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Distribution of active and inactive forms of endorphins in rat pituitary and brain

(C-Fragment, β-endorphin/processing/C′-Fragment/acetylation/immunofluorescent immunoassay)

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ABSTRACT **The recent isolation and identification of α-*N*-acetyl forms of the C-Fragment of lipotropin (β-endorphin, residues 61–91) and the C′-Fragment (residues 61–87) [Smyth, D. G., Massey, D. E., Zakarian, S. & Finnie, M. (1979) *Nature (London)* 279, 252–254] has led to a study of their distribution in the pituitary and brain of the rat. Regions were mapped by the method of immunofluorescent staining and the reactive peptides were determined by immunoassay after extraction, gel filtration, and ion exchange chromatography. The major immunoreactive peptides in both lobes of the pituitary were found to be C′-Fragment and *N*-acetyl C′-Fragment, which are weakly active or inactive as opiates; the C-Fragment and its *N*-acetyl derivative represented minor components. This indicates that in the rat the circulating "endorphins" released from pituitary would have little morphinomimetic activity. The same four immunoreactive peptides were observed in rat brain. In the hippocampus the C′-Fragment was the principal component; in the midbrain there was more C-Fragment but C′-Fragment predominated; in the hypothalamus the C-Fragment was the major peptide, almost to the exclusion of the other peptides. The results demonstrate that the processing of lipotropin is under differential control in anatomically distinct regions of the central nervous system. The processing of lipotropin in the hypothalamus is directed specifically to the production of lipotropin C-Fragment.**

Understanding of the full physiological role of the endorphins must start from the identification of these peptides and elucidation of their distribution in regions of pituitary and brain. Present in picomolar concentrations, they can be detected and measured by the application of highly sensitive immunological techniques involving the use of specific antibodies. In studies of β-endorphin [lipotropin (LPH) C-Fragment] the antisera employed have exhibited COOH-terminal specificity (1–4) and would react with the α-*N*-acetyl derivatives of the C- and C′-Fragments of LPH, as well as with a series of peptides including the 31,000-M_r endorphin prohormone, LPH, the C-Fragment (residues 61–91), and the C′-Fragment (residues 61–87). All these peptides occur naturally, but previous studies on the distribution of β-endorphin by immunofluorescence (5, 6) or by radioimmunoassay of tissue extracts after gel filtration (7–10) have not differentiated between them. Because only the C-Fragment exhibits potent opiate activity—the C′-Fragment is $\frac{1}{500}$th as active as an analgesic agent (11) and the acetyl peptides (12) and LPH (13, 14) are inert—it is essential that the immunoreactive peptides should be precisely identified. We give here a brief description of our detailed mapping of the pituitary and brain of the rat by immunofluorescence using antibodies raised against homogeneous porcine C-Fragment and we present the results of experiments that have allowed characterization of the fluorescing substances in specific regions. The data reveal strikingly different patterns of distribution of the known peptides, reflecting controlled processing of the 31,000-M_r endorphin prohormone.

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MATERIALS AND METHODS

LPH, the C-Fragment of LPH, the C′-Fragment, the acetyl derivatives of the C- and C′-Fragments, the nonadecapeptide (LPH-61–79), and corticotropin were obtained by extraction from porcine pituitary (12, 15). Methionine enkephalin was prepared by synthesis.

Preparation and Characteristics of Antibody to LPH C-Fragment. The C-Fragment (5 mg) isolated from porcine pituitary (15) was coupled to bovine gamma globulin (25 mg) by incubation with 60 mg of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulphonate. A small amount of ^{125}I-labeled C-Fragment (^{125}I-C-Fragment) (about 100 μg, 10,000 cpm) was added to permit estimation of the coupling efficiency. The reaction mixture was desalted on Sephadex G-75 in 50% (vol/vol) acetic acid and the solvent was removed from the coupled peptide by evaporation under reduced pressure. Rabbits were immunized intramuscularly at multiple sites with 100 μg of C-Fragment (coupled to bovine gamma globulin) in complete Freund's adjuvant. The immunization was repeated four times at 2-week intervals by using incomplete Freund's adjuvant. The rabbits were bled 2 weeks after the last injection and the sera were tested for C-Fragment binding by the charcoal radioimmunoassay procedure (16). Antisera were collected over a period of 6 months.

