location can affect the above described tegmental area without affecting surrounding mesencephalic structures. If the mesencephalic structures are affected, the resulting impairment to the patient's overall arousal level (*18*) will override a selective memory deficit.

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Characterization of a 41-Residue Ovine Hypothalamic Peptide That Stimulates Secretion of Corticotropin and β-Endorphin

Abstract. *A peptide with high potency and intrinsic activity for stimulating the secretion of corticotropin-like and β-endorphin–like immunoactivities by cultured anterior pituitary cells has been purified from ovine hypothalamic extracts. The primary structure of this 41-residue corticotropin- and β-endorphin–releasing factor has been determined to be:*

H-Ser-Gln-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Thr-Lys-Ala-Asp-Glu-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Leu-Asp-Ile-Ala-NH_2

The synthetic peptide is active in vitro and in vivo.

Experimental and clinical observations have supported the concept that the hypothalamus plays a key role in the regulation of the secretory functions of adenohypophyseal corticotropic cells (*1*). More than 25 years ago, Guillemin and Rosenberg and Saffran and Schally independently demonstrated the presence of factors in hypothalamus that would increase the rate of corticotropin secretion by the pituitary gland incubated in vitro or maintained in organ culture (*2*). Several characterized substances present in hypothalamic extracts release corticotropin in vitro or in vivo including vasopressins, norepinephrine, fragments of proteins such as hemoglobin or myelin basic protein, and modified amino acids (*3, 4*). None of the previously characterized secretagogs, with the possible exception of norepinephrine which has yet to be fully evaluated, meets the criteria (*5*) expected of a physiologic corticotropin-releasing factor (CRF). We report here the purification, sequence analysis, and total synthesis of a 41-residue peptide that stimulates the secretion of corticotropin-like and β-endorphin–like immunoactivities in vitro and in vivo.

Throughout our purification program we used an in vitro method for assaying the ability of a putative CRF to stimulate the secretion of corticotropin (ACTH) by primary cultures of rat pituitary cells (*6*). Concentrations of ACTH and β-endorphin (β-End) in culture fluids were assayed by double antibody radioimmunoassays (RIA's) (*7*). Although the terms ACTH and β-End are used in this report, it is recognized that these RIA's detect multiple forms of the peptides; for example, the β-endorphin RIA measures pro-opiocortin, β-lipotropin, and β-endorphin and its acylated forms.

Starting material for this purification was a side fraction of 490,000 fragments of ovine hypothalamus (initially processed in the Laboratories for Neuroendocrinology at the Salk Institute) during the program to characterize gonadotropin-releasing hormone. As described (*8*), tissues were extracted in a mixture of ethanol, acetic acid, and chloroform, defatted with a mixture of ether and petroleum ether and partitioned in the system consisting of 0.1 percent acetic acid, *n*-butanol, and pyridine (11:5:3). Although ACTH-releasing activity was found in portions of both upper and lower phases, only the lower phases were available to us for further purification and thus were used as starting material for the project we describe.

After ultrafiltration (Amicon UM-10) or dialysis (Spectrapor 3) against 2*N* acetic acid, about 350,000 fragment equivalents of the retained fractions (weighing 15 g) were subjected to gel filtration on Sephadex G-50. The bulk of material was chromatographed at 4°C (*9*) in successive runs on a G-50 column, 3.1 by 150 cm, eluted with 2*N* acetic acid. Two zones of ACTH-releasing activity were detected: zone 1 eluting at about 1.3 V_e/V_o and zone 2 eluting at about 2.0 V_e/V_o. Multiple ACTH-releasing zones, including "large CRF's," have been described (*4, 10*). The two zones showed

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different intrinsic activities in that zone 1 elicited a much higher secretory V_{max} (secretory rate at maximum concentration of added substance) than did zone 2. The activity in zone 2 was similar to that of vasopressin and, expectedly, further purification of the ACTH-releasing activity of this zone yielded an active fraction with the same amino acid composition as [Arg^8]vasopressin (*11*). Because of the high intrinsic activity of zone 1 and encouraging results of a series of in vitro studies (*4*), we focused on the further purification of the ACTH-releasing substance in this higher molecular weight fraction.

