Proc. Nat. Acad. Sci. USA
Vol. 68, No. 7, pp. 1428–1433, July 1971

Regulation of Formation and Proposed Structure of the Factor Inhibiting the Release of Melanocyte-Stimulating Hormone

(oxytocin/C-terminal tripeptide/estrous cycle/rat)

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Communicated by Vincent du Vigneaud, February 18, 1971

ABSTRACT **Microsomal preparations from the stalk median eminence of female rats are shown to contain an enzymic activity that is responsible for the formation of MSH-release-inhibiting factor (MSH-R-IF). The amount of this activity remains constant throughout the estrous cycle. The corresponding mitochondrial preparations from the stalk median eminence contain another enzymic principle, estrous cycle-dependent, which competes with the enzyme present in the microsomal preparation for the same "substrate", and can thereby prevent the formation of MSH-R-IF.**

Several neurohypophyseal hormones, analogs, and peptide intermediates have been tested for their intrinsic MSH-R-IF activity and for their ability to be transformed into MSH-R-IF by incubation with microsomal preparations of stalk median eminence from male rats; it is concluded that the enzyme responsible for the formation of MSH-R-IF is an exopeptidase and that the release-inhibiting factor itself is a tripeptide. Oxytocin is converted by the incubation to L-prolyl-L-leucylglycinamide; nanogram amounts of this tripeptide inhibit the release of MSH from the pituitary both *in vivo* and *in vitro*.

Rat hypothalamus contains a factor (MSH-R-IF) that inhibits the release of melanocyte-stimulating hormone (1, 2). Evidence for the enzymic formation of this factor has been presented (3). In male rats the pituitary content of MSH remains constant, whereas in females it fluctuates as a function of the estrous cycle (4).

This last-mentioned finding was the point of departure for investigating the manner in which pituitary MSH content reflects a change in the enzymic activities responsible for the control of MSH-R-IF formation. We show that a microsomal exopeptidase in the hypothalamus degrades oxytocin, liberating the hormonal C-terminal tripeptide, prolyl-leucyl-glycinamide; this tripeptide inhibits the release of pituitary MSH *in vivo* and *in vitro* in nanogram amounts per rat.

This work has been performed in Córdoba and New York. Therefore, different strains of rats and of toads (*Bufo arenarum, Bufo marinus*) were employed. Since the results obtained in the two laboratories showed no discrepancies, combined publication was agreed upon.

Abbreviations of amino-acid derivatives and peptides are in accordance with the IUPAC–IUB *Tentative Rules* on Biochemical Nomenclature, *Biochemistry*, **5**, 2485, 1445 (1966); **6**, 362 (1967). The amino acids (except glycine) had the L configuration unless otherwise stated. SME, stalk median eminence. MSH-R-IF, melanocyte-stimulating hormone release-inhibiting factor.

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MATERIALS

Oxytocin (5), lysine-vasopressin (6) arginine-vasopressin (7), crystalline [1-β-mercaptopropionic acid]-oxytocin (deamino-oxytocin) (8), oxytocinoic acid (9), [4-proline]-oxytocin (10), [8-phenylalanine]-oxytocin (11) and [8-serine]-oxytocin (12) were prepared by the solid-phase method of peptide synthesis (13). [4-Valine]-oxytocin (14), [7-glycine]-oxytocin (15), [8-alanine]-oxytocin (16), [9-sarcosinamide]-oxytocin (17) and crystalline deamino-dicarba-oxytocin (an oxytocin analog in which the N-terminal amino group is replaced by a hydrogen atom and the disulfide bridge by an ethylene moiety, ref. 18), were prepared by classical methods of peptide synthesis. The neurohypophyseal hormones and synthetic analogs were checked for their fowl vasodepressor (19) or rat pressor (20) activities; the biological potencies found correspond to those cited in the above references. The C-terminal acyclic peptide intermediates of oxytocin (the tripeptide, prolylleucylglycinamide·$^1/_2H_2O$, and the tetrapeptide, *S*-benzyl-cysteinyl-prolyl-leucylglycinamide; the hydrobromide of the hexapeptide, glutaminyl-asparaginyl-*S*-benzyl-cysteinyl-prolyl-leucylglycinamide; the octapeptide, tyrosyl-isoleucyl-glutaminyl-asparaginyl-*S*-benzyl-cysteinyl-prolyl-leucylglycinamide; and the nonapeptide, *S*-benzyl-cysteinyl-tyrosyl-isoleucyl-glutaminyl-asparaginyl-*S*-benzyl-cysteinyl-prolyl-leucylglycinamide) were prepared and used in the course of earlier investigations (21, 22); just prior to use for this investigation they were checked and found to exhibit the same melting points and optical rotations as in the earlier work. In addition, *N*-Z-prolyl-leucylglycinamide (23) was tested as well as *Z*-prolyl-N^G-Tos-arginylglycinamide (24), prolyl-N^G-Tos-arginyl-glycinamide (24), leucylglycinamide, *D*-leucylglycinamide, prolyl-D-leucylglycinamide, and pyroglutaminyl-leucylglycinamide, as prepared by Drs. S. Hase and J. Chakravarty in this laboratory.

