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Kidney Antihemophilic Factor. Partial Purification and Some Properties*

Emily M. Barrow and John B. Graham

ABSTRACT: Antihemophilic factor activity has been isolated from leucine aminopeptidase preparations of porcine, human, and canine kidneys. The antihemophilic factor activity can be separated from the leucine aminopeptidase activity by Sephadex G-200 gel filtration. Removal of contaminating proteins by this single step has resulted in a large increase in antihemophilic factor activity, the specific activity increasing more than 800 times. No other clotting factors have been detected in the purified preparations of kidney antihemophilic factor. $MgCl_2$ is required for preservation of the antihemophilic factor activity during purification, and maximum activation of antihemophilic factor before assay requires incubation at 40° in the presence of $MnCl_2$. The molecular weight of kidney antihemophilic factor, estimated by gel filtration methods, is consistent with that of proteins with molecular weights between 25,000 and 28,000. This is in contrast to plasma antihemophilic factor which is excluded from G-200 with the proteins of molecular weights exceeding 200,000. Kidney antihemophilic factor is greatly retarded by DEAE-Sephadex, is eluted under starting conditions from CM-Sephadex, and moves as a prealbumin on polyacrylamide and agar gels. A specific neutralizing antibody against kidney antihemophilic factor has been prepared by injecting the purified kidney antihemophilic factor into rabbits. The antibody which neutralizes kidney antihemophilic factor activity does not influence the hydrolysis of L-leucinamide by crude kidney preparations.

The differences in molecular weight of plasma antihemophilic factor and kidney antihemophilic factor suggest that kidney antihemophilic factor may be the monomeric form or one of the subunits of plasma antihemophilic factor.

We reported earlier that the antihemophilic factor activity of normal plasma could be protected from proteolytic destruction by trypsin and thrombin if the plasma were pretreated with $MnCl_2$, and that this antihemophilic factor was not different from the antihemophilic factor of untreated plasma by several criteria (Barrow *et al.*, 1966a,b). The unsuspected relationship between Mn^{2+} and antihemophilic factor suggested that antihemophilic factor might be a Mn^{2+}-dependent enzyme which would be expected to be present in mammalian tissues and susceptible of purification. In searching for a tissue enzyme which requires Mn^{2+} for stabilization or activation and which might be identical with antihemophilic factor, we have examined brain, kidney, and red cells of man, dog, and hog.

Antihemophilic factor activity has been found in leucine aminopeptidase preparations of kidney of all three species. Most of our experiments have been performed

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on hog kidney because of availability in quantity. Many of the experiments have been performed on dog and human kidneys also. This report describes the separation of the antihemophilic factor activity from the leucine aminopeptidase activity and some of the characteristics of the kidney antihemophilic factor after further purification.

Materials and Methods

Preparation of Enzymes. Prolinase was prepared from hog kidney and prolidase from dog kidneys and dog and human erythrocytes according to Smith (1955). Glutamine synthetase was made from human brain by the method of Elliott (1955). Leucine aminopeptidase was prepared from hog, dog, and human kidneys exactly by the method of Hill *et al.* (1958) through the acetone precipitation step. This material is referred to as "crude leucine aminopeptidase" and was the starting material for further purification by gel filtration, ion-exchange chromatography, or electrophoresis.

Measurement of Peptidase Activities. The alcoholic titration method of Grassman and Heyde (1929) and the colorimetric ninhydrin method of Moore and Stein (1954) were used for the estimation of α-NH_2 groups liberated from the substrates glycyl-L-proline and L-leucinamide hydrochloride after incubation of the peptides and the enzyme preparations with $MnCl_2$ at 40°. Glycl-L-proline was obtained from Mann Research Laboratories, and L-leucinamide hydrochloride was purchased from Sigma Chemical Co.

Estimation of Clotting Factor Activities. Specific assays for clotting factors V, VIII, IX, X, and XII were performed using the partial thromboplastin time technic of Langdell *et al.* (1953). Each deficient human plasma substrate (except the XII deficient) was activated with 5 mg of kaolin/ml of plasma for 10 min at 28°, then stored at 0° during the assay period without removal of the kaolin. Factor VII assays were performed using a factor VII deficient plasma, thromboplastin, and $CaCl_2$. For detection of maximum antihemophilic factor activity in kidney preparations 0.1 ml of a 0.02 M solution of $MnCl_2$ was added to 0.9 ml of test material. The mixture was incubated for 15 min at 40°, then diluted, and assayed.

Purification by Gel Filtration. Crude leucine aminopeptidase preparations were purified by gel filtration using Sephadex G-200. The protein content of the effluent was monitored with an LKB Uvicord absorptiometer and recorder at 254 mμ. Protein concentration was estimated by reading the fractions at 280 mμ in a Beckman DU spectrophotometer.

Estimation of Molecular Weight. Nonenzymatic protein markers of known molecular size were applied to columns of Sephadex G-200, G-100, and G-75. The markers used were crystalline bovine serum albumin, twice-crystallized ovalbumin, six-times-crystallized chymotrypsinogen, crystalline sperm whale myoglobin, and horse heart cytochrome *c;* all were obtained from Mann Research Laboratories. Samples (2 ml), each containing 5 mg/ml of protein, were added separately to the columns and the volume at which peak absorption at 254 mμ occurred was recorded for each protein marker. Crude hog kidney LAP (2 ml) containing protein (5 mg/ml) was then applied to the columns and the elution volume of the maximum antihemophilic factor activity from each column was also recorded. The peak elution volumes of the protein markers were found to have a linear relationship when they were plotted on an arithmetic scale against the molecular weights on a logarithmic scale (Andrews, 1965).