A portion of zone 1 (equivalent to 130,000 fragments) was subjected to ion exchange chromatography on SP-Sephadex. The sample was applied in 0.01*M* ammonium formate buffer, *p*H 3.2, to a column containing 21 ml of gel at 4°C and eluted with a linear gradient of the application buffer up to 1.5*M* ammonium formate, *p*H 7.0. The ACTH-releasing activity was weakly adsorbed. The ACTH-releasing fraction from SP-Sephadex and the remainder of zone 1 from Sephadex G-50 were each dissolved in a mixture of 6*M* guanidine · HCl and acetic acid, *p*H 2.5, heated for 5 minutes at 90°C, then separately chromatographed at room temperature on Bio-Gel P-10, and eluted with a mixture of 4*M* guanidine · HCl and acetic acid, *p*H 2.5. The ACTH-releasing fractions were pooled and purified further by successive high-pressure liquid chromatographic (HPLC) steps which included (i) reverse phase HPLC on μBondapak CN (Waters Associates) with a gradient of acetonitrile (15 to 39 percent) in 0.25*N* triethylammonium phosphate (TEAP); (ii) reverse phase HPLC on μBondapak C_{18} with a mixture of TEAP and acetonitrile (30.6 to 38.4 percent) at 0°C, followed by reverse phase HPLC on μBondapak CN with gradients of acetonitrile (30.6 to 39 percent) in 0.25*N* triethylammonium formate; (iii) other recently developed systems which were successfully used for the purification of CRF after step (i) employed columns having large pore sizes (300 to 330 Å) from Perkin-Elmer or Vydac and the TEAP or 0.1 percent trifluoroacetic acid and acetonitrile solvent systems (*12*) (Fig. 1, lower panel). Lyophilized active zones from either HPLC steps (ii) or (iii) were used for composition and sequence analysis.

The primary structure of the major component was determined by Edman degradation with the use of a Beckman 890C spinning cup sequencer modified according to Wittmann-Liebold (*13*). The phenylthiohydantoin derivatives of the amino acids were identified by reverse phase HPLC (*14*). Several analyses of 0.6 to 3.6 nmole of peptide were performed. Residues 1 to 39 were unambiguously determined by this approach. Residue 40 (Ile) was established by spinning cup sequencing of the tryptic digest of the peptide. The COOH-terminal alaninamide was identified by HPLC after digestion of CRF with thermolysin and carboxypeptidase Y and confirmed by COOH-terminal tritiation experiments (*14*). The sequence of this 41-residue amidated peptide which we hereafter refer to as CRF is:

H-Ser-Gln-Glu-Pro-Pro-Ile-Ser-Leu-
Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-
Glu-Val-Leu-Glu-Met-Thr-Lys-Ala-
Asp-Gln-Leu-Ala-Gln-Gln-Ala-His-
Ser-Asn-Arg-Lys-Leu-Leu-Asp-Ile-
Ala-NH_2

Fully protected CRF was synthesized in a stepwise manner onto a benzhydrylamine resin according to the general solid phase approach of Merrifield (*15*) by means of experimental procedures described in (*16*). After complete deprotection and cleavage by hydrofluoric acid, the crude preparation of CRF was partially purified by gel permeation on G-25F with a mixture of 25 percent acetic acid and 0.01 percent β-mercaptoethanol as eluant. This product was further purified by semipreparative HPLC techniques (*12*). [Met(O)^{21}]-CRF was obtained by mild oxidation (H_2O_2 and 0.1 percent acetic acid) of CRF and further purification by HPLC. Synthetic [Met(O)^{21}]-CRF and native CRF, as it was isolated, were found to elute with identical retention times under the conditions shown in Fig. 1. Under the same

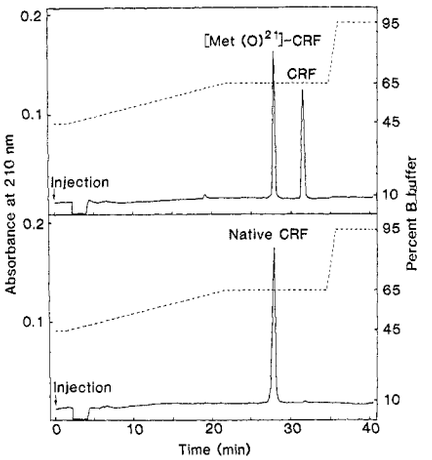

Fig. 1. Reverse phase HPLC profile of synthetic CRF and [Met(O)^{21}]-CRF (methionine sulfoxide) (upper panel) and purified native CRF (lower panel). Purified native CRF and CRF plus [Met(O)^{21}]-CRF (about 0.4 μg each) were successively chromatographed under identical conditions. Buffer A was 0.1 percent trifluoroacetic acid (TFA), buffer B was 0.24 percent aqueous TFA and acetonitrile (40:60). Gradient conditions are shown by the dotted line. Column support was Vydac C_{18} end-capped (5 μm, 300 Å pore size). Flow rate was 1.2 ml/min at room temperature. Injection volume was 1.5 ml.