METHODS

Preparation of rat hypothalamic extracts

Rats (200–250 g) were killed by placing them in a small ether-saturated container. Immediately after death the brain was removed and the hypothalamus excised as described (2) by cutting anteriorly just in front of the optic chiasma,

laterally at the level of the hypothalamic fissures, and posteriorly through the mammillary bodies. A horizontal cut placed about 2 mm above the surface separates the hypothalamic block from the rest of the brain. Two fractions were obtained from this block. A horizontal cut separated the stalk median eminence (SME) and adjacent areas. Two oblique cuts extending from the midline of the anterior to the level of the premammillary area separated the supraoptic nuclei. SME and supraoptic nuclei of several animals were homogenized at 4°C (in 0.3 ml of 0.25 M sucrose per hypothalamus). The homogenate was centrifuged for 15 min at 1000 × *g*. The supernate was collected and recentrifuged for 60 min at 17,000 × *g* in a Sorvall centrifuge model RC-2B. The supernate and precipitate were separated and each was dialyzed (the precipitate after resuspension in 1 ml of 0.05 M phosphate buffer, pH 7.0) against distilled water for 24 hr at 4°C. The nondialyzable materials of supernate and precipitate were used in separate experiments and are referred to as "microsomal" and "mitochondrial" preparations, respectively.

Incubation of microsomal preparation with neurohypophyseal hormones and related peptides

Aliquots of the microsomal homogenate, equivalent to three hypothalami of male rats, were incubated in 2 ml of phosphate buffer, pH 7.0 (final buffer concentration 0.05 M) at 37°C for 2.5 hr with 300 ng of each peptide sample (this amount of peptide is in excess of that required to give complete inhibition of MSH release, based on preliminary incubation experiments of oxytocin with the microsomal homogenate). The reaction was stopped by 5-min heat treatment (~100°C), and the mixture was centrifuged at 1000 × *g* for 10 min; the clear supernate, referred to as "incubation mixture," was collected.

Incubation of oxytocin with SME preparations of female rats

The SME of adult female rats at different stages of the estrous cycle was removed, homogenized, and separated into microsomal and mitochondrial fractions as described for male rats. (*a*) A microsomal preparation equivalent to three SME derived from rats in proestrus was incubated in the phosphate buffer (pH 7.0, final volume 2 ml) at 37°C with 150 mU (300 ng) of oxytocin. The reaction was stopped after 2.5 hr by heat treatment, and MSH-R-IF activity was determined in the *in vivo* assay. Identical experiments were performed with preparations from rats in estrus and diestrus. (*b*) A mitochondrial preparation equivalent to three SME derived from female rats in proestrus was incubated as just described with 150 mU of oxytocin in the presence of a microsomal preparation derived from three SME of male rats. Identical experiments were again carried out with rats in estrus and diestrus. MSH-R-IF activity was determined by the *in vivo* assay (below). Oxytocin was incubated as control, under identical conditions, with the microsomal preparation derived from the SME of three male rats.

***In vivo* assay for MSH-release-inhibiting factor (MSH-R-IF)**

The injection of the homogenate of SME from two male rats into recipient animals induces the depletion of pituitary MSH content to some 40–50% of control values (25). The ability of agents to block such depletion was taken as an index of MSH-R-IF activity. Consequently, the assay consisted of injecting intravenously an aliquot of "incubation mixture" derived from male rats (see above), together with the homogenate of SME from two male rats, in a total volume of about 1 ml, into the jugular vein of a male rat (180–200 g) anesthetized with ether. Control animals were injected with 1 ml of the phosphate buffer (pH 7.0); these animals were used in the determination of the normal pituitary content of MSH. Simultaneously, we injected male rats with SME homogenate derived from two other male rats in order to determine "residual" pituitary MSH levels, i.e., the amount of MSH per pituitary remaining after release has been induced.