Procedure for Radioimmunoassay. Portions of fractions from chromatographed tissue extracts were evaporated under reduced pressure and taken up in 200 μl of 0.05 M sodium phosphate (pH 7.6) containing 0.25% human serum albumin. ^{125}I-C-Fragment (20,000 cpm, approximately 10 pg) in 50 μl of the phosphate buffer and then 50 μl of C-Fragment antiserum at a dilution of 1:16,000 were added, giving 60–70% binding of the radioactive peptide. The mixture was incubated overnight at 4°C. The bound peptide was separated from unbound material by the addition of 200 μl of a suspension of activated charcoal [3 g of activated charcoal, 0.73 g of dextran (M_r 73,000), 10 ml of 0.5 M sodium phosphate (pH 7.6), and 60 ml of horse serum, the total being diluted with H_2O to 100 ml]. The mixture was centrifuged at 3000 rpm for 30 min at 4°C and the supernatant, which contained the bound peptide, was transferred and assayed for radioactivity in a Wallach gamma counter.

Antibody Specificity. Radioimmunoassay of the antibody to C-Fragment showed that the antisera reacted with porcine LPH and with porcine C′-Fragment, in addition to the C-Fragment; the molar potencies of these peptides were, respectively, one-fourth and one-sixth of the potency exhibited by the C-Fragment. The affinity of the antibody for the α-*N*-

Abbreviations: LPH, lipotropin; C-Fragment (β-endorphin), residues 61–91 of LPH; C′-Fragment, residues 61–87 of LPH.

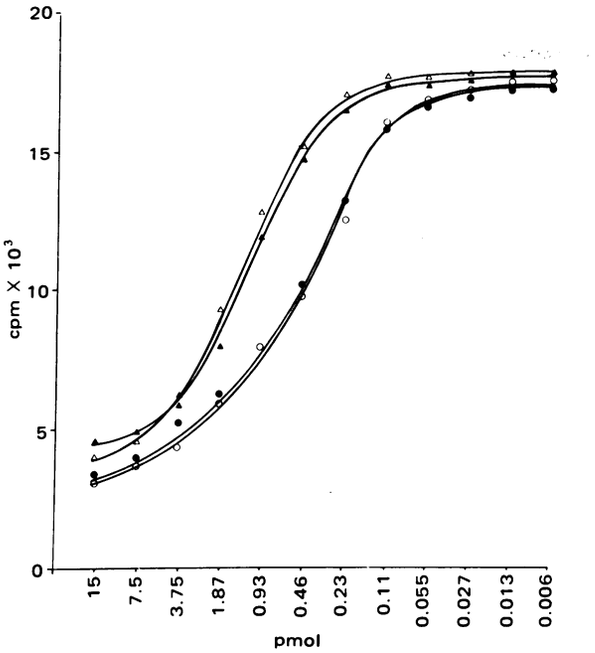

FIG. 1. Radioimmunoassay standard curves for LPH C-Fragment (○), *N*-acetyl C-Fragment (●), C′-Fragment (△), and *N*-acetyl C′-Fragment (▲).

acetyl peptides was the same as that for the parent unacetylated peptides (Fig. 1). It should be noted that these relative potencies may not be identical to the potencies of the corresponding peptides from the rat because of a minor difference in COOH-terminal sequence (17). The antiserum did not react with the nonadecapeptide (LPH-61–79), γ-endorphin (LPH-61–77), methionine enkephalin (LPH-61–65), or corticotropin.

Immunofluorescence. Lewis rats (100–150 g) were perfused through the aorta with phosphate-buffered saline and then with cold 4% depolymerized paraformaldehyde in 0.1 M sodium phosphate at pH 7.6. Brain and pituitary were removed, incubated in the perfusion solution for 4 hr and washed for 2 days in 0.3 M sucrose in phosphate-buffered saline. The tissue was quick frozen in a Frigocut 2700 cryostat, 10-μm serial sections were cut, and the sections were mounted on gelatin-coated slides. Staining was by the indirect immunofluorescence method (18).

