and trypsin also brought about the activation of inactive renin. Little is known, however, about either the biochemical characteristics or physiological significance of inactive renin from human amniotic fluid. The aim of the present study was to employ a variety of chromatographic techniques in an effort not only to purify inactive renin but also to determine some of the characteristics of the material.

MATERIALS AND METHODS

Materials. Human amniotic fluid was obtained at 20–24 weeks of gestation. Tetradecapeptide renin substrate (TDP) was obtained from Schwarz/Mann, Orangeburg, NY. [^{125}I]Angiotensin I was purchased from New England Nuclear, Boston, MA. Angiotensin I for the radioimmunoassay was obtained from the National Institute for Biological Standards, Holy Hill, London. AH-Sepharose 4B and Sephacryl S-200 (superfine) were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Glycosylex A was purchased from Miles Laboratories, Elkhart, IN. Pepstatin was obtained from the Protein Research Foundation, Osaka, Japan. Affi-Gel Blue and the protein assay dye reagent were obtained from Bio-Rad Laboratories, Richmond, CA. Bovine serum albumin, ovalbumin, pepsinogen myoglobin, cytochrome *c*, pepsin, α-methyl-D-mannoside, and morpholinopropane sulfonic acid (MOPS) were all purchased from Sigma Chemical Co., St. Louis, MO.

Purification of human inactive renin. The chromatographic columns used in the purification of inactive renin were all equilibrated with 10 mM sodium phosphate buffer, pH 7.0, 0.15 M NaCl and 5 mM sodium azide (hereafter referred to as PNA buffer). The elution of protein from the columns was observed by measuring the absorbance of the eluate at 280 nm. The fractions were assayed, as described below, for renin activity both before and after pepsin treatment. Concentration of the pooled fractions was carried out using an Amicon PM-10 filter. All steps in the purification scheme were carried out at 4°.

Human amniotic fluid (330 ml), obtained at 23 weeks of gestation, was centrifuged at 7,000 rev/min for 15 min. The supernatant fraction was decanted and treated with ammonium sulfate until a concentration of 3 M was obtained. The mixture (pH 6.9) was then centrifuged for 15 min at 10,000 rev/min. The precipitate was dissolved in PNA buffer and then dialyzed against this same buffer system for 20 hr.

The above solution was applied to an Affi-Gel Blue column (2.5 × 60 cm). The column was washed with PNA buffer. Fractions of 8 ml were collected. When 480 ml eluant had been collected, elution was begun with 1.4 M NaCl in PNA buffer. After an additional 560 ml had been collected, the column was eluted with 6 M urea. The fractions obtained from the 1.4 M NaCl elution were pooled, concentrated, and dialyzed against PNA buffer.

The concentrated solution obtained from the Affi-Gel Blue column was next applied to a Glycosylex A column [agarose–concanavalin A (Con A), 1.5 × 18 cm]. The column was washed with starting buffer until 66 ml eluant had been collected, at which

time elution was begun with 0.3 M α-methyl-D-mannoside in PNA buffer. Fractions 3–9 (non-retained material) were combined and concentrated. Fractions 16–19 (retained material) were also pooled and concentrated and the concentrated material was dialyzed against PNA buffer.

The non-retained material from the Glycosylex A column was applied to a pepstatin affinity column (1.0 × 22 cm). This column was prepared by coupling pepstatin to AH-Sepharose 4B using the procedure previously described by Kazakova and Orekhovich [4]. Based on amino acid analysis of the wet gel, the amount of pepstatin incorporated into the column was 3.2 μmoles/ml. The column was washed with PNA buffer. Fractions of 6 ml were collected. After 75 ml eluant had been collected, the column was eluted with 1 M NaCl in PNA buffer. This elution step was followed by elution with 50 mm Tris–HCl, pH 9.5. Fractions 3–9 were combined, concentrated and then applied to a calibrated Sephacryl S-200 superfine gel filtration column (1.6 × 89 cm). The column was eluted in an ascending manner with PNA buffer at a flow rate of 0.6 ml/min. Fractions of 2 ml were collected. The fractions eluting between 90 and 112 ml were pooled and concentrated.

Inactive renin molecular weight. The molecular weight of inactive renin was estimated by comparing the elution volume of inactive renin from the calibrated Sephacryl S-200 superfine gel filtration column with the elution volumes of the following molecular weight standards: bovine serum albumin, ovalbumin, pepsinogen, myoglobin and cytochrome *c*. A semilogarithmic plot of elution volume versus molecular weight gave a standard curve from which the molecular weight of inactive renin was calculated.

