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PARTIAL PURIFICATION AND CHARACTERIZATION OF POST-PROLINE CLEAVING ENZYME:

ENZYMATIC INACTIVATION OF NEUROHYPOPHYSEAL HORMONES BY KIDNEY PREPARATIONS OF VARIOUS SPECIES

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Summary

The inactivation of the neurohypophyseal hormones arginine vasopressin and oxytocin, both ^{14}C-labelled in the C-terminal glycine residue, by enzymes present in kidney homogenates of various species has been investigated, and some of the enzymes responsible have been partially purified and characterized. The Leu-Gly peptide bond of oxytocin is generally most effectively cleaved by kidney homogenates, although with certain species enzymic activity hydrolyzing the Pro-Leu bond is significant. Degradation of arginine vasopressin is slower than oxytocin in all species studied, and appears to occur by a different overall mechanism since cleavage of the Pro-Arg bond is more significant than hydrolysis of the Arg-Gly bond. The enzyme releasing glycinamide from oxytocin and the "Post-Proline Cleaving Enzyme", which releases C-terminal dipeptide from oxytocin and arginine vasopressin, were partially purified from lamb kidney by ammonium sulfate fractionation and column chromatography. The two enzymes are shown to be separate entities with different pH profiles. The prolyl peptidase activity released the C-terminal dipeptides from oxytocin and arginine vasopressin at similar rates and was inhibited by *p*-chloromercuriphenylsulfonic acid, 1,10-phenanthroline, L-1-tosylamido-2-phenylethylchloromethyl ketone, Co^{2+}, Ca^{2+}, and Zn^{2+}, but significantly enhanced by dithiothreitol. The prolyl peptidase preparation cleaves proline-containing peptide

Abbreviations follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature, J. Biol. Chem. (1967) 242, 555; J. Biol. Chem. (1975) 250, 3215; Biochem. J. (1975) 149, 1. All optically active amino acids are of the L-configuration unless otherwise stated. Additional abbreviations: CHO_A, a carbohydrate moiety on a peptide fragment of ovine lutropin (subscript designation is in accord with nomenclature proposed for the glycopeptide in Gen. Comp. Endocrinol. (1968) 11, 444—457).

substrates at the Pro-X bond. The rate of cleavage is dependent on the nature of residue X and with the conditions used there is no cleavage when X equals Pro; however, cleavage occurs when X is a D isomer: [Mpr^1, D-Arg^8] vasopressin is inactivated at a rate similar to [Mpr^1, Arg^8]- and [Mpr^1, Lys^8]vasopressin, suggesting that the known prolonged biological action of [Mpr^1, D-Arg^8]vasopressin is not due to resistance to the prolyl peptidase. In all characteristics tested the lamb kidney prolyl peptidase was identical to the post-proline cleaving enzyme isolated earlier from human uterus. In vivo experiments in the cat suggested that both the glycinamide-releasing enzyme and post-proline cleaving enzyme are present and effective in inactivating neurohypophyseal hormones in the intact animal.

Introduction

The mammalian kidney is a target organ for neurohypophyseal hormones [1,67—69] and also one of the major tissues responsible for their inactivation [2—7]. In rat kidney, we found the predominant mode of enzymatic inactivation of oxytocin involves the release of glycinamide both in in vivo and in vitro experiments by hydrolysis of the peptide linkage between the C-terminal and the penultimate amino acid residue of the hormone by a chymotrypsin-like enzyme [5,6,8]. Recent evidence suggests that the over-all inactivation pattern of arginine vasopressin by rat kidney differs from that of oxytocin and that the direct release of glycinamide, by an enzyme distinct from that catalyzing the release of glycinamide from oxytocin, plays only a minor role [9]. Moreover, in preliminary studies it was found that, while kidney homogenates of all species were able to release glycinamide from the hormones, in homogenates of certain species, an enzyme which catalyzes the release of the dipeptide Leu-Gly-NH_2 from oxytocin and Arg-Gly-NH_2 from arginine vasopressin could be demonstrated.

These developments indicate that the complete picture of the inactivation of neurohypophyseal hormones in kidney is far from being understood. In order to gain further insight the inactivation studies of oxytocin and arginine vasopressin have been extended from rat to include mouse, hamster, rabbit, pig, lamb, cat, dog, chicken, pigeon and human. Lamb kidney was among the preparations containing both the enzyme which releases glycinamide from oxytocin and the post-proline cleaving enzyme, which releases the C-terminal dipeptide from oxytocin and vasopressin. Kidney of lamb was therefore chosen as a readily available source to demonstrate that both enzymes can be separated. Both enzymes have been partially purified and the post-proline peptidase activity of lamb kidney in particular has been tested in this study for its substrate specificity, pH optimum, and susceptibility to ions, sulfhydryl reagents and inhibitors. The properties of this enzyme preparation are compared to an enzyme present in human uterus [10—12] possessing the same substrate specificity. In addition, some studies have been performed to evaluate the physiological significance of these enzymes in vivo.

Materials and Methods

[9-*Glycinamide*-1-^{14}C]oxytocin and [9-*Glycinamide*-1-^{14}C]arginine vasopressin possessed a specific radioactivity of 30 Ci/mol and exhibited an avian vasodepressor activity [13,14] and a rat pressor activity [15] of 482 units/mg and 416 units/mg, respectively [16]. The unlabelled peptides used in this study were as follows: oxytocin [17], (1,6-aminosuberic acid)oxytocin [18], (7-glycine)oxytocin [19], (1,6-aminosuberic acid, 7-glycine)oxytocin [20], (8-alanine)oxytocin [21], (1-β-mercaptopropionic acid, 8-alanine)oxytocin [21], arginine vasopressin [22], (1-β-mercaptopropionic acid, 8-arginine)vasopressin [23,70], (1,6-aminosuberic acid, 8-arginine)vasopressin [24], lysine vasopressin [25], (1-β-mercaptopropionic acid, 8-lysine)vasopressin [26], Cys(Bzl)-Pro-Leu-Gly-NH_2 [27], and H-Pro-Leu-Gly-NH_2 · ½H_2O [28] were available from earlier studies. The syntheses of (7-alanine)oxytocin, (1-β-mercaptopropionic acid, 7-alanine)oxytocin and (1-β-mercaptopropionic acid, 7-glycine)oxytocin were carried out in our laboratory (Walter, R., unpublished). Analogs of oxytocin with the following residues in position 8: valine [29], proline, serine, threonine, phenylalanine [30], and glutamine [31] were gifts of Dr. M. Manning, Medical College of Ohio, Toledo, Ohio. (8-(N^{ϵ}-Formyl)lysine)oxytocin and (8-citrulline)oxytocin [32] were supplied by Dr. K. Lübke, Schering AG, Berlin, Germany. (1-β-mercaptopropionic acid, 8-D-arginine)vasopressin [33] was a gift of Dr. M. Zaoral, Czechoslovak Academy of Sciences, Prague, Czechoslovakia. <Glu-Trp-Pro-Arg-Pro [34] was supplied by Dr. M.A. Ondetti, Squibb Institute, New Brunswick, New Jersey; H-Ala-Tyr-Pro-Thr-Pro-Ala-Arg [35] and H-Pro-Ile-Asn(CHO_A)-Ala-Thr-Leu [36] by Dr. D. Ward, University of Texas, Houston, Texas; <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 by Dr. H. McGregor, Wyeth Laboratories, Philadelphia, Penn. and tuftsin (H-Thr-Lys-Pro-Arg) [37] by Dr. V.A. Najjar, Tufts University, Boston, Mass. H-Pro-Pro-Pro-Pro [38] was a gift of Dr. M. Rothe, Universität Ulm, Ulm, G.F.R. Analogs of (5-valine)angiotensin II (H-Asp-Arg-Val-Tyr-Val-His-Pro-Phe) with the following amino acid residue in position 8: leucine [39], valine [39], isoleucine [40] as well as (4-histidine, 6-tyrosine)angiotensin II [40], (6-arginine)angiotensin II [40], (4-phenylalanine,8-alanine)angiotensin II, (5-valine)-angiotensin II and Ile-His-Pro-Phe were gifts of Dr. R. Smeby, Cleveland Clinic, Cleveland, Ohio and (5-valine)angiotensin I, synthesized according to the method of Freer and Stewart [41], was supplied by Dr. J.M. Stewart, University of Colorado Medical School, Denver, Colo. (5-Isoleucine)angiotensin II was purchased from Calbiochem. Z-Gly-Leu-Gly was purchased from Peninsula Laboratories; H-Phe-Phe-Val-Pro-Pro-AlaOBu^t, H-Gln-Ala-Pro-Lys(Boc)-Gly-NH_2, Z-Pro-His-Phe, Pro-Pro-Ala-OBu^t and Z-Phe-Phe-Pro-Pro were gifts of Drs. R. Geiger and W. König, Farbwerke Hoechst Laboratories, G.F.R. Post-proline cleaving enzyme of human uterus was prepared as described previously [12] and according to the method detailed in this paper. Amino acid analysis was carried out according to the general method of Spackman et al. [42].