Sections were incubated with rabbit anti-C-Fragment IgG at a dilution of 1:400 for 24 hr at 4°C. Antibody dilution was in 0.2 M sodium phosphate (pH 7.6) containing 0.3% Triton X-100 [0.1 M L-lysine hydrochloride was added to reduce nonspecific binding of antibody to the paraformaldehyde-fixed tissue (S. de Petris, personal communication)]. Excess antibody was removed from the sections by washing three times with phosphate (5 min each) and the sections were incubated for 30 min at room temperature with rhodamine-conjugated goat anti-rabbit IgG at a dilution of 1:32.* Excess conjugate was washed three times (5 min each) with phosphate-buffered saline and mounted in a 1:9 phosphate-buffered saline containing 0.1% azide/glycerine mixture. Sections were examined through a Leitz Orthoplan fluorescence microscope; photographic exposure time in all cases was 2 min. Control sections were incubated either with normal rabbit IgG or with rabbit anti-C-Fragment IgG absorbed with porcine C-Fragment; in these experiments no fluorescence was seen.

* Antibody to C-Fragment and the rhodamine-conjugated goat anti-rabbit IgG were absorbed with rat liver powder or rat brain homogenate to prevent nonspecific association.

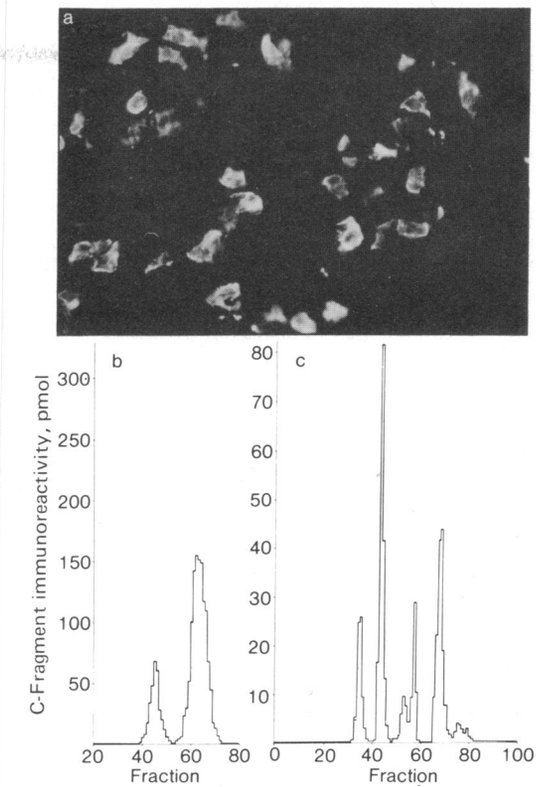

FIG. 2. (*a*) Localization of peptides with LPH COOH-terminal immunoreactivity in rat anterior pituitary by immunofluorescence (×80). (*b* and *c*) Resolution of peptides in rat anterior pituitary that have LPH COOH-terminal immunoreactivity. Extraction was carried out on 60 freshly dissected anterior pituitary lobes and the peptides were resolved by chromatography. (*b*) Gel filtration on Sephadex G-75 in 50% acetic acid. Aliquots (100 μl) of each 3.4-ml fraction were immunoassayed with rabbit antisera raised against porcine C-Fragment. (*c*) Ion exchange chromatography of the "β-endorphin" peak from *b* on SP-Sephadex C-25. Aliquots (5 μl) of each 1.8-ml effluent fraction were immunoassayed by using anti-porcine C-Fragment antiserum. The amounts of the peptides shown in the figures have not been corrected for their relative immunoreactivities.

Extraction and Gel Filtration of Peptides. Freshly dissected anterior lobes and pars intermedia plus posterior lobes of rat pituitary, or regions of rat brain, were homogenized one by one at 4°C in approximately 30 ml of acid acetone [acetone/water/concentrated hydrochloric acid (40:5:1)]. The suspension was clarified by centrifugation (18,000 rpm for 30 min) and the supernatant was concentrated under reduced pressure at 20°C. The moist residue was dissolved in 3 ml of 50% acetic acid and gel filtration was performed on a column (70 × 2.4 cm) of Sephadex G-75 in 50% acetic acid. Aliquots of each fraction were dried in a vacuum desiccator and immunoassay was carried out with antiserum to C-Fragment. In some cases further gel filtration was carried out on a column (60 × 1 cm) of Sephadex G-100 in 4 M guanidine and immunoassay was carried out on 10-μl aliquots.