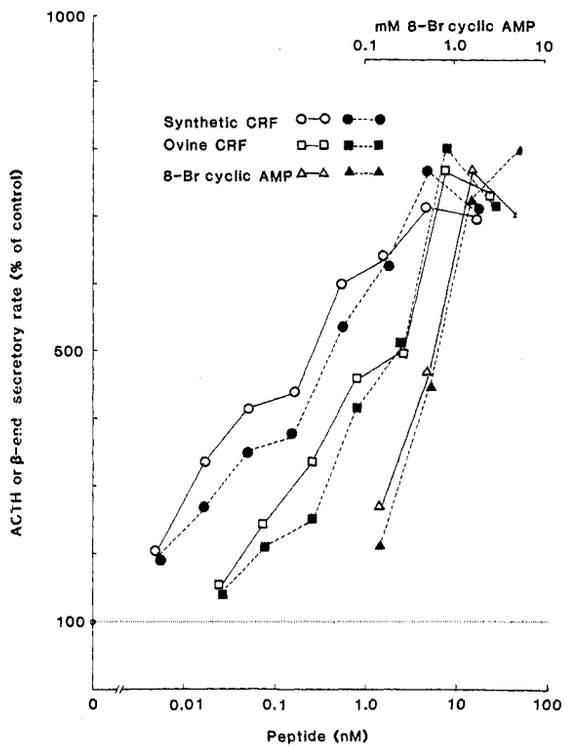

Fig. 2. Effect of synthetic CRF (○ and ●), purified native ovine CRF (□ and ■), and 8-Br-adenosine 3′,5′-monophosphate (△ and ▲) on secretion of ACTH (solid line) and β-endorphin–like immunoactivity (dashed line) by anterior pituitary cells in primary culture. Concentrations of synthetic and purified native CRF were determined by amino acid analysis.

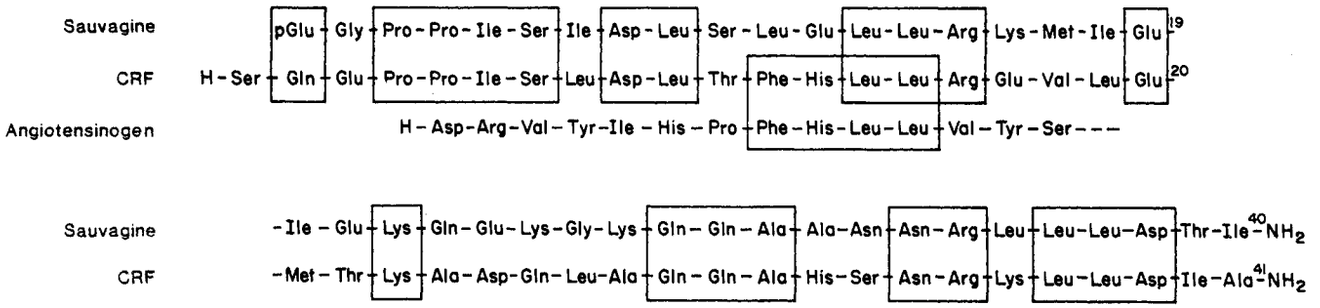

Fig. 3. Primary sequence (*11*) of ovine CRF. Residues homologous with sauvagine (*22*) or angiotensinogen are boxed (*21*).

experimental conditions we could show that [Met(O)^{21}]-CRF, CRF, CRF(1–40)-NH_2, CRF(1–39)-NH_2, and the COOH-terminal free acid eluted as distinct peaks (*12*). Because purified ovine CRF exhibited behavior on HPLC (system iii) similar to that of synthetic [Met(O)^{21}]-CRF, it is probable that the methionine may have been oxidized to methionine sulfoxide during the extraction or purification of the native peptide.

