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# PARTIAL PURIFICATION AND CHARACTERIZATION OF THE ANTIDIURETIC HORMONE-INACTIVATING ENZYME FROM RENAL PLASMA MEMBRANES

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## Summary

An antidiuretic hormone-inactivating peptidase located in renal plasma membranes of porcine kidney medulla has been studied. Treatment of antidiuretic hormone (lysine vasopressin) with renal plasma membranes resulted in a progressive loss of biological activity as measured by the rat pressor assay. The reaction of 2,4,6-trinitrobenzenesulfonic acid with released amino groups was employed to follow the peptidase-catalyzed hydrolysis of the hormone. An 83-fold purification of the membrane-bound peptidase was achieved by Lubrol PX solubilization of the membranes followed by DEAE-cellulose, hydroxylapatite, and 8% agarose column chromatography. The molecular weight of the peptidase was 442 000 as determined by 8% agarose gel filtration. An analysis of the antidiuretic hormone hydrolysis products by thin-layer chromatography revealed the presence of trinitrophenyl-glycinamide. The release of glycinamide from the hormone as a function of time was demonstrated. $Mg^{2+}$ had a slight inhibitory effect and $Ca^{2+}$ had a strong inhibitory effect on the peptidase activity.

## Introduction

The degradation of polypeptide hormones by inactivating mechanisms within their target tissues has been suggested by the results of several investigations [1—5]. Since the initial site of action of the antidiuretic hormone appears to be the renal plasma membrane, it was considered that this organelle might also contain an inactivating mechanism for the hormone. Such as mechanism in the vicinity of the antidiuretic hormone receptor might serve to regulate the concentration of hormone at its site of action and to act as major site of clearance of antidiuretic hormone without requiring penetration of the hormone into the cell.

Experiments in vivo and in vitro have indicated that the antidiuretic hormone may be inactivated by the following enzymatic mechanisms: reduction of the disulfide bond of cystine [6—8]; aminopeptidase-like cleavage of the cystinyltyrosine bond followed by sequential hydrolysis of the next two peptide bonds ending at the glutamine residue [7,9,10]; trypsin-like hydrolysis of the terminal lysylglycinamide bond [10—12].

In the present investigation an antidiuretic hormone-inactivating system has been located in the renal plasma membranes of porcine kidney medulla, and inactivation has been related to a membrane-bound peptidase.

## Experimental

*Purification of antidiuretic hormone (lysine vasopressin)*

Lysine vasopressin (Grade IV, synthetic powder, 101 units/mg) obtained from Sigma Chemical Co. was further purified by the method of Campbell et al. [13]. The purified product gave a single spot upon analysis by paper electrophoresis by the procedure of Campbell et al. [11], and by descending paper chromatography according to Schally and Guillemin [14]. The extinction coefficient of the purified material was $E^{1\%}_{1\,cm}$ = 12.33 at 280 nm. The specific biological activity was 260—280 units/mg as determined by the rat pressor assay.

*Preparation of plasma membranes, adenylate cyclase and ($Na^+$,$K^+$)ATPase assays*

Adenylate cyclase and ($Na^+$,$K^+$)-ATPase assays were carried out as reported by Campbell et al. [13]. Renal plasma membranes were also isolated according to previously reported procedures [13]. In a typical preparation the medulla was separated from cortex material following a mid-sagittal cut dividing the kidney into equal halves. Approximately 100—200 g of medulla tissue were collected for each preparation. The medulla tissue was placed in a homogenizing mixture of 0.25 M sucrose and 1 mM EDTA in the ratio of 1 part tissue to 4 parts sucrose—EDTA (w/v). The suspension was homogenized for 30 s using a Polytron homogenizer (Kinematica, GmbH., Luzern-Schweiz, Type PT-20-OD). The highly minced material was then homogenized with six complete strokes using a Potter—Elvehjem teflon-glass homogenizer (Arthur H. Thomas Co., 4288-B,C). The product of this step is referred to as homogenate. The homogenate was filtered though one layer or cheese-cloth, and the residue on the cheese-cloth was rinsed one time with 1vol. of 0.25 M sucrose—1 mM EDTA. The residue was discarded and the filtrate from this step is referred to as filtered homogenate. The following fractionation procedures were carried out in a Sorvall RC2-B refrigerated centrifuge using an SS-34 rotor. The filtered homogenate was centrifuged at 1475 × *g* for 10 min. The supernatant was discarded and the pellet resuspended in 1 vol. of 2 M sucrose with two strokes in the teflon-glass homogenizer. This fraction is referred to as the 1475 × *g* pellet. The resuspended pellet was centrifuged at 13 000 × *g* for 10 min, and the supernatant collected and the pellet discarded. This fraction is referred to as the 13 000 × *g* supernatant. The supernatant was diluted to 0.25 M sucrose with 7 vol. of cold deionized water and centrifuged a third time at 35 000 × *g*