Ion-Exchange Chromatography. The anion exchanger DEAE-Sephadex A-50 was used. The dry gel was swollen in 1.0 M Tris-HCl buffer (pH 8.0) containing 5 mM $MgCl_2$ and packed into a column. A rectangular Buchler Instruments Varigrad gradient device was used to develop a KCl gradient after introduction of the sample to the column, and a Sigmamotor peristaltic pump maintained a constant flow through the column. The first five chambers of the Varigrad contained 100 ml of 0.2 M Tris-HCl buffer (pH 8.0) containing 5 mM $MgCl_2$. Chamber 6 contained 100 ml of 3.0 M KCl dissolved in the same buffer. Fractions were dialyzed against 5 mM Tris-Mg^{2+} buffer (pH 8.0) for 24 hr before testing. The cation exchanger CM-Sephadex was used in the same manner, except that the buffer used was 50 mM Tris-HCl–5 mM $MgCl_2$ (pH 8.0). It was not necessary to use a salt gradient for elution of antihemophilic factor from CM-Sephadex, because the antihemophilic factor activity emerged from the column in the early samples.

Electrophoresis. Separation of kidney leucine aminopeptidase or plasma proteins on polyacrylamide gel was accomplished in a vertical preparative electrophoresis cell (EC Apparatus). Leucine aminopeptidase (1 ml) containing 20–30 mg of protein was applied to the gel. Electrophoresis was carried out using a current of 50 mA for 2–3 hr. The buffer used was 50 mM Tris-HCl containing 5 mM $MgCl_2$ (pH 8.6). The proteins were eluted from the unstained gel slab in an elution–convection (E–C) cell for 6–8 hr, using the same buffer. The current applied was 50–80 mA.

Analytical disc electrophoresis on polyacrylamide gel was performed by the method of Davis (1964). Human plasma, crude leucine aminopeptidase, antihemophilic factor containing fractions after Sephadex filtration, and preparative polyacrylamide gel electrophoresis samples were analyzed for purity by this method.

Preparation of an Antibody against Purified Hog Kidney Antihemophilic Factor. Crude hog kidney leucine aminopeptidase (5 ml) was dissolved in a 0.3% solution of Seravac Agarose gel (Gallard-Schlesinger Corp.) and poured into the cathodal end of an LKB preparative electrophoresis column which contained solidified 0.3% Agarose gel. The buffer used in the gel was sodium barbital (0.5 M, pH 7.6), containing 0.1 M glycine and 6% sucrose. The electrode vessels contained the same buffer without the sucrose. The electrophoresis was carried out for 17 hr at 4° with a current of 25–30 mA. After the electrophoretic run, the gel cylinder was gently extruded from the column and cut into 1-cm slices and the individual slices were placed into tubes and frozen at −20°. Each tube was assayed for antihemophilic factor after the gel–protein mixtures were thawed. The area containing the fastest migrating proteins had the antihemophilic factor activity. The "fast" component (10 ml) was pooled and

TABLE I: The Effect of Kaolin on Antihemophilic Factor Activity in Four Separate Kidney Leucine Aminopeptidase Preparations.

Preparation	Untreated Kidney Antihemophilic Factor		Kaolin-Treated Kidney Antihemophilic Factor[a]	
	Kaolin-Activated Substrate A	Nonactivated Substrate B	Kaolin-Activated Substrate C	Nonactivated Substrate D
1. Crude leucine aminopeptidase	100	10	25	<5
2. Crude leucine aminopeptidase	63	<5		
3. Hog kidney leucine aminopeptidase after G-200	20	<5	<5	<5
4. Human kidney leucine aminopeptidase after G-200	98	<5		

[a] Antihemophilic factor activity, units per milliliter. One unit is $^1/_{100}$ of the antihemophilic factor activity in a pooled sample of normal human plasma.

coupled overnight with ethylenemaleic anhydride (Levin and Katchalski, 1967). The coupled protein–polymer (20 ml) was mixed with 10 ml of Freund's complete adjuvant and injected subcutaneously into eight paravertebral areas of each of two rabbits. In 2 weeks the rabbits were bled and the serum was tested for precipitating antibody.

Purification of Rabbit Antiserum. The rabbit antiserum was freed of nonspecific clotting factors by ion-exchange chromatography on DEAE-Sephadex. Rabbit antiserum (15 ml), dialyzed against 50 mM Tris-HCl buffer (pH 7.5), was applied to a 20 × 2.5 cm column of the anion exchanger and eluted with 50 mM Tris buffer, the immunoglobulins emerging from the column in the first protein peak. Each 3-ml fraction was tested for specificity by double diffusion on Agarose-coated microscope slides using goat antirabbit γ-globulin antiserum. Those fractions which showed precipitin lines were pooled and frozen at −20° in small aliquots for use in antihemophilic factor neutralization experiments.