Chromatographic properties of activated inactive renin. A portion (0.8 ml) of the material obtained from the Sephacryl S-200 gel filtration step was incubated with 0.2 ml pepsin (240 μg/ml) in 0.48 M sodium acetate buffer, pH 4.8. After 30 min at 37° the incubation mixture was treated with 1 ml of 0.5 M Tris–HCl buffer, pH 7.4. The solution was then dialyzed overnight against PNA buffer.

One ml of the dialyzed material was placed on the pepstatin affinity column. The column was washed with PNA buffer until 50 ml eluant was obtained. At this time the column was eluted with 4 M urea in PNA buffer. The fractions (5 ml) collected were dialyzed against PNA overnight and then assayed for renin activity, as described below.

The remaining volume of activated material was applied to the calibrated Sephacryl S-200 superfine gel filtration column. The column was eluted in the same manner as described above for the gel filtration chromatography of inactive renin. Fractions of 2 ml were collected and assayed for renin activity. The fractions eluting between 90 and 112 ml were pooled and concentrated.

The pooled and concentrated fractions from the Sephacryl S-200 column were applied to the Glycosylex A column. The column was washed with PNA buffer until 55 ml eluant had been collected. The column was then eluted with 0.3 M α-methyl-D-mannoside, after which time the fractions were assayed for renin activity.

Activation of inactive renin with pepsin. A modification of the method described by Morris [3] was used in this study. Aliquots (80 μl) of samples containing inactive renin were treated with 20 μl of a solution of pepsin (240 μg/ml) in 0.48 M sodium acetate buffer, pH 4.8. This mixture was incubated for 30 min at 37°. After this time, the reaction was placed in an ice bath and the reaction stopped by the addition of 0.1 ml of 0.5 M Tris–HCl buffer, pH 7.4. Control samples were treated in the same manner except that 20 μl of 0.48 M sodium acetate buffer, pH 4.8, was used instead of the pepsin solution. Mixtures that contained pepsin and no sample gave the same low values as those without pepsin.

Acid activation of inactive renin. Aliquots (0.1 ml) of samples containing inactive renin were treated with 12.5 μl of 3 M glycine hydrochloride, pH 3.3, and then incubated at either 4 or 25° for 20 hr. After this time the acidic mixtures were neutralized with 87.5 μl of 3 M MOPS, pH 7.0. The neutralized mixtures were diluted with 0.2 ml of PNA buffer and then assayed for renin activity. Control samples were treated with 12.5 μl of 3 M glycine, pH 7.0, instead of the 3 M glycine hydrochloride, pH 3.3, solution.

Time course of acid activation. Inactive renin from amniotic fluid obtained at 20 weeks of gestation was partially purified by ammonium sulfate preciptation, as described above. Aliquots (0.1 ml) of this preparation were acid activated using the acid activation method described above. The acid activation was carried out at either 4 or 25° for 1, 2, 4, 8, 16, 24 and 32 hr. Renin activity of these samples was measured and expressed as ng angiotensin I generated/hr/mg of protein.

pH Optimum studies. The pH optimum for renin activity of the non-retained material from the Glycosylex A chromatography step was determined using TDP as the substrate. Thirty μl of either a 3- or 12-fold dilution of the non-retained material was incubated at 37° with 20 μl TDP (10 nmoles/ml in 0.15 M NaCl) and 50 μl of either 0.15 M sodium acetate buffer (pH 4.0, 4.5, 5.0 or 5.5), 10 mM EDTA or 0.15 M sodium phosphate buffer (pH 6.0, 6.5, 7.0, or 7.5), 10 mM EDTA. After 30 min the enzymatic reaction was stopped by placing the incubation mixtures on ice and diluting them with 0.8 ml of 0.1 M Tris–acetate buffer, pH 7.4. Each sample was assayed in duplicate and the angiotensin I produced was assayed by radioimmunoassay. The enzymatic activity was expressed as μg angiotensin I generated/ml/hr.

The pH optimum for angiotensin I generation of the retained material, as well as the pH optimum for angiotensin I generation of both the non-retained and retained material after pepsin activation, was determined in the same manner.