for 15 min. The resultant pellet from this step was composed of a dark brown bottom layer and a fluffy pink top layer. The top layer was collected by adding 3 ml of 0.05 M Tris—HCl buffer at pH 7.6 to the centrifuge tube and swirling the contents. The fluffy pink upper layer distributed in the buffer, and the brown layer remained at the bottom of the tube. The upper layer was collected and washed twice by centrifuging at 35 000 × *g* after resuspending in the Tris—HCl buffer. This final fraction is referred to as plasma membranes.

*Biological assay of the antidiuretic hormone*

The rat pressor assay described by Dekanski [15] was modified as follows. Male rats (Sprague—Dawley) weighing between 250—350 g were anesthetized with a sodium nembutal solution (50 mg/ml of normal saline) by injecting intraperitoneally 0.1 ml per 100 g body weight. The rats were maintained under anesthesia by periodically injecting one-tenth the original dose, either intraperitoneally or intravenously. The anesthetized rat was mounted on an operating board, and a trachea cannula was inserted to insure proper breathing. A carotid artery was isolated and cannulated with polyethylene No. 50 tubing, and a femoral vein near the inguinal ligament was cannulated with polyethylene No. 10 tubing. The carotid cannula was connected to a Statham pressure transducer filled with a solution of sodium heparin in normal saline (1000 U.S.P. units of heparin/50 ml saline). The pressure transducer was attached to a Grass No. 7 Polygraph calibrated to give a 0.1 cm pen deflection per 5 mm Hg pressure.

A dose—response relationship was established by injecting known quantities of vasopressin standard into the venous cannula and recording the increase in blood pressure at the carotid artery. Pitressin obtained from Parke-Davis Company at a concentration of 20 pressor units/ml was employed in serial dilutions with normal saline as a standard for the dose—response measurements. A linear dose—response relationship was obtained by plotting the log of the dose administered in milliunits versus the response in mm Hg. The biological activity of the antidiuretic hormone in a sample was determined by comparing its measured pressor response to the standard dose-response curve obtained from the same rat under the same conditions.

*Peptidase assay*

The reaction of 2,4,6-trinitrobenzenesulfonic acid with primary amino groups was employed to follow peptidase-catalyzed hydrolysis of the antidiuretic hormone. It has previously been shown that trinitrobenzenesulfonic acid reacts selectively with primary amino groups of amino acids and peptides to yield trinitrophenyl products in a nearly quantitative reaction [16,17]. In a typical assay 0.20 ml of $5.0 \cdot 10^{-4}$ M antidiuretic hormone in 0.01 M sodium phosphate at pH 7.4 was added to 0.50 ml of enzyme preparation in the same buffer. The reaction mixture was maintained at 37 °C in 12 ml conical centrifuge tubes with gentle shaking. At various time intervals 0.50 ml of 10% trichloroacetic acid was added to stop the reaction. After the addition of trichloroacetic acid, the precipitate was allowed to form for 5 min, and then the tubes were centrifuged at maximum speed in a desk top International clinical centrifuge for 10 min. Aliquots of 1 ml from the resulting supernatant were pipetted

into 2 ml of 8% $NaHCO_3$ to neutralize the trichloroacetic acid. To the neutralized aliquots was added 0.1 ml of 0.4% trinitrobenzenesulfonic acid, and the color reaction was allowed to proceed at 40°C for 2 h in the dark. The reaction was stopped by adding 0.2 ml of 10 M HCl, and the $CO_2$ bubbles were removed by swirling the tubes in a Vortex mixer. The absorbance of the solutions were then measured at 340 nm using the zero time tube (the tube in which the enzymic reaction was stopped immediately after mixing reactants) as a blank. In the more impure preparations a correction for hydrolysis of endogenous proteins was made by employing a control that contained no antidiuretic hormone. The protein concentration of the peptidase preparations was determined by the method of Lowry et al. [18]. Fractions from the DEAE-cellulose and hydroxylapatite columns were dialyzed against 20 vol. of 0.01 M sodium phosphate buffer at pH 7.4 with two changes of buffer to remove excess salt. Aliquots of the dialyzed fractions were then assayed for peptidase activity against antidiuretic hormone using the trinitrobenzenesulfonic acid reaction as described above.