Neutralization of Antihemophilic Factor in Kidney Leucine Aminopeptidase by Rabbit Antikidney Antihemophilic Factor. Mixtures were prepared of equal parts of crude kidney leucine aminopeptidase and rabbit immunoglobulin. Antihemophilic factor assays were performed immediately after mixing, and after incubation for 30 min at 37°. Controls in the antihemophilic factor assays consisted of mixtures of crude leucine aminopeptidase with buffer, and rabbit immunoglobulin with either human plasma or buffer incubated 30 min at 37°. Mixtures were assayed for residual antihemophilic factor activity by the assay procedure described earlier.

Immunoelectrophoresis of Rabbit Antihemophilic Factor Antibody and Kidney Antihemophilic Factor Antigens. This procedure was performed with LKB equipment utilizing a modified procedure of Grabar and Williams (1953). Electrophoresis of crude kidney fractions was performed for 1 hr on agar-coated microscope slides. Troughs were then cut in the slides and rabbit antiserum was added. After overnight diffusion in a humid atmosphere, the slides were examined for formation of visible precipitin lines.

Results

Antihemophilic Factor Activity in Enzyme Preparations. The testing of kidney enzyme preparations for antihemophilic factor activity presents problems that do not exist when plasma or plasma fractions are assayed. $MgCl_2$ (5 mM) must be present during the final stages of purification and $MnCl_2$ (2 mM) is required for maximal antihemophilic factor activation before assay. Preparations of crude kidney leucine aminopeptidase filtered through Sephadex G-200 had no antihemophilic factor *or* leucine aminopeptidase activity if the $MgCl_2$ were omitted from the Tris buffer. Dialysis of crude leucine aminopeptidase preparations against Tris buffer *without* $MgCl_2$ for 48 hr also resulted in loss of both activities. Furthermore, kidney antihemophilic factor cannot be measured in an assay which is based on shortening of the clotting time of hemophilic plasma unless this substrate plasma has been activated with kaolin and the kaolin is allowed to remain in the activation mixture.

Several types of enzymes were tested for antihemophilic factor activity once these conditions had been defined. Glutamine synthetase preparations from brain were unsatisfactory because of the large quantities of "thromboplastic" activity. Prolinase (iminodipeptidase) preparations had no measurable antihemophilic factor activity. Prolidase (imidodipeptidase) preparations from kidney were slightly active but erythrocyte prolidase lacked antihemophilic factor activity. Leucine amino-

TABLE II: Coagulation Factor Assays of Seven Crude Hog Kidney Leucine Aminopeptidase Preparations.

Prepn	Clotting Factor Act. (U/ml)[a]						Protein (mg/ml)	Antihemophilic[b] Sp Act.
	V	VII	VIII	IX[c]	X	XII		
1	<5	<5	60	9	<5	<5	64	0.93
2	<5	<5	110	15	<5	<5	15	7.30
3			135				32	4.21
4	<5	<5	105	<5	<5	<5	43	2.44
5	<5		22	<5	<5		14	1.57
6			50				53	0.94
7			155				22	5.22

[a] One unit is $^1/_{100}$ of the activity in a pooled sample of normal human plasma. [b] Specific activity = (units of antihemophilic factor/ml)/(mg of protein/ml). [c] Factor IX activity not detectable in antihemophilic factor peak after Sephadex G-200 filtration.

TABLE III: A Comparison of the Efficiency of Three Methods for Purification of Kidney Antihemophilic Factor.

Procedure	Vol (ml)	Antihemophilic Factor (U/ml)	Total Units	Protein[a] (mg/ml)	Sp Act. (U/mg)	Yield (%)	Leucine Aminopeptidase, % Hydrolysis
1. Crude leucine aminopeptidase[b]	30	60	1800	64.00	0.93	100	100
2. Sephadex G-200	15	160	2400	0.19	842	132	0
3. DEAE-Sephadex	20	55	1100	0.32	109	61	90
4. Polyacrylamide gel electrophoresis	10	47	470	0.42	112	26	75

[a] Protein quantitated by the method of Lowry *et al.* (1951). [b] Starting material for methods 2–4.

peptidase preparations from human, hog, and dog kidneys proved to be rich in antihemophilic factor activity when tested in specific antihemophilic factor assays using kaolin-activated human hemophilic plasma as substrate.

Columns A and B of Table I show the antihemophilic factor activities of four LAP preparations and demonstrate the necessity of adding kaolin to the substrate. The activator effect of kaolin appears to be expressed through the substrate rather than through the enzyme preparations; prior incubation of the enzyme preparations with kaolin apparently diminishes the antihemophilic factor activity (columns C and D).

Table II displays the clotting profiles of seven preparations of crude hog kidney leucine aminopeptidase. Antihemophilic factor activities ranged from 22 to 135 units per ml and sometimes there were traces of PTC (factor IX). Factors V, VII, X, and XII activities could not be detected by the assay methods. In no instance was thromboplastic or fibrinolytic activity found and purified fibrinogen was not clotted after 24-hr incubation with the enzyme preparations. Canine and human preparations gave similar results.