Assay of renin activity. The fractions obtained from the above chromatographic columns were assayed for renin activity before and after pepsin activation of the inactive renin. Fifty μl of an appropriate dilution of each fraction was incubated at 37° with 50 μl of bovine serum substrate in 150 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA and 20 mM dithiotreitol. Bovine serum substrate, containing 2.07 nmoles of angiotensin I equivalents/ml, was prepared according to the method of Lever *et al.* [5]. After 30 min the enzymatic reaction was stopped by placing the incubation mixtures on ice and diluting them with 0.8 ml of 0.1 M Tris–acetate buffer, pH 7.4. The angiotensin I pro-

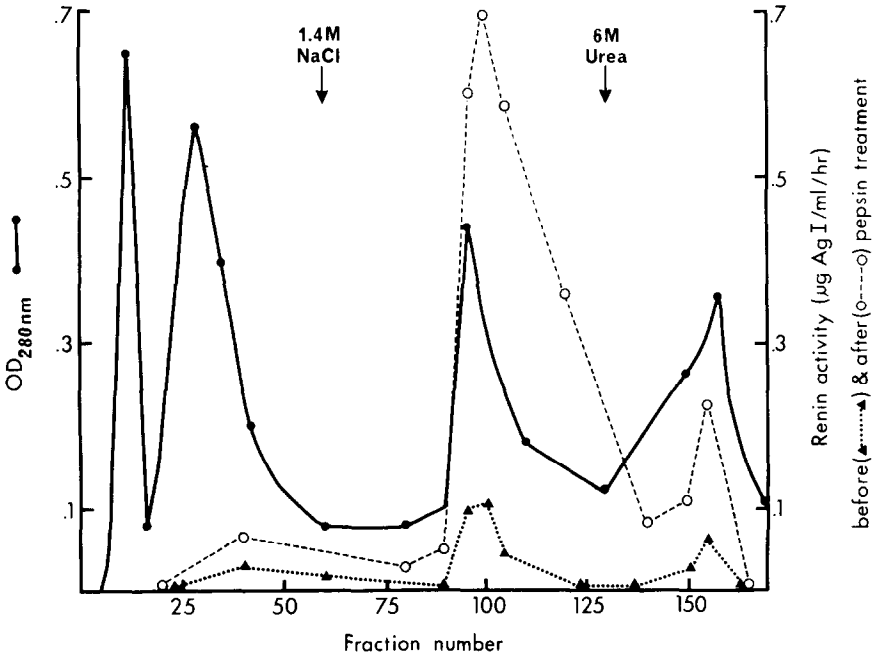

Fig. 1. Affi-Gel Blue column chromatography of the 3 M ammonium sulfate fraction from human amniotic fluid. Elution was with 10 mM sodium phosphate buffer, pH 7.0, 0.15 M NaCl and 5 mM NaN_3. At fraction numbers 60 and 130, 1.4 M NaCl and 6 M urea were added to the eluting buffer respectively.

duced was assayed using the radioimmunoassay described below. Each fraction was assayed in duplicate. The enzymatic activity was expressed as μg angiotensin I generated/ml/hr. Renin activity of the pooled and concentrated fractions was measured before and after either acid or pepsin activation of the inactive renin. Each enzyme solution was assayed in duplicate at two concentrations of enzyme. The amount of inactive renin was calculated by subtracting the renin activity in the sample before pepsin treatment from the renin activity after pepsin treatment. The per cent of inactive renin in the concentrated fractions was determined by the following formula

$$\% \text{ Inactive renin} = \frac{\text{Inactive renin}}{\text{Total renin}} \times 100$$

Specific activities of the concentrated fractions were expressed as μg antiotensin I/mg protein/hr. The protein concentrations of the concentrated fractions were determined using the Bio-Rad protein assay method [6].

Radioimmunoassay of angiotensin I. The method used in this study was analogous to that described by us previously [7] except that the supernatant fractions were counted with a Packard gamma counter instead of by liquid scintillation.

RESULTS

Amniotic fluid was found to have approximately 85 per cent of its renin activity in the form of inactive renin. Ammonium sulfate precipitation of this material resulted in a 5-fold purification. When the 3 M ammonium sulfate fraction was applied to an Affi-Gel Blue column, most of the active and inactive renin activity was found to be retained on the column (Fig. 1). Elution of the inactive renin was achieved with 1.4 M NaCl and resulted in an additional 3-fold purification. A small amount of active and inactive renin was also eluted from the column with 6 M urea.