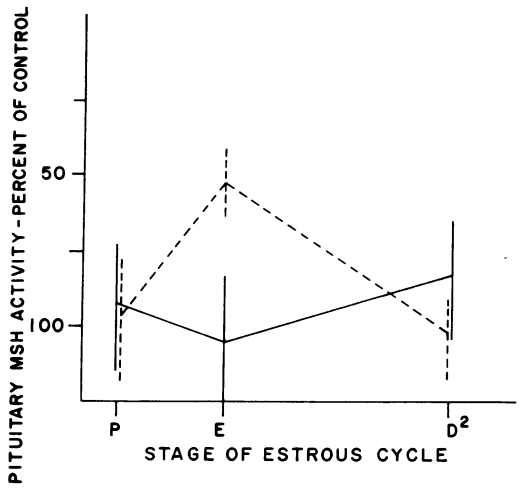

FIG. 1. Interrelationship between the formation and inhibition of the MSH-release-inhibiting factor as a function of the estrous cycle of female rats. P, E, D^2, stand for values during proestrus, estrus, and the second day of diestrus, respectively, always determined during the morning. Control values were derived from incubates of oxytocin with microsomal preparations of three male rats. Solid line refers to amount of enzymic activity present in microsomal preparations of stalk median eminence (SME) capable of forming MSH-R-IF upon incubation with oxytocin; dashed line refers to another enzymic activity, present in mitochondrial preparations of SME, which prevents formation of MSH-R-IF. Data are the result of two independent experiments; the vertical bars are 95% confidence limits. "Residual" pituitary MSH (after injection of SME of two male rats) was approximately 54.4 (43.1–70.3) %.

The animals were killed 20 min later. The hypophyses were removed, homogenized in distilled water (5 mg pituitary tissue per ml of water), and kept frozen at −20°C until further use. MSH activity in the pituitary was determined by a toad assay for MSH (25). Residual MSH content in the experimental animals is expressed as a percentage of pituitary MSH content in control animals; confidence limits were calculated according to the method of Bliss (26).

***In vitro* assay for MSH-R-IF**

Male rats were killed by decapitation and the pituitary glands were immediately removed and divided in halves. Both halves were first incubated at 37°C under air in a Dubnoff metabolic shaker in 1 ml of Krebs–Ringer bicarbonate buffer, pH 7.4 (27). After 1 hr the gland portions were placed in 1 ml of fresh buffer. Prolyl-leucylglycinamide (15 ng) was added to one of the gland halves and incubation

TABLE 1. *Evidence for presence of MSH-release-inhibiting factor after incubation of hypothalamic microsomal preparations of male rats with neurohypophyseal hormones, analogs, and intermediate peptides all having a leucine residue in the penultimate position*

	Compound	Rat 1*	Rat 2*	Mean
	None (SME homogenate alone)	61.1 (40.5–77.0)	65.3 (56.0–76.1)	64.6 (58.6–73.4)
1.	Oxytocin	97.8 (80.4–118.9)	101.0 (81.0–126.0)	99.2 (86.7–113.5)
2.	Deamino-oxytocin	49.8 (42.1–59.0)	48.0 (39.0–59.3)	50.9 (43.0–57.3)
3.	Deamino-dicarba-oxytocin	65.3 (56.0–76.1)	61.1 (48.5–77.0)	64.6 (58.6–73.4)
4.	[4-Valine]-oxytocin	96.7 (77.4–120.8)	112.6 (89.1–142.3)	103.6 (89.5–1198)
5.	[4-Proline]-oxytocin	44.8 (35.7–56.2)		
6.	[7-Glycine]-oxytocin	56.0 45.1–69.6)		
7.	[9-Sarcosine]-oxytocin	62.9 (54.1–73.2)	60.1 (47.4–75.9)	62.0 (56.3–71.9)
8.	Oxytocinoic acid	34.9 (27.2–56.3)	62.6 (53.4–73.5)	51.8 (46.0–59.6)
9.	*S*-Bzl-Cys-Tyr-Ile-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-GlyNH$_2$·HBr	98.5 (82.8–117.3)	98.9 (85.0–130.2)	98.9 (90.1–108.5)
10.	Tyr-Ile-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-GlyNH$_2$·HBr	102.0 (88.4–117.7)	98.8 (77.0–123.9)	101.2 (92.7–113.3)
11.	Gln-Asn-*S*-Bzl-Cys-Pro-Leu-GlyNH$_2$·HBr	98.2 (85.6–112.8)	100.8 (86.2–117.8)	99.4 (90.5–109.1)
12.	*S*-Bzl-Cys-Pro-Leu-GlyNH$_2$	115.0 (88.8–148.9)		
13.	*N*-Z-*S*-Bzl-Cys-Pro-Leu-GlyNH$_2$	45.0 (35.0–57.8)	43.1 (31.9–58.2)	44.2 (39.4–51.9)
14.	Pro-Leu-GlyNH$_2$	98.9 (83.3–117.3)	99.2 (83.6–118.2)	99.0 (91.2–110.2)

