Degradation of Neurohypophyseal Hormones by Brain Extracts and Purified Brain Enzymes[1] (37045)

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Recent *in vitro* and *in vivo* studies in our laboratories suggest that the initial cleavage of the peptide chain of neurohypophyseal hormones between residues 8 and 9 (with a concomitant release of H-Gly-NH_2) (1) or between 7 and 8 (release of H-Leu-Gly-NH_2 from oxytocin or H-Arg-Gly-NH_2 from arginine-vasopressin) (2, 3) represent important mechanisms for the enzymic inactivation of these hormones. Therefore, in future investigations these mechanisms will have to be considered in addition to the well-known degradation of neurohypophyseal hormones involving the hydrolysis of the peptide bond of the N-terminal half-cystine residue (4). Less clear at present is the contribution of disulfide bond scission (5) or oxidation of the tyrosine side chain (6) to the enzymic inactivation of neurohypophyseal hormones.

The present study represents the first attempt to identify the sites of inactivation of oxytocin and arginine-vasopressin by enzymes present in rat brain. It has been reported that uptake of radioactivity in the brain occurred after intravenous injections of labeled neurohypophyseal hormones (7) or incubation with brain tissue (8). Moreover, brain tissues or extracts degrade oxytocin and vasopressin (8, 9). An investigation of enzymic activities present in brain is also desirable in light of the suggestion of Celis, Taleisnik and Walter (10) that H-Pro-Leu-Gly-NH_2, a metabolite derived from oxytocin by brain tissue, may be the natural inhibiting factor for the release of melanocyte-stimulating hormone (MSH). Moreover, H-Cys-Tyr-Ile-Gln-Asn-OH, the N-terminal pentapeptide of oxytocin, acts like a MSH-releasing factor (11).

Materials and Methods. Substrates. [9-Glycinamide-1-^{14}C]-oxytocin and [9-glycinamide-1-^{14}C]-arginine-vasopressin (specific radioactivity of each of these hormones was 30.7 mCi/mmole) (12), oxytocin (13), lysine-vasopressin, arginine-vasopressin and arginine vasopressinoic acid (an analog in which the glycinamide is replaced by glycine) (14) were prepared by the solid-phase method of peptide synthesis (15). Crystalline deamino-oxytocin (16) and [1,6-aminosuberic acid]-lysine-vasopressin (an analog in which the terminal amino group and the disulfide group have been replaced, respectively, by a hydrogen atom and an ethylene bridge) (17), were prepared by the classical methods of peptide synthesis. The neurohypophyseal hormones and analogs were checked for their fowl vasodepressor (18) or rat pressor (19) activities; the biological potencies found corresponded to those cited in the above references. The C-terminal acyclic peptide intermediates of oxytocin (the dipeptide, leucylglycinamide; the tripeptide, prolylleucylglycinamide; the octapeptide, tyrosyl-isoleucyl-glutaminyl-asparaginyl-*S*-benzyl-cysteinyl-prolylleucylglycinamide and the nonapeptide, *S*-benzyl-cysteinyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl-*S*-benzyl-cysteinyl-prolylleucylglycinamide, were the same as those used in an earlier investigation (20).

Enzymes. Pig brain arylamidase and aminopeptidase were purified according to the method of Marks, Datta and Lajtha (21). The arylamidase is characterized by its ability to hydrolyze chromogenic arylamides, especially arginyl-β-naphthylamide; the

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TABLE I. Degradation of Labeled Oxytocin by Tissue Extracts and Various Subcellular Fractions of Rat Brain.[a]

Brain preparation	H-Leu-Gly-NH_2	H-Gly-NH_2	Oxytocin degradation (%)
Homogenate	+	++	34
Nuclear	Trace	+	20
Mitochondrial	Trace	+	20
Microsomal	+	Trace	5
Supernatant (cytosol)	+	+++	60
Median eminence homogenate	Trace	+++	60

[a] Incubation time was 2 hr. Since the oxytocin is specifically ^{14}C-labeled in the glycine residue, both metabolites in the table are radioactive.

aminopeptidase by its ability to cleave the N-terminal amino acid from the tripeptide, leucylglycylglycine.

Homogenization of rat brain in sucrose and preparation of subcellular fractions. Brain tissue (10 g), obtained from young Wistar albino rats of both sexes, was homogenized in an Aldrich-type homogenizer for 1 min at 1000 rpm in 10 vol of 0.32 *M* sucrose. Subcellular fractions were prepared as detailed previously (22). In brief, this homogenate was centrifuged at 700*g* for 10 min, and the resulting supernatant was isolated. This crude extract was then centrifuged at 900*g* for 10 min to sediment the nuclear fraction, and the supernatant fluid was recentrifuged at 14,000*g* to sediment the mitochondrial fraction. Microsomes and the final supernatant fractions were separated by a further centrifugation at 100,000*g* for 1 hr.

