BIOSYNTHESIS AND RELEASE OF β -ENDORPHIN-, N-ACETYL β -ENDORPHIN-, β -ENDORPHIN-(1-27)-, AND N-ACETYL β -ENDORPHIN-(1-27)-LIKE PEPTIDES BY RAT PITUITARY NEUROINTERMEDIATE LOBE: β -ENDORPHIN IS NOT FURTHER PROCESSED BY ANTERIOR LOBE¹

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Abstract

Continuous labeling and pulse-chase techniques were employed to study the synthesis and secretion of multiple forms of immunoreactive β -endorphin by cultured dispersed rat anterior lobe cells and intact neurointermediate pituitary lobe. Cell and medium extract immunoreactive β -endorphin (specific immunoprecipitation and radioimmunoassay) exhibiting a K_{av} similar to authentic β -endorphin upon gel filtration was characterized further by nonequilibrium isoelectric focusing, cation exchange chromatography, reverse phase high pressure liquid chromatography, and partial tryptic and chymotryptic mapping.

Intact neurointermediate lobes incorporated radiolabeled amino acids into four to six forms of immunoreactive β -endorphin. Four of these forms were physicochemically similar to authentic β -endorphin, N-acetylated β -endorphin, β -endorphin-(1-27), and N-acetylated β -endorphin-(1-27). Pulse-chase studies indicated that a β -lipotropin-like molecule served as a metabolic intermediate for a β -endorphin-like molecule. As β -endorphin-like material accumulated in the cell, some of it was N-acetylated (~18% at 2 hr chase and ~65% at 18 hr chase). At later chase times, β -endorphin-(1-27)- and N-acetylated β -endorphin-(1-27)-like peptides were the predominant molecular species detected. All endorphin forms were detected in unlabeled tissue maintained in culture or tissue continuously labeled for 72 hr and were released into the medium under basal, stimulatory (10^{-8} M norepinephrine), or inhibitory (10^{-7} M dopamine) incubation conditions. In all cases, β -endorphin-(1-27)-like species were the predominant forms (more than 70% of total) present in the cells and released into the medium.

In contrast, approximately 90% of radiolabeled immunoreactive β -endorphin extracted from anterior lobe cells and medium similarly incubated appeared to represent the authentic β -endorphin molecule. Continuous labeling (72 hr) revealed the β -lipotropin/ β -endorphin molar ratio to be approximately 4. We conclude that, in anterior lobe, most of the β -endorphin is not processed further and is released intact, while in neurointermediate lobe, it serves as a biosynthetic intermediate.

It has now been firmly established that adrenocorticotrophic hormone (ACTH)- and β -lipotropin-related peptides are derived from a common precursor molecule(s) in the pituitaries of several species thus far studied. Within a species, anterior pituitary corticotrophic cells and intermediate lobe cells synthesize identical or very similar common precursor molecules. However, the post-translational processing of the precursor in the two lobes differs. In rat anterior pituitary (AP), ACTH-(1-39) (and

its glycosylated derivative) and β -lipotropin (β -LPH) appear as major end products, with lesser amounts of β -endorphin (β -EP) present, while in neurointermediate lobe (NIL), relatively little intact ACTH or β -LPH is found. In this lobe, ACTH is processed further to α -melanotropin (α -MSH)- and ACTH-(18-39)-like peptides. Likewise, β -LPH is processed further into β -EP-like and γ -LPH-like peptides (see Herbert et al., 1980; Eipper and Mains, 1980; and Chretien et al., 1979, for a review).

Until recently, β -EP (the 31 COOH-terminal amino acids of β -lipotropin) was generally thought to represent a major secretory product in the rat neurointermediate lobe, derived from a trypsin-like cleavage of β -LPH at a pair of basic residues immediately preceding the β -en-

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dorphin sequence. Smyth et al. (1978, 1979) have isolated four peptides from pig pituitary which they identified as β -EP, β -EP-(1-27), and their α -N-acetyl derivatives on the basis of chromatographic behavior and amino acid analysis. Very recently, Zakarian and Smyth (1979) identified β -EP-(1-27) and α -N-acetyl β -EP-(1-27) as the major components of immunoreactive (IR) β -EP in extracts of freshly obtained rat anterior and intermediate pituitary lobe tissue; authentic β -EP and its α -N-acetyl derivative were minor components. Subsequently, we have confirmed this finding with respect to neurointermediate lobe (Liotta and Krieger, 1980). Since the major species detected exhibit no or only weak opiate agonist activity (Smyth et al., 1979), these findings directly bear on the possible physiological role(s) which these peptides subserve. However, the mere detection of a peptide in a tissue extract cannot be taken as direct evidence that such a peptide represents an actual physiological product. Incomplete inactivation of pituitary enzymes during extraction or postmortem autolysis could produce some of the detected peptides artifactually. Furthermore, an intracellular mechanism may exist that, under certain metabolic conditions, degrades peptides otherwise destined for secretion (e.g., lysosomal fusion with secretory vesicles).

We therefore sought to determine if the multiple forms of immunoreactive β -endorphin detected in rat pituitary did indeed represent secretory products. We reasoned that, if this were so, (1) their biogenesis should be demonstrable *in vitro* when pituitary is incubated in the presence of radiolabeled amino acids utilizing pulse-chase and continuous labeling paradigms and (2) they should be released into the medium when tissue is maintained in culture under the appropriate conditions.