Ion-Exchange Chromatography. Peptide fractions obtained by gel filtration of Sephadex G-75 were concentrated under reduced pressure at 20°C and the moist residue was dissolved in 2 ml of 50% acetic acid. The solution was added to a column (60 × 0.6 cm) of SP-Sephadex C-25 equilibrated in 50% acetic acid, a 0–0.6 M sodium chloride gradient was applied (mixer volume, 100 ml), and fractions (1.8 ml) were collected. To locate and measure the eluted peptides, aliquots of each fraction were dried under reduced pressure and the residues were analyzed by immunoassay.

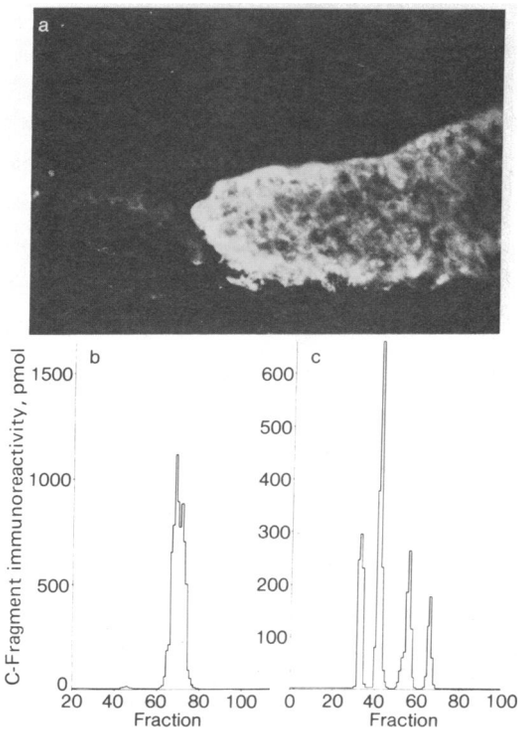

FIG. 3. (*a*) Localization of peptides with LPH COOH-terminal immunoreactivity in rat pars intermedia plus posterior pituitary by immunofluorescence (×80). (*b* and *c*) Resolution of peptides in rat pars intermedia plus posterior pituitary. Extraction was carried out on 60 freshly dissected tissues and the peptides were resolved by chromatography. (*b*) Gel filtration on Sephadex G-75. Aliquots (20 μl) of each 3.4-ml fraction were immunoassayed with rabbit antisera raised against porcine C-Fragment. (*c*) Ion exchange chromatography of the "β-endorphin" peak from *b*. Aliquots (1 μl) of 1.8-ml effluent fractions were immunoassayed. The amounts of the peptides shown in the figures have not been corrected for their relative immunoreactivities.

RESULTS

Analysis of rat pituitary by immunofluorescence showed that isolated cells were reactive in the anterior lobe (Fig. 2*a*) whereas in the pars intermedia there was dense staining in all the cells (Fig. 3*a*). No immunoreactive material was seen in the posterior pituitary. To identify the fluorescent substances, the peptides were extracted and resolved by gel filtration and ion exchange chromatography and the amounts were determined by immunoassay.

Gel filtration of the material extracted from the anterior pituitary revealed the presence of two groups of peptides which differed in molecular size (Fig. 2*b*). The major fraction emerged in the position of porcine β-endorphin, but subsequent ion exchange chromatography showed that it contained four immunoreactive components which differed in electrical charge (Fig. 2*c*). They emerged in the positions of authentic markers of the porcine peptides—acetyl C′-Fragment, C′-Fragment, acetyl C-Fragment, and C-Fragment. After correction of the results for immunoreactivity the principal peptides in the β-endorphin fraction were seen to be *N*-acetyl C′-Fragment (peak 1) and C′-Fragment (peak 2). In addition to the "β-endorphin" peak on the Sephadex G-75 column there was a fraction that contained larger polypeptides (peak 1, Fig. 2*b*). Further gel filtration of this fraction on Sephadex G-100 in 4 M guanidine demonstrated a significant amount of immunoreactive peptide emerging at the exclusion volume, attributable to the 31,000-M_r corticotropin–LPH precursor (19), as well as a retarded peak which emerged in a similar position to a porcine LPH marker.