*Analysis of the products of peptidase cleavage of antidiuretic hormone*

The trinitrophenyl derivatives of the products released during enzymatic digestion of antidiuretic hormone were isolated from the reaction mixtures described above. Following measurement of the absorbance of the reaction mixtures at 340 nm, the assay mixtures were extracted four times with 5 ml of peroxide-free diethyl ether. The diethyl ether extracts were combined, washed twice with 5 ml of demineralized water, and evaporated to dryness. The residue was dissolved in a small volume of acetone and chromatographed on thin-layer chromatographic plates.

Pre-coated thin-layer chromatographic plates (Silica Gel 60 F-254, 20 cm × 20 cm) of 0.25 mm thickness were spotted with sample and heated at 90—95°C for 10 min prior to chromatography. Two solvent systems were employed: pyridine—toluene—acetic acid (10 : 80 : 5; by vol.) and chloroform—benzyl alcohol—acetic acid (70 : 30 : 3; by vol.). To quantitate the amount of trinitrophenylglycinamide produced during reaction, the trinitrophenylglycinamide spot was scraped from the plate, extracted with ethyl acetate, the absorbance measured at 337 nm and the nmoles of trinitrophenylglycinamide calculated from the extinction coefficient. Standard trinitrophenylglycinamide was synthesized by reaction of glycinamide with trinitrobenzenesulfonic acid and recrystallized from 75% methanol. The melting point, absorption maximum, and extinction coefficient of the synthesized derivative were determined. A known amount of trinitrophenylglycinamide was added to a simulated reaction mixture, extracted with diethyl ether, chromatographed on thin-layer chromatographic plates, and quantitated to correct for the amount lost during these procedures. The recovery of trinitrophenylglycinamide was determined in three separate reaction mixtures. The percent recoveries were 15.6, 14.2, and 16.2; the corresponding correction factors were 6.4, 7.0 and 6.5, respectively (average 6.5). By this method it was possible to detect trinitrophenylglycinamide in a reaction mixture at a level of 1 nmole.

*Lubrol PX solubilization of membrane-bound peptidase*

A renal plasma membrane suspension at a concentration of 4.6 mg mem-

brane protein/ml in 0.01 M sodium phosphate buffer, pH 7.4, was stirred briskly at 4°C prior to the slow addition of 10% Lubrol PX (Imperial Chemical Industries Inc., Charlotte, N.C.) until a final Lubrol PX concentration of 0.5% was achieved. Stirring was continued for 1 h, and then the mixture was centrifuged at 113 000 × *g* for 1 h in a Beckman L2-65B ultracentrifuge. The supernatant which contained the solubilized peptidase was decanted and maintained at 4°C or frozen at −20°C prior to use.

*DEAE-cellulose chromatography*

A DEAE-cellulose column, 4 cm × 9 cm, was equilibrated with 0.01 M sodium phosphate buffer at pH 7.4. To this column were added 687 mg of Lubrol PX solubilized plasma membrane protein in 550 ml of the equilibrating buffer. The column was developed by stepwise elution with 0.01 M phosphate buffer at pH 7.4 containing: no KCl, 0.05 M KCl, and 0.50 M KCl. The absorbance of the fractions collected was measured at 280 nm.

*Hydroxylapatite chromatography*

Hypatite C (Clarkson Chemical Company Inc., Williamsport, Pa.) with a binding capacity of 80 mg of serum albumin per gram was employed in this procedure. The active peptidase fraction eluted from the DEAE-cellulose column with 0.5 M KCl was dialyzed against 0.01 M sodium phosphate buffer at pH 7.4 prior to application to the hydroxylapatite column (4 cm × 8 cm) which had been equilibrated with the same buffer. The peptidase fraction contained 188 mg protein in 220 ml of the phosphate buffer (Lubrol PX was added to make the final Lubrol PX concentration 0.5%). The column was eluted stepwise with the following phosphate buffers at pH 7.4: 0.01 M, 0.10 M and 0.20 M.