Separation of Antihemophilic Factor from Leucine Aminopeptidase Activity. Filtration of crude leucine aminopeptidase preparations of hog and human kidneys through Sephadex G-200 resulted in complete separation of leucine aminopeptidase and antihemophilic factor activities. Figure 1 shows that the leucine aminopeptidase activity emerges with the first and second protein peaks while the antihemophilic factor peak occurs after all the leucine aminopeptidase activity has emerged from the column, and is in the area with the lowest protein concentration.

Purification of Antihemophilic Factor from Leucine Aminopeptidase Preparations. In the procedure for preparation of kidney leucine aminopeptidase, many contaminating proteins are removed. However, the crude leucine aminopeptidase still contains several proteins which have properties other than antihemophilic factor and leucinamide-hydrolyzing activities. In order to further purify the antihemophilic factor fraction of this heterogeneous preparation, we utilized techniques of gel filtration, ion-exchange chromatography, and electrophoresis.

Table III gives the pertinent data for estimating spe-

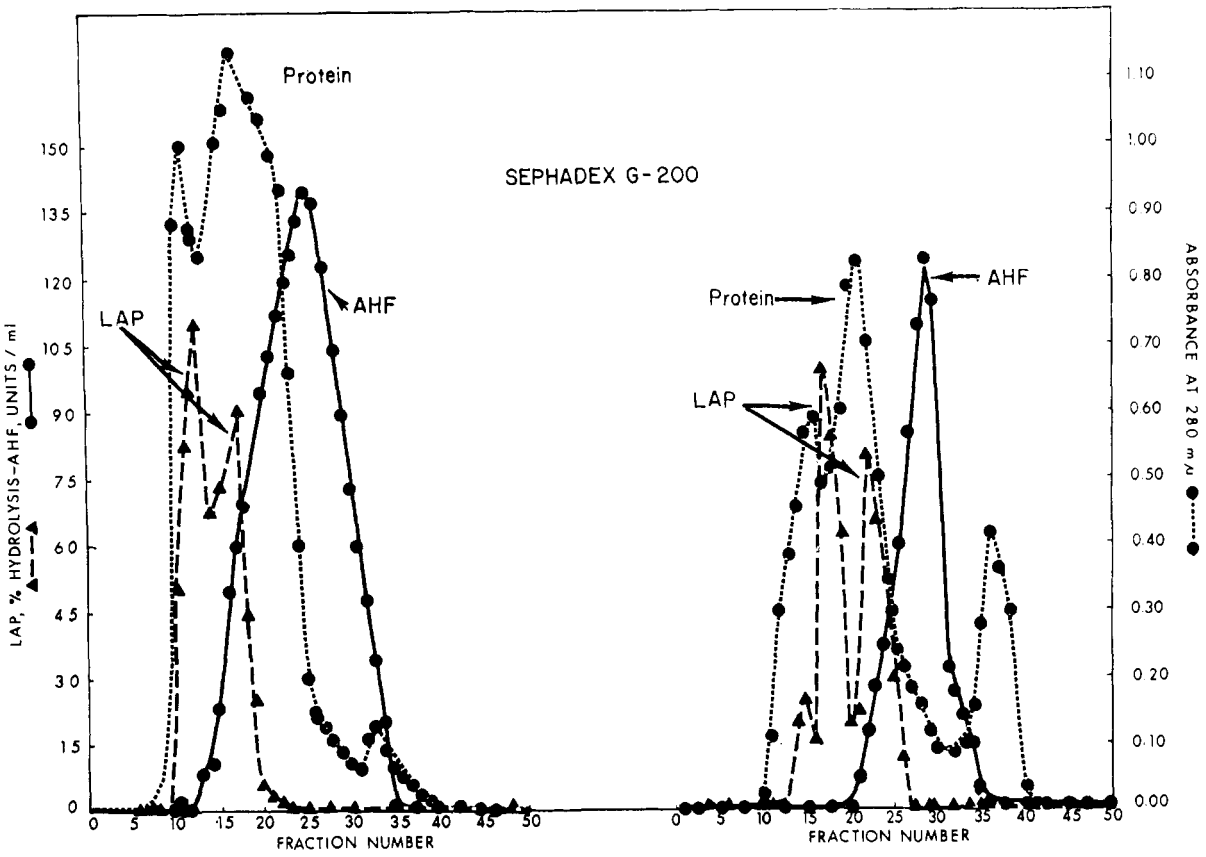

FIGURE 1: Sephadex G-200 filtration of crude leucine aminopeptidase preparations made from hog kidneys, on the left, and human kidneys, on the right. Each sample (5 ml) was added separately to a 2.5 × 40 cm column containing 200 ml of swollen gel. The elution buffer was 5 mM Tris-HCl, containing 5 mM $MgCl_2$ (pH 8.0). Fractions were collected in 5.0-ml aliquots. The flow rate was approximately 30 ml/hr. Leucine aminopeptidase activity is expressed as per cent hydrolysis of L-leucinamide hydrochloride estimated by the Grassman and Heyde (1929) alcoholic titration method. Antihemophilic factor activity is measured as described in the text. Protein concentration is indicated as absorbance at 280 mμ.

cific activities and yield of antihemophilic factor in a hog kidney leucine aminopeptidase preparation. It is obvious that Sephadex G-200 filtration gave the best results. The other two procedures resulted in much lower yields and they did not separate leucine aminopeptidase from antihemophilic factor activities.