Gel filtration of the material extracted from the pars intermedia plus posterior pituitary showed the presence of a single group of peptides of similar molecular size, emerging in the position of β-endorphin (Fig. 3*b*). The amount of immunoreactive peptide in this fraction was approximately 6 times the amount in the corresponding fraction from the anterior pituitary. There was a negligible amount of larger immunoreactive peptides. Ion exchange chromatography of the "β-endorphin" fraction revealed the same four components that were resolved in the experiments with anterior pituitary; the peptide that emerged in the position of C-Fragment was present in the least amount (Fig. 3*c*).

Further evidence confirming our identification of the four immunoreactive peptides was obtained in a parallel study conducted with porcine pituitary tissue. Immunoassay of the analogous fractions from this large-scale experiment (100 pituitaries) showed four components that chromatographed in the same positions as the corresponding immunoreactive peptides extracted from rat tissue. Each of the four porcine peptides was obtained pure after a second ion exchange step and characterized by amino acid analysis with acid or enzymic hydrolysis. The values obtained corresponded to the values given by authentic peptides that had been isolated in homogeneous form by other procedures (12). Close correlation was observed between the amounts of the four peptides determined by chromatography and immunoassay and the amounts determined by chromatography and amino acid analysis.

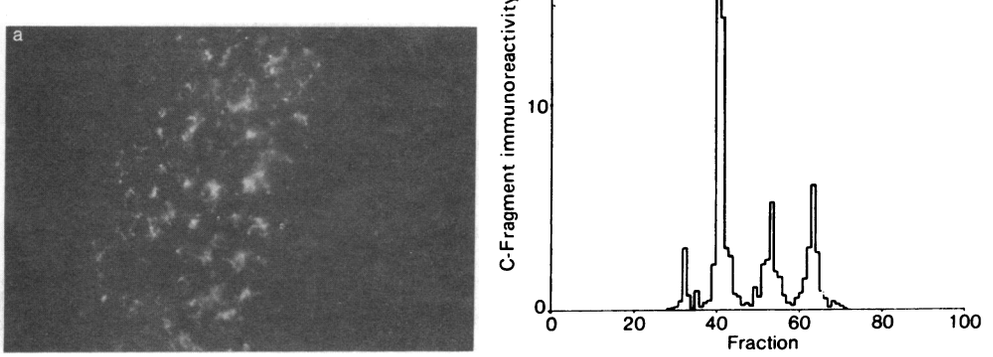

FIG. 4. (*a*) Localization of LPH fragments with COOH-terminal immunoreactivity in rat hippocampus by immunofluorescence (×80). (*b*) Resolution of peptides in rat hippocampus. The peptides extracted from the hippocampus of 60 rats were, after gel filtration on Sephadex G-75, separated on a column of SP-Sephadex C-25 as in Fig. 2*c*. Aliquots (100 μl) of each 1.8-ml fraction were immunoassayed.