The application of the 1.4 M NaCl fraction from the Affi-Gel Blue column to the Glycosylex A column resulted in the separation of this material into two fractions (Fig. 2). One fraction consisted of those fractions not retained on the column while the second fraction consisted of those fractions which were eluted with 0.3 M α-methyl-D-mannoside. Both fractions were found to contain active and inactive renin. While approximately 66 per cent of the active renin activity recovered from the column was found in the non-retained fractions, almost 91 per cent of the inactive renin was found here. Although both fractions produced angiotensin I when tetradecapeptide was used as the substrate, their pH optima were different. These results are depicted in Fig. 3. The pH optimum for the non-retained material both before and after pepsin activation was found to be around 6.5 while that of the retained material was 4.5.

Inactive renin was also not retained on the pepstatin affinity column (Fig. 4). A large portion of the active renin originally present was removed by the column since the per cent of inactive renin present rose from 87 to 95 per cent during this chromatography step. The active renin could not be eluted with either 1 M NaCl or with 50 mM Tris–HCl, pH 9.5. Urea (4 M), however, was capable of removing the active renin from the pepstatin column (not shown in Fig. 4). This renin activity was not activated by pepsin treatment.

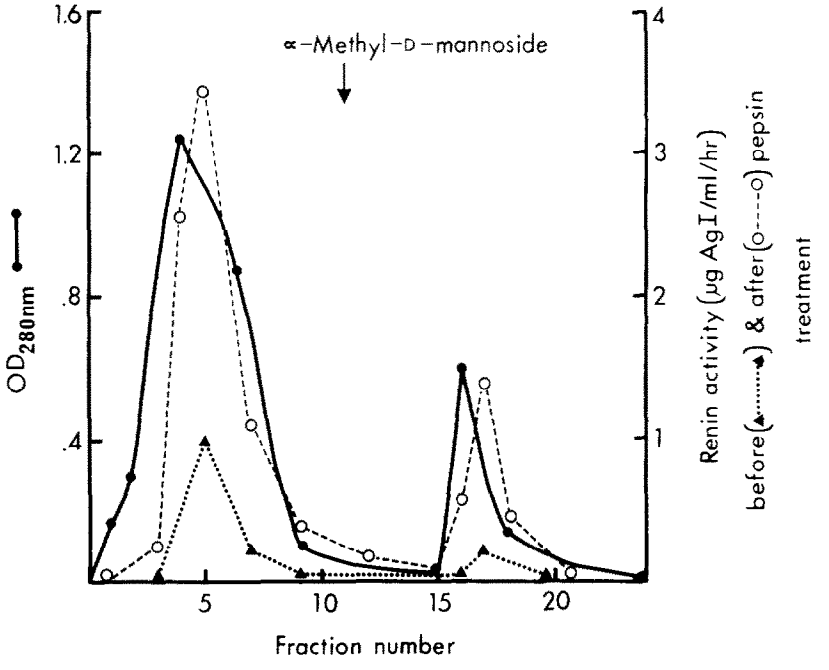

Fig. 2. Glycosylex A column chromatography of the 1.4 M NaCl fraction off the Affi-Gel Blue column. The initial elution was carried out with 10 mM sodium phosphate buffer, pH 7.0, 150 mM NaCl and 5 mM NaN_3. After twelve fractions had been collected, the eluting buffer was changed to 0.3 M α-methyl-D-mannoside.

Sephacryl S-200 gel filtration of the inactive renin which passed through the pepstatin column is depicted in Fig. 5. The inactive renin was eluted as a single peak with an elution volume of 102 ml. The active renin present also eluted at this elution volume. When compared to the elution position of proteins of known molecular weight (Fig. 6), this elution volume was found to correspond to a molecular weight of 39,000. Several additional determinations employing other batches of amniotic fluid have given us this same molecular weight. An additional 3-fold purification was achieved during this step. This material, when reapplied to the pepstatin affinity column, was found not to be retained on the column.