Materials and Methods

Preparation of tissue and conditions of culture. Anterior pituitary cells were prepared as described (Liotta et al., 1979) and plated at 6 to 9×10^5 cells/well. To ensure that no cross-contamination of lobes occurred during dissection, the whole pituitary was removed intact and anterior lobe not in contact with NIL was removed with a pair of iris scissors for dispersion. The remaining anterior tissue adhering to NIL was removed and discarded. Neurointermediate lobe tissue was not dispersed but incubated intact, one per well.

Medium 199 supplemented with nonessential amino acids ($200 \mu\text{M}$), 5% normal rat serum, 5% fetal calf serum, penicillin G (50 units ml^{-1}), and streptomycin ($100 \mu\text{g ml}^{-1}$) was employed. Several batches of serum had to be tested prior to use, since some preparations contained considerable proteolytic activity (added β -EP and α -MSH were recovered poorly). Anterior pituitary cells and neurointermediate lobes were incubated with $300 \mu\text{l}$ of medium, except for delivery of the 30-min "pulse" of radioactive amino acids, in which case, $100 \mu\text{l}$ was used. When 72-hr prelabeled or unlabeled cells were used to characterize IR- β -EP released into medium (see below), the serum was replaced with 0.8% defatted rat albumin.

Incubation of pituitary tissue with radioactive amino acids. Anterior pituitary cells were incubated with [^{35}S]methionine (final specific activity, 23 Ci mmol^{-1} at

a final concentration of $150 \mu\text{M}$) continuously for 72 hr. Neurointermediate lobe tissue was similarly incubated with [^{35}S]methionine and [^3H]tyrosine (Tyr; final specific activity, 22 Ci mmol^{-1} at a final concentration of $200 \mu\text{M}$). For pulse-chase studies, NIL was incubated for 30 min with [^{35}S]methionine at a final specific activity of 100 Ci mmol^{-1} and a final concentration of $200 \mu\text{M}$, after which, the medium was removed and the tissue was washed (1 min/wash) three times with Medium 199 devoid of [^{35}S]methionine (unlabeled methionine was present at 1 mM) and then incubated (chase) in such medium for 1, 2, 4, 6, or 18 hr.

Release studies. Anterior pituitary cells were incubated for 1 hr with 0.1 equivalent ($\sim 80 \mu\text{g}$ of protein) of a rat medial basal hypothalamic extract (rHME; HE-RP-1 obtained from National Institute of Arthritis, Metabolism, and Digestive Diseases). Neurointermediate lobes were incubated 1 or 2 hr with 10^{-7} M dopamine (DA) or 10^{-8} M norepinephrine (NE). Then IR- β -EP released into the medium under these conditions was characterized and compared to IR- β -EP released when tissue was incubated in medium only (basal release). Subsequent to such incubations, the tissue was extracted and likewise characterized. In addition, total IR- α -ACTH and total IR- α -MSH was determined on cell and media extracts for anterior cells and NIL tissue, respectively.

Radioimmunoassays (RIA). The ACTH and β -endorphin assays were performed as previously described in our laboratory (Yamaguchi et al., 1980). The ACTH (West) antiserum was obtained from National Institute of Arthritis, Metabolism, and Digestive Diseases. The β -EP antiserum cross-reacts on an equimolar basis with camel β -EP, camel β -EP-(1-27), and the human forms of these peptides and their α -N-acetyl derivatives as well as with the common precursor molecules. It does not react at all with α - or γ -EP. Since the antiserum reacted equally with the human acetylated peptides and since the sequences of rat and camel β -EP are identical (Rubinstein et al., 1977), we assume that all related rat peptides would be recognized likewise. The α -MSH antiserum was a gift from Dr. H. Vaudry (Université de Rouen, Mont-Saint-Aignan, France). It possesses less than 0.1% cross-reactivity with synthetic porcine or human ACTH. The procedure utilized was the same used for β -EP assay.

Immunoprecipitation. Antiserum raised to synthetic porcine β -EP was affinity-purified as previously described (Liotta et al., 1979, 1980). This is the same antiserum employed for β -EP radioimmunoassay. Immunoprecipitation was performed as described (Comstock et al., 1979). Immunoprecipitates were dissolved in $100 \mu\text{l}$ of solution containing 0.1 M HCl , 0.2 M formic acid, and 8 M urea (overnight incubation at room temperature).

Gel filtration. Gel filtration was performed on $0.9 \times 60 \text{ cm}$ columns packed with Sephadex G-50 fine beads as described (Liotta et al., 1979, 1980), except that 1% formic acid was included in the eluent. Recoveries from these columns always exceeded 94%.

NaDodSO₄-polyacrylamide gel electrophoresis (SDS-PAGE). This procedure was employed as previously described (Liotta et al., 1980). Recovery of applied radioactivity always exceeded 93%.

Concentration and desalting of samples. C-18 Sep-

Pak cartridges (Waters Associates) were utilized. For elution of peptides, 80% CH_3CN in 0.05 M HCl or 0.06 M H_2SO_4 was employed.

Cation exchange chromatography. IR- β -EP obtained from the gel filtration studies was applied to a 0.9×15 cm column of SP-Sephadex equilibrated with 0.04 M HCl. A linear gradient of NaCl in 0.04 M HCl was established utilizing a three-channel peristaltic pump and a mixer volume of 100 ml. The flow rate to the column was 0.25 ml min^{-1} ; 0.75 M NaCl was pumped to the mixer vessel at $0.125 \text{ ml min}^{-1}$ and 1-ml fractions were collected. In order to avoid any carry-over of peptides, these columns were used only once. Recovery from these columns always exceeded 90%.