* 300 ng of each compound was incubated with microsomal preparation of stalk median eminence. The clarified incubation mixture, mixed with the total SME homogenate of male rats, was injected into the recipient rat. Values given are the pituitary MSH content 20 min later, expressed as percentage of the pituitary MSH content of controls injected with phosphate buffer. A toad skin assay was used (25); 50 melanophores were graded in each of 5–10 pieces of skin immersed for 1 hr in the homogenate. Values in parentheses are 95% confidence limits (26).

was continued at 37°C for 1 hr, when the gland portions were removed and weighed. The amount of MSH released into the incubation medium was determined by direct comparison with α-MSH standard in the toad assay.

RESULTS

Microsomal preparations of SME of three female rats in either proestrus, estrus, or diestrus, when incubated with oxytocin, showed no significant differences in MSH-R-IF formation between the three hormonal states (Fig. 1). The same figure shows that *mitochondrial* preparations of female rats at various stages of the estrous cycle, incubated with a mixture of microsomal preparation and oxytocin, prevented the formation of IF only when the female was in estrus.

TABLE 2. *Intrinsic MSH-release-inhibiting activity of oxytocin, analogs, and other peptides*

Compound	Pituitary MSH content (% of control)
None (SME homogenate alone)	42.6 (32.4–55.5)
Oxytocin (compound 1 of Table 1)	40.7 (31.5–50.9)
[4-Valine]-oxytocin (cpd. 4)	39.9 (34.4–48.4)
S-Bzl-Cys-Tyr-Ile-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-GlyNH$_2$·HBr (cpd. 9)	48.7 (37.5–63.2)
Tyr-Ile-Gln-Asn-*S*-Bzl-Cys-Pro-Leu-GlyNH$_2$·HBr (cpd. 10)	45.5 (36.4–56.8)
Gln-Asn-*S*-Bzl-Cys-Pro-Leu-GlyNH$_2$·HBr (cpd. 11)	41.9 (32.6–53.7)
S-Bzl-Cys-Pro-Leu-GlyNH$_2$ (cpd. 12)	41.4 (32.4–52.7)
Pro-Leu-GlyNH$_2$ (cpd. 14)	99.2 (83.6–118.2)
Arginine-vasopressin (ref. 29)	46.3 (23.0–63.0)
Lysine-vasopressin	41.2 (32.2–50.3)
Z-Pro-N^G-Tos-Arg-GlyNH$_2$	43.1 (31.9–58.2)
Pro-N^G-Tos-Arg-GlyNH$_2$	81.5 (65.0–102.1)
Z-Pro-Leu-GlyNH$_2$	48.3 (37.0–64.6)
Pro-D-Leu-GlyNH$_2$	52.4 (44.7–62.1)
PyroGlu-Leu-GlyNH$_2$	47.2 (40.1–57.0)
PyroGlu-His-ProNH$_2$ (TRH)	51.7 (41.6–64.3)
Leu-GlyNH$_2$	59.0 (50.4–69.2)
D-Leu-GlyNH$_2$	42.7 (35.0–52.0)

40 ng of each compound was injected, together with SME homogenate, into a recipient rat. Controls received phosphate buffer.

To elucidate the nature of the inactivation of neurohypophyseal hormones and to identify the structure of IF formed during incubation of the hormone with the microsomal preparation, we incubated 300 ng each of several highly purified, synthetic neurohypophyseal hormones, structural analogs, and peptide intermediates with microsomal preparations of male rats and tested the incubated mixture for MSH-R-IF activity *in vivo*. Table 1 lists the peptides possessing a leucine residue in the penultimate position. Oxytocin and some other peptides (compounds 4, 9–12, 14) so incubated prevented the fall in pituitary MSH content that injection of the microsomal preparation alone would have evoked. Significantly, the degree of IF activity formed was identical, within experimental error, for all these peptides. The remaining neurohypophyseal hormone analogs and protected intermediates did not yield IF activity.