Source of kidney tissue. Kidney (frozen in dry ice) from ICR Swiss Webster mice, Syrian golden hamsters, domestic cat, lamb, pig and pigeon were purchased from Rockland Farms, Gilbersville, Pa. Lamb kidneys used in en-

zyme preparations were purchased dry-ice frozen from Max Insel Cohen, Newark, N.J. Female Sprague-Dawley rats and White Leghorn roosters were decapitated, and the kidneys were removed immediately and frozen in dry ice. Dog kidneys were obtained from sodium pentobarbital-anesthetized mongrel dogs and rabbit kidneys from New Zealand White rabbits killed by intravenous air injection. Human tissue came from malignant nephrosclerotic kidney removed in surgery; only that part of the kidney was used which appeared to be free of disease. All tissues were stored frozen at −20°C.

Preparation of kidney extract. One to 5 g (wet weight) of kidney tissue from each species was homogenized at 4°C in 10 vol. of 25 mM sodium phosphate buffer containing 0.5 mM EDTA, pH 6.8 (phosphate buffer), in a Virtis model 45 homogenizer for four 15-s periods with resting periods of 1 min. The homogenate was centrifuged at 105 000 × *g* at 4°C for 1 h using a Beckman Model L ultracentrifuge. The clear supernatant (referred to as kidney extract) was used for further studies.

Determination of enzymic activity. Kidney extract (0.2 ml) of each species was incubated with 5 μg of substrate ([9-*Glycinamide*-1-^{14}C])oxytocin or [9-*Glycinamide*-1-^{14}C]arginine vasopressin diluted to a total volume of 0.5 ml with 25 mM phosphate buffer. Incubation times, at 37°C, were 0, 5, 10, 15, 30, and 60 min for oxytocin and 15 min for vasopressin. The reaction was terminated by boiling for 10 min. The heat-denatured suspension was centrifuged at 2000 × *g* for 10 min.

In later purification steps of the lamb kidney prolyl peptidase, Z-Gly-Pro-Leu-Gly was used in addition to, or instead of, hormone as a substrate for locating the enzymic activity in column fractions. The protected tetrapeptide is useful as a substrate for the prolyl peptidase because it is water soluble, is resistant to aminopeptidase action, but is readily cleaved by the prolyl peptidase to yield Leu-Gly as the only ninhydrin-active product. The product was identified by high voltage electrophoresis of an incubation mixture using authentic Leu-Gly as standard; material located on the electrophoretogram in the area corresponding to the standard dipeptide was eluted, hydrolyzed and subjected to amino acid analysis to give Leu and Gly (1 : 1 molar ratio). During column chromatography approximately 20 μmol of synthetic peptide was incubated for 15 min at 37°C with 0.05—0.1 ml of column eluate in 0.5 ml of 0.05 M sodium phosphate buffer, pH 7.4. The reaction was stopped by addition of ninhydrin reagent (see below). During later purification steps of lamb kidney Gly-NH_2-releasing enzyme, the peptide Z-Cys(Bzl)-Pro-Leu-Gly-NH_2 was used as substrate. The peptide (5 mg) was dissolved in a solution of 0.3 ml glacial acetic acid, 0.45 ml H_2O and 0.02 ml Triton X-100, the solution neutralized with NaOH, and the volume adjusted to 5.0 ml. Aliquots of column eluent (0.01 ml) were incubated with 0.1 ml of the substrate solution at 37°C for 2, 4, and 24 h, and products were identified after high voltage electrophoresis with ninhydrin spray.

Qualitative and quantitative determinations of enzymic digest products. Aliquots (50 ml) of the protein-free supernatant from each enzyme-^{14}C-la-

belled hormone digest were applied to Whatman 3 MM paper strips (4 × 60 cm) and subjected to high voltage electrophoresis in 5% acetic acid/0.5% pyridine, pH 3.5, for 1.5 h at 1500 V, 2.5 mA/cm as described for earlier studies of [9-*Glycinamide*-1-^{14}C]oxytocin [43] and [9-*Glycinamide*-1-^{14}C]arginine vasopressin [12]. Zero-time incubation, as well as [9-*Glycinamide*-1-^{14}C]oxytocin and [9-*Glycinamide*-1-^{14}C]arginine vasopressin incubated in buffer alone, served as controls. Unlabelled compounds were developed with 0.2% ninhydrin in acetone and radioactive marker and products were identified on a Packard 7201 chromatogram scanner. When the substrate Z-Gly-Pro-Leu-Gly was used, 0.5 ml ninhydrin reagent [44] was added to the incubation mixture, which was then boiled for 15 min. After cooling, 2.5 ml of 50% ethanol was added and absorption at 570 nm was determined. For quantification a standard curve of Leu-Gly was constructed.

Fractionation of lamb kidney extract by ammonium sulfate. Crude lamb kidney extract was prepared as described above except that 25 g wet kidney tissue (≈ one-half kidney was homogenized in 100 ml of 25 mM phosphate buffer pH 6.8 containing 1 mM EDTA (for purification studies all buffers contained 0.05 mM dithiothreitol). The homogenate was centrifuged for 10 min at 12 000 × *g* at 4° C and the supernatant subjected to further centrifugation at 105 000 × *g* for 1 h at 4° C. The supernatant (80 ml) was collected and subjected to ammonium sulfate fractionation as follows: to 80 ml supernatant, 16 ml of a cold saturated $(NH_4)_2SO_4$ solution containing 1 mM EDTA and adjusted to pH 6.8 with NaOH (referred to as $(NH_4)_2SO_4$ solution) was added over a period of 15 min with constant stirring. The suspension was transferred to centrifuge tubes, allowed to stand 30 min at 4° C and then centrifuged for 15 min at 20 000 × *g*. The pellet obtained is referred to as fraction I (0 to 20% ammonium sulfate saturation). Similarly, six additional fractions were obtained by repeating the precipitation process by successive addition of the same $(NH_4)_2SO_4$ solution to the respective collected supernatants: fraction II (20—30%), fraction III (30—40%), fraction IV (40—50%), fraction V (50—60%), fraction VI (60—70%), fraction VII (70—80%) $(NH_4)_2SO_4$. Fraction VIII (80—100%) was obtained by the addition of solid $(NH_4)_2SO_4$. All precipitates obtained were dissolved in phosphate buffer and aliquots were diluted to a protein concentration of ≈1 mg/ml determined by the method of Warburg and Christian [45]. For pH studies aliquots (0.1 ml) of appropriate fractions were incubated with substrate as described for 15 min. Buffers used, at a final concentration of 0.05 M, were sodium acetate (pH 4—5.5); sodium phosphate (pH 5.5—8.0); and tris(hydroxymethyl)aminomethane (pH 8.0—9.0).

Gel filtration of enzymes from lamb kidney. The clear supernatants of fractions VI and VII, which contained the prolyl peptidase, were pooled and dialyzed against 500 ml of phosphate buffer for 3 h before application to a Sephadex G-100 column (2 × 90 cm) equilibrated with phosphate buffer. The total amount of protein applied was 250 mg [45]. The column was eluted with phosphate buffer at a flow rate of 20 ml/h. Enzymic activity was measured as described in aliquots of the 5-ml fractions. Fractions corresponding to the region of the chromatogram which releases Leu-[^{14}C]Gly-NH_2 from [9-*Gly-*

cinamide-1-^{14}C] oxytocin and Arg-[^{14}C] Gly-NH_2 from [9-*Glycinamide*-1-^{14}C] arginine vasopressin were pooled. Ammonium sulfate fractions II—IV, which contained most of the activity releasing Gly-NH_2 from oxytocin, were subjected to gel filtration on the same G-100 column. Eluted fractions which released Gly-NH_2 from oxytocin and deamino-oxytocin, were pooled.

Ion exchange chromatography of lamb kidney enzymes. Pooled eluates from gel filtration were applied to a DEAE cellulose (Cellex-D) column (1.5 × 18 cm) prepared as described by Koida et al. [5]. The unabsorbed material was washed from the column with 100 ml phosphate buffer. For the prolyl peptidase the column was eluted with a linear gradient of NaCl (0—0.2 M) in a total of 200 ml of phosphate buffer; for the Gly-NH_2-releasing enzyme, with a stepwise NaCl gradient (0.01 M, 0.25 M, 1 M). Finally, the column was washed with 50 ml of 1 M NaCl. Eluent was monitored for protein concentration and enzymic activity as described above and, in addition, hydrolysis of Z-Gly-Pro-Leu-Gly or Z-Cys(Bzl)-Pro-Leu-Gly-NH_2 was monitored. The protein fractions which hydrolyzed the Leu-Gly bond were pooled and used for further study, while those which hydrolyzed the Pro-X bond were pooled and rechromatographed as described for the first ion-exchange step on a column (1.5 × 20 cm) of Whatman DE 52 DEAE cellulose which had been washed with dilute H_3PO_4 (pH 4.5) and equilibrated with phosphate buffer, pH 6.5, containing 1 mM EDTA and 0.5 mM dithiothreitol. The eluent was monitored and pooled as described.