Homogenization of rat brain in phosphate buffer. Rat brain (4 g) was homogenized in 10 vol of 30 m*M* phosphate buffer, pH 7.4. The tissue suspension was then centrifuged at 14,000*g* to remove debris, and at 100,000*g* for 1 hr to yield the final supernatant fraction.

Preparation of rat median eminence extract. Fifteen rats were killed by decapitation, the cerebellar hemisphere was carefully exposed, and the brain was excised from its cavity starting with the frontal end to expose its ventral surface. The median eminence, a defined morphological entity of about 1 mg weight, lying on the ventral surface of the hypothalamic region caudal to the optic chiasm, was removed. The tissue was homogenized in 1 ml of 0.32 *M* sucrose buffer with a small glass homogenizer and centrifuged at 30,000*g*. The supernatant was used for further study.

Incubation of labeled hormones with rat enzyme preparations and identification of digest products. Different fractions of the sucrose and phosphate buffer preparations were diluted to contain approximately 1 mg of protein/ml, as measured by the method of Lowry *et al.* (23). Samples (0.1 ml) were incubated at 37° for the time periods indicated with 2 μg of labeled oxytocin or arginine-vasopressin dissolved in 0.2 ml of 30 m*M* phosphate buffer, pH 7.4. Incubations were terminated by boiling for 5 min and the mixture was centrifuged at 30,000*g* for 20 min. Aliquots of the supernatant were applied to Whatman 3MM paper strips and subjected to high-voltage electrophoresis in 5% pyridine acetate buffer (pH 3.5) at 1500 V as reported previously, to identify glycinamide (24) and leucylglycinamide (2, 3). Oxytocin, arginine-vasopressin (both incubated with buffer alone as control), glycinamide, leucylglycinamide and prolylleucylglycinamide served as markers. Percentage of degradation is based upon the known amount of labeled hormone subjected to enzymic digestion and the amount of radioactivity present as metabolite(s) after incubation and electrophoretic separation.

Incubation of peptides with purified pig brain peptidases and identification of metabolites. Arylamidase and aminopeptidase diges-

TABLE II. Release of C-Terminal Products from Labeled Oxytocin and Arginine-Vasopressin by Rat Brain Preparations.

	Time of incubation (hr)	Radioactive products released (%)	
		H-Leu-Gly-NH_2	H-Gly-NH_2
1. Sucrose method			
Homogenate	1	21	33
	24	0	>90
Supernatant	1		3
	3	10	40
	24	15	>90
2. Phosphate buffer			
Homogenate	1	tr	>90
	24	tr	>90
Supernatant	5 min	4	6
	1	12	38
	24	tr	>90
Supernatant[a]	5 min	4[a]	24
	24	12[a]	>90

[a] Arginine-vasopressin served as substrate and labeled H-Arg-Gly-NH_2 was identified as described (3).

tions were carried out in 0.3 ml of Tris–HCl buffer (40 m*M*, pH 7.6) containing 0.1 m*M* Cleland's reagent (dithiothreitol) at 37° for 24 hr unless otherwise stated. Incubation mixtures contained 0.2 μmole of substrate and 25 μg of pig brain aminopeptidase or arylamidase (peak II) (21). Reactions were stopped by freezing the samples. Amino acids released during the digestions were identified and quantitated by ion-exchange chromatography of incubation mixtures using the short-column procedure of Catravas (25).

Results and Discussion. Table I summarizes the relative enzymic activities present in crude sucrose homogenate of rat brain and in its subcellular fractions in terms of fragments released from [^{14}C]-labeled oxytocin after a 2-hr incubation. Whole rat brain extract incubated with the substrate led to the rapid appearance of [^{14}C]-glycinamide, but to only small amounts of the dipeptide, leucyl-[^{14}C]-glycinamide. Most of the activity was associated with the 100,000*g* supernatant which gave 54% of the radioactivity in the form of glycinamide under the conditions noted, but again only small amounts of the C-terminal dipeptide. Incubation with the other fractions gave considerably less release of glycinamide and trace amounts of dipeptide. Crude extracts of rat median eminence were characterized by a rapid breakdown of oxytocin to yield largely the C-terminal glycinamide as characterizeable product.

The appearance of two radioactive metabolites was suggestive of the presence in some fractions of more than one enzymic principle which inactivates neurohypophyseal hormones. An effort was made to obtain an indication of whether the ratios of the enzymic activities responsible could be changed by simple cell extraction procedures. Brain homogenates were prepared by two methods, first with isotonic sucrose and second with hypotonic phosphate buffer, in order to induce disruption of organelles. The relative enzymic activities of both preparations were compared in terms of release of the products from labeled oxytocin (Table II). There seems indeed to be some separation of the activities. In terms of accumulation of glycinamide in the digest mixture, the most active fractions were those prepared with hypotonic phosphate buffer; as much as 40–90% of glycinamide is released from oxytocin within the first hour of incubation (the data shown in Table II suggest that glycinamide release from arginine-vasopressin may be even more rapid than from oxytocin). In terms of