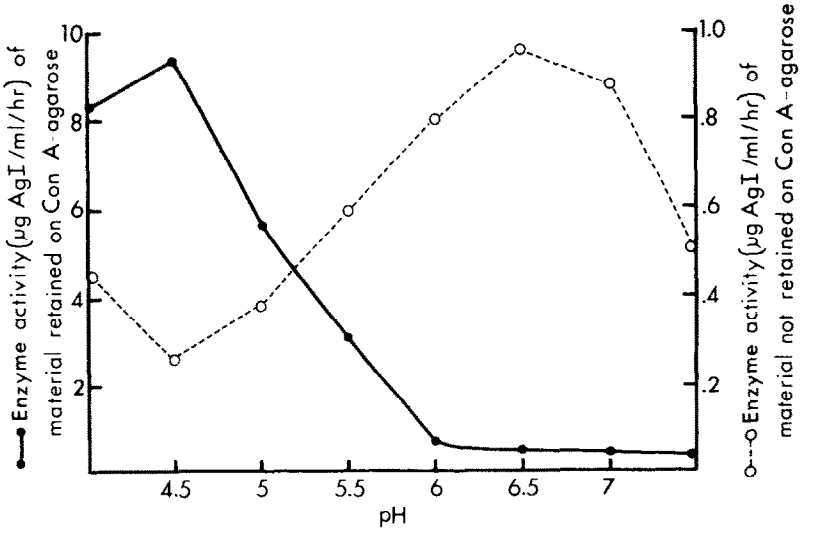

Fig. 3. Effect of pH on the enzymatic activity of the non-retained (O- - -O) and retained (●—●) material from the Glycosylex A chromatography step using tetradecapeptide as the substrate.

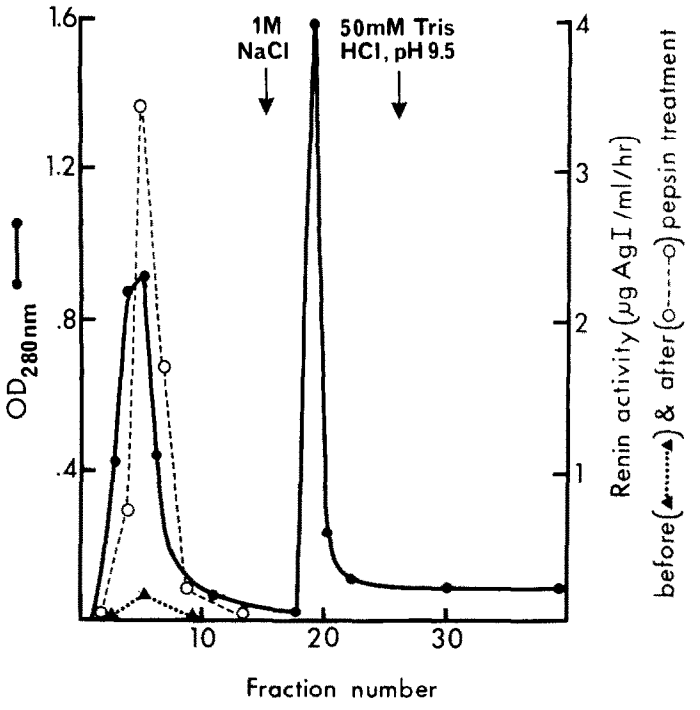

Fig. 4. Pepstatin affinity chromatography of the non-retained material from the Glycosylex A column. The column was equilibrated with 10 mM sodium phosphate buffer, pH 7.0, 150 mM NaCl and 5 mM NaN_3.

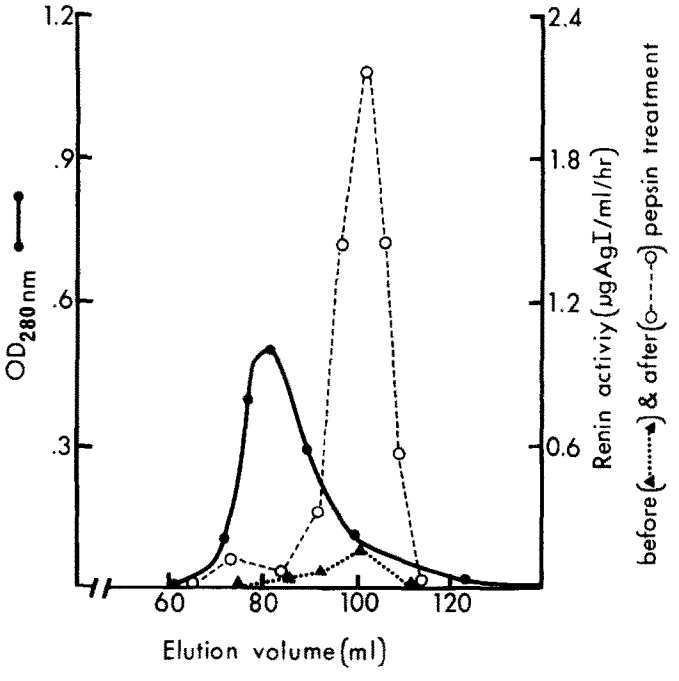

Fig. 5. Sephacryl S-200 gel filtration of the inactive renin from the pepstatin affinity column. Elution was carried out in 10 mM sodium phosphate buffer, pH 7.0, 150 mM NaCl and 5 mM NaN_3.