Characterization of post-proline cleaving enzyme. All studies except the pH profile were carried out in duplicate in 0.05 M phosphate buffer, pH 7.4, containing 1 mM EDTA and 0.5 mM dithiothreitol. pH studies were carried out as described for the crude ammonium sulfate fractions. Aliquots of purified lamb kidney enzyme (0.1 ml, 35 μg) were incubated with 2 μg [9-*Glycinamide*-1-^{14}C] arginine vasopressin in a total volume of 0.5 ml of 0.05 M phosphate buffer for 15 min at 37°C in the presence or absence of 1.0 or 0.1 mM EDTA, dithiothreitol and the chlorides of Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ca^{2+} and Zn^{2+}. The enzyme preparation (0.1 ml, 10 μg) was also incubated with 100 μg of Z-Gly-Pro-Leu-Gly in a total volume of 0.5 ml in the presence of 0.1 and 1.0 mM dithiothreitol. To study the effect of various functional reagents on enzyme activity, aliquots (containing 0.1 mg protein) of lamb kidney or human uterus enzyme were preincubated in the presence or absence of 0.1 or 1.0 mM *p*-chloromercuriphenylsulfonic acid, 1,10-phenanthroline, iodoacetic acid or L-1-tosylamido-2-phenylethylchloromethyl ketone in a total volume of 0.45 ml for 30 min at 4°C. The substrate specificity of the post-proline cleaving enzyme was tested with a series of unlabelled peptides which are listed in Table V. Peptides were incubated for 4 h at 37°C [43].

Preparation of cats for in vivo studies. Female cats (2.5—3.0 kg) were anesthetized by intraperitoneal injection of sodium pentobarbital (36 mg/kg). One femoral vein was cannulated for intravenous hormone injection (and additional anesthetic). Tracheotomy was performed, and the urinary bladder was cannulated for urine collection. Water diuresis was induced by oral hydration (8%

of body weight) and maintained approximately constant throughout the experiment. The hydration fluid consisted of 0.15% NaCl solution containing 0.5% dextrose and 2% ethanol.

Hormone injection and renal excretion. Labelled hormone (3 or 10 μg) was injected after the rate of urine flow reached a constant value. Urine was then collected and radioactive products determined as described [8].

Results

On the basis of release of [^{14}C]Gly-NH_2, kidney extracts of rat, mouse, hamster and pig inactivate [9-*Glycinamide*-1-^{14}C]oxytocin most rapidly, amounting to more than 60% within a 10 min incubation period. Extracts of human, rabbit, dog, cat, lamb, chicken and pigeon kidney inactivate [9-*Glycinamide*-1-^{14}C]oxytocin at a slower rate (Table I). While with extracts which degrade [9-*Glycinamide*-1-^{14}C]oxytocin rapidly it is difficult to detect any products apart from Gly-NH_2 (e.g., rat, Fig. 1A), substantial levels of Leu-Gly-NH_2 are present (except in the case of rabbit) with extracts of species which inactivate [9-*Glycinamide*-1-^{14}C]oxytocin at a slower rate (e.g., cat, Fig. 1B). With extracts of certain of these latter species we found that in addition to the above metabolites measurable amounts (after 30 min incubation about 10%) of Pro-Leu-Gly-NH_2 are released from oxytocin (e.g., chicken, Fig. 1C). With extract of lamb kidney, both labelled Gly-NH_2 and Leu-Gly-NH_2 are formed as long as [9-*Glycinamide*-1-^{14}C]oxytocin is available in the incubation mixture (first 10 to 15 min). Continuation of incubation leads to a reduction of labelled Leu-Gly-NH_2 with increase of [^{14}C]Gly-NH_2. Similar data are obtained with species other than lamb and pigeon (Table I) (provided the incubation is carried out for 2 h instead of the 1 h shown in the table).

The inactivation of [9-*Glycinamide*-1-^{14}C]arginine vasopressin is generally slower than that of [9-*Glycinamide*-1-^{14}C]oxytocin with kidney extract of a given species if one compares 15-min incubation periods (Table I). In contrast to oxytocin greater amounts of the labelled C-terminal dipeptide (Arg-Gly-NH_2) rather than [^{14}C]Gly-NH_2 are released from the antidiuretic hormone.

Fractionation of lamb kidney extract with ammonium sulfate. The extract of lamb kidney was fractionated into eight fractions (I—VIII) (Fig. 2). Fractions II—V (referred to as peak A) contained the enzymatic activity catalyzing the release of Gly-NH_2 from [9-*Glycinamide*-1-^{14}C]oxytocin but not from [9-*Glycinamide*-1-^{14}C]arginine vasopressin. The activity which releases labelled Leu-Gly-NH_2 from [9-*Glycinamide*-1-^{14}C]oxytocin and Arg-Gly-NH_2 from [9-*Glycinamide*-1-^{14}C]arginine vasopressin, was present in fractions VI and VII. There was some overlap of the activities in fractions V and VI.

Gel filtration of lamb kidney enzyme activities. Pooled fractions from the ammonium sulfate fractionation were subjected to gel filtration on a Sephadex G-100 column in order to achieve further purification of the prolyl peptidase or the Gly-NH_2-releasing enzyme. In the case of the former activity, considerable amounts of inert protein were separated, but the enzymatic activity releas-

TABLE I

ENZYMATIC RELEASE OF GLY-NH_2 AND LEU-GLY-NH_2 FROM OXYTOXIN AND OF GLY-NH_2 AND ARG-GLY-NH_2 FROM VASOPRESSIN *

Species	L—G	G	L—G	G	L—G	G	L—G	G	L—G	G	A—G	G
	(5 min, ^{14}C-Oxy)		(10 min, ^{14}C-Oxy)		(15 min, ^{14}C-Oxy)		(30 min, ^{14}C-Oxy)		(60 min, ^{14}C-Oxy)		(15 min, ^{14}C-AVP)	
Mouse	neg	40	3	60	7	70	8	90	8	90	8	7
Rat	neg	62	neg	70	trace	85	trace	86	—	—	42	35
Hamster	neg	66	neg	90	neg	90	neg	99	neg	99	28	10
Rabbit	neg	30	neg	50	3	60	7	80	—	—	—	—
Pig	2	55	neg	70	neg	95	neg	95	—	—	20	15
Lamb	14	35	30	45	25	70	15	82	1	95	45	10
Cat **	7	17	10	24	18	34	20	34	35	44	18	2
Dog	10	14	13	27	18	42	18	53	25	57	—	—
Pigeon	5	30	6	45	12	70	15	80	9	87	22	9
Chicken **	11	12	15	21	35	36	32	50	—	—	30	11
Human	5	17	7	35	9	43	12	75	15	80	9	5

* Radioactively labelled neurohypophyseal hormone ([9-*Glycinamide*-1-^{14}C] oxytocin or [9-*Glycinamide*-1-^{14}C] arginine vasopressin) was incubated with kidney extract as described in the text. The labelled metabolites (Gly-NH_2, G; Leu-Gly-NH_2, L-G; Arg-Gly-NH_2, A-G) were identified by high voltage electrophoresis. Results of a typical experiment are given in percent of total radioactivity recovered.

** Homogenates also release Pro-Leu-Gly-NH_2 from [9-*Glycinamide*-1-^{14}C] oxytocin.

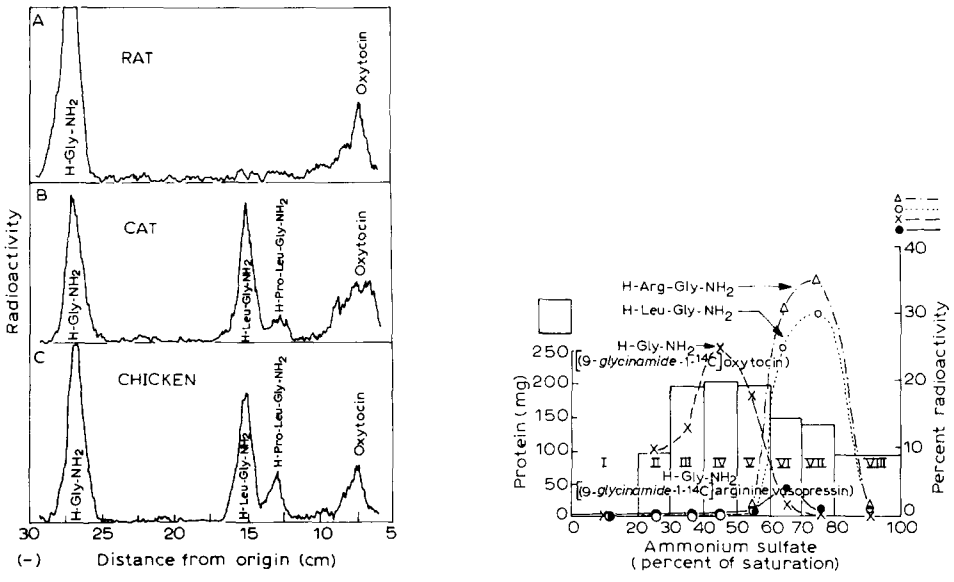

Fig. 1. Electrophoretic separation of radioactive products resulting from incubation of [9-*Glycinamide*-1-^{14}C]oxytocin (5 μg) with kidney extracts (0.2 ml) from various species. After varying lengths of time at 37° C, an aliquot of digest mixture was subjected to high-voltage electrophoresis at pH 3.5 for 1.5 h at 1500 V. Products were located on a chromatogram scanner and identified by comparison with authentic markers. For details of incubation and electrophoresis see text. A, Rat kidney extract, 5-min incubation; B, cat kidney extract, 60-min incubation; C, chicken kidney extract, 30-min incubation.