In a second group of experiments oxytocin, several of its analogs and peptide intermediates (all at 40 ng, an amount still considerably in excess of that required, *see later*, for complete inhibition of pituitary MSH release by the most potent of these compounds, prolyl-leucylglycinamide) were tested for their intrinsic IF activity, without prior incubation with microsomal preparations (Table 2). Only compounds 10 and 12 were active. The most potent compound, prolyl-leucylglycinamide (which corresponds to the C-terminal tripeptide of oxytocin), effectively inhibited MSH release in amounts as low as 5 ng per rat. Moreover, as shown in Fig. 2*a*, a dose-dependent relationship was obtained in the inhibition of MSH release in the range of 3–10 ng of prolyl-leucylglycinamide. The effect of hypertonic saline (2% NaCl), which is known to induce the release of pituitary MSH (28), was likewise

TABLE 3. *Inhibiting activity of prolylleucylglycinamide on the release of MSH from pituitary gland in vitro*

Amount of MSH released*		Percent
Control	Experimental†	inhibition‡
33.4 (3.86)	19.2 (3.20)	32
31.6 (3.89)	25.9 (3.50)	9
42.7 (3.25)	20.3 (4.06)	63
41.8 (4.09)	21.2 (4.80)	57
39.4 (3.59)	18.5 (3.53)	53

* MSH released into the incubation medium was compared with α-MSH standard and expressed in ng of MSH per pituitary per hour. Numbers in parentheses are weights (mg) of the incubated pituitary halves.

† Prolyl-leucylglycinamide (15 ng) was added to the incubation medium.

‡ Expressed in percent per mg of pituitary tissue.

counteracted by prolyl-leucylglycinamide, although somewhat higher doses were required (Fig. 2*b*).

The data in Table 3 illustrate that the C-terminal tripeptide of oxytocin is a potent inhibitor of MSH release *in vitro* as well. At a concentration of 15 ng of prolyl-leucylglycinamide per half pituitary the MSH release was inhibited about 50%. The concentration of MSH was the same in *homogenates* of pituitary incubated as described in the presence or absence of the tripeptide, i.e., the tripeptide did not affect the rate of synthesis of MSH.

Table 4 summarizes analogous studies with neurohypophyseal hormones and derivatives with amino-acid substitutions in position 8. It is apparent that the incubation of all these peptides with microsomal preparations gives rise to IF activity, though in differing degrees.

DISCUSSION

Celis and Taleisnik (3) presented evidence that the incubation of male SME homogenates with oxytocin results in the enzymic formation of MSH-R-IF. This factor prevents the release of pituitary MSH caused by injection of SME homogenate without stimulating the synthesis of MSH (29). Conversely, no MSH-R-IF was detected when a homogenate of paraventricular nuclei was added to an oxytocin-containing SME homogenate (30). In male rats pituitary MSH remains at constant levels, and microsomal preparations of different male rats incubated under standard conditions with heat-denatured SME extracts of males or with oxytocin, which appears to be the initial substrate present in the denatured homogenate, yield approximately equal amounts of IF (3). In female rats, pituitary MSH content is a function of the estrous cycle of the animal: it is low during estrus and high during proestrus and diestrus (4). In this study we found that in female rats—just as in microsomal preparations of male rats—the enzymic activity responsible for the formation of IF remains constant throughout the cycle; however, during estrus a second enzymic activity emerges which is more effective in the degradation of oxytocin than the activity responsible for the formation of MSH-R-IF (30). Thus it appears that IF concentrations (which in turn affect pituitary MSH content) are the result of the dualistic action of two enzymes: one (referred to as E_1), present in the microsomal fraction, is responsible for the formation of IF; the

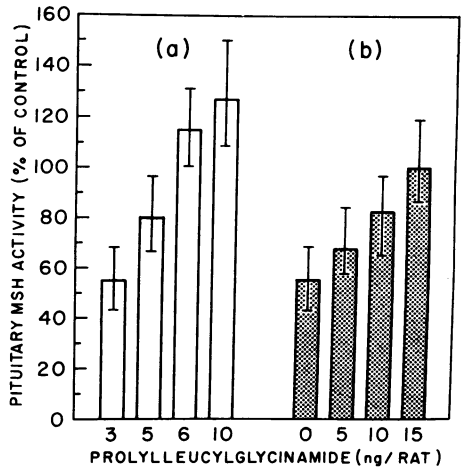

FIG. 2. Typical experiments of *in vivo* MSH-release-inhibiting activity of prolyl-leucylglycinamide. Part (*a*) shows the increasing inhibition of MSH depletion in the presence of increasing doses of prolyl-leucylglycinamide (in nanograms per rat). The pituitary MSH content of a recipient male rat 20 min after the injection of an aliquot of a SME homogenate from male rats equivalent to 1 hypothalamus, which stimulates the release of pituitary MSH, was found to be 48.0 (39.0–59.3). The first bar in part (*b*) shows the residual pituitary MSH content after i.v. injection of 2 ml of hypertonic saline (2% NaCl) per rat. The other bars show the progressive blockade of saline-induced MSH release as reflected by the increasing pituitary MSH content with increasing doses (5, 10, and 15 ng) of the tripeptide. The vertical bars are 95% confidence limits.