Estimation of the Molecular Weight of Antihemophilic Factor. The antihemophilic factor from crude hog kidney leucine aminopeptidase preparations emerged from a carefully calibrated G-200 column as a protein of mol wt 28,500 (Figure 2). Sephadex G-100 and G-75 filtration of the same preparation gave molecular weights of 26,000 and 25,000, respectively. Antihemophilic factor of plasma emerges from Sephadex G-200 columns with proteins of >200,000 molecular weight (Lewis, 1964a), a value consistent with previous ultracentrifugal data (Wagner and Thelin, 1957). The molecular weight of leucine aminopeptidase is reported to be 300,000 (Spackman *et al.*, 1955) but this may represent aggregation of a smaller molecule. In our hands leucine aminopeptidase activity of kidney preparations emerges from Sephadex G-200 early, but in two protein peaks, suggesting that it may exist in more than one molecular form (Figure 1).

Elution of Antihemophilic Factor from Ion-Exchange Media. Early experiments with DEAE-Sephadex A-50 using a KCl gradient with a limit concentration of 1.0 M, or even 2.0 M, failed to elute antihemophilic factor. Leucine aminopeptidase activity was, however, found in the only major protein peak eluted under these conditions. Figure 3 shows the protein distribution and location of antihemophilic factor activity of a crude hog kidney leucine aminopeptidase preparation after ion-exchange chromatography on DEAE-Sephadex A-50 using a KCl gradient with 3 M limit concentration. The antihemophilic factor activity was eluted from the column in the last protein peak. Leucine aminopeptidase activity did not separate from antihemophilic factor under these conditions.

The cation exchanger CM-Sephadex was also used in an attempt to purify the kidney antihemophilic factor of crude leucine aminopeptidase preparations. The antihemophilic factor and leucine aminopeptidase activities were eluted with the starting buffer in the first protein peak. The behavior of kidney antihemophilic factor on anion and cation exchangers is consistent with the idea that it is an acidic protein.

Electrophoretic Separation of Antihemophilic Factor. Preparative vertical electrophoresis of crude leucine aminopeptidase on polyacrylamide gel followed by elution of the proteins and assay for antihemophilic factor showed *kidney antihemophilic factor* activity to be

TABLE IV: Neutralization of Antihemophilic Factor by Rabbit Antikidney Antihemophilic Factor Antiserum in Crude Leucine Aminopeptidase Preparations.

Incubation Time (min):	0	30	0	30
Test and Control Mixtures	Antihemophilic Factor Activity[a]		Leucine Aminopeptidase Activity[b]	
Crude leucine aminopeptidase + buffer	100	100	100	100
Crude leucine aminopeptidase + rabbit antibody	100	45	100	100
Human plasma + rabbit antibody	100	100	0	0
Buffer + rabbit antibody	0	0	0	0

[a] The concentration of the antihemophilic factor activity of the test material is defined as 100%. [b] Per cent hydrolysis of L-leucinamide hydrochloride as determined by Moore and Stein's (1954) ninhydrin method.

in the fastest migrating protein area, the prealbumin zone. *Plasma antihemophilic factor* activity was found only in the α-globulin area and the prealbumin band was void of antihemophilic factor activity.

The results of analytical disc electrophoresis on polyacrylamide gel are shown in Figure 4. Both human plasma and crude kidney leucine aminopeptidase preparations can be seen to be heterogeneous. Kidney leucine aminopeptidase consists of at least seven protein bands and shares several areas with human plasma. Gel filtration reduces the number of protein bands to two as shown in tubes C and D. Each shows a distinct prealbumin and a weakly staining albumin band. Pattern E is the reelectrophoresis of the antihemophilic factor active fraction obtained by preparative vertical gel electrophoresis and elution. The single protein-staining band in the prealbumin area suggests that we achieved high purification of kidney antihemophilic factor.

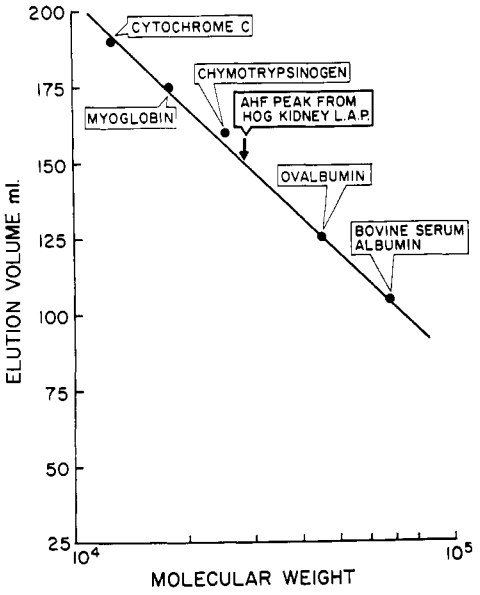

FIGURE 2: Calibration of a G-200 Sephadex column (2.5 × 40 cm) for estimation of molecular weight of the antihemophilic factor obtained from hog kidney leucine aminopeptidase preparation. Details are given in the text.