Fig. 2. Separation of neurohypophyseal hormone inactivating activities in lamb kidney extract by ammonium sulfate fractionation. Bars represent total amount of protein in each fraction, determined according to ref. 45. Hormone inactivating activity, determined by incubating 0.2 mg protein with 2 μg [9-*Glycinamide*-1-^{14}C]oxytocin or [9-*Glycinamide*-1-^{14}C]arginine vasopressin for 15 min at 37° C followed by electrophoretic identification of products is expressed as percent of total radioactivity present as product. [^{14}C]Gly-NH_2 from [9-*Glycinamide*-1-^{14}C]oxytocin (X———X); [^{14}C]Gly-NH_2 from [9-*Glycinamide*-1-^{14}C]arginine vasopressin (●———●); Leu-[^{14}C]Gly-NH_2 (○- - - - -○); Arg-[^{14}C]-Gly-NH_2 (△— . — .△).

ing labelled Gly-NH_2 from [9-*Glycinamide*-1-^{14}C]arginine vasopressin was not separated from the prolyl peptidase activity; in addition, small amounts of activity releasing Gly-NH_2 from [9-*Glycinamide*-1-^{14}C]oxytocin were present. The prolyl peptidase activity was present in fractions corresponding to an elution volume of 140—160 ml (K_{av} = 0.28). Post-proline cleaving activity isolated from human uterus as described [12] possessed an identical elution volume when subjected in this study to gel filtration, and the elution volumes of these enzymes correspond to an approximate molecular weight of 60 000, estimated on the basis of comparison with ribonuclease, chymotrypsinogen, ovalbumin and bovine albumin [46].

The major enzymic activity responsible for the release of labelled Gly-NH_2 from [9-*Glycinamide*-1-^{14}C]oxytocin emerged from the column in an elution volume of 110—135 ml (K_{av} = 0.13).

Further purification of lamb kidney enzymes by ion exchange chromatography. The pooled fractions from gel filtration containing either prolyl pep-

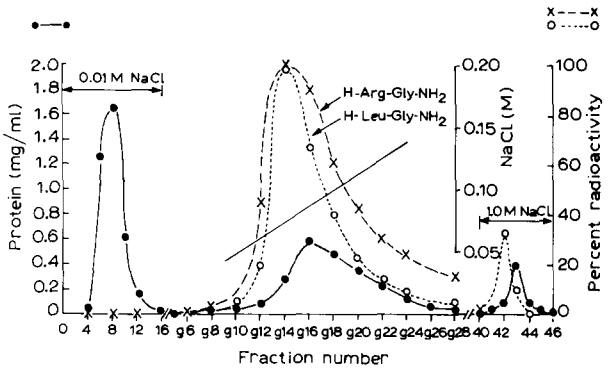

Fig. 3. Chromatography of lamb kidney post-proline cleaving enzyme on DEAE cellulose. Enzymatic activity was eluted using a gradient of 0.0—0.2 M NaCl in phosphate buffer, as described in the text. Protein content was determined according to ref. 45 (•———•). Enzyme activity was measured as described in the text by incubation of 0.2 ml aliquots of alternate fractions with 2 μg [9-*Glycinamide*-1-^{14}C]oxytocin or [9-*Glycinamide*-1-^{14}C]arginine vasopressin and electrophoretic identification of products. Amount of inactivation is given as percent of total radioactivity present as Arg-[^{14}C]Gly-NH_2 or Leu-[^{14}C]Gly-NH_2. Leu-Gly-NH_2 (○-----○); Arg-[^{14}C]Gly-NH_2 (X-----X).

tidase or Gly-NH_2-releasing enzyme were subjected to DEAE-cellulose column chromatography. Essentially all of the post-proline cleaving activity was recovered in the range of 0.06 to 0.1 M NaCl (Fig. 3) and upon rechromatography was recovered in the same area. Throughout the chromatogram no enzymatic activity was detected which catalyzed the release [^{14}C]Gly-NH_2 from [9-*Glycinamide*-1-^{14}C]oxytocin or from [9-*Glycinamide*-1-^{14}C]arginine vasopressin, suggesting that these enzymes are inactivated, absorbed or merely diluted out during these purification steps. The final preparation failed to release significant amounts of N- and C-terminal amino acid residues from a number of acyclic peptides not containing proline. The results of the purification procedures of the peptidase are summarized in Table II.

When the fractions corresponding to the elution volume of 110—135 ml were subjected to ion-exchange chromatography, essentially all of the activity releasing Gly-NH_2 from [9-*Glycinamide*-1-^{14}C]oxytocin and from Z-Cys(Bzl)-

TABLE II

PURIFICATION AND RECOVERY OF POST-PROLINE CLEAVING ENZYME

Unit = 1 μmol/mg protein/min of Arg-Gly-NH_2 released from arginine vasopressin.

	Total protein (mg)	Total activity (units)	Yield of activity (%)	Specific activity (unit/mg)	Degree of purification
Supernatant	11 600	348	100	0.030	1
60—80% $(NH_4)_2SO_4$	840	300	86	0.36	12
G-100	240	208	60	0.86	29
DEAE-cellulose pH 6.5 chromatography	50	160	46	3.2	107
DEAE-cellulose pH 6.5 rechromatography	15	80	23	5.4	180

Pro-Leu-Gly-NH_2 was recovered in the fraction eluted with 0.25 M NaCl. This fraction also released Gly-NH_2 from deamino-oxytocin and [8-phenylalanine]-oxytocin, but no detectable amounts of Gly-NH_2 were liberated when [8-glutamine]-, [8-serine]-, [8-valine]-, [8-alanine]-oxytocin, or [9-*Glycinamide*-1-^{14}C]arginine vasopressin were the substrates; the procedure for testing for the release of Gly-NH_2 from the unlabelled hormone analogs was the same as described [5].

Effect of pH on the activity of partially purified lamb kidney enzymes. Initially the pH profiles of the enzymatic activities present in lamb kidney were carried out using fractions IV and V, as well as VI and VII (Fig. 2). The enzyme which removes [^{14}C]Gly-NH_2 from [9-*Glycinamide*-1-^{14}C]oxytocin exhibited in sodium phosphate buffer its pH optimum between 6.0 and 6.5 both as the crude ammonium sulfate fraction and after purification steps. The pH optimum for the prolyl peptidase was found to be between 7.0 and 7.5; the pH profiles for the release of the labelled C-terminal dipeptides from [9-*Glycinamide*-1-^{14}C]oxytocin and [9-*Glycinamide*-1-^{14}C]arginine vasopressin were identical (Fig. 4). Moreover, after further purification of the prolyl peptidase by gel filtration and DEAE chromatography the pattern of pH dependence was retained. The same pH optimum is also obtained for the post-proline cleaving enzyme purified from human uteri. In addition, the time course of release of labelled C-terminal dipeptide from [9-*Glycinamide*-1-^{14}C]oxytocin and [9-*Glycinamide*-1-^{14}C]arginine vasopressin was nearly identical over 60 min for both the lamb kidney enzyme and the human uterine enzyme.

Effect of divalent metal ions, EDTA and dithiothreitol on the activity of lamb kidney prolyl peptidase. The activity of post-proline cleaving enzyme from lamb kidney is neither inhibited nor stimulated significantly by 10^{-4} M—10^{-3} M EDTA Mg^{2+}, Mn^{2+}, or Fe^{2+}, but is inhibited to different de-

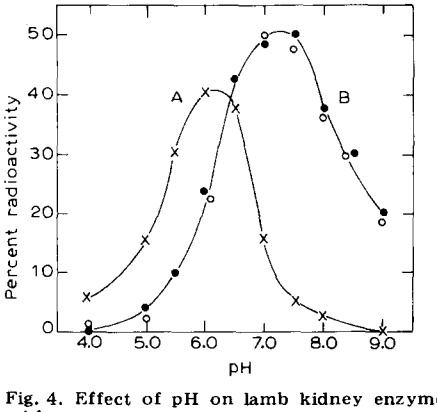

Fig. 4. Effect of pH on lamb kidney enzymes which release (A) [^{14}C]Gly-NH_2 from [9-*Glycinamide*-1-^{14}C]oxytocin (X———X); or (B) C-terminal dipeptide from [9-*Glycinamide*-1-^{14}C]oxytocin (●———●) or [9-*Glycinamide*-1-^{14}C]arginine vasopressin (○———○). Aliquots of ammonium sulfate fractions were incubated with 2 μg [9-*Glycinamide*-1-^{14}C]oxytocin or [9-*Glycinamide*-1-^{14}C]arginine vasopressin for 15 min at 37°C and products identified by high voltage electrophoresis. Inactivation is expressed as percent of total radioactivity present as [^{14}C]Gly-NH_2 or labelled C-terminal dipeptide.