Table 1. Purification of inactive renin from human amniotic fluid

Fraction	Total protein (mg)	Sp. ac. (μg AgI/mg/hr)	Recovery (%)	Purification	Per cent inactive renin
Amniotic fluid	5035	0.063	100	1.0	85
Ammonium sulfate precipitate	808	0.307	78	4.9	85
Affi-Gel Blue	148	0.995	46	15.9	84
Glycosylex A	82	1.04	27	16.6	87
Pepstatin	39	1.17	14	18.7	95
Sephacryl S-200	9	3.87	11	61.6	91

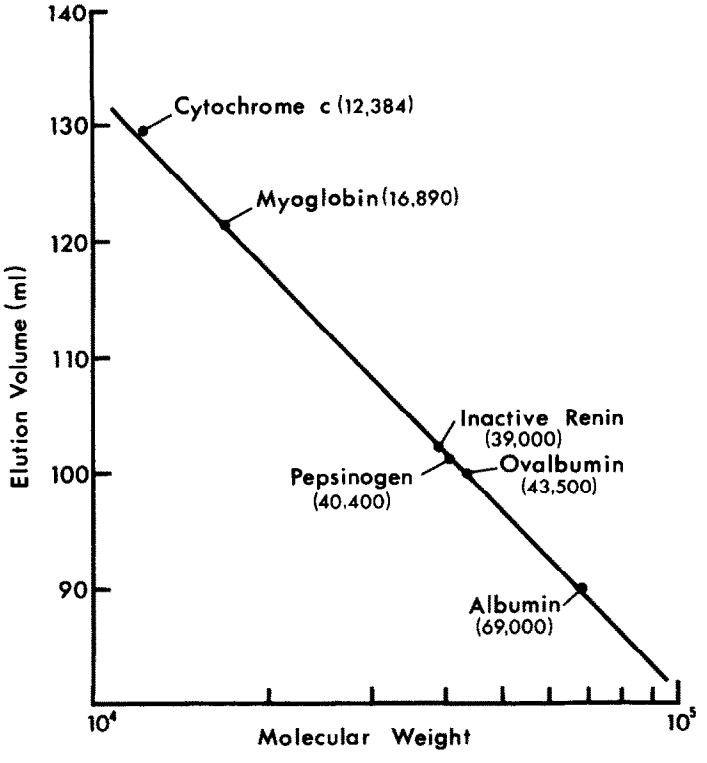

Fig. 6. Molecular weight estimation of inactive renin from human amniotic fluid by chromatography on a Sephacryl S-200 gel filtration column.

Table 2. Comparison of pepsin versus acid activation of inactive renin

Fraction	Renin activity (μg AgI/mg/hr)		Ratio pepsin/acid
	Pepsin activation	Acid activation	
Ammonium sulfate precipitation	0.36	0.19	1.89
Affi-Gel Blue	1.19	0.72	1.65
Glycosylex A	1.20	0.68	1.76
Pepstatin	1.24	0.71	1.75
Sephacryl S-200	4.24	2.04	2.08

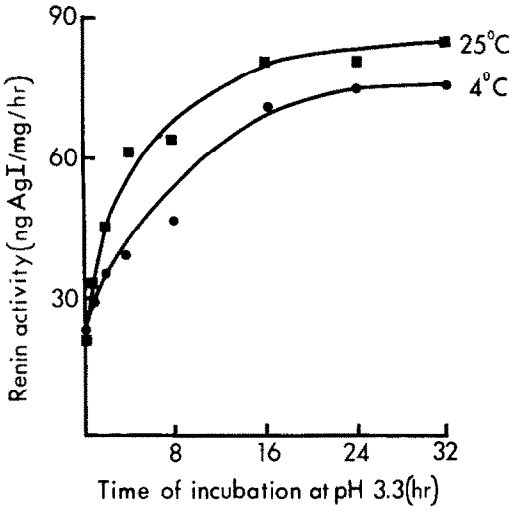

Fig. 7. Time course of acid activation of inactive renin from amniotic fluid. Acid activation was carried out at either 4° (●—●) or 25° (■—■) for 1, 2, 4, 8, 16, 24 and 32 hr.

The results from the purification of inactive renin are summarized in Table 1. The chromatographic procedures employed resulted in a 62-fold increase in specific activity of the inactive renin. The overall recovery was approximately 11 per cent.