Behavior of the Rabbit Antibody against Kidney Antihemophilic factor. Figure 5 shows the results of immunoelectrophoresis of crude kidney antigens against the rabbit antiserum prepared as described. Only a single precipitin line forms when rabbit antikidney antihemophilic factor reacts with a water-soluble extract of hog kidney. Against a crude leucine aminopeptidase preparation, there are either two lines or a very elongated single precipitin line. The results indicate that the antigen used to immunize the rabbits had been highly purified and that the antibody is highly specific against a mixture of antigens.

Table IV demonstrates that the rabbit antibody against the fast electrophoretic component is a neutralizing antibody specifically directed against kidney antihemophilic factor. It reduced the antihemophilic factor activity of a hog kidney extract without affecting its leucine aminopeptidase activity. It had no effect on neutralization of nondissociated human plasma antihemophilic factor.

The rabbit antihog kidney antihemophilic factor antibody was tested by immunoelectrophoresis for formation of precipitin lines against plasma from humans who were normal, humans with hemophilia A or von Willebrand's disease, normal and hemophilic dogs, and

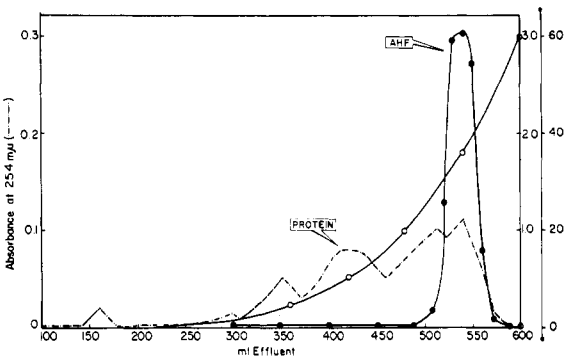

FIGURE 3: DEAE-Sephadex A-50 elution profile of crude hog kidney leucine aminopeptidase. Sample (15 ml) was added to a 2.5 × 20 cm column. Details of elution are described in the text. (●—●) Units per milliliter of antihemophilic factor; (○—○) molarity of KCl.

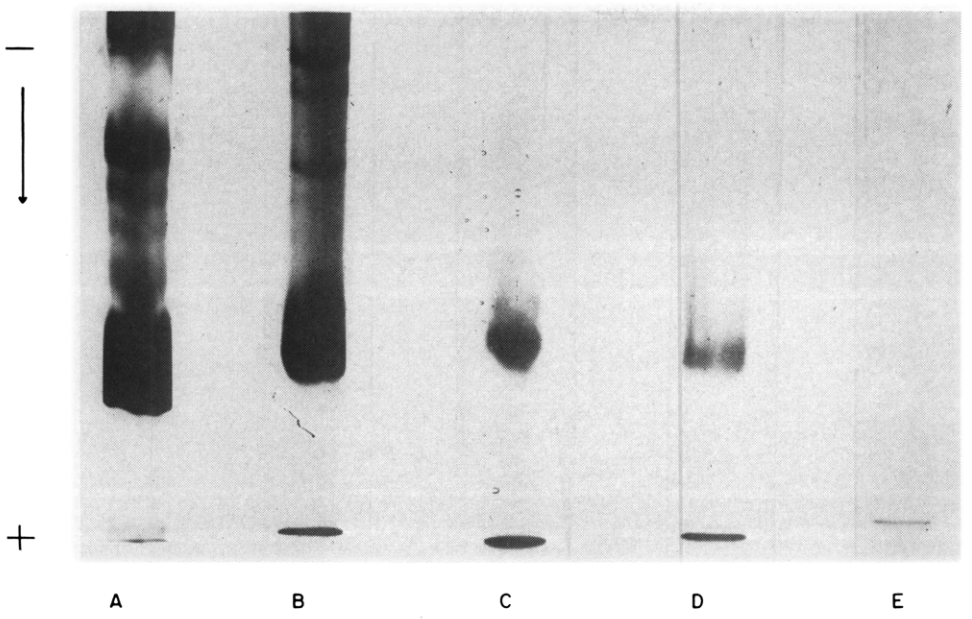

FIGURE 4: Analytical disc electrophoresis in polyacrylamide gel. (A) Normal human plasma; (B) crude hog kidney leucine aminopeptidase; (C) antihemophilic factor active peak after G-200 filtration of crude leucine aminopeptidase; (D) antihemophilic factor active peak after G-75 filtration of crude leucine aminopeptidase; and (E) antihemophilic factor active fraction after preparative polyacrylamide gel electrophoresis of crude leucine aminopeptidase. Note the prominent prealbumin area in each sample.

normal hogs. Precipitation was observed only against normal hog plasma, and the pattern was identical with the precipitin line(s) formed against normal crude leucine aminopeptidase as seen in the top of Figure 5.

Discussion

The "cascade" or "waterfall" theory of blood coagulation (Macfarlane, 1964) postulates that antihemophilic factor after activation is an enzyme which acts on the substrate factor X (Stuart factor). Since we had demonstrated in earlier studies that Mn^{2+} had an unusual relationship to plasma antihemophilic factor, it occurred to us that antihemophilic factor might be a Mn^{2+}-dependent enzyme. A search was initiated for its tissue source because all plasma proteins are synthesized in the cells of one or more organs. Several different types of crude enzymes were prepared from kidney and examined for antihemophilic factor activity since a number of Mn^{2+}-dependent enzymes had been isolated from this organ. Leucine aminopeptidase preparations were richest in antihemophilic factor activity, and we have demonstrated kidney antihemophilic factor activity in leucine aminopeptidase preparations from the kidneys of hog, dog, and man.