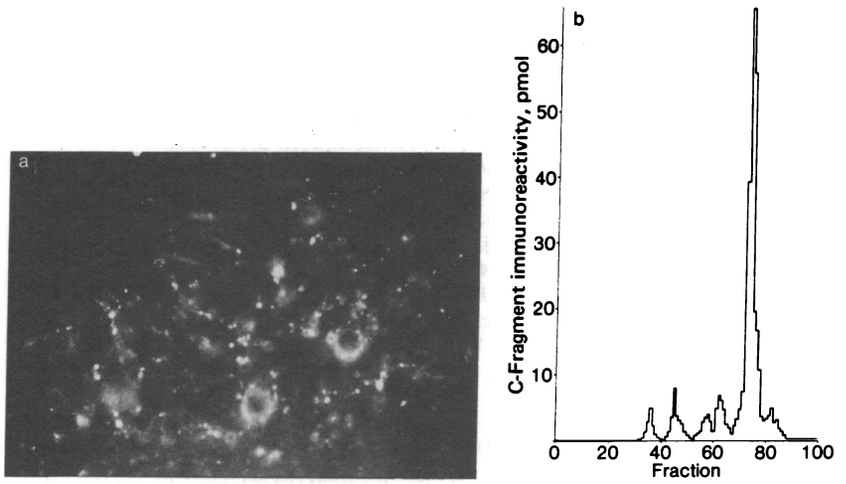

FIG. 5. (*a*) Localization of peptides with LPH COOH-terminal immunoreactivity in rat hypothalamus by immunofluorescence (×120). (*b*) Resolution of peptides in rat hypothalamus. The peptides extracted from the hypothalamus of 30 rats were, after gel filtration on Sephadex G-75, separated on a column of SP-Sephadex C-25 as in Fig. 2*c*. Aliquots (100 μl) of each 1.8-ml fraction were immunoassayed.

In the brain, the regions shown by fluorescence to contain peptides with LPH COOH-terminal immunoreactivity were the hypothalamus, midbrain, hippocampus, amygdala, and periaqueductal gray. The main concentrations of "β-endorphin" were in the hypothalamus; cell bodies were located ventromedial to the third ventricle and long beaded axons and terminals were lateral to the third ventricle (Fig. 4*a*). In addition, there were bundles of terminals in the hippocampus (Fig. 5*a*) and cell bodies and dense terminals in the amygdala. There was light staining of beaded axons in the periaqueductal gray, the fluorescence being confined to the ventral regions of the aqueduct, and the midbrain contained long axons sparsely distributed. The three regions that contained the highest concentrations of fluorescent material were extracted and chromatographed to allow identification of the fluorescing peptides.

Chromatography of the peptides obtained from whole brain, using gel filtration on Sephadex G-75 and ion exchange on SP-Sephadex C-25, demonstrated the presence of the same four peptides that were present in the pituitary, and dissected regions of the brain showed an organized distribution of these peptides. In the hippocampus the principal immunoreactive component was the C′-Fragment (Fig. 4*b*); in the midbrain the four peptides occurred in more equivalent proportions; in the hypothalamus the C-Fragment was the major peptide, almost to the exclusion of the other peptides (Fig. 5*b*).

Intact LPH was seen in small quantity during gel filtration of the peptides extracted from whole brain; negligible quantities were observed in the hypothalamus, midbrain, or hippocampus.

The results of these experiments, which establish the concentrations of peptides with LPH COOH-terminal immunoreactivity, are summarized in Table 1.

DISCUSSION

It is generally assumed that C-Fragment (β-endorphin) occurs in high concentration in the anterior lobe and pars intermedia of the pituitary. However, the present results demonstrate that the principal immunoreactive peptide in both lobes of rat pituitary is not the C-Fragment: it is the C′-Fragment together with its *N*-acetyl derivative. In the anterior pituitary C-Fragment represents <8% of the peptides with LPH COOH-terminal immunoreactivity, and in the pars intermedia it represents only 4%. Because the C′-Fragment and the acetyl peptides are the predominant "endorphins" in the pituitary and are only weakly active or inactive as opiates, it seems likely that the circulating endorphins derived from pituitary in the rat are essentially inert.

The biological significance of the C′-Fragment and the acetylated peptides is unknown. The C′-Fragment retains a negligible fraction of the analgesic activity of C-Fragment and it has a similar duration of action (11, 20); it is therefore unlikely to be elaborated in order to perform a similar function to the C-Fragment. It might, of course, have an entirely different biological activity but so far there is no evidence for this. Similarly, the acetyl derivatives of the C- and C′-Fragments are inert as opiates (12). They are probably formed concomitantly with α-melanotropin because these acetylated peptides are concentrated in the same region of pituitary (this communication and ref. 21) and originate from the same 31,000-M_r prohormone (19). Because the acetyl group is important for the biological activity of α-melanotropin (22) but eliminates the activity of C-Fragment, the acetylation reaction may provide a means for selective activation of different regions of the endorphin prohormone.