*Agarose gel filtration*

An 8% agarose (Bio-Gel A-1.5 m, 200—400 mesh) column, (2.5 cm × 91 cm) was equilibrated with 0.01 M sodium phosphate buffer at pH 7.4. The active fraction from the hydroxylapatite column was dialyzed against 0.01 M sodium phosphate buffer, pH 7.4, and applied to the column (30 mg of protein in 10 ml). Elution with the same buffer was carried out, and 5—10 ml fractions were collected at 5°C over a period of 24 h. The gel filtration step was standardized for comparison of the gel filtration properties of the peptidase with that of proteins of various molecular weights by the method of Andrews [19].

**Results and Discussion**

The incubation of renal plasma membranes with biologically active antidiuretic hormone resulted in a progressive loss of biological activity with time of incubation. The results presented in Fig. 1 indicate that the ability of the membrane preparation to inactivate the hormonal response was lost upon heating the membranes at 100°C for 30 min. To estimate the precision of the results reported in Fig. 1, the pressor response of five equal injections of purified antidiuretic hormone was measured, and the standard deviation was calculated to be 10% of the mean. The inactivation curves for antidiuretic

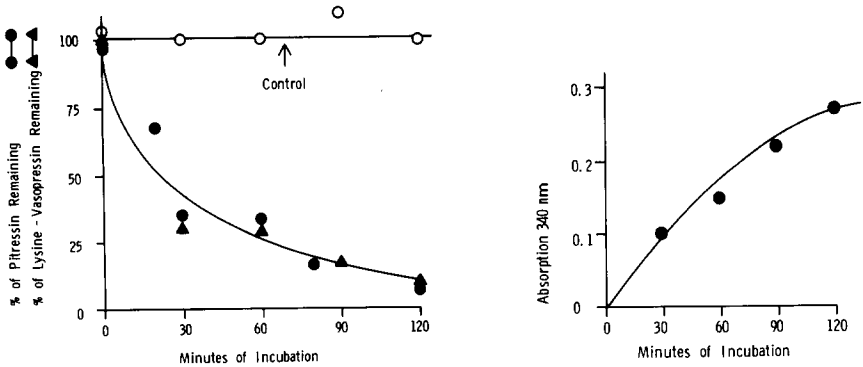


Fig. 1. The inactivation of pitressin and lysine vasopressin by isolated plasma membranes. 6 ml of pitressin (2.4 units) and 6 ml of lysine vasopressin ($1.04 \cdot 10^{-6}$ M) in normal saline were separately incubated with 6 ml of plasma membrane suspension (12.6 mg protein) at 37°C. 1-ml aliquots were assayed for their pressor response. The percent of hormone remaining at $x$ min was calculated by dividing the munits of hormone at $x$ min by the munits of hormone at 0 min multiplied by 100. For the control 6 ml of lysine vasopressin ($1.04 \cdot 10^{-6}$ M) was incubated with 6 ml of plasma membranes (12.6 mg) that had been boiled for 30 min. Each point represents an average of two to three injections given in randomized injection schedule. The standard error of the mean for five equal injections was determined to be ±10%.

Fig. 2. The hydrolysis of lysine vasopressin by isolated plasma membranes. 1 ml of lysine vasopressin solution ($5.1 \cdot 10^{-4}$ M) in 0.01 M sodium phosphate buffer, pH 7.4, was incubated with 3.5 ml of membrane suspension (1.84 mg protein) in the same buffer at 37°C. The time-dependent release of free amino groups was measured by their reaction with 2,4,6-trinitrobenzenesulfonic acid.

hormone and pitressin presented in Fig. 1 show a residual pressor activity of approximately 10% of the original activity for both hormones. Similar results have been reported for the inactivation of pitressen by crude homogenates of rat kidney over a period of 120 min [20].