The various fractions obtained throughout these chromatographic procedures were also capable of being activated by acid treatment. Our method of acid activation involved the direct addition of 3 M glycine hydrochloride, pH 3.3, to the fractions. The time course of acid activation using this method is shown in Fig. 7. A slightly higher degree of acid activation was obtained at 25° vs 4°. In both cases, however, the rate of activation reached a plateau at around 20 hr. When this same amniotic fluid was activated by acid dialysis for 24 hr at 4°, the renin activity reached a value of 66 ng/mg/hr. This value was similar to that obtained with our direct addition method. A comparison of acid versus pepsin activation as illustrated in Table 2 shows that acid activation does not give the same results as activation with pepsin. In all cases pepsin treatment gave a greater degree of activation than did acid treatment. Interestingly, the ratio of pepsin to acid activation was relatively constant.

The chromatographic properties of the purified inactive renin after it had been activated with pepsin were

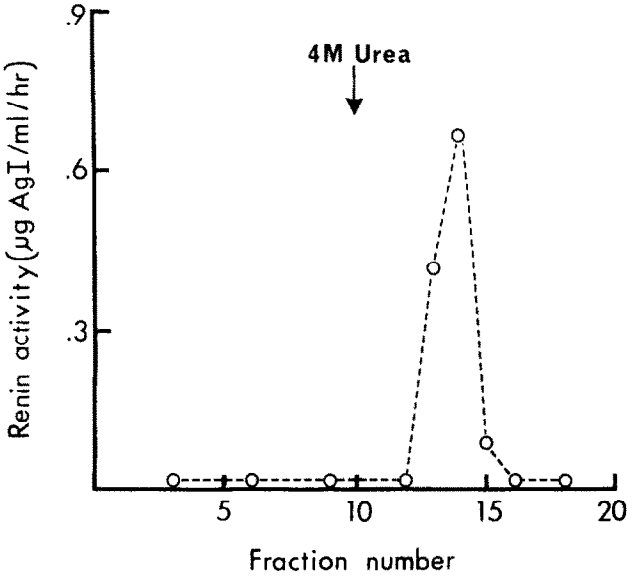

Fig. 8. Pepstatin affinity chromatography of the material from the Sephacryl S-200 gel filtration step after activation with pepsin. Column was equilibrated with 10 mM sodium phosphate buffer, pH 7.0, 150 mM NaCl and 5 mM NaN_3. After ten fractions had been collected, the column was eluted with 4 M urea. Renin activity, O- - -O.

determined on the Sephacryl S-200, pepstatin, and Glycosylex A columns. When the activated material was applied to the pepstatin column, no activity was seen in the wash fractions. Upon elution of the column with 4 M urea, however, a single peak of renin activity was obtained (Fig. 8). Sephacryl S-200 chromatography of the activated material yielded a single peak of renin activity with an elution volume of 102 ml. As indicated above, this elution volume corresponds to a molecular weight of 39,000. When the activated material was applied to the concanavalin A affinity column, 62 per cent of the renin activity was not retained on the column. The remaining 38 per cent was retained and could be eluted with 0.3 M α-methyl-D-mannoside. Similar results were obtained when the inactive renin was activated with acid instead of pepsin.

DISCUSSION

Although the presence of an inactive form of renin in human amniotic fluid was demonstrated several years ago, no attempts have, as yet, been reported on the purification of this material. In the present study we have employed a variety of affinity chromatography columns in an effort not only to purify inactive renin, but also to determine some of the characteristics of this material.

The binding of inactive renin to the Affi-Gel Blue affinity column represents a new and interesting finding. Affi-Gel Blue consists of an agarose matrix to which has been coupled the blue dye Cibacron Blue F3GA. Because of the structural similarity between Cibacron Blue F3GA and the nucleotides, this gel has a high affinity for enzymes that possess nucleotide-binding sites [8,9]. This property has thus made Affi-Gel Blue very useful in the purification of nucleotide-requiring enzymes. It has also been found to be useful in removing albumin from serum [10]. It was because of this latter property that we originally employed this affinity column since we saw this as a means to remove albumin, the major protein of amniotic fluid [11]. To our surprise, however, our experiments showed that most of the inactive renin was also bound to the column. This could indicate that inactive renin might also have a nucleotide-binding site. We are currently conducting experiments to determine if this is indeed the case and if so what significance such a site might have. It should be mentioned that in the present purification scheme most of the albumin is removed in the ammonium sulfate step.