Nonequilibrium isoelectric focusing. We define nonequilibrium isoelectric focusing as an electrophoretic procedure utilizing conventional isoelectric focusing equipment and reagents but terminating the run before the molecular species under study have migrated to their respective isoelectric points. This procedure was used rather than isoelectric focusing due to the very basic isoelectric points of β_c -EP and β_c -EP-(1-27). Under equilibrium conditions, these peptides cannot be resolved, since they migrate to the cathodal end of the focusing gel. Since the pIs of β_c -EP-(1-27) and its α -N-acetyl form should fall somewhere between the pK_a of the α - NH_2 and the pK_a of the ϵ - NH_2 of lysine and the pK_a of the histidine imidazolium function, respectively, termination of the focusing run prior to reaching the neutral region of the gel results in a greater separation of these peptides.

A Bio-Rad model 150A vertical tube gel apparatus and 10-cm 7.5% acrylamide gels containing 2% Bio-Lytes, pH range 3 to 10, were employed. Samples were dissolved in 25 to 100 μl of 0.06 N H_2SO_4 containing 1% Bio-Lytes and 8 M urea. Samples were applied to the anode side of the gel and focused for 2.5 hr with a limiting voltage of 200 V. The pH gradient of each gel was determined using a surface electrode (Bio-Rad Laboratories). Gels were sliced at 2-mm intervals and eluted overnight at 10°C with 0.5 to 1 ml of a solution containing 0.25% Triton X-100, 0.8% human serum albumin, 0.1 M Na_2HPO_4 and 22 mM Na_2EDTA , pH 7.5. Marker peptides were run on separate gels. These included camel β -endorphin, camel β -EP-(1-27), and the human forms of β -EP, β -EP-(1-27) and their α -N-acetylated derivatives. The human peptides were run for comparative purposes only, since they possess lower charge-to-mass ratios than their rat analogues. Recoveries from the gels always exceeded 96%.

High pressure liquid chromatography. Reverse phase high pressure liquid chromatography (HPLC) was performed utilizing a Beckman model 332 two-pump, microprocessor-controlled apparatus and a 0.46×25 cm Altex ODS column (C-18; $5 \mu\text{m}$). Two solvent systems were employed: (1) 0.05 M formic acid/triethylamine (0.25 M formic acid stock solution adjusted to pH 3.0 with triethylamine) using a gradient of acetonitrile and (2) 0.5 M formic acid, 0.14 M pyridine using 1-propanol as the organic modifier (Lewis et al., 1979).

Prior to injection, all samples were incubated at 45°C in the presence of 1 M dithiothreitol (DTT) (Houghten and Li, 1979; R. A. Houghten, personal communication). Recovery of IR- β -EP always exceeded 87%.

Trypsinization of radiolabeled IR- β -endorphin-(1-9). Sample was dissolved in 200 μl of 0.1 M sodium phosphate, pH 7.8, containing 50 μg of human serum albumin. Ten micrograms of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)/trypsin was added and incubated for 16 to 20 hr at 37°C .

Chymotrypsinization of radiolabeled IR- β -endorphin-(1-9). The same procedure was employed as for trypsinization.

Source of synthetic peptides. Synthetic camel β -endorphin (β_c -EP) and synthetic β_c -EP-(1-27) were obtained from Peninsula Laboratories and Bio-Flex Laboratories, respectively.

Dr. R. A. Houghten (Division of Endocrinology, Mount Sinai School of Medicine) kindly provided the following synthetic peptides: β -EP-(1-9), human [^3H]Tyr 1 - β -EP, human β -EP, β -EP-(1-27), and their α -N-acetyl derivatives. β -[^3H]EP-(1-4) was obtained by chymotrypsinization of [^3H]Tyr 1 - β -EP in the presence of 1 mg of unlabeled β_c -endorphin; it was purified by HPLC and its identity was confirmed by amino acid analysis and by liberation of [^3H]Tyr with aminopeptidase M. α -N-Acetyl β -EP-(1-9) was prepared by tryptic digestion of α -N-acetyl β_c -EP; its amino acid sequence was confirmed by amino acid analysis (Bio-Flex Laboratories) and the presence of the acetyl group was confirmed indirectly by its resistance of aminopeptidase M (as suggested by D. G. Smyth, National Institute for Medical Research, The Ridgeway, Mill Hill, London). β -EP-(5-9) was obtained by chymotrypsinization of β -EP-(1-9) and its sequence was confirmed by microsequencing.

Analysis of immunoreactive β -endorphin. Since the purpose of this study was to characterize forms of IR- β -EP with the approximate molecular size of the authentic β -EP molecule, all tissue and medium extracts were submitted to molecular sieve chromatography (Sephadex gel filtration) and material eluting in the region (with a similar K_{av}) of the synthetic camel β -EP marker was pooled, lyophilized, and reconstituted in the appropriate vehicle, and aliquots were analyzed further by nonequilibrium isoelectric focusing, SP-Sephadex cation exchange chromatography, and reverse phase HPLC. Synthetic camel β -EP and β -EP-(1-27) were used as marker peptides in all separation systems. The α -N-acetyl derivatives were not available to us, but their expected behavior could be estimated on theoretical grounds.²

IR- β -EP radiolabeled during the 72-hr incubation of tissue in medium containing [^{35}S]methionine and [^3H]tyrosine was analyzed and purified according to the scheme depicted in Figure 1. The radiolabeled IR- β -EP forms obtained by this purification procedure also were subjected to trypsinization. The β -EP-(1-9)-like peptides generated were identified by (1) HPLC analysis, (2) immunoprecipitation with a methionine enkephalin antibody (Liotta and Krieger, 1979) that recognizes β -EP-(1-9) but no other tryptic fragment of β -EP, (3) detection

² For instance, the acetylated peptides possess one less positive charge and should migrate more slowly than the corresponding unacetylated peptides in the electrophoretic procedure, elute just before them in the cation exchange chromatographic system, and be retained longer in the reverse phase HPLC system.

Figure 1. Schematic representation of the purification procedure. IR- β -EP extracted from pituitary incubated for 72 hr in medium containing [35 S]methionine and [3 H]tyrosine was purified in the following manner. Immunoprecipitated β -EP-like material which eluted in the region of the authentic β -EP peptide was analyzed by cation exchange chromatography. Any radiolabeled peptides that eluted at the position of β -EP (I), β -EP-(1-27) (III), or the expected positions of their α -N-acetyl derivatives (II and IV, respectively) were analyzed individually in the isoelectric focusing system. The region of each gel corresponding to peaks I to IV was analyzed separately by reverse phase HPLC. The regions of the HPLC chromatograms corresponding to I to IV were rerun on separate SP-Sephadex columns. See "Materials and Methods" for details of each separation system used.

of both 35 S and 3 H radioactivity (cells incubated with [35 S]methionine and [3 H]tyrosine) as an indication of the presence of both methionine and tyrosine residues, and (4) submission of HPLC-purified IR- β -EP-(1-9)-like material to chymotrypsinization and analysis of digests by HPLC.

In addition to the above characterization, aliquots of cell extracts obtained from the pulse-chase experiments also were immunoprecipitated with the β -EP antiserum and analyzed by NaDodSO₄-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (Liotta et al., 1980).

Results

Behavior of pituitary tissue in culture (Table I)

NIL. Over a 5-day culture period, the cellular content of immunoassayable β -EP ranged between 82 and 111% ($n = 3$ on days 1, 3, and 5) of the average content found in freshly extracted tissue. Basal release was linear with time (tested over a 72-hr period) and averaged $0.4\% \text{ hr}^{-1}$ of the cell content. Recovery of synthetic β -EP added to

TABLE I

Immunoreactive β -EP content of neurointermediate lobe (NIL)

Freshly removed NIL tissue obtained from 180- to 220-gm Sprague-Dawley rats was either extracted immediately or maintained in tissue culture for 1 to 5 days prior to quantification of IR- β -EP content of tissue.

	Day	Pmol Lobe ⁻¹ ^a
Fresh NIL extracts ^b		385 $\pm$ 18
NIL tissue in culture ^c	1	378 $\pm$ 14
	2	354 $\pm$ 6
	3	331 $\pm$ 9

^a Mean $\pm$ SEM.

^b $n = 12$.

^c $n = 3$ each day.

the medium in the presence of tissue averaged 86% after 12 hr of incubation.

Anterior lobe cells. Cellular content of immunoassayable β -EP³ ranged between 69 and 86% ($n = 3$ on days 2, 3, and 5) of the average content found in acutely dispersed cells or freshly extracted tissue. Basal release was linear from days 2 to 5 and averaged $0.5\% \text{ hr}^{-1}$ of the corresponding cell content. Recovery of added synthetic β -EP and hACTH (1 ng each) to representative cells averaged 89 and 81%, respectively, after 12 hr incubation.

These data show that the pituitary tissue was stable under our culture conditions, since both NIL and anterior lobe cells were able to maintain near normal (relative to fresh tissue extracts) intracellular content of IR- β -EP. Furthermore, the low rates of basal release noted probably reflect the integrity of the plasma membranes. We thus concluded that the culture technique was suitable for use in our incubation studies.

Characterization of immunoreactive β -endorphin content of pituitary extracts

Gel filtration of anterior pituitary extracts ($n = 6$) revealed two predominant IR- β -EP peaks: One eluted just after the human β -LPH marker and probably corresponded to rat β -LPH (the rat molecule is smaller than the human form; Eipper and Mains, 1979), and the other peak co-eluted with the β -EP marker. The IR- β -LPH peak always predominated, with an average IR- β -LPH/ β -EP molar ratio of approximately 4. In contrast, gel filtration of NIL extracts ($n = 6$) revealed only one major peak of IR- β -EP, which co-eluted with the β -EP marker (Fig. 2).

Aliquots of IR- β -EP (K_{av} of β -EP) then were analyzed further as described under "Materials and Methods." NIL IR- β -EP was separated into four to six components in each system. Four of these components, designated peaks I to IV for descriptive purposes, were tentatively identified as authentic β -EP (I), N-acetyl β -EP (II), β -EP-(1-27) (III), and N-acetyl β -EP-(1-27) (IV). Figure 3 illustrates a typical cation exchange chromatogram. Peaks I and III eluted at the same positions as synthetic camel β -EP and β -EP-(1-27), respectively. IR- β -EP peaks II and IV eluted at the expected positions of the α -N-acetyl derivatives of β -EP and β -EP-(1-27), respec-

³ RIA was performed on unchromatographed tissue extracts. Therefore, these values reflect IR- β -LPH and β -EP.

Figure 2. Sephadex G-50 gel filtration of freshly prepared rat anterior pituitary (—) and neurointermediate lobe (---) extracts. IR- β -EP was determined for each fraction by radioimmunoassay. Ninety-six percent of the total IR- β -EP applied to column was recovered. Peak elution positions of β _h-LPH and β _c-EP correspond to fractions 18 and 28, respectively.

Figure 3. Comparison of rat anterior and neurointermediate lobe IR- β -EP: Cation exchange chromatography. IR- β -EP obtained from the gel filtration analysis was subjected to SP-Sephadex ion exchange chromatography as described under "Materials and Methods." NIL IR- β -EP (---) was partially resolved into multiple components, four of which (peaks I to IV) were tentatively identified as β -EP, *N*-acetyl β -EP, β -EP-(1-27), and *N*-acetyl β -EP-(1-27). Synthetic camel β -EP and β -EP-(1-27) displayed the same mobility as peaks I and III, respectively. The respective *N*-acetyl camel peptides would be expected to migrate to the same positions as II and IV, based on theoretical considerations. Note that peak I comprises most of anterior pituitary IR- β -EP (—). All fractions from 10 to 120 were assayed for IR- β -EP. Limit of detection was 0.05 pmol tube⁻¹. Ninety-seven percent of anterior pituitary IR- β -EP and 92% of NIL IR- β -EP applied to the columns was recovered.

tively. Similar analysis of 18 individual NIL extracts revealed that peak III usually predominated (11 of 18); peak IV was the second most abundant in 11 of 18 extracts (and was the largest peak in the other 7 instances). Peak II always contained more IR- β -EP than peak I.

Approximately 90% of the anterior pituitary IR- β -EP behaved like the authentic β -EP molecule in all three separation systems (see Fig. 3 for a typical cation exchange elution profile).

Detection and characterization of radiolabeled immunoreactive β -endorphin: 72-hr continuous incubation

Gel filtration and SDS-PAGE of radiolabeled β -EP immunoprecipitates of anterior pituitary cells and NIL tissue extracts yielded profiles that were very similar to profiles obtained for the fresh tissue extracts. Similar to fresh tissue extracts, further characterization of anterior pituitary radiolabeled β -EP revealed the presence of only one major component physicochemically resembling the authentic β _c-EP molecule. All four IR- β -EP components detected in fresh NIL extracts were radiolabeled in the 72-hr incubates; the (1-27)-like species (peaks III and IV) again predominated. Figure 4 shows the cation exchange profiles obtained. All four species (I to IV) also were detected in approximately the same absolute quantities (after correction for recovery) by nonequilibrium isoelectric focusing and HPLC, eluting at positions consistent with the structures of β -EP, β -EP-(1-27), and their α -*N*-acetyl derivatives.

The radiolabeled peaks obtained from the cation exchange column were subjected to the electrophoretic procedure separately. Figure 5 shows that, in each instance, the majority of ³⁵S-labeled IR- β -EP migrated appropriately. Peaks I and III migrated to the same positions as the β _c-EP and β _c-EP-(1-27) marker peptides. Peak II migrated midway between them, and peak IV was the slowest moving component (the behavior of peaks II and IV is consistent with the structures of *N*-acetylated β -EP and β -EP-(1-27), since these peptides

Figure 4. Continuous labeling experiment: Cation exchange chromatographic characterization of immunoreactive β -endorphin. Rat anterior pituitary cells (A) and intact rat neurointermediate lobes (B) were incubated for 72 hr in medium containing [³⁵S]methionine and [³H]tyrosine as described under "Materials and Methods." An aliquot of each tissue extract was immunoprecipitated with the affinity-purified β -EP antiserum and subjected to gel filtration, and radiolabeled material eluting with a *K*_{av} similar to β _c-EP was analyzed on separate SP-Sephadex columns. The elution positions of synthetic β _c-EP and β _c-EP-(1-27) are indicated; the expected elution positions of their α -*N*-acetyl derivatives (II and IV, respectively) are shown also. The ³⁵S profiles are shown. Almost identical patterns were obtained when ³H was counted. All fractions (1 to 120) were counted. Greater than 91% of radioactivity applied to columns was recovered.

Figure 5. Nonequilibrium isoelectric focusing analysis of neurointermediate lobe β -endorphin-like peptides. Fractions 50 to 64 (A), 65 to 75 (B), 85 to 95 (C), and 100 to 110 (D) eluted from the cation exchange column (see Fig. 4) were pooled separately, concentrated, and applied to separate tube gels. At the completion of the run, gels were sliced at 2-mm intervals and ^{35}S and ^3H activity was quantified. The ^{35}S profile is shown. The pH gradient is shown in panel A. The migration position of β -EP (I), β -EP-(1-27) (III), as well as the expected positions of their α -N-acetyl derivatives (II and IV, respectively) are indicated.

each possess one less positive charge (below the pK_a of the α - NH_2 group) than their unacetylated counterparts). The small amount of radioactivity evident in the first few slices of each gel probably represents peptide that precipitated near the origin of the gel (the same observation was made for the synthetic peptides). Greater than 97% of the radioactivity applied to these gels was accounted for in the run depicted.

The radiolabeled peaks obtained from two separate nonequilibrium isoelectric focusing runs were pooled separately, and after reduction (see "Materials and Methods"), were subjected to HPLC analysis. In each instance, the major portion of the radioactivity eluted appropriately. Figure 6 depicts the results for peaks I and III. When peak I, from the electrophoretic gel, was analyzed, most of the ^{35}S activity ($\sim 88\%$) was present in a single peak which exhibited the same retention time (t_R) as synthetic β -EP (Fig. 6A). Since 91% of the ^{35}S activity injected into the HPLC system was recovered, the existence of another major peak in this profile is not possible. When peak III was similarly analyzed (Fig. 6B), 90% of the recovered ^{35}S activity eluted at the t_R of β -EP-(1-27). Overall recovery from the column was 93% for this peak. Peaks II and IV eluted at positions consistent with the structures of N -acetyl β -EP and N -acetyl β -EP-(1-27), respectively (data not shown).

Peak II eluted with a t_R nearly identical to β -EP-(1-27); this was an expected result since, in this HPLC system, the loss of two lysine residues or the addition of an acetyl group both increase t_R . (Analysis of the corresponding synthetic human peptides revealed that N -acetyl β -EP and β -EP-(1-27) eluted within 15 sec of each other.) Peak IV (83% of ^{35}S -labeled material applied) eluted 3.9 min after β -EP-(1-27); analysis of the human forms showed that N -acetyl β -EP-(1-27) eluted 3.6 min

Figure 6. HPLC analysis of neurointermediate lobe β -EP-like and β -EP-(1-27)-like peptides. ^{35}S -labeled IR- β -EP which exhibited the elution and migration characteristics of β -EP (peak I) and β -EP-(1-27) (peak III) in both the SP-Sephadex cation exchange chromatographic (see Fig. 4) and nonequilibrium electrophoretic focusing systems (see Fig. 5) was subjected to reverse phase HPLC analysis. A, Peak I material from the SP-Sephadex chromatogram was subjected to the electrophoretic procedure; the 5.4- to 6.2-cm portions of two representative gels were eluted, concentrated, incubated with DTT, and subjected to reverse phase HPLC analysis. B, Peak III similarly derived was treated similarly, except that the 4.4- to 5.0-cm segment of the focusing gels was analyzed. The formic acid/triethylamine/ CH_3CN gradient is indicated by the dashed line. The flow rate was 1 ml min^{-1} and 0.5-ml fractions were collected. The elution positions of β -EP and β -EP-(1-27) are indicated. Results for peaks II and IV are not shown.

after β -EP-(1-27). Finally, the labeled peak from each HPLC run was rerun in the original cation exchange system. All four peaks ran true (data not shown).

Similar analysis of anterior pituitary ^{35}S -labeled IR- β -EP (peak I from Fig. 4A) revealed that, in each subsequent separation system, $>84\%$ of the material behaved as the authentic β -EP molecule (data not shown).

Partial tryptic and chymotryptic mapping

NIL peaks I to IV from the final cation exchange column of the rerun experiments (tissue labeled with [^{35}S]methionine and [^3H]tyrosine) were trypsinized individually. Peaks I and III yielded a dual-labeled fragment that possessed the identical t_R (26.3 min) of synthetic β -EP-(1-9) upon HPLC analysis (Fig. 7A). Peaks II and IV yielded a dual-labeled fragment that exhibited a t_R (29.8 min) nearly halfway between β -EP-(1-9) and methionine enkephalin (32.9 min) (Fig. 7B). This time corresponded precisely with the t_R of α -N-acetyl β -EP-(1-9). All four of the generated β -EP-(1-9)-like fragments were shown to be specifically immunoprecipitable with the affinity-purified methionine enkephalin antiserum.

Chymotryptic fragments of the β -EP-(1-9)-like fragments obtained from both peptides I and III (Fig. 8) yielded a major ^3H -labeled fragment which exhibited the

Figure 7. HPLC analysis of tryptic digests. Neurointermediate lobe tissue was incubated for 72 hr in medium containing [35 S]methionine and [3 H]tyrosine. Radiolabeled IR- β -EP was fractionated sequentially by cation exchange chromatography, nonequilibrium electrophoretic focusing, and reverse phase HPLC. The four purified IR- β -EP peptides (peaks I to IV) then were trypsinized separately and the resulting digests were analyzed in the formate/pyridine/1-propanol solvent system. *A*, The profile obtained for peak III material; *B*, the profile for digest of peak IV. The total 35 S of each fraction (flow rate 1 ml min $^{-1}$, 0.5 ml fraction $^{-1}$) is shown. A 30-min linear gradient of 1-propanol, from 0 to 10% was followed by a 20-min isocratic period at 10% 1-propanol. Results for tryptic digestions of peaks I and II not shown. Met-Enk, methionine enkephalin.

t_R of synthetic β -[3 H]EP-(1-4). Two minor 3 H-labeled fragments were present also; the peak at 8.2 min eluted with [3 H]tyrosine;⁴ the nature of the small peak (~6% of total activity) at t_R = 16.5 min is not known. The major

⁴ Under the conditions of digestion employed, the Tyr¹—Gly² bond of β -EP-(1-9) is cleaved with approximately 10% efficiency (relative to the Met⁴—Phe⁵ bond). It has been reported that the NH₂-terminal tyrosine of β -EP is unusually resistant to cleavage by aminopeptidases or α -chymotrypsin (Austen and Smyth, 1977; Graf et al., 1977). These authors postulated that a preferred conformation of the β -EP molecule (postulated to involve interaction of residues within the NH₂-terminal segment of the molecule with some residues within the COOH terminus) makes the Tyr¹—Gly² and Phe⁴—Met⁵ bonds relatively inaccessible. Consistent with this notion, Austen and Smyth (1977) have shown, under mild conditions of digestion, that β -EP-(1-18) is the major NH₂-terminal chymotryptic fragment generated, while under more rigorous conditions (at 20°C, 16 hr; enzyme/peptide ratio (w/w) of 1/100), β -EP-(1-4) is obtained in approximately 95% yield. Since a β -EP-(1-9)-like fragment served as substrate in our digestion experiments, it would not be unreasonable to expect the Tyr¹—Gly² bond to be relatively more accessible to the enzyme than when this bond occurs in the intact molecule. When the Tyr¹ residue is α -N-acetylated, it appears to be removed easily by α -chymotrypsin (Mains and Eipper, 1980).

Figure 8. HPLC analysis of chymotryptic digests of β -EP-(1-9)-like fragments. The HPLC-purified dual-labeled β -EP-(1-9)-like fragment obtained from the tryptic digestion of peak III (see Fig. 7*A* and text for details) was digested with α -chymotrypsin and subjected to reverse phase HPLC analysis using the formic acid/triethylamine/CH₃CN solvent system. The shape of the CH₃CN gradient is shown by the dashed line. Both the 3 H and 35 S activities were quantified and individually plotted (*A* and *B*, respectively). The positions of synthetic peptide fragments are indicated. Data for digestion of peak I is not shown, since virtually identical results were obtained.

35 S-labeled peak, which comprised ~89% of such activity, eluted with the t_R (10.4 min) of synthetic β -EP-(5-9). The peak (~3% of total) at t_R = 8.5 min may be the methionine sulfoxide form of β -EP-(5-9).⁵

Similar analysis of material obtained from tryptic digests of peaks II and IV yielded two major radiolabeled fragments; a peak comprising >93% of the total 3 H activity eluted with the t_R of synthetic α -N-acetyl tyrosine and a peak comprising >92% of the total 35 S activity injected onto the column eluted at the position of synthetic β -EP-(5-9) (Fig. 9).

Neurointermediate lobe pulse-chase experiments

After the 30-min pulse, more than 90% of the 35 S label immunoprecipitated by the β -EP antiserum exhibited an apparent M_r of more than 30,000. The intracellular bio-

⁵ In earlier experiments (January–February, 1980) in which we did not include the dithiothreitol reduction step prior to HPLC analysis, this peak (t_R = 8.5 min) comprised approximately 11% of the total 35 S activity; the β -EP-(5-9)-like peak comprised approximately 80% of such activity.

Figure 9. HPLC analysis of chymotryptic digests of *N*-acetyl β -EP-(1-9)-like fragments. The HPLC-purified dual-labeled *N*-acetyl β -EP-(1-9)-like fragment obtained from the tryptic digestion of peak IV (see Fig. 7B and text for details) was digested with α -chymotrypsin and analyzed by reverse phase HPLC as described in Figure 8. Data for peak II not shown. A, ^3H activity; B, ^{35}S activity; dashed line, shape of the CH_3CN gradient.

synthetic processing of this precursor and the kinetics of appearance of β -EP-like material have been described extensively (Mains and Eipper, 1979; Herbert et al., 1980; Chretien et al., 1979). Therefore, we will state our findings only briefly, since the major purpose of our study was to further physicochemically characterize the generated β -EP-like material as a function of chase time. After the 30-min pulse, only material exhibiting apparent $M_r > 30,000$ was immunoprecipitable with β -EP antiserum. After a 1-hr chase period, a β -LPH-like peak ($M_r = 10,500$) was detected, in addition to the high molecular weight material. After a 2-hr chase period, a β -EP-like

peak ($M_r = 3,500$) was first detected. The majority of the ^{35}S label was present in the $M_r = 3,500$ peak in the 4-hr chase sample. At 6 and 18 hr, virtually all of the specific immunoprecipitable material was contained in the β -EP-like peak.

IR- β -EP obtained (by gel filtration) from the 2-, 6-, and 18-hr chase samples then was subjected to cation exchange chromatography. The majority of the ^{35}S -labeled material eluted in the four designated peak areas. Peaks I and II (β -EP and *N*-acetyl β -EP-like) comprised approximately 88% of this total after the 2-hr chase period; peak I contained approximately 4 times more label than peak II (Fig. 10A). At this time, the putative acetylated peptides comprised $\sim 18\%$ of the label in the four peaks. At 6 hr of chase, all four peaks were detected again; peaks I and II comprised approximately 65% of the label in the four peaks. At 18 hr of chase, the majority

Figure 10. Pulse-chase experiment: Characterization of IR- β -EP. Cell extracts obtained from the 2- and 18-hr chase incubations were immunoprecipitated and applied to a gel filtration column (after dissolution of immune complex). Then, IR- β -EP was analyzed by SP-Sephadex cation exchange chromatography. A, At 2 hr of chase, most of the newly synthesized IR- β -EP eluted at the position of camel β -EP (peak I). B, At 18 hr of chase, ^{35}S -labeled IR- β -EP was separable into multiple molecular species (peaks I to IV) in proportions similar to the total cellular IR- β -EP profile. Similar to fresh NIL extract profiles (see Fig. 3), the β -EP-(1-27)-like species (peaks III and IV) predominate.

(more than 70%) of the ^{35}S label was in peaks II and IV. Approximately 66% of the label was present in the putative acetylated peptides (Fig. 10B). Thus, this ^{35}S -labeled IR- β -EP profile is very similar to the profile obtained for the fresh NIL tissue extracts (see Fig. 3).

Release studies

Anterior pituitary cells. rHME caused a 7.8 ± 1.1 -fold increase ($p < 0.001$) in release of IR- β -EP (assay of cell and medium extracts prior to gel filtration) compared to control ($n = 5$). Gel filtration revealed that the IR- β -LPH/ β -EP molar ratio was approximately 4 in both cell and medium extracts. Further characterization of the IR- β -EP peak showed that almost all (more than 92%; $n = 5$) of this material behaved like the authentic β -EP molecule (data not shown). Essentially equimolar amounts of IR- α -ACTH and IR- β -LPH + β -EP were contained in the cells and released into the medium (Fig. 11A).

Neurointermediate lobe. The 2-hr incubation with 10^{-7} M DA ($n = 3$) resulted in a significant decrease (IR- α -MSH, 0.15 ± 0.001 versus 0.10 ± 0.007 pmol; IR- β -EP, 0.17 ± 0.008 versus 0.09 ± 0.009 pmol; $p < 0.05$ in both cases), while similar incubation with 10^{-8} M NE ($n = 3$) was associated with a significant increase ($p < 0.001$) in release of IR- β -EP and α -MSH compared to basal control. Concentrations of IR- β -EP and α -MSH were approximately equimolar in both cell and media extracts (Fig. 11B). Chromatographic and electrophoretic analysis (data not shown) demonstrated that β -EP-related peaks I to IV were present in extracts of incubated cells and media in similar relative concentrations to the profiles obtained for fresh NIL extracts. When 72-hr prelabeled NIL tissue was used for release experiments, both

the total IR-EP profiles and the immunoprecipitable radiolabeled β -EP profile were also similar. Therefore, the presence of NE or DA did not result in any change in either the cell or medium profiles under the short term conditions of incubation employed.

Discussion

The present data demonstrate that cells within the rat neurointermediate lobe synthesize multiple forms of immunoreactive β -endorphin. Such material eluted with a K_{av} similar to authentic camel β -EP upon gel filtration and was resolved into multiple molecular species in three different chromatographic and electrophoretic systems. We have tentatively identified four of these immunoreactive species as authentic β -endorphin, β -endorphin-(1-27), and their α -N-acetyl derivatives.

Analysis of freshly extracted neurointermediate lobes revealed that the two β -EP-(1-27)-like peptides always comprised at least 70% of total immunoreactive β -EP. This result confirms the findings of Zakarian and Smyth (1979). A similar distribution of endorphin forms also was obtained for ^{35}S -labeled material synthesized during a 72-hr continuous incubation of NIL tissue in the presence of [^{35}S]methionine.

The NIL pulse-chase experiments indicate that β -endorphin is present in the pathway leading to the formation of α -N-acetyl β -EP and the β -EP-(1-27)-like analogues, since immunoreactive material physicochemically similar (with respect to the parameters of characterization performed) to authentic β -EP was the major β -EP-like species detected after a 2-hr chase period. At 18 hr of chase, these (1-27)-like forms were the predominant species present. Thus, the removal of the four COOH-terminal amino acids from the β -EP-(1-31) forms (probably by the sequential action of a trypsin-like enzyme at the carboxyl side of lysine at position 28, followed by removal of this lysine residue by a carboxypeptidase B-like action) appears to be the last step in the biogenesis of the β -EP-(1-27)-like peptides. These studies do not exclude the possibility that at least some of the β -EP-(1-27)-like peptides are generated by the removal of the COOH-terminal amino acids from the β -LPH-like material prior to the enzymatic liberation of the β -EP-sized species (since β -LPH-like material was not characterized beyond SDS-PAGE). Therefore, we have not demonstrated that the authentic β -EP molecule serves as an obligatory metabolic intermediate in this pathway.

NIL release studies demonstrated that all IR- β -EP forms were released into the medium under basal, inhibitory, and stimulatory incubation conditions. No preferential release of any given species was noted in any of these experiments. The medium profile always reflected the cell profile and the β -EP-(1-27)-like forms predominated. Furthermore, total cell or medium IR- β -EP was always approximately equimolar to the total cell or medium IR- α -MSH.⁶

While the pulse-chase data indicate that formation of

Figure 11. Secretion studies. *A*, Coordinate release of total immunoreactive β -endorphin (shaded bar) and immunoreactive ACTH (open bar) from anterior pituitary cells maintained in culture. Cells were incubated for 2 hr with 0.1 equivalent of a rat medial basal hypothalamus (HME). An approximately 8-fold increase in both activities occurred. *B*, Coordinate release of total IR- β -endorphin (shaded bar) and immunoreactive α -MSH (open bars) from neurointermediate lobe tissue maintained in culture. Incubation for 2 hr with 10^{-8} M DA caused a significant decrease in the concentration of IR- β -endorphin in the medium. A similar incubation with 10^{-7} M NE caused a significant increase in the concentration of IR- β -endorphin released into the medium, compared to incubation without test substances (basal). The response of IR- α -MSH paralleled and was approximately equimolar to that of IR- β -endorphin in all instances. * $p < 0.05$; ** $p < 0.001$.

⁶ These data are consistent with the notion that all of these molecular species are co-sequestered within the same secretory granules. Cell and medium IR- α -MSH profile did not mirror the cell profile. Preferential release of one form was apparent in preliminary studies (A. S. Liotta, H. Yamaguchi, and D. T. Krieger, manuscript in preparation).

the β -EP-(1-27)-like forms is a relatively late post-translational event, these times may not simulate *in situ* kinetics, wherein the innervation of the neurointermediate lobe may be involved in the activation/inactivation or induction/repression of cleaving enzymes. Alternatively, the tissue culture conditions employed may have altered processing times.

Previous investigations of the biosynthetic pathway of β -endorphin-related peptides in rat intermediate lobe (Roberts et al., 1978; Mains and Eipper, 1979; Seidah et al., 1978) failed to detect these multiple endorphin species. In all of these studies, techniques were employed that would either exclude all but the authentic β -EP molecule from ultimate detection (e.g., cation exchange chromatography followed by microsequencing) or that would not resolve them at all (e.g., SDS-PAGE). Both Roberts et al. and Mains and Eipper have commented on the inadequacy of the SDS-PAGE procedures. The latter authors noted that "more detailed analyses of the peptide products would be required to determine whether subtle changes in processing occurred . . . such as *N*-acetylation . . ."

However, evidence that rat pituitary " β -EP" represented a heterogeneous mixture of peptides can be fathomed from the study of Rubinstein et al. (1977). In that study, β -EP-like material obtained by gel filtration was purified by HPLC methodology using both ion