In Fig. 2 is presented the time course of hydrolysis of the antidiuretic hormone catalyzed by renal plasma membranes. The reaction was followed by measuring the release of free amino groups during the course of the inactivating reactions. This peptidase assay was employed to determine the peptidase activity against antidiuretic hormone of fractions leading to the purification on renal plasma membranes. At the same time the activities of $(Na^+,K^+)$-ATPase and adenylate cyclase were measured in the same fractions. The results shown in Table I show an increase in the activities of the three enzymes as the membrane fraction was purified. Furthermore, the antidiuretic hormone peptidase activity of the plasma membrane fraction was 40-fold greater than that of the homogenate.

The release of free amino groups from antidiuretic hormone promoted by peptidase activity was also employed as an assay in a number of procedures leading to the partial purification of the enzyme. A summary of results of peptidase purification is presented in Table II. In the DEAE-cellulose procedure the protein fractions that eluted in steps prior to elution with 0.01 M phosphate buffer at pH 7.4 in 0.5 M KCl were devoid of peptidase activity. When the column was eluted with phosphate buffer in 0.5 M KCl, the peptidase was released from the exchanger. The large loss of peptidase activity on DEAE-cellulose was probably due to irreversible binding of peptidase-active high molecular weight material. The data in Table II indicate a simultaneous loss of

TABLE I

CHARACTERIZATION OF THE PLASMA MEMBRANE ISOLATION STEPS

The specific activity of adenyl cyclase is expressed in terms of pmoles of cyclic AMP produced/mg of protein per 10 min. The specific activity of $(Na^+, K^+)$-ATPase is in $\mu$moles of $P_i$ formed/mg protein per 20 min. The specific activity of the antidiuretic hormone peptidase is expressed in terms of the increase in absorbance at 340 nm/mg of protein per minute multiplied by $10^5$.

| Fraction | Protein concn (mg/ml) | Volume (ml) | Total protein (mg) | Spec. Act. adenyl cyclase | Spec. Act. $(Na^+, K^+)$-ATPase | Spec. Act. peptidase | Total units adenyl cyclase | Total units $(Na^+, K^+)$-ATPase | Total units peptidase |
|---|---|---|---|---|---|---|---|---|---|
| Homogenate | 20.2 | 700 | 14140 | 21.1 | 0.75 | 17 | 298000 | 10600 | 240000 |
| Filtered homogenate | 17.4 | 715 | 12400 | 28.3 | 0.70 | 55 | 351000 | 8680 | 682000 |
| 1475 × *g* pellet | 15.6 | 345 | 5380 | 41.6 | 2.22 | 69 | 224000 | 11900 | 371000 |
| 13000 × *g* supernatant | 4.7 | 165 | 775 | 50.4 | 1.31 | 114 | 39000 | 1020 | 884000 |
| Plasma membranes | 1.52 | 50 | 76 | 59.4 | 3.23 | 678 | 451 | 245 | 515000 |
| Plasma membranes + $10^{-5}$ M antidiuretic hormone | — | — | — | 154.2 | — | — | 1170 | — | — |

TABLE II

SUMMARY OF THE PEPTIDASE PURIFICATION STEPS

The specific activity of the peptidase is expressed in terms of the increase in absorbance at 340 nm per mg of protein multiplied by $10^5$.

| Fraction | Volume (ml) | Protein concn (mg/ml) | Total protein (mg) | Spec. Act. of peptidase | Total units of peptidase |
|---|---|---|---|---|---|
| Isolated plasma membranes | 560 | 4.6 | 2576 | 351 | 904000 |
| Lubrol-solubilized plasma membranes | 560 | 1.25 | 700 | 640 | 448000 |
| DEAE-cellulose | 128 | 0.85 | 109 | 590 | 64300 |
| Hydroxylapatite | 118 | 0.62 | 73 | 915 | 66800 |
| 8% agarose "C" fraction | 17.8 | 0.38 | 6.8 | 1410 | 9590 |