TABLE III

EFFECT OF DIVALENT CATIONS, EDTA AND DITHIOTHREITOL ON THE ACTIVITY OF POST-PROLINE CLEAVING ENZYME FROM LAMB KIDNEY

Enzyme fractions (35 μg of protein) obtain from ion exchange chromatography were incubated in duplicate with 2 μg of [9-*Glycinamide*-1-^{14}C]arginine vasopressin in the absence and presence of metal chlorides, EDTA or dithiothreitol in 50 mM phosphate buffer pH 7.4 (except see under [a]. For procedures for incubation and analysis of digest see text.

Compound Added	Concentration (mM)	Percent activity
None		100; 100
Mg^{2+}	0.1	105; 100
	1.0	110; 100
Mn^{2+}	0.1	100; 95
	1.0	116; 95
Fe^{2+}	0.1	100; 105
	1.0	100; 95
Co^{2+}	0.1	60; 55
	1.0	45; 50
Ca^{2+}	0.1	60; 40 [a]
Zn^{2+}	0.1	40; 20 [a]
EDTA	0.1	95; 95 [a]
	1.0	105; 100 [a]
Dithiothreitol [b]	0.1	100; 105
	1.0	125; 125

[a] Incubations were carried out in tris(hydroxymethyl)aminomethane buffer, pH 7.8.
[b] Activity of the enzyme preparation with Z-Gly-Pro-Leu-Gly (100 μg) as substrate was enhanced by 20% in the presence of 0.1 and 1.0 mM dithiothreitol.

grees by 10^{-4} M Co^{2+}, Ca^{2+}, and Zn^{2+}. The activity of the enzyme is stimulated by dithiothreitol (Table III).

Effect of enzyme inhibitors on the activity of prolyl peptidase. The post-proline cleaving activities from both lamb kidney and human uterus are almost

TABLE IV

EFFECT OF ENZYME INHIBITORS ON THE ACTIVITY OF HUMAN UTERUS AND LAMB KIDNEY POST-PROLINE CLEAVING ENZYME

Enzyme preparations (0.1 mg protein) were incubated with 5 μg [9-*glycinamide*-1-^{14}C] arginine vasopressin (uterine preparation) or 5 μg [9-*glycinamide*-1-^{14}C]oxytocin (kidney preparation) in the presence or absence of inhibitor in 50 mM phosphate buffer, pH 7.4. See text for details of incubation and analysis.

Inhibitor	Concentration (mM)	Enzyme inhibition *	
		Human uterus	Lamb kidney
None	—	—	—
p-Chloromercuriphenyl sulfonic acid	1.0	++	++
	0.1	++	++
1,10-Phenanthroline	1.0	++	+
	0.1	+	+
Iodoacetic acid	1.0	+	+
	0.1	+	—
L-1-Tosylamido-2-phenylethyl-chloromethyl ketone	1.0	++	++
	0.1	++	++

* Lack of inhibition —; up to 50% inhibition +; 80—100% inhibition ++.

TABLE V

DEGRADATION OF PEPTIDES BY POST-PROLINE CLEAVING ENZYME [a]

Substrate	Product(s) identified
1 Oxytocin	Leu-Gly-NH_2 [b]
2 [1,6-aminosuberic acid] oxytocin	Leu-Gly-NH_2 [b]
3 [8-arginine] vasopressin	Arg-Gly-NH_2 [b]
4 [1,6-aminosuberic acid, 8-arginine] vasopressin	Arg-Gly-NH_2 [b]
5 H-Asp-Arg-Val-Tyr-Val-His-Pro-Phe ([Val^5] angiotensin II)	Phe[c]
6 H-Cys(Bzl)-Pro-Leu-Gly-NH_2	Leu-Gly-NH_2 [b]
7 H-Glu-Tyr-Pro-Arg-Pro	Arg, Pro[d, f]
8 Z-Gly-Pro-Leu-Gly	Leu-Gly[b, d]
9 [7-glycine] oxytocin	None
10 [1,6-aminosuberic acid, 7-glycine] oxytocin	None
11 [8-alanine] oxytocin	H[b, e]
12 [1-β-mercaptopropionic acid, 8-alanine] oxytocin	Ala, Gly [d]
13 [8-serine] oxytocin	H[b, e]
14 [8-threonine] oxytocin	H[b, e]
15 [8-phenylalanine] oxytocin	H[b, e]
16 [8-valine] oxytocin	H[b, e]
17 [8-glutamine] oxytocin	H[b, e]
18 [8-(N^{ε}-formyl)lysine] oxytocin	H[b, e]
19 [8-citrulline] oxytocin	H[b, e]
20 [8-proline] oxytocin	None
21 [1-β-mercaptropropionic acid, 7-glycine] oxytocin	None
22 [1-β-mercaptopropionic acid, 8-arginine] vasopressin	Arg-Gly-NH_2 [b]
23 [1-β-mercaptopropionic acid, 8-D-arginine] vasopressin	D-Arg-Gly-NH_2 [b]
24 [8-lysine] vasopressin	Lys-Gly-NH_2 [b]
25 [1-β-mercaptopropionic acid, 8-lysine] vasopressin	Lys-Gly-NH_2 [b]
26 [7-alanine] oxytocin	None
27 [1-β-mercaptopropionic acid, 7-alanine] oxytocin	None
28 ‹Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (LRH)	Gly-NH_2 [b]
29 H-Thr-Lys-Pro-Arg(Tuftsin)	Arg[c, f]
30 H-Phe-Phe-Val-Pro-Pro-AlaOBu[t]	Ala[d]
31 H-Gln-Ala-Pro-Lys(Boc)-Gly-NH_2	Lys, Gly[d]
32 H-Glu-Trp-Pro-Arg-Pro	Arg-Pro[b, d]
33 H-Ala-Tyr-Pro-Thr-Pro-Ala-Arg	Ala, Arg[d, g]
34 H-Leu-Arg-Pro-Gly-NH_2	Gly-NH_2 [b]
35 Z-Pro-His-Phe	His-Phe[h]
36 H-Pro-Pro-AlaOBu[t]	Ala[d]
37 H-Ile-His-Pro-Phe	Phe[c]
38 H-Pro-Ile-Asn(CHO_A)-Ala-Thr-Leu	None
39 Z-Phe-Phe-Pro-Pro	None
40 H-Pro-Pro-Pro-Pro	None
41 H-Pro-Leu-Gly-NH_2 · $\frac{1}{2}H_2O$	None
42 Z-Pro-Leu-Gly-NH_2	Leu-Gly-NH_2 [b]
43 H-Pro-Gly-NH_2	None
44 Z-Pro-Gly-NH_2	None
45 [5-valine] angiotensin I	Phe, His[d], Leu[c]
46 [8-leucine] angiotensin II	Leu[c]
47 [8-isoleucine] angiotensin II	Ile[c]
48 [8-valine] angiotensin II	Val[b, c]
49 [4-phenylalanine, 8-alanine] angiotensin II	Ala[c]
50 [4-histidine, 6-tyrosine] angiotensin II	Phe[c]
51 [6-arginine] angiotensin II	Phe[c]

[a] Substrates 1—10 were incubated with prolyl peptidase from both lamb kidney and human uterus. In both cases identical products were released. Substrates 11—51 were incubated with lamb kidney enzyme only.

[b] Products were identified by high voltage electrophoresis of the enzyme digest and comparison with authentic samples. Products were located by scanning in the case of radioactive substrates or by ninhydrin spray.

[c] In cases were Pro is the penultimate amino acid, digest mixtures were subjected directly to amino acid analysis to determine release of free amino acids.

[d] Products were identified by elution from paper after high voltage electrophoresis, followed by hydrolysis and amino acid analysis.

(continued on p. 151)

TABLE VI

RATE OF RELEASE OF C-TERMINAL AMINO ACIDS FROM ANGIOTENSIN II AND ANALOGS BY LAMB KIDNEY POST-PROLINE CLEAVING ENZYME

Hormone	Release of C-Terminal Residue (nmol/h)
[Ile^5] Angiotensin II	16
Ile-His-Pro-Phe	16
[Arg^6] Angiotensin II	17
[His^4, Tyr^6] Angiotensin II	17
[Phe^4, Ala^8] Angiotensin II	3
[Ile^8] Angiotensin II	3
[Leu^8] Angiotensin II	3
[Val^8] Angiotensin II	1

totally inhibited by *p*-chloromercuriphenyl sulfonic acid and L-tosylamido-2-phenylethylchloromethyl ketone at 0.1 or 1.0 mM and by 1.0 mM 1,10-phenanthroline. Both enzymes were somewhat inhibited by iodoacetic acid (Table IV).