Our first attempts to separate antihemophilic factor and leucine aminopeptidase were unsuccessful and it appeared for a time that antihemophilic factor might be leucine aminopeptidase. Both the leucine aminopeptidase and the antihemoliphilic factor obtained from hog kidneys are acidic proteins, migrating in an electric field toward the anode at pH 8.6. Both seem to require Mg^{2+} for stabilization and Mn^{2+} for maximum activation. In carefully controlled Sephadex G-200 filtration, however, the activities emerged at different rates from the column and were completely separated.

Kidney antihemophilic factor has been found by gel filtration methods to have an estimated molecular weight of approximately 26,000. This is in sharp contrast to the reported molecular weight of leucine aminopeptidase (300,000) and plasma antihemophilic factor (>200,000).

The kidney antihemophilic factor migrates as a prealbumin in polyacrylamide and agar gels at pH 8.6. It is eluted from the anion exchanger DEAE-Sephadex only at high salt concentrations, but from CM-Sephadex under starting conditions. These data are consistent and indicate that kidney antihemophilic factor is an acidic protein with a net negative charge at alkaline pH's. The differences between the molecular weights of plasma and kidney antihemophilic factor suggest that the antihemophilic factor activity we are obtaining from the kidney *may* be the monomer or a subunit of plasma antihemophilic factor. Of course, the possibility that plasma antihemophilic factor is kidney antihemophilic factor bound to a large molecular carrier has not been excluded. We are attempting to dissociate plasma antihemophilic factor in search of an activity resembling kidney antihemophilic factor. In this connection it is interesting that the prealbumin of *plasma* lacks antihemophilic factor activity.

A specific antibody has been prepared against hog kidney antihemophilic factor which neutralizes kidney antihemophilic factor activity. The fact that this antibody is capable of neutralizing more than half of the antihemophilic factor activity in kidney preparations without inhibiting the hydrolysis of L-leucinamide from the same preparations indicates its high specificity and

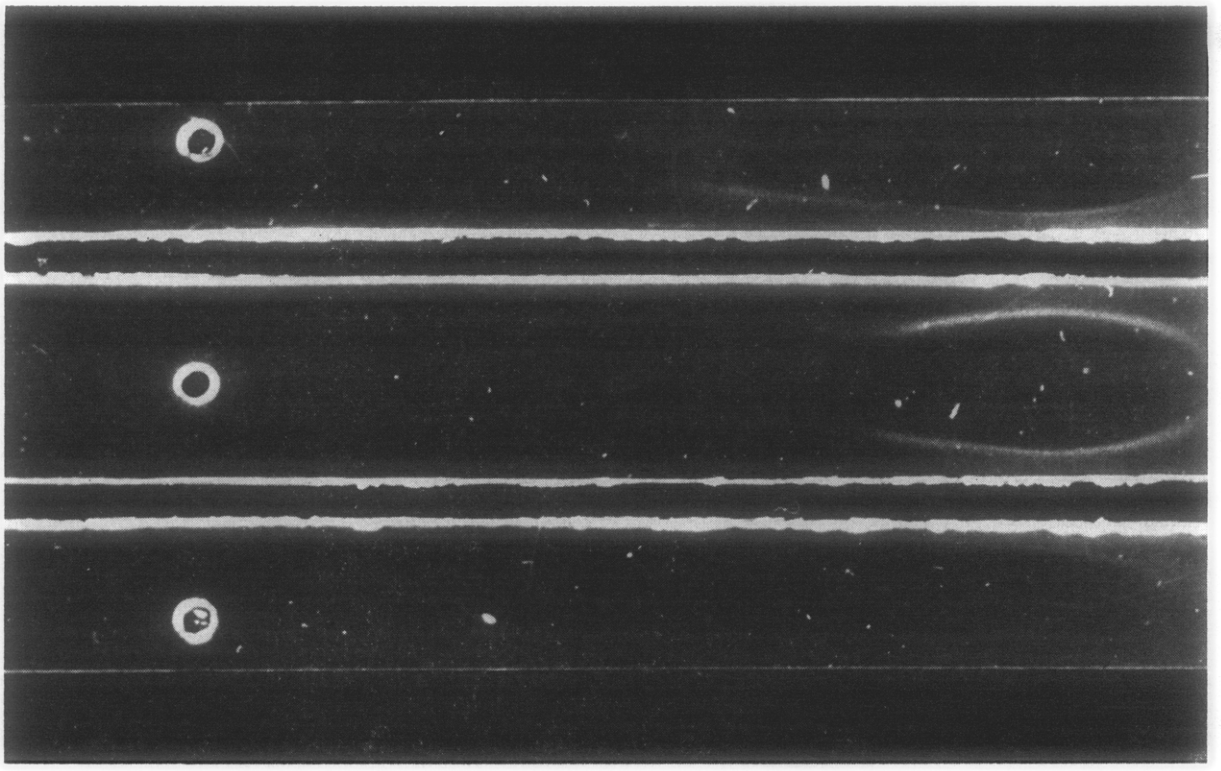

FIGURE 5: Immunoelectrophoresis pattern produced by rabbit antihog kidney antihemophilic factor antibody. The top and bottom wells contained crude hog kidney leucine aminopeptidase. The central well contained a water-soluble extract of acetone-dried hog kidney. After electrophoresis, rabbit antikidney antihemophilic factor antiserum was placed in the troughs and allowed to diffuse overnight.

confirms the separation of antihemophilic factor and leucine aminopeptidase activities by gel filtration.