86% of the total peptidase units and 84% of the total protein on the DEAE-cellulose column. These losses are most likely due to tightly bound, high molecular weight aggregates of peptidase which are active prior to binding to DEAE-cellulose and inactive following DEAE-cellulose binding. The presence of these high molecular aggregates has been demonstrated by agarose filtration. When the Lubrol-solubilized membrane preparation was passed over 8% agarose columns prior to DEAE-cellulose chromatography, a substantial peptidase-active fraction appeared in the void volume suggesting a molecular weight range near 1 500 000 or greater. This peak containing high molecular weight aggregates was virtually absent when the solubilized membrane preparation was treated with DEAE-cellulose prior to 8% agarose filtration. The high molecular weight active material was not amenable to further separation in subsequent procedures. The DEAE-cellulose step was, therefore, employed to remove the active, high molecular weight aggregates of peptidase from the lower molecular weight species which could be further purified. The elution curve for the hydroxylapatite chromatographic step is shown in Fig. 3. The major portion of the peptidase was eluted with the 0.10 M phosphate buffer, although a small amount of activity could be detected in the fraction eluted with 0.2 M phosphate buffer.

The major active fraction from the hydroxylapatite column was concentrated by ultrafiltration prior to gel filtration. Concentration of this fraction to levels above approximately 1 mg/ml caused aggregation of the protein. Therefore, at this step the solution was adjusted to 0.5% Lubrol PX by adding Lubrol PX to the solution, and concentration was carried out without observable aggregation effects. Results obtained from the gel filtration of peptidase and

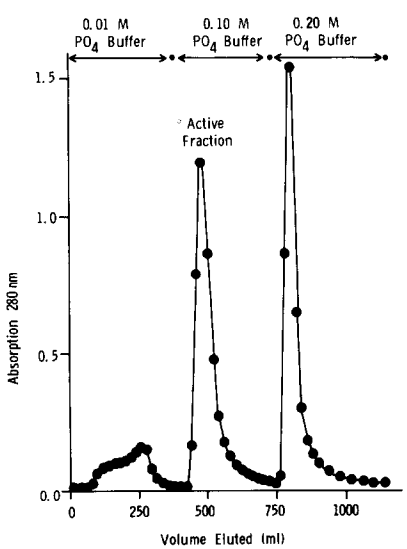


**Fig. 3. Hydroxylapatite column chromatography of the DEAE-cellulose active peptidase fraction. 220 ml of dialyzed fraction from DEAE-cellulose column (188 mg protein containing 0.5% Lubrol PX) was applied to a 4 cm × 8 cm bed of hydroxylapatite equilibrated with 0.01 M sodium phosphate buffer, pH 7.4. The column was eluted stepwise with the following sodium phosphate buffers, pH 7.4: 0.01 M, 0.10 M and 0.20 M.**

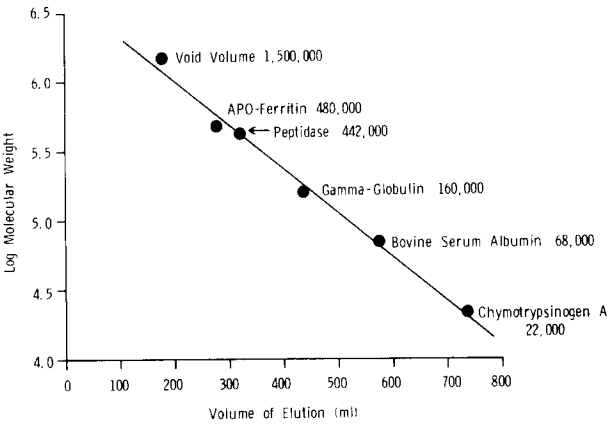


**Fig. 4. Molecular weight determination by 8% agarose gel filtration. An 8% agarose column (91 cm × 2.5 cm) operating in reverse flow was calibrated by dissolving Dextran blue 2000 and proteins of known molecular weight in 10 ml of 0.01 M sodium phosphate buffer, pH 7.4, and applying these solutions to the column. The peptidase was chromatographed in 10 ml of the same buffer and its elution volume was calculated from the center of its activity peak. The activity peak of the peptidase was determined by incubating 0.250 ml of each aliquot eluted with 0.100 ml of $1.0 \cdot 10^{-3}$ M antidiuretic hormone at 37 °C for 2 h and assaying for the release of free amino groups with 2,4,6-trinitrobenzenesulfonic acid.**

proteins of various known molecular weights on the 8% agarose column are reported in Fig. 4. The molecular weight of the peptidase estimated according to the method of Andrews [19] is approximately 442 000. A 4-fold increase in the activity of the peptidase was achieved by the procedures outlined in Table II. The rat pressor assay was employed to compare the antidiuretic hormone-inactivating effect or renal plasma membranes with that of purified peptidase. The specific inactivation was measured as the munits of antidiuretic hormone inactivated/h per mg of protein. Renal plasma membranes exhibited a specific inactivation of 14 munits/h per mg of protein while peptidase from the 8% agarose column exhibits a specific inactivation of 379 munits/h per mg of protein.