TABLE 4. *Evidence for MSH-release-inhibiting activity after incubation of hypothalamic microsomal preparations of male rats with neurohypophyseal hormones and analogs modified in the 8-position*

Compound	Pituitary MSH content, as percentage of buffer-injected controls		
	Rat 1	Rat 2	Mean
None (SME homogenate alone)	47.5 (34.8–64.8)	55.7 (42.2–73.7)	54.9 (45.6–66.2)*
1. Oxytocin	93.9 (73.0–120.9)	107.7 (87.3–132.8)	101.7 (88.0–117.5)
2. Lysine-vasopressin	93.5 (80.9–108.0)	100.2 (84.3–119.2)	96.2 (88.4–110.4)
3. Arginine-vasopressin	69.4 (50.1–96.0)	69.0 (51.7–92.1)	73.1 (60.7–88.1)
4. [8-Serine]-oxytocin	82.3 (66.2–102.0)	83.0 (67.3–102.5)	83.0 (70.3–95.8)
5. [8-Phenylalanine]-oxytocin	87.0 (72.3–104.7)	83.4 (64.3–101.0)	84.1 (73.8–95.8)
6. [8-Alanine]-oxytocin	81.3 (64.4–105.0)	76.5 (59.0–99.1)	80.9 (68.8–95.1)

See Table 1 for details of method. * $P < 0.001$.

other (referred to as E_2), present in the mitochondrial preparation, prevents the formation of IF catalyzed by E_1 (Fig. 2). With the present data it cannot be decided whether E_2 has a higher affinity for the common substrate, oxytocin, or whether it is present at higher concentrations than E_1 at times of inhibition of IF formation.

Oxytocin is not the only compound capable of yielding MSH-R-IF. Certain oxytocin analogs and intermediates can likewise give rise to MSH-R-IF when exposed to the enzyme present in SME of male rats. Notably, all of these active peptides inhibit the MSH release to the same degree at the amount tested; a comparison of their primary structure reveals that they have in common a prolyl-leucylglycinamide sequence. Compounds in which this sequence is lacking are inactive: [7-glycine]-oxytocin, in which a glycine residue replaces the proline residue of oxytocin, [9-sarcosine]-oxytocin, which possesses an additional methyl group in the glycinamide residue, and oxytocinoic acid, in which the glycinamide is replaced by glycine (Table 1, compounds 6–8). Furthermore, only peptides with an unprotected terminal amino group are capable of forming IF: deamino-oxytocin, deamino-dicarba-oxytocin and *N*-Z-*S*-benzyl-cysteinyl-prolyl-leucylglycinamide (Table 1, compounds 2, 3, and 13) are ineffective, while [4-valine]-oxytocin (compound 4) and the oxytocin intermediates 9–12 and 14 all give rise to the same degree of IF potency. These results lead to the conclusion that SME of male rats contains an exopeptidase which degrades oxytocin to yield prolyl-leucylglycinamide.

The exopeptidase appears to hydrolyze the peptide bond at the carboxyl side of the proline residue at a slow rate or not at all. This explains the accumulation of prolyl-leucylglycinamide (and possibly of other tripeptides possessing a prolyl-X-glycinamide sequence, see below) and moreover, the inability of [4-proline]-oxytocin (Table 1, compound 5) to give rise to IF activity when incubated with the SME preparation; from the above argument it was expected that the degradation of [4-proline]-oxytocin would result in the inactive prolyl-asparaginyl-prolyl-leucylglycinamide. The preliminary substrate specificity data available argue against leucine aminopeptidase (EC 3.4.1.1, refs. 31–33) as the active principle in microsomal SME. This aminopeptidase, which has been shown to possess an exceedingly broad specificity, cleaves the peptide bond of an amino terminal residue just prior to proline to a negligible degree; thus if leucine aminopeptidase were the critical enzymic principle, *S*-benzyl-cysteinyl-prolyl-leucylglycinamide, which is inactive upon direct injection (Table 2) would, contrary to our finding in this study, not be expected to give rise to prolyl-leucylglycinamide upon incubation with SME (see Table 1, compound 12), nor would oxytocin be converted to this tripeptide. An important finding was that none of the active peptides listed in Table 1, except compound 14, inhibited MSH release when injected intravenously into male rats without prior incubation with SME homogenate (Table 2). Several di- and tripeptides, including those with amino-acid residues that are acylated or of D configuration, were tested for intrinsic IF activity. In addition, thyrotropin-releasing hormone (37, 38) was tested; this hormone has distinct structural similarities with the C-terminal tripeptide fragment of oxytocin in that it is also a tripeptide possessing an N-terminal amino acid with a secondary nitrogen and a C-terminal residue protected by a carboxamide. Significantly, all these peptides were found to be inactive, with the exception of two compounds in Table 2: prolyl-N^G-tosyl-arginyl-glycinamide (see below) and prolyl-leucylglycinamide. The latter was found to inhibit the release of MSH *in vitro* (Table 3).