The amount of the active form of C-Fragment found in the

Table 1. Peptides with LPH COOH-terminal immunoreactivity in regions of rat pituitary and brain

	pmol/g of tissue				
Tissue	LPH plus 31,000-M_r	Acetyl-C′-Fragment	C′-Fragment	Acetyl-C-Fragment	C-Fragment
Anterior pituitary	3570 (38.1%)	1868 (19.9%)	3168 (33.8%)	39.2 (0.4%)	729 (7.8%)
Pars intermedia plus posterior pituitary	3000 (0.9%)	103,600 (31.3%)	186,400 (56.4%)	24,910 (7.5%)	12,790 (3.9%)
Hippocampus	2.7 (11.9%)	10.4 (7.5%)	92.5 (66.8%)	8.5 (6.3%)	10.5 (7.6%)
Midbrain	6.3 (3.4%)	32.0 (17.4%)	95.4 (52.0%)	15.3 (8.3%)	34.6 (18.9%)
Hypothalamus	4.5 (1.9%)	29.1 (12.1%)	53.2 (22.1%)	21.9 (9.1%)	131.5 (54.7%)

The amounts of the peptides have been corrected for their individual immunopotencies. The LPH values were obtained by gel filtration on Sephadex G-75 and the values of the four peptides the size of β-endorphin were obtained by chromatography on SP-Sephadex C-25. The weights of tissues refer to wet weights.

anterior lobe was small but the amount of the other immunoreactive peptides behaving like C-Fragment during gel filtration was substantial. Previous reports (23–25) have identified 'β-endorphin' in the anterior pituitary of the rat (and of mouse ATt-20 cells) without distinguishing the four peptides and in two cases (26, 27) it was reported that rat anterior pituitary contains essentially no β-endorphin. Indeed, β-endorphin was described as an artifact generated by freezing and thawing of the tissue prior to extraction. It is important to note that the present results were obtained under conditions chosen to prevent *post mortem* autolysis. Each of the 60 pituitary lobes was homogenized immediately after dissection and without freezing, and samples of dissected anterior lobes were sectioned histologically to ensure that there was no contamination from pars intermedia. Furthermore, the use of acid acetone as a homogenizing medium does not give rise to isolation artifacts (28) and it gives high yields during extraction of peptides the size of C-Fragment.

Because C-Fragment appears to be formed from LPH *in vivo* (29, 30), the concentrations of the C- and C′-Fragments in different regions of pituitary and brain probably reflect the action of enzymes with specificity for the lysylarginine residues at positions 59 and 60 and lysyllysine residues at positions 88 and 89 in the LPH sequence. Furthermore, on comparing the relative amounts of the C- and C′-Fragments in the anterior pituitary with the same peptides in the pars intermedia—the ratios are 1:4 and 1:16, respectively—it becomes clear that the manner of processing of LPH differs in the two regions.

The finding that C-Fragment is the predominant endorphin in rat hypothalamus stands in contrast to the results which show C′-Fragment and its acetyl derivative as the major peptides in the pituitary. This implies that brain endorphins do not originate in the pituitary but are biosynthesized in brain, in agreement with conclusions drawn from experiments with hypophysectomized rats (7, 31). Furthermore, our results indicate that different mechanisms are responsible for the formation of the C- and C′-peptides. One possibility is that different enzymes are involved; another is that a single enzyme is held under inhibition with respect to cleavage between positions 89 and 90 but not to cleavage between positions 60 and 61. In either case, the results point to the existence in the hypothalamus of a highly specific processing mechanism for production of the potent opiate, LPH C-Fragment.

The C′-Fragment is the major immunoreactive peptide in the hippocampus and in the midbrain, but it is unlikely to fulfill an active role as an opiate. Because the formation of this peptide must take place at the expense of C-Fragment, the C′-Fragment may be considered, like the acetyl peptides, to be an inactivated form of β-endorphin that is stored intracellularly. The degree to which C-Fragment is made to undergo inactivation during processing *in vivo* may represent one mechanism by which the normal levels of opiate activity are maintained in the central nervous system. Malfunction in the regulation of the inactivating enzymes could lead to abnormal concentrations of C-Fragment which may underlie certain neurological disorders.