Since the measurement of hormonal inactivation by the rat pressor assay indicates a 27-fold increase in activity going from plasma membranes to partially purified enzyme while comparison of the same fractions by the trinitrobenzenesulfonic acid reaction shows only a 4-fold increase in activity, it is apparent that one measurement of activity does not quantitatively reflect the other. It is probably not justified to refer to the increasing specific activity of the peptidase-enriched fractions since the results show that the most active fraction produces four products indicating multiple peptide bond cleavage. It is also possible that additional inactivating mechanisms [7—10] are concentrated by the purification procedure which would account for the lack of quantitative correlation between the rat pressor assay and the peptidase assay. Nevertheless, the use of the peptidase assay provides a facile technique for qualitative localization of peptidase activity against the antidiuretic hormone during the purification procedures.

The kinetics of peptidase-catalyzed hydrolysis of antidiuretic hormone were compared with the kinetics of the loss of biological activity of the hormone as measured by the rat pressor assay. The results presented in Fig. 5

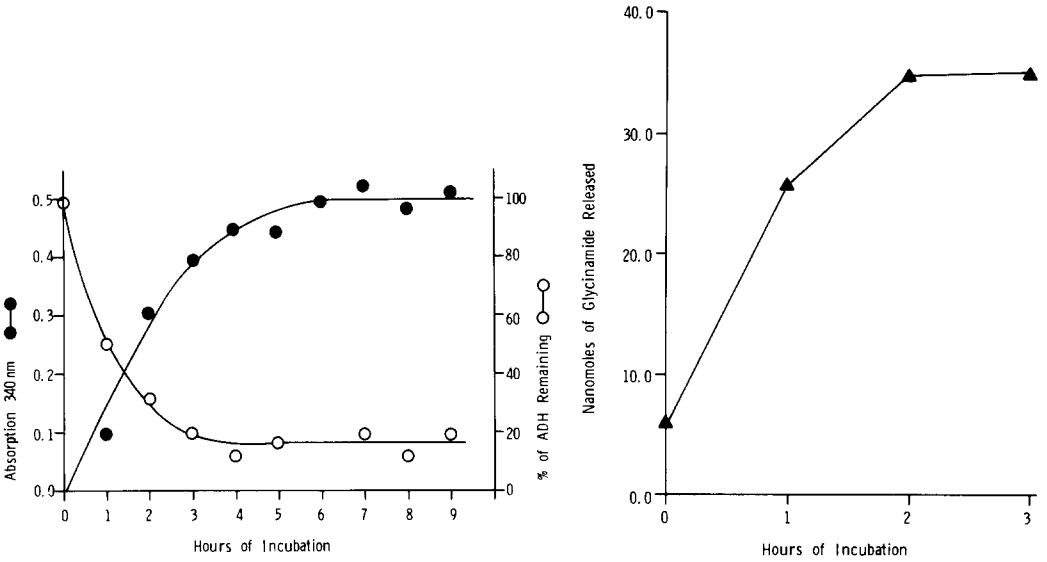


Fig. 5. The kinetics of lysine vasopressin hydrolysis and inactivation. 0.2 ml of antidiuretic hormone ($5.0 \cdot 10^{-4}$ M) was incubated with 0.5 ml of peptidase (189 μg eluted in the active fraction from 8% agarose) in 0.01 M sodium phosphate buffer, pH 7.4, at 37 °C. The time-dependent release of free amino groups during hydrolysis was measured by their reaction with 2,4,6-trinitrobenzenesulfonic acid. The time-dependent loss of biological activity was followed by measuring the munits of vasopressin remaining at $x$ min and dividing by the munits of vasopressin at 0 min times 100 using the rat pressor assay.