The behavior of inactive renin on the concanavalin A affinity column suggests that it is not a glycoprotein or that, if it is, the residues are either not recognized by concanavalin A or not exposed while renin is in the inactive form. It appears that the latter explanation may in part be true since after the material is activated with pepsin, a portion of it, when reapplied to the Con A column, is now retained by the column. Most of it, however, is not bound by the concanavalin A affinity column. Inactive renin thus differs from human kidney renin since the latter is retained by a concanavalin A affinity column [12]. The behavior of the activated material suggests that the renin present in amniotic fluid is heterogeneous. Despite the fact that concanavalin A chromatography did not result in any significant purification, it did serve a useful purpose in that it removed from the preparation material which was capable of generating angiotensin I but which had a pH optimum of 4.5 against TDP. In contrast, the renin activity which was not retained by the Con A column possessed a pH optimum of 6.5. The material retained by the Con A column may consist in part of cathepsin D. Human cathepsin D is known to be a glycoprotein [13] and we have previously shown that cathepsin D from bovine spleen is bound by a concanavalin A affinity column [10]. In addition, the pH optima obtained in this study are very similar to those observed by Hackenthal *et al.* [14] for cathepsin D and kidney renin respectively.

In the present study inactive renin was found not to be bound to a pepstatin affinity column. The active renin that was present in the preparation before the pepstatin chromatography was, however, removed during this chromatographic step. This was expected since renin is known to bind to a pepstatin affinity column [15]. Although a small amount of active renin was observed in the wash fractions from the pepstatin column, the active renin present in these samples was probably due to the conversion of some of the inactive renin after the fractions had been collected. Since it is known that the binding of renin to pepstatin involves the active site of the enzyme [16], it appears that in inactive renin the active site is blocked or altered such that pepstatin is no longer capable of interacting with the enzyme. The activation of inactive renin with acid or pepsin treatment, however, must somehow alter the molecule so that the active site becomes exposed, because the activated material is completely retained on the pepstatin affinity column. If the active site of renin is indeed blocked in inactive renin, then it seems unlikely that inactive renin would be able to interact with the much larger substrate molecule. This thus suggests that inactive renin has no inherent enzymatic activity but is truly an inactive form of renin which only shows enzymatic activity after the appropriate activation has occurred.

A previous report [17] indicated that the molecular weight of inactive renin from amniotic fluid was around 63,000. A more recent study [18], however, has reported a molecular weight of 44,000 both before and after acid treatment. Our results are in agreement with this latter study since we have found that the molecular weight of inactive renin both before and after activation with either pepsin or acid is 39,000. The fact that the molecular weight of inactive renin does not change upon activation is somewhat surprising. One expects that, upon converting inactive renin to active renin with pepsin, some peptide fragment might be lost. It may be, however, that this fragment is so small that the molecular weight difference between inactive and active cannot be detected by gel filtration. It should be noted that similar discrepancies in the molecular weight of inactive renin from human plasma have also been reported. Whereas Day *et al.* [17,19] report a molecular weight of 63,000 for inactive renin in human plasma, Boyd [20] reports a molecular weight of 43,000 for inactive renin and a molecular weight of 41,000 after inactive renin has been treated with acid.

The mechanism by which inactive renin is activated remains unknown. *In vitro* studies with inactive renin from amniotic fluid have demonstrated that, besides being activated by acid treatment, inactive renin can be

activated by such proteolytic enzymes as pepsin, trypsin, and cathepsin D [2,3]. In the present study we observed that at every step of the purification of inactive renin, acid activation still took place. The extent of acid activation, however, was always less than observed with pepsin treatment. Moreover, the ratio of pepsin to acid activation remained relatively constant during the course of purification. This finding suggests that the activation that occurs with acid treatment is different somehow from that which occurs with pepsin treatment. If, as has been proposed, acid treatment allows a proteolytic enzyme to act on inactive renin, then this enzyme must have characteristics that are very similar to inactive renin in order for it to still be present after several chromatographic steps. Morris [3] has suggested that cathepsin D may be responsible for the activation of inactive renin *in vivo*. Although cathepsin D is present in amniotic fluid, our results argue against the idea that cathepsin D is responsible for the activation after acid treatment *in vitro* since this enzyme was probably removed from the preparation by the concanavalin A chromatography step. Although there could still be another enzyme which is responsible for the activation that occurs after acid treatment, we feel that our results are also consistent with the idea that inactive renin may be capable of activating itself much the same way as pepsinogen does [21,22]. Whether or not this mechanism actually takes place remains to be shown, however.

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