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1. Smyth, D. G. & Zakarian, S. (1979) in *Endorphins in Mental Health Research*, eds., Usdin, E., Bunne, W. C. & Kline, N. S. (MacMillan, New York), pp. 84–92.
2. Guillemin, R., Ling, N. & Vargo, T. M. (1977) *Biochem. Biophys. Res. Commun.* **77**, 361–366.
3. Li, C. H., Rao, A. J., Doneen, B. A. & Yamashiro, D. (1977) *Biochem. Biophys. Res. Commun.* **74**, 656–662.
4. Höllt, V., Przewlocki, R. & Herz, A. (1978) *Nauyn-Schmiedeberg's Arch. Pharmacol.* **303**, 171–174.
5. Watson, S. J., Barchas, J. D. & Li, C. H. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5155–5158.
6. Bloom, F., Battenberg, E., Rossier, J., Ling, N. & Çuillemin, R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1591–1595.
7. Rossier, J., Vargo, T. M., Minick, S., Ling, N., Bloom, F. E. & Guillemin, R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5162–5165.
8. Krieger, D. T., Liotta, A., Suda, T., Palkovits, M. & Brownstein, M. J. (1977) *Biochem. Biophys. Res. Commun.* **76**, 930–936.
9. Lissitsky, J. C., Morin, O., Dupon, A., Labrie, F., Seidah, N. G., Chretien, M., Lis, M. & Coy, D. H. (1978) *Life Sci.* **22**, 1715–1722.
10. Yoshimi, H., Matsukara, S., Sueoka, S., Fukase, M., Yokata, M., Hirata, Y. & Imura, H. (1978) *Life Sci.* **22**, 2189–2196.
11. Geisow, M. J., Deakin, J. F. W., Dostrovsky, J. O. & Smyth, D. G. (1977) *Nature (London)* **269**, 167–169.
12. Smyth, D. G., Massey, D. E., Zakarian, S. & Finnie, M. (1979) *Nature (London)* **279**, 252–254.
13. Loh, H. H., Tseng, L. F., Wei, E. & Li, C. H. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2895–2898.
14. Graf, L., Szekely, J. I., Ronai, A. Z., Dunai-Kovacs, Z. & Bajusz, S. (1976) *Nature (London)* **263**, 240–241.
15. Smyth, D. G., Snell, C. R. & Massey, D. E. (1978) *Biochem. J.* **175**, 261–270.
16. Herbert, V., Lau, K., Gottlieb, C. W. & Bleicher, S. J. (1965) *J. Clin. Endocrinol.* **25**, 1375–1384.
17. Rubinstein, M., Stein, S., Gerber, L. D. & Udenfriend, S. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4969–4972.
18. Coons, A. H., Creech, G. J., Jones, R. N. & Berliner, E. (1942) *J. Immunol.* **45**, 159–170.
19. Mains, R. E., Eipper, B. A. & Ling, N. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3014–3018.
20. Feldberg, W. & Smyth, D. G. (1977) *Br. J. Pharmacol.* **60**, 445–454.
21. Lowry, P. J. & Scott, A. P. (1975) *Gen. Comp. Endocrinol.* **26**, 16–23.
22. Eberle, A., Leukart, O., Schiller, P., Fauchere, J. L. & Schwyzer, R. (1977) *FEBS Lett.* **82**, 325–328.
23. Mains, R. E. & Eipper, B. A. (1978) *J. Biol. Chem.* **253**, 651–655.
24. Roberts, J. L., Phillips, M., Rosa, P. A. & Herbert, E. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3609–3618.
25. Przewlocki, R., Höllt, V. & Herz, A. (1978) *Eur. J. Pharmacol.* **51**, 179–183.
26. Rubinstein, M., Stein, S., Gerber, L. D. & Udenfriend, S. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3052–3055.
27. Liotta, A. S., Suda, T. & Krieger, D. T. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2950–2954.
28. Smyth, D. G., Austen, B. M., Geisow, M. J. & Snell, C. R. (1977) in *Molecular Endocrinology*, Proceedings of the 6th International Congress of Endocrinology, (Elsevier/North-Holland, Amsterdam), pp. 327–336.
29. Bradbury, A. F., Smyth, D. G. & Snell, C. R. (1976) *Biochem. Biophys. Res. Commun.* **69**, 950–956.
30. Seidah, N. G., Gianoulakis, C., Çrine, P., Lis, N., Benjannet, S., Routhier, R. & Chretien, M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3153–3157.
31. Cheung, A. & Goldstein, A. (1976) *Life Sci.* **19**, 1005–1008.