Next we assessed the role of the antidiuretic principle of the rat, arginine-vasopressin (39), in forming IF activity. Like oxytocin, arginine-vasopressin upon incubation with SME homogenates prevented the depletion of pituitary MSH, but the inhibitory effect was less pronounced. When injected directly arginine-vasopressin was inactive, while the synthetic tripeptide fragment, prolyl-N^G-tosyl-arginylglycinamide, exhibited some IF activity. This finding suggests that it is again—as was observed with oxytocin—the C-terminal tripeptide of the pituitary hormone which is the component inhibiting pituitary MSH depletion in these experiments and, moreover, that side-chain replacements of the penultimate leucine residue in prolyl-leucylglycinamide are permitted. To explore this matter further we tested the SME homogenate digests of the neurohypophyseal hormone analogs listed in Table 4. All of them are active to various degrees; this indicates a considerable tolerance to structural modifications in position 8.

All the above results argue, but do not prove, that the enzymic principle present in SME homogenates of male rats converts oxytocin to prolyl-leucylglycinamide, which acts as a natural MSH-R-IF. Additional support for this contention comes from earlier studies in which it was shown that the destruction of paraventricular nuclei, which contain considerably more oxytocin than arginine-vasopressin (40, 41), results in the decrease of MSH (42); thus oxytocin is depleted and no prolyl-leucylglycinamide can be produced *in vivo*.

Further work is in progress to study the enzymatically controlled release of prolyl-leucylglycinamide from oxytocin, which in this capacity represents the precursor molecule or prohormone, and to isolate MSH-R-IF from hypothalamic tissue.

The authors thank Drs. S. Hase and J. Chakravarty for the preparation of several di- and tripeptides used in this study, and Dr. R. T. Havran for supplying oxytocinoic acid and [9-sarcosine]-oxytocin; Drs. D. Gillessen for pyroGlu-His-ProNH$_2$; M. Manning and W. H. Sawyer for [4-proline]-oxytocin, [8-phenylalanine]- and [8-serine]-oxytocin; J. Meienhofer for [8-lysine]-vasopressin; S. Sakakibara for crystalline deamino-dicarba-oxytocin; and S. Lande for α-MSH standard. We thank Mrs. Dora de Rosales for expert technical assistance, and Drs. H. Sachs and V. Pliška for their interest in this work.

This work was supported in part by the U.S. Public Health Service Grants AM-13567, AM-10080, NB 04732, by the U.S. Atomic Energy Commission; and by the Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina. This work was presented at the 15th Biophysical Society Meeting, New Orleans, La., February 15–18, 1971.

1. Kastin, A. J., and A. V. Schally, *Gen. Comp. Endocrinol.*, **7**, 452 (1966).
2. Taleisnik, S., and M. E. Tomatis, *Endocrinology*, **81**, 819 (1967).
3. Celis, M. E., and S. Taleisnik, *Int. J. Neuroscience*, **1**, 1 (1970).
4. Tomatis, M. E., and S. Taleisnik, *Acta Physiol. Lat. Amer.*, **18**, 96 (1968).
5. Bodanszky, M., and V. du Vigneaud, *J. Amer. Chem. Soc.*, **81**, 5688 (1959).
6. Meienhofer, J., and Y. Sano, *J. Amer. Chem. Soc.*, **90**, 2996 (1968).
7. Meienhofer, J., A. Trezeciak, R. T. Havran, and R. Walter, *J. Amer. Chem. Soc.*, **92**, 7199 (1970).
8. Ferrier, B. M., D. Jarvis, and V. du Vigneaud, *J. Biol. Chem.*, **240**, 4264 (1965).