The synthetic 41-residue peptide has been shown to be highly active in stimulating the secretion of ACTH and β-End in vitro (Fig. 2) from anterior pituitary cell cultures. The fact that synthetic CRF is ten times more potent than purified native ovine CRF can be attributed to the presence of methionine sulfoxide in the latter peptide. In other experiments we have found purified native CRF to be equipotent with synthetic [Met(O)^{21}]-CRF. The minimal and half-maximal responses to synthetic CRF are routinely observed at concentrations of < 10 p*M* and 50 to 200 p*M*, respectively. As expected from experiments with partially purified CRF fractions (*4*), the secretory response to maximal (> 5 n*M*) concentrations of CRF is equal to that due to maximal concentrations of the cyclic AMP derivative, 8-Br cyclic AMP (Fig. 2), and is greater than those attributable to maximal concentrations of norepinephrine or [Arg^8]-vasopressin, which exhibit 30 to 50 percent and 10 to 20 percent of the intrinsic activity of CRF. As observed earlier with native CRF (*17*), synthetic CRF stimulates the secretion of both ACTH and β-End. Chromatography of media of anterior pituitary cell cultures reveals that approximately one third of the β-endorphin released by synthetic CRF co-elutes with β-endorphin while the bulk of the remainder co-elutes with β-lipotropin.

Glucocorticoids are established as physiologic regulators of ACTH secretion. Prior treatment of cultured anterior pituitary cells for 24 hours with 2 to 200 n*M* dexamethasone-21 phosphate results in a dose-related inhibition of CRF-mediated release of ACTH and β-End. The calcium dependency of CRF-mediated hormone secretion is supported by the observation that co-addition of 2 m*M* $CoCl_2$ in the presence of 2.5 m*M* calcium completely abolishes the secretory response to CRF by cultured pituitary cells.

This synthetic CRF is a powerful stimulator of ACTH and β-End secretion in vivo in several rat preparations. Plasma concentrations of ACTH and β-End are elevated 5 to 20 minutes after the intravenous administration of CRF to male rats anesthetized with Nembutal, to rats treated with chlorpromazine, morphine, and Nembutal (*18*) or to quiescent male or female rats with indwelling intravenous cannulas. In these preparations, doses ranging from 30 to 3000 ng per kilogram of body weight rapidly elevate plasma ACTH and β-End 5- to 20-fold. We have shown (*19*) that CRF also stimulates ACTH and β-End secretion in rats with hypothalamic deafferentations in the frontal and lateral retrochiasmatic area, a procedure that abolishes the secretion of ACTH due to surgical stress (*20*).

The potencies of CRF in vitro and in vivo are of the same order of magnitude as the potencies observed for the other hypothalamic hypophysiotropic regulatory peptides—thyrotropin-releasing hormone, gonadotropin-releasing hormone, and somatostatin. The relation between this CRF and those partially purified molecules responsible for the activities, reported by other groups cannot be determined without direct chemical and biological comparison (*10*). This CRF is not similar to the partially characterized α-CRF or β-CRF (*5*), but may be related to the larger ACTH-releasing substances (*10*).

The entire COOH-terminal region in CRF is required for full potency as shown by our findings of the less than 0.1 percent potency of the synthetic CRF COOH-terminal free acid and CRF(1–39)-NH_2. In contrast we have shown that, while synthetic CRF(10–41) is less than 0.1 percent as potent as CRF, CRF(4–41) and *N*-acetyl CRF are fully active in vitro. These results and the presence of an unblocked NH_2-terminus in this ovine CRF raises the possibility of heterogeneity in the NH_2-terminal region both within and between species. Thus, NH_2-terminally extended forms, blocked peptides such as *N*-acetyl-CRF, or even smaller CRF-like moieties might exist; for example, p-Glu, formed from Gln in the second position in CRF, might be the NH_2-terminus of some mammalian CRF-like species.

CRF contains regions homologous with other known peptides: angiotensinogen, sauvagine, and urotensin I. The presence of a common tetrapeptide sequence -Phe-His-Leu-Leu- (Fig. 3) in both CRF and angiotensinogen (*21*) may reflect a distant ancestral relatedness between these two peptides, each of which can ultimately control different functions of the adrenal cortex. It is possible that renin might act to cleave CRF and thereby be implicated in either the inactivation of CRF or the generation of fragments with other activities. The presence of two consecutive basic residues, Arg^{35}-Lys^{36}, suggests still another potential cleavage site in CRF.