Fig. 6. The kinetics of glycinamide release from lysine vasopressin. 0.2-ml aliquots of antidiuretic hormone ($5.0 \cdot 10^{-4}$ M) were incubated with 0.5 ml of peptidase (189 μg protein from the 8% agarose column) in sodium phosphate buffer, pH 7.4, at 37 °C for 0, 1, 2 and 3 h. The 2,4,6-trinitrobenzenesulfonic acid treated incubation mixtures were diethyl ether extracted and chromatographed on silica gel thin-layer plates using a chloroform—benzyl alcohol—acetic acid (70 : 30 : 3; by vol.) solvent system. The spots corresponding to trinitrophenyl glycinamide were scraped from the plates and quantitated for the nanomoles of glycinamide in each 0.70 ml reaction mixture. Each reaction mixture contained a total of 102 nmoles of antidiuretic hormone substrate. Each point represents the average of two complete and independent analyses.

suggest that under these assay conditions approximately 50% of the biological activity is lost within the first hour of treatment while 50% of the peptidase-catalyzed reaction is complete at approximately 1.75 h. Furthermore the curves suggest that peptidase-catalyzed hydrolysis continues after the biological activity of the hormone is reduced to its residual level. It seems likely that this effect is produced by peptidase-catalyzed hydrolysis of inactive peptide fragments.

Analysis of the digestion products of thin-layer chromatography revealed the presence of trinitrophenylglycinamide as well as three other unidentified spots. The quantitative release of glycinamide as a function of time is shown in Fig. 6. The synthesized trinitrophenylglycinamide which was used as a standard in these experiments melted at 234—235 °C, exhibited an absorption maximum at 334 nm, and had an $\epsilon = 1.66 \cdot 10^4\ M^{-1} \cdot cm^{-1}$. The presence of 5.7 nmoles of glycinamide at zero time probably represents the very rapid release of product at the initial rate before the enzymatic reaction was stopped. Inactivation of antidiuretic hormone by peptidase cleavage of the bond linking glycinamide to the intact hormone has been previously reported in extracts of toad bladder [11] and of rat kidney [12]. It should be noted, however, that these enzymes

TABLE III

EFFECTS OF CALCIUM AND MAGNESIUM UPON PEPTIDASE-CATALYZED HYDROLYSIS OF ANTIDIURETIC HORMONE

The data are expressed as relative activity and are calculated by dividing the specific activity of the peptidase with addition of metal ion by the specific activity in the absence of metal ion multiplied by 100. The activity was measured by the trinitrobenzenesulfonic acid-peptidase assay described in Experimental.

| Metal ion | Metal ion concentration | | |
|---|---|---|---|
| | 0.1 mM | 1.0 mM | 10 mM |
| $Mg^{2+}$ | 90 | 73 | 80 |
| $Ca^{2+}$ | 83 | 43 | 7 |

are soluble enzymes while the peptidase described in this work is bound to the hormone-sensitive renal plasma membrane.

The effects of increasing concentration of calcium and magnesium upon peptidase-catalyzed hydrolysis of the antidiuretic hormone are reported in Table III. The results demonstrate a pronounced inhibitory effect of calcium with 93% inhibition occurring at a concentration of 10 mM calcium. Magnesium at the same concentration caused only 20% inhibition. These results support the investigations reported by Thorn and Willumsen [21] who measured the loss in biological activity of antidiuretic hormone produced by incubation of the hormone with rat kidney slices. These workers determined that an increase of calcium concentration from 2.5 to 12.5 mM in the incubation medium produced a 51% reduction in the inactivation of the hormone.

In conclusion, the reaction of 2,4,6-trinitrobenzenesulfonic acid with primary amino groups released from antidiuretic hormone has proved useful in localizing, partially purifying and characterizing the antidiuretic hormone-inactivating enzyme bound to renal plasma membranes. The same reaction was also useful in identifying and following the release of glycinamide from the C-terminus of the hormone. In these experiments it was necessary to use relatively high concentrations of antidiuretic hormone ($3 \cdot 10^{-4}$ M) in order to obtain reliable color yields and to isolate products from the reaction mixture. In future work the development of an adenylate cyclase—antidiuretic hormone sensitive assay will permit investigations within the physiological range ($10^{-6}$—$10^{-10}$ M antidiuretic hormone) of the membrane receptor locus. Complete characterization of the breakdown products from peptidase cleavage of antidiuretic hormone must await the availability of larger quantities of purified polypeptide.

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