9. Ferrier, B. M., and V. du Vigneaud, *J. Med. Chem.*, **9**, 55 (1966).
10. Sawyer, W. H., T. C. Wuu, J. W. M. Baxter, and M. Manning, *Endocrinology*, **85**, 385 (1969).
11. Sawyer, W. H., J. W. M. Baxter, M. Manning, E. Heinicke, and A. M. Perks, *Gen. Comp. Endocrinol.*, **15**, 52 (1970).
12. Baxter, J. W. M., T. C. Wuu, M. Manning, and W. H. Sawyer, *Experientia*, **25**, 1127 (1969).
13. Merrifield, R. B., *Science*, **150**, 178 (1965).
14. Du Vigneaud, V., G. Flouret, and R. Walter, *J. Biol. Chem.*, **241**, 2093 (1966).
15. Bodanszky, M., and R. J. Bath, *Chem. Commun.*, 766 (1968).
16. Walter, R., and V. du Vigneaud, *Biochemistry*, **5**, 3720 (1966).
17. Cash, W. D., L. M. Mahaffey, A. S. Buck, D. E. Nettleton, C. Romas, and V. du Vigneaud, *J. Med. Pharm. Chem.*, **5**, 413 (1962).
18. Yamanaka, T., S. Hase, S. Sakakibara, I. L. Schwartz, B. M. Dubois, and R. Walter, *Mol. Pharmacol.*, **6**, 474 (1970).
19. Munsick, R. A., W. H. Sawyer, and H. B. van Dyke, *Endocrinology*, **66**, 860 (1960).
20. *The Pharmacopeia of the United States of America*, 17th rev. (Mack Publishing Co., Easton, Pa., 1965), p. 750.
21. Overweg, N. I. A., I. L. Schwartz, B. M. Dubois, and R. Walter, *J. Pharmacol. Exp. Ther.*, **161**, 342 (1968).
22. Glass, J. D., B. M. Dubois, I. L. Schwartz, and R. Walter, *Endocrinology*, **87**, 730 (1970).
23. Cash, W. D., *J. Org. Chem.*, **27**, 2136 (1961).
24. Huguenin, R. L., and R. A. Boissonnas, *Helv. Chim. Acta*, **45**, 1629 (1962).
25. Taleisnik, S., and R. Orías, *Amer. J. Physiol.*, **208**, 293 (1965).
26. Bliss, C. I., *The Statistics of Bioassay* (Academic Press, New York, 1952).
27. *Biochemists' Handbook*, ed. C. Long (E. & F. Spon Ltd., London, 1961), p. 58.
28. Orías, R., *Acta Physiol. Lat. Amer.*, **16**, Suppl. 95 (1966).
29. Taleisnik, S., and M. E. Tomatis, *Amer. J. Physiol.*, **212**, 157 (1967).
30. Celis, M. E., dissertation, Universidad Nacional de Córdoba, Instituto de Ciencias Quimicas, 1969; Celis, M. E., and S. Taleisnik, in preparation.
31. Smith, E. L., and R. L. Hill, in *The Enzymes*, ed. P. D. Boyer, H. Lardy, and K. Myrbäck (Academic Press, 1960), Vol. 4, p. 37.
32. Hanson, H., D. Glaeser, and H. Kirschke, *Z. Physiol. Chem.*, **340**, 107 (1965).
33. Himmelhoch, S. R., and E. A. Peterson, *Biochemistry*, **7**, 2085 (1968).
34. Smith, E. L., and D. H. Spackman, *J. Biol. Chem.*, **212**, 271 (1955).
35. Schroeder, W. A., J. R. Shelton, J. B. Shelton, and J. Cormick, *Biochemistry*, **2**, 1353 (1963).
36. Light, A., and J. Greenberg, *J. Biol. Chem.*, **240**, 258 (1965).
37. Burgus, R., T. E. Dunn, D. Desiderio, D. N. Ward, W. Vale, R. Guillemin, A. M. Felix, D. Gillessen, and R. O. Studer, *Endocrinology*, **86**, 573 (1970).
38. Bowers, C. Y., L. V. Schally, F. Enzmann, J. Bøler, and F. Folkers, *Endocrinology*, **86**, 1143 (1970).
39. Du Vigneaud, V., H. C. Lawler, and E. A. Popenoe, *J. Amer. Chem. Soc.*, **75**, 4880 (1953).
40. Brooks, E. McC., T. Ishikawa, K. Koizumi, and H.-H. Lu, *J. Physiol.*, **182**, 217 (1966).
41. Lederis, K., in Neurosecretion, ed. H. Heller, and R. B. Clark (Academic Press, New York, 1962), p. 227.
42. Taleisnik, S., J. de Olmos, R. Orías, and M. E. Tomatis, *J. Endocrinol.*, **39**, 485 (1967).