Degradation of peptides by post-proline cleaving enzyme from lamb kidney. The purified kidney enzyme releasing dipeptide amide from oxytocin and vasopressin was found to hydrolyze only the Pro-X bond(s) of the peptides studied (Tables V and VI). N-Terminal acylated proline-containing dipeptides, free dipeptides and tripeptides are not cleaved, while N-terminal protected tripeptides can be hydrolyzed. Incubation of [1-β-mercaptopropionic acid, 8-D-arginine]vasopressin with the lamb kidney enzyme led to release of D-Arg-

TABLE VII

COMPARISON OF INACTIVATION RATES OF [MPR^1, D-ARG^8]VASOPRESSIN WITH THOSE OF [MPR^1, ARG^8]VASOPRESSIN AND [MPR^1, LYS^8]VASOPRESSIN BY POST-PROLINE CLEAVING ENZYME FROM LAMB KIDNEY *

	Percent Inactivation		
	[Mpr^1, D-Arg^8]vasopressin	[Mpr^1, Arg^8]vasopressin	[Mpr^1, Lys^8]vasopressin
Zero time (control)	0	5	0
15 min incubation	30	25	20
60 min incubation	75	70	60

* Peptides were incubated with lamb kidney enzyme as described in the text and residual hormonal activity was determined by the pressor assay [15], using either the 4-point method or matches (in which samples are diluted to approximately the same level of activity as the USP Posterior Pituitary Reference Standard). At least 3 rats were used for each determination. Values are percentage of inactivation, and represent the mean of two independent experiments, in which values agreed within 10%.

[e] H = N-terminal hepatapeptide of oxytocin.

[f] Arg on the high voltage electrophoretogram was detected with Sakaguchi reagent [65].

[g] Two other spots were seen on high voltage electrophoresis; on elution followed by hydrolysis and amino acid analysis, each spot was found to contain Ala, Tyr, 2 Pro and Thr.

[h] The spot on the electrophoretogram was His-positive as judged by the Pauly reagent [66]; on elution followed by amino acid analysis without hydrolysis no free amino acids were found.

TABLE VIII

COMPOUNDS EXCRETED INTO URINE OF CAT FOLLOWING INTRAVENOUS INJECTION OF [9-*GLYCINAMIDE*-1-^{14}C] OXYTOCIN AND [9-*GLYCINAMIDE*-1-^{14}C] ARGININE VASOPRESSIN*

Hormone injected	Radioactivity excreted	Time of urine collection after injection of [^{14}C] hormone (in min)								
		0-2	2-5	5-7	7-10	10-15	15-20	20-30	30-40	40-50
	total cpm/fraction	2680	55 500	24 240	34 738	36 400	33 500	45 600	26 600	18 816
^{14}C-Oxy (10 μg)	Leu-Gly-NH_2	n.d.	15	15	16	16	15	16	17	n.d.
	Gly-NH_2	n.d.	11	12	15	14	14	15	14	n.d.
	Oxytocin	n.d.	45	68	53	53	56	50	60	n.d.
	total cpm/fraction	320	26 950	25 168	22 650	28 470	21 420	38 525	18 905	13 539
^{14}C-AVP (10 μg)	Arg-Gly-NH_2	n.d.	2	3	5	5	6	8	9	8
	Gly-NH_2	n.d.	2	3	5	6	12	15	30	23
	Vasopressin	n.d.	63	62	53	53	55	57	37	38

* Amounts of intact hormones and metabolites are expressed in percent of radioactivity excreted.

Gly-NH_2 as shown in Table V. A time-course study of the inactivation of this analog was also carried out, and residual activity was measured by the rat pressor assay [15]. The data in Table VII reveal that the analog is inactivated fairly rapidly by the enzyme, with about 70% of the activity lost after a 60 min incubation.

In vivo studies: excretion of radioactivity in the urine of cat. Urine flow and excretion of radioactivity into the urine of the cat were measured for a period of 100 min following intravenous administration of 10 μg of [9-*Glycinamide*-1-^{14}C]oxytocin or [9-*Glycinamide*-1-^{14}C]arginine vasopressin. Independent of the degree of antidiuretic response observed with different cats, the excretion rate of the radioactivity had a maximum from 2 to 10 min after intravenous injection. In experiments with both [9-*Glycinamide*-1-^{14}C]oxytocin and [9-*Glycinamide*-1-^{14}C]arginine vasopressin, both labelled Gly-NH_2 and C-terminal dipeptide were excreted into the urine (Table VIII). With [9-*Glycinamide*-1-^{14}C]oxytocin, labelled Leu-Gly-NH_2 accounted for about 15% of the total radioactivity in each fraction, as was the case with [^{14}C]Gly-NH_2. With [9-*Glycinamide*-1-^{14}C]arginine vasopressin only a small percentage of radioactivity is associated with metabolites and, in contrast to [9-*Glycinamide*-1-^{14}C]oxytocin, they seem to increase in each fraction with time. Note that the percentage of identified compounds does not fully account for the total cpm in each fraction. Because of low radioactive counts and other technical problems it was not possible to extend a determination of individual components present in each fraction beyond a period of 50 min. The data shown in Table VIII are typical results obtained with one cat. Experiments using 3 and 10 μg injections of hormone were carried out with a total of 5 animals.

Discussion

The first specific mechanism of enzymatic inactivation in vivo of neurohypophyseal hormones was the finding of the susceptibility of the cysteinyltyrosyl peptide bond of oxytocin to hydrolytic cleavage by a group of enzymes frequently referred to by the generic name of oxytocinase [47] present in placenta and plasma of pregnant primates [48,71,72]. It appears that among the several aminopeptidases present in colostrum [43,49,50] one actually in certain instances is the placental cystine aminopeptidase [51]. Nevertheless, the physiological role of the inactivation of oxytocin by cystine aminopeptidase in relation to the regulation of pregnancy and parturition of primates is at present unclear [52]. A potentially physiologically significant mechanism of oxytocin inactivation involving the stepwise removal of N-terminal amino acid residues by an exopeptidase present in hypothalamic preparations results in the formation of Pro-Leu-Gly-NH_2 [53,54]; this C-terminal tripeptide of oxytocin has been suggested to be a natural factor inhibiting the release of pituitary melanocyte-stimulating hormone [53,55] and it has been shown to possess several extrapituitary effects [56].

Consideration of the preferred three-dimensional structure of oxytocin in solution [57] suggested that peptide bonds located outside the 20-membered ring component of neurohypophyseal hormones should be more susceptible to

hydrolytic cleavage by enzymes than those within the ring [58]. Cleavage of the peptide chain of hormones between residiues 8 and 9 (with a concomitant release of Gly-NH_2) or between 7 and 8 (release of Leu-Gly-NH_2 from oxytocin or Arg-Gly-NH_2 from arginine vasopressin) could represent important mechanisms for enzymic inactivation of these hormones (see ref. 43). In view of our emerging awareness of the distribution among numerous tissues of high levels of different enzymic activities which rapidly release C-terminal fragments from neurohypophyseal hormones, it was thought appropriate to investigate the enzymatic content of one of the target and inactivating organs of neurohypophyseal hormones, i.e., the kidney, of several species.

With kidney preparations of species which release 50% or more of labelled Gly-NH_2 from [9-*Glycinamide*-1-^{14}C]oxytocin witin the first 10-min incubation period, the C-terminal amino acid moiety was the only radioactively labelled digest product present in large quantities. In contrast, with kidney preparations which release less than 50% of [^{14}C]Gly-NH_2 from [9-*Glycinamide*-1-^{14}C]oxytocin during the first 10-min incubation period, labelled Leu-Gly-NH_2 was present at significant levels (Table I).

The data given in Table I for Gly-NH_2 represent the summation of [^{14}C]Gly-NH_2 released from intact [9-*Glycinamide*-1-^{14}C]oxytocin as well as the amount formed, in secondary enzymatic reactions, from Leu-[^{14}C]Gly-NH_2. Therefore, even with kidney extracts of species in which practically only Gly-NH_2 was found, it is probable that the post-proline cleaving enzyme is present and contributes to the inactivation of hormone. However, the post-proline cleaving enzyme is less efficient in inactivating [9-*Glycinamide*-1-^{14}C]-oxytocin than the enzyme releasing Gly-NH_2 and, moreover, the Leu-Gly-NH_2 formed is rapidly cleaved by aminopeptidases present in the extracts [9]. Since the rates of cleavage of the Pro-Leu bond in [9-*Glycinamide*-1-^{14}C]oxytocin and the Pro-Arg bond in [9-*Glycinamide*-1-^{14}C]arginine vasopressin by post-proline cleaving enzyme are similar [12], in kidney extracts of those species which slowly inactivate [9-*Glycinamide*-1-^{14}C]arginine vasopressin by direct release of Gly-NH_2 the substantial levels of Arg-Gly-NH_2 formed during a 15-min incubation period with arginine vasopressin as substrate should represent a fairly accurate indication of the level of enzymatic activity capable of cleaving the peptide bond between residues 7 and 8 of neurohypophyseal hormones. Results depicted in Table I support our earlier conclusions that the overall mechanism of inactivation of arginine vasopressin in rat kidney in vitro differs from that of oxytocin inactivation [9]; apparently the enzyme which releases Gly-NH_2 directly from arginine vasopressin is present at low concentrations or is not active in rat kidney, thereby allowing predominant expression of other inactivating activities (e.g., cleavage of the Cys-Tyr and Pro-Arg bonds). This same type of pattern holds when comparing inactivation of oxytocin by kidneys of various species, i.e., while both enzymes (Gly-NH_2-releasing or post-proline cleaving) are present, the ratio of enzyme concentrations or degree of activities determines the major pathway of hormone inactivation. Since lamb kidney clearly contains high levels of activity of post-proline cleaving enzyme in addition to the Gly-NH_2-releasing activity, this readily available tissue was chosen for purification of the two enzyme activities.

The results of fractionation of the crude lamb kidney enzyme mixture

show that the enzyme which releases Gly-NH_2 from oxytocin and the post-proline cleaving enzyme are separate entities. Both preparations appear to be free of significant amounts of aminopeptidase and carboxypeptidase activities, although analytical disc electrophoresis revealed that both preparations are contaminated by protein without enzymatic activity. Results of incubation of this preparation with neurohypophyseal peptides with substitutions in position 8 suggest that the Gly-NH_2-releasing enzyme has a chymotrypsin-like substrate specificity as was found for the Gly-NH_2-releasing activity of rat kidney [5]. The different pH profiles of the post-proline cleaving enzyme and the glycinamide-releasing enzyme, both in the crude and partially purified states, further confirms their distinct identities.

The purified prolyl peptidase of lamb kidney was inhibited by $Zn^{2+} > Ca^{2+} > Co^{2+}$, while Mg^{2+}, Mn^{2+} and Fe^{2+} were ineffective (Table III). Interestingly, dithiothreitol at a concentration of 1 mM enhanced the enzyme activity. The enhancement of enzyme activity is independent of the presence of the disulfide bridge in the substrate since it is also observed with Z-Gly-Pro-Leu-Gly in the presence of 0.1 and 1.0 mM dithiothreitol. By way of comparison of properties of post-proline cleaving enzyme from lamb kidney and human uterus, the stimulatory effect of dithiothreitol had also been observed with the enzyme purified from the latter source as were some of the ion effects [12]. In view of the observed stimulatory effect of dithiothreitol, prolyl peptidase from both sources was treated with a number of enzyme inhibitors including sulfhydryl-specific reagents. Both enzyme preparations respond in a similar manner to various enzyme inhibitors (Table IV). The effects of iodoacetic acid and *p*-chloromercuriphenyl sulfonic acid may indicate the presence in the enzymes of a free sulfhydryl group. The fact that L-1-tosylamido-2-phenylethylchloromethyl ketone abolishes activity of post-proline cleaving enzyme (Table IV) warrants further attention, although it appears premature to reach any conclusions concerning the active site of the enzyme. Under identical gel filtration conditions, the enzymes behave identically, indicating a similar molecular weight (≈60 000). Furthermore, the substrate specificity of both enzyme preparations is identical as far as a direct comparison was made. The specificity of the prolyl peptidase from human uterus was previously tested using oxytocin, arginine vasopressin, [Ile^5]angiotensin II as well as some analogs of these hormones and some smaller peptides [12,43]. Only the peptide bond at the carboxyl side of proline was cleaved in these substances.

In the present study the specificity of the purified lamb kidney enzyme has been determined with a larger number of substrates. Neurohypophyseal hormone analogs which do not contain Pro are not cleaved as was the case with a number of additional peptides used as controls. In all peptides containing the Pro-X element the peptide bond at the carboxyl side of proline was cleaved irrespective of the nature of X provided it is not Pro; note, however, that no Gly-NH_2 was released from (8-proline)oxytocin, which contains a Pro-Pro-X sequence. The Pro-X peptide bond is not cleaved when a free Pro occupies the N-terminal position, but is cleaved when the Pro residue is further within the peptide chain; or occupies the penultimate C-terminal position, although in this case the nature of the residue at the carboxyl side of proline influences the rate of cleavage. This is illustrated by the experiments in Table VI, in which pep-

tides of the angiotensin family possessing substitutions in the C-terminus (position 8) were incubated for various time intervals with lamb kidney enzyme. Analogs with aliphatic residues at the carboxyl side of Pro are hydrolyzed more slowly than those with Phe at this position.

(1-β-Mercaptopropionic acid, 8-D-arginine)vasopressin, a synthetic analog of the natural mammalian antidiuretic principle, has been subject in recent years to clinical studies for its usefulness in the management of severe diabetes insipidus in human [59—63]. It was hypothesized [59,62] that the D-Arg residue in place of the L-Arg in position 8 may protect the analog from enzymatic inactivation and explain its long half-life. However, in the light of new information this does not seem to be the case. Surprising to us was the finding that (1-β-mercaptopropionic acid, 8-D-arginine)vasopressin (Table V, compound 23) released upon treatment with the post-proline cleaving enzyme the D-Arg-Gly-NH_2 moiety, i.e., the L-Pro-D-Arg peptide bond is hydrolyzed. In fact, when the inactivation of this analog was compared with that containing the L-arginine residue ((1-β-mercaptopropionic acid, 8-arginine)vasopressin) and with (1-β-mercaptopropionic acid, 8-lysine)vasopressin, it was found by bioassay that all three peptides lose their activity at approximately the same rate (Table VII). Thus, to whatever degree that the prolyl peptidase may contribute to the inactivation in vivo of neurohypophyseal peptides, it cannot be responsible for the prolonged antidiuretic responses observed with (1-β-mercaptopropionic acid, 8-D-arginine)vasopressin [59,63]. Indeed, it was reported [64] that substitution of D-Arg for the L residue had little influence on the duration of action in diabetes insipidus rats. Moreover, there is recent evidence [9] that hydrolysis of the peptide bond between Arg and Gly-NH_2 does not play a major role in the inactivation of vasopressin (at least in rat) and it appears unlikely that the D-Arg-Gly-NH_2 bond is a primary target for enzymatic attack. It seems then that the Pro-Arg bond and N-terminus of arginine vasopressin are important sites for enzymatic inactivation. The latter contention is in accord with studies showing that replacement of the terminal amino group by a hydrogen is the structural change that has the greatest effect in prolonging duration of antidiuretic action [64]. However, the duration of action of a hormone or analog is not only governed by enzymatic inactivation in the target organ and other sites but also by relative hydrophobicity of the peptide influencing affinity parameters for various binding sites and the distribution pattern; unfortunately at present the degree to which such factors contribute to duration of action is little understood.

In earlier studies we found a direct correlation in the inactivation of neurohypophyseal hormones by rat kidney tissue at three levels of complexity: in vivo, in vitro in the homogenate, and in the isolated perfused kidney. We were interested in a similar comparison with a species that inactivated neurohypophyseal hormones slower than the rat, as it was hoped that the prolyl peptidase activity would be more apparent. Kidney homogenate of cat inactivates both oxytocin and arginine vasopressin slowly (Table I). With both hormones a relatively large amount of C-terminal dipeptide is released, and oxytocin is inactivated to a greater degree than vasopressin. The result of in vivo studies in the cat suggest that in this animal both enzymes responsible for degradation of [9-*Glycinamide*-1-^{14}C]oxytocin in the kidney extract in vitro

are also functional in vivo. The amount of total radioactivity recovered after oxytocin injection is about 50—60% and 40—50% for arginine vasopressin irrespective of the dose injected. While in the rat 50—60% of the recoverable radioactivity after oxytocin injection was in the form of [^{14}C] Gly-NH_2 and no labelled Leu-Gly-NH_2 was detected [8], in the cat Gly-NH_2 accounted for only 15%, and Leu-Gly-NH_2 for another 15% of radioactivity recovered. In cat, much of the recoverable radioactivity was in the form of intact hormone, and arginine vasopressin was inactivated to a lesser degree than oxytocin. The difference in inactivation, as well as the fact that much of the hormone is excreted intact both in the case of [9-*Glycinamide*-1-^{14}C] oxytocin and [9-*Glycinamide*-1-^{14}C] arginine vasopressin, may also result from the fact that the doses used are 1000—10 000 times greater than the physiological concentrations. As seen in the perfused rat kidney in vitro [6], arginine vasopressin can produce extreme vasoconstriction, predominantly on the afferent vessels, and these hemodynamic responses could affect the metabolism of the hormone.

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References

1 Sawyer, W.H. (1974) in Handbook of Physiology (Greep, R.O. and Astwood, E.B., eds), Section 7, Vol. 4, Part I, pp. 443—468, Waverly Press, Baltimore
2 Chaudhury, R.R. and Walker, J.M. (1959) J. Endrocrinol. 19, 189—192
3 Aroskar, J.P., Chan, W.Y., Stouffer, J.E., Schneider, C.H., Murti, V.V.S. and du Vigneaud, V. (1964) Endocrinology 92, 189—193
4 Sjöholm, I. and Rydén, G. (1969) Acta Endocrinol. (Kbh) 61, 432—440
5 Koida, M., Glass, J.D., Schwartz, I.L. and Walter, R. (1971) Endocrinology 88, 633—643
6 Walter, R. and Bowman, R. (1973) Endocrinology 92, 189—193
7 Lauson, H.D. (1974) in Handbook of Physiology (Greep, R.O. and Astwood, E.B., eds), Section 7, Vol. 4, Part I, pp. 287—393, Waverly Press, Baltimore
8 Walter, R. and Shlank, H. (1971) Endocrinology 89, 990—995
9 Walter, R. and Shlank, H. (1975) Endocrinology 96, 811—814
10 Walter, R., Glass, J.D., Dubois, B.M., Koida, M. and Schwartz, I.L., (1972) in "Progress in Peptide Research", Vol. II, Proc. 2nd Amer. Peptide Symp. 1970 (Lande, S., ed.), pp. 327—333, Gordon and Breach, New York
11 Walter, R., Shlank, H., Glass, J.D., Schwartz, I.L. and Kerenyi, T.D. (1971) Science 173, 827—829
12 Shlank, H. and Walter, R. (1972) Proc. Soc. Exp. Biol. Med. 141, 452—455
13 Munsick, R.A., Sawyer, W.H. and Van Dyke, H.B. (1960) Endocrinology 66, 860—871
14 Coon, J.M. (1939) Arch. Int. Pharmacodyn. 62, 79—99
15 The Pharmacopeia of the United States of America, (1965) 17th rev., p. 749, Mack Publ. Co., Easton Pa.
16 Walter, R. and Havran, R.T. (1971) Experientia 27, 645—646
17 Hase, S. and Walter, R. (1973) Int. J. Peptide Prot. Res. 5, 283—288
18 Yamanaka, T., Hase, S., Sakakibara, S., Schwartz, I.L., Dubois, B.M. and Walter, R. (1970) Mol. Pharmacol. 6, 474—480
19 Bespalova, Zh.D., Martynov, V.F. and Titov, M.I. (1968) Zh. Obshch. Khim. 38, 1684—1687
20 Yamanaka, T. and Sakakibara, S. (1974) Bull. Chem. Soc. Jap. 47, 1228—1232; Walter, R., Yamanaka, T. and Sakakibara, S. (1974) Proc. Natl. Acad. Sci. U.S. 71, 1901—1905
21 Walter, R. and du Vigneaud, V. (1966) Biochem. 5, 3720—3727
22 Meienhofer, J., Trzeciak, A., Douša, T., Hechter, O., Havran, R.T., Schwartz, I.L. and Walter, R. (1971) in "Peptides 1969" (Scoffone, E., ed.), pp. 157—160, North-Holland Publ. Co., Amsterdam

23 Huguenin, R.L. and Boissonnas, R.A. (1966) Helv. Chim. Acta 49, 695—705
24 Hase, S., Sakakibara, S., Wahrenburg, M., Kirchberger, M., Schwartz, I.L. and Walter, R. (1968) J. Am. Chem. Soc. 94, 3590—3600
25 Meienhofer, J. and Sano, Y. (1968) J. Am. Chem. Soc. 90, 2996—2997
26 Havran, R.T. (1973) Experientia 29, 520—521
27 Ressler, C. and du Vigneaud, V. (1954) J. Am. Chem. Soc. 76, 3107—3109
28 Zaoral, M. and Rudinger, J. (1955) Coll. Czech. Chem. Commun. 20, 1183—1189; Overweg, N.I.A., Schwartz, I.L., Dubois, B.M. and Walter, R. (1968) J. Pharm. Exp. Ther. 161, 342—347
29 Jaquenod, P.-A. and Boissonnas, R.A. (1961) Helv. Chim. Acta 44, 113—117
30 Baxter, J.W.M., Manning, M. and Sawyer, W.H. (1969) Biochem. 8, 3592—3597
31 Baxter, J.W.M., Wuu, T.C., Manning, M. and Sawyer, W.H. (1969) Experientia 25, 1127—1128
32 E. Schillinger, O. Loge, E. Schröder, E. Kleiger and K. Lübke (1972) Eur. J. Biochem. 27, 473—481
33 Zaoral, M. and Šorm, F., (1967) Coll. Czech. Chem. Common. 32, 1250—1257
34 Bodanszky, A., Ondetti, M.A., Ralofsky, C.A. and Bodanszky, M. (1971) Experientia 27, 1269—1270
35 Liu, W.-K., Nahm, H.S., Sweeney, C.M., Lamkin, W.M., Baker, H.N. and Ward, D.N. (1972) J. Biol. Chem. 247, 4351—4364
36 Liu, W.-K., Nahm, H.S., Sweeney, C.M., Lamkin, W.M., Baker, H.N. and Ward, D.N. (1972) J. Biol. Chem. 247, 4365—4381
37 Najjar, V.A. and Nishioka, K. (1970) Nature 228, 672—673
38 Rothe, M., Theyson, R. and Steffens, K.D. (1970) Tet. Lett. (47), 4063—4066
39 Khosla, M.C., Leese, R.A., Maloy, W.L., Ferreira, A.T., Smeby, R.R. and Bumpus, F.M. (1972) J. Med. Chem. 15, 792—795
40 Khosla, M.C., Kumar, S., Smeby, R.R. and Bumpus, F.M. (1972) J. Med. Chem. 15, 627—629
41 Freer, R.J. and Stewart, J.M. (1973) J. Med. Chem. 16, 733—735
42 Spackman, D.H., Stein, W.H. and Moore, S. (1958) Anal. Chem. 30, 1190—1206
43 Walter, R. (1973) in "Peptides 1972, Proc. 12th Eur. Pept. Symp.", (H. Hanson and H.-D. Jakubke, eds), pp. 363—368, North-Holland Publ. Co., Amsterdam
44 Moore, S. and Stein, W.H. (1948) J. Biol. Chem. 176, 367—388
45 Warburg, O. and Christian, W. (1941) Biochem. Z. 310, 384—421
46 Andrews, P. (1964) Biochem. J. 91, 222—233
47 Tuppy, H. and Nesvadba, H. (1957) Monat. Chem. 88, 977—988
48 Werle, E., Hevelke, A. and Buthmann, K. (1941) Biochem. Z. 309, 270—282
49 Werle, E. (1960) Polypeptides Which Affect Smooth Muscles and Blood Vessels (Schachter, M. ed.), pp. 89—91, Pergamon Press, Oxford
50 Brouet-Yager, M., Kleiner, H., Van Bogaert, E. and Graff, G.L.A. (1974) Clin. Chim. Acta 54, 387—390
51 Watkins, W.B., Small, C.W. and Walter, R. (1976) Pharmacol. Res. Commun., in the press
52 Sjöholm, I. (1969) FEBS Lett. 4, 135—139
53 Celis, M.E., Taleisnik, S. and Walter, R. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1428—1433
54 Walter, R., Griffiths, E.C. and Hooper, K.C. (1973) Brain Res. 60, 449—457
55 Nair, R.M.G., Kastin, A.J. and Schally, A.V. (1971) Biochem. Biophys. Res. Commun. 43, 1376—1381
56 Kastin, A.J., Plotnikoff, N.P., Nair, R.M.G., Redding, T.W. and Anderson, M.S. (1973) Hypothalamic Hypophysiotropic Hormones: Physiological and Clinical Studies (C. Gual, ed.), pp. 159 ff, Excerpta Medica, Amsterdam
57 Urry, D.W. and Walter, R. (1971) Proc. Natl. Acad. Sci. U.S. 68, 956—958
58 Walter, R. (1971) in Structure-Activity Relationships of Protein and Polypeptide Hormones (Margoulies, M. and Greenwood, F.C., eds), pp. 181—193, Excerpta Medica, Amsterdam
59 Vávra, J., Machová, A., Holeček, V., Cort, J.H., Zaoral, M. and Šorm, F. (1968) Lancet 1, 948—952
60 Andersson, K.-E. and Arner, B. (1972) Acta Med. Scand. 192, 21—27
61 Aronson, A.S., Andersson, K.-E., Bergstrand, C.G. and Mulder, J.L. (1973) Acta Paed. Scand. 62, 133—140
62 Edwards, C.R.W., Kitau, M.J., Chard, T. and Besser, G.M. (1973) Br. Med. J., 375—378
63 Nementhova, V. and Lichardus, B. (1974) Endokrinol. 63, 137—141
64 Manning, M., Balaspiri, L., Judd, J., Acosta, M. and Sawyer, W.H. (1974) FEBS Lett. 44, 229—232
65 Jepson, J.B. and Smith, I. (1953) Nature 172, 1100—1101
66 Pauly, H. (1904) Hoppe-Seyler's Z. Physiol. Chem. 42, 508—518
67 Douša, T., Hechter, O., Schwartz, I.L. and Walter, R. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1693—1697
68 Grantham, J.J. and Burg, M.B. (1966) Am. J. Physiol. 211, 255—259
69 Schwartz, I.L., Schlatz, L.J., Kinne-Saffran, E. and Kinne, R. (1974) Proc. Natl. Acad. Sci. U.S. 71, 2595—2599
70 Huguenin, R.L., Stürmer, E., Boissonnas, R.A. and Berde, B. (1965) Experientia 21, 68
71 Page, E.W. (1946) Am. J. Obstet. Gyn. 52, 1014—1022
72 Werle, E. and Enzenbach, R. (1950) Arch. Gynäk 177, 211—217