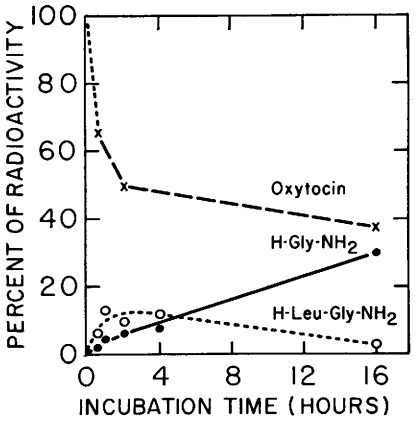

FIG. 1. Inactivation of labeled oxytocin by supernatant of phosphate extract of rat brain as a function of time.

accumulation of dipeptide during the first hour of incubation the most active preparations were the sucrose homogenate and 100,000*g* supernatant fractions. Leucylglycinamide released by the supernatant of the phosphate buffer preparation accounted for about 10% of the total radioactivity.

Figure 1 depicts the inactivation of oxytocin and formation of leucylglycinamide and glycinamide during a 16-hr digestion period. There was an initial drop of 35% in the radioactivity associated with the electrophoretic mobility of oxytocin as soon as the hormone was added to the enzyme preparation. A similar loss of activity has been observed previously when oxytocin was added to a homogenate of estrous rat uteri, and it was suggested that this effect was due to nonspecific binding of the hormone to protein (26). We did observe an increase in radioactivity at the origin of the electrophoretogram even at zero time experiments of hormone-digest mixtures, which would account for the difference in total recovery of leucylglycinamide and glycinamide on the one hand and loss of hormone on the other. Figure 1 shows an initial increase and subsequent drop in the formation of leucylglycinamide, while the amount of glycinamide steadily increases.

The above data reveal that one of the mechanisms of inactivation of neurohypophyseal hormones by rat brain preparations involves the hydrolysis of the peptide bond between residues 7 and 8, giving rise to leucylglycinamide in the case of oxytocin and arginylglycinamide from arginine-vasopressin. Whether the extracts contained enzyme(s) which remove the glycinamide moiety from intact hormone remains to be shown; we found that purified arylamidase from pig brain rapidly hydrolyzes leucylglycinamide (Table III, footnote *a*) and, therefore, glycinamide could derive from the dipeptide once released from the hormone as well as

TABLE III. Breakdown of Neurohypophyseal Hormones, Analogs and Peptide Intermediates by Purified Pig Brain Peptidases.

Peptide	Aminopeptidase	Arylamidase	
		Amino acids released	N-Terminal amino acid released (%)[a]
H-Leu-Gly-NH_2	nt[b]	Leu	100
H-Pro-Leu-Gly-NH_2	0	Pro, Leu	92
Octapeptide	nt	Tyr, Ile	60
Nonapeptide	nt	Cys, Tyr	70
Oxytocin	0	Tyr, Ile	1
Arginine-vasopressin	0	Tyr, Phe	4
Lysine-vasopressin	0	Tyr, Phe	8
Deamino-oxytocin	0	—	0
Arginine-vasopressinoic acid	0	Tyr, Phe	4
[1,6-Aminosuberic acid]-lysine-vasopressin	0	—	0

[a] Incubation time was 24 hr in all experiments; in the case of H-Leu-Gly-NH_2 and H-Pro-Leu-Gly-NH_2 a breakdown of 60 and 50%, respectively, was observed upon 2-hr incubation.

[b] Not tested.

from the intact hormone *per se*. In this context it will be noted that the arylamidase degrades prolylleucylglycinamide, while the pig brain aminopeptidase does not (Table III); this latter finding indicates that this aminopeptidase differs from leucine aminopeptidase (EC 3.4.1.1) which inactivates prolylleucylglycinamide readily (27). In quantitative terms, the inactivation by N-terminal cleavage of neurohypophyseal hormones does not seem to be a major route of inactivation by brain tissue peptidases, as may be deduced from the data of Pliska, Thorn and Vilhardt (8). Moreover, the rapid appearance of C-terminal fragments of oxytocin and arginine-vasopressin on incubation with rat brain extracts is in distinct contrast to the very slow inactivation of these hormones by purified pig brain aminopeptidases (Table III).

Summary. Rat brain tissue inactivates oxytocin and arginine-vasopressin by the release of the C-terminal dipeptide and glycinamide. Most of the enzymic activities are present in the 100,000*g* supernatant fraction although some activity is bound to particulates. Extracts prepared in isotonic sucrose exhibit a higher ratio of dipeptide to glycinamide released than do comparable preparations in hypotonic medium. Inactivation of oxytocin is also demonstrated in homogenates of the median eminence region. Purified arylamidase from pig brain inactivates neurohypophyseal hormones negligibly and pig brain aminopeptidase inactivates not at all. It is suggested that enzymes present in brain inactivate neurohypophyseal hormones primarily by hydrolysis of peptide bonds in the acyclic portion, and to a minor degree by initial cleavage of the N-terminal half-cystine residue.

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