The specificity of the purified kidney antihemophilic factor on correcting the clotting defect in hemophilic plasma but not in plasmas deficient in factors XII or IX is striking. Among other things, it indicates that this activity is not related to the urinary or kidney procoagulant substances described by others (Aoki and von Kaulla, 1966; Lewis, 1964b). von Kaulla's "Substance P" from urine acts as a thromboplastin, migrates as a β-globulin, and is excluded from Sephadex G-200.

Work in progress is attempting to determine the enzymatic specificity of kidney antihemophilic factor and its relationship to plasma antihemophilic factor. If a specific synthetic substrate can be found which is hydrolyzed by kidney antihemophilic factor, this will prove its enzymatic nature and may provide a more suitable assay than the present ones which depend upon the clotting times of hemophilic plasma.

Acknowledgments

We wish to express our thanks to Miss Harolyn Sparks and Mrs. Vivian Burnette for excellent and devoted technical assistance. We are also indebted to Dr. Ivan I. Dejanov, now at the Institute for Blood Transfusion, Skopje, Yugoslavia, for the preparation of the antihemophilic factor–ethylenemaleic anhydride antigen for immunization of rabbits.

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Dimensions of Protein Random Coils*

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ABSTRACT: The dimensions of protein random coils have been calculated for a variety of proteins of known amino acid sequence. As was anticipated from the work of Flory and coworkers, glycine and proline contribute to reducing the dimensions of random coil proteins. Branched side chains expand the chain only slightly more than unbranched side chains. Side chains represented as structured to the γ position were compared with structureless representations. It was demonstrated that the two approaches give comparable chain dimensions. The effect of sequence was investigated. Although the distribution of glycine and proline along the chain was of some importance, the mean-square end-to-end distance based on a knowledge of composition alone and assuming a randomized sequence was generally within 10% of the value obtained when sequence was taken into account. The calculated dimensions were compared with the experimental values of Tanford and coworkers determined from viscosity and osmotic pressure data in 6 M guanidine hydrochloride. The agreement is substantial with the experimentally deduced values tending to be slightly smaller than the calculated ones. An approximate accounting was made of the effect of regions of highly associated side chains within a randomly coiling molecule. The chain dimensions were found rather insensitive to reasonably large knots of associated residues. Thus it cannot be ruled out that proteins in 6 M guanidine hydrochloride have sizeable amounts of highly associated though not regularly ordered regions.

The measurement and understanding of thermodynamic and kinetic changes accompanying conformational changes in proteins has advanced to the point where very detailed models are being proposed. The reference state generally chosen is a folded or near-native conformation, giving a unique reference state for each protein. It would be desirable, if possible, to have a well-defined reference state that is common to all proteins.

Tanford *et al.* (1967a) have investigated the intrinsic viscosities and sedimentation coefficients of proteins in concentrated guanidine hydrochloride solutions and concluded "that protein polypeptide chains, in the solvent medium employed, are true random coils, retaining no elements of their original native conformation." Tanford and coworkers have presented extensive additional experimental evidence in an effort to support this conclusion (Nozaki and Tanford, 1967; Tanford *et al.*, 1967b; Lapanje and Tanford, 1967; Aune *et al.*, 1967).

The configuration of polymer random coils is a well-studied area of synthetic polymer chemistry. The theory leading to predictions of the average dimensions of polymer random coils has been quite successful in that values in agreement with experiment may be calculated for numerous synthetic polymer random coils (Volkenstein, 1963). Thus a polymer random coil as conformational reference state for proteins is attractive in that it is theoretically tractable.

The configuration of polymer random coils is well known to depend upon hindrance potentials for rotation about the main-chain bonds, as well as on bond angles, bond distances, and long-range interactions (excluded volume). Appropriate though approximate forms for the potential functions have been given for peptide-bond polymers (Brant and Flory, 1965b; DeSantis *et al.*, 1965; Ramakrishnan and Ramachandran, 1965; Leach *et al.*, 1966; Scott and Scheraga, 1966; Brant *et al.*, 1967; Schimmel and Flory, 1967, 1968). Utilizing hindrance potentials, bond angles, and bond distances (Sasisekharan, 1962; Leung and Marsh, 1958) applicable to specific types of amino acid residues, unperturbed random coil dimensions, *i.e.*, dimensions neglecting long-range interactions such as the chain crossing back on itself, have been computed for homopolymers having an alanine type (*cf. seq.*) amino side chain (Brant and Flory, 1965b; Brant *et al.*, 1967), for poly-L-proline (Schimmel and Flory, 1967), for random sequence copoly (glycine, alanine) (Miller *et al.*, 1967), and for copoly (glycine, proline), copoly (alanine, proline),

* From the Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received June 26, 1968*. This work was aided by a U. S. Public Health Service grant (GM-15903).