Sauvagine (Fig. 3) is a 40-residue peptide recently isolated from the skin of the South American frog *Phylomedusa sauvagei* (*22*). Almost half of the amino acids in sauvagine occur in an equivalent region in CRF while 12 additional amino acids could represent single base changes. The closest homologies between sauvagine and CRF are toward the NH_2- and COOH-termini of each molecule. Sauvagine has been reported to release ACTH and β-End in vivo and in vitro and inhibit thyrotropin (TSH), somatotropin (GH), and prolactin secretion as well (*22*). We have compared some of the hypophysiotropic actions of CRF and synthetic sauvagine, prepared in this laboratory, which indeed appears to be a potent stimulator of ACTH and β-End in our in vitro assay. We find no effect, however, of CRF on basal or stimulated

secretion of TSH and GH by cultured anterior pituitary cells. CRF has effects similar to those of sauvagine (*22*) on blood pressure. Mean carotid blood pressure in a urethan-anesthetized rat fell from 87 mmHg to 72 ± 2 mmHg and remained at that level for 30 minutes after injections of 8 μg of CRF per kilogram of body weight; the subsequent administration of 80 μg of CRF per kilogram of body weight lowered mean blood pressure to 42 ± 3 mmHg for more than 2 hours. The doses of CRF required to lower blood pressure are at least 100 times greater than the amounts that stimulate ACTH and β-End secretion.

Another nonmammalian hypotensive peptide, urotensin I, isolated from teleost urohypophysis (*23*) is closely related structurally (*24*) to sauvagine and thus to CRF.

Although physiologic roles for CRF have yet to be established, the high potency and intrinsic activity to stimulate ACTH and β-End secretion and CRF's presence in the hypothalamus would be consistent with this peptide functioning as a regulator of adenohypophyseal corticotropic cells (*25*). Studies of the interactions of this CRF and known modulators of ACTH and β-End secretion such as glucocorticoids, prostaglandins, norepinephrine, and vasopressin may provide insight concerning corticotropic cells' secretory activities under a variety of physiologic and pathophysiologic circumstances. As with other regulatory peptides, this CRF will probably be found to be distributed outside of the hypothalamus and to possess various extra-hypophysiotropic roles. The ability of CRF to release neurotropic substances such as ACTH and β-endorphin as well as its possible direct brain actions lead us to speculate that this peptide might be a key signal in mediating and integrating an organism's endocrine, visceral, and behavioral responses to stress.

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25. It is premature to refer to this CRF as *the* CRF, corticoliberine or corticotropin-releasing hormone (CRH). A trivial name such as amunine, derived from the Greek ἀμύνειν (to ward off) might be appropriate were this CRF established to play a role in the acute defense of homeostasis.
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Suppression of Immunity by Stress: Effect of a Graded Series of Stressors on Lymphocyte Stimulation in the Rat

Abstract. *In rats a graded series of stressors produced progressively greater suppression of lymphocyte function, as measured by the number of circulating lymphocytes and by phytohemagglutinin stimulation of lymphocytes in whole blood and isolated cultures. This evidence suggests that stress suppresses immunity in proportion to the intensity of the stressor.*

Stress may contribute to the development and course of a range of illnesses by inducing changes in immune function (*1*). Indirect evidence that stress can influence immunity has come from studies of the effect of stressful conditions on infectious processes, neoplasia, and tissue rejection (*2*). There is also recent direct evidence for this phenomenon (*3*). The present study was undertaken to determine the relation between a graded series of stressors and a direct in vitro measure of immune function, mitogen-induced lymphocyte proliferation.

Forty-eight W/Fu rats (Microbiological Associates) were divided into four groups of 12 each: home-cage controls, apparatus controls, low-shock animals, and high-shock animals. Home-cage controls were housed four to a cage and not disturbed during the experimental period. Apparatus controls were placed in the shock apparatus with electrodes attached to their tails (*4*), but no current was applied. The procedure involved mild restraint, since the animal's tail was kept outside the cage by means of a ring taped to the tail. The two shock groups were placed into the apparatus with electrodes attached to their tails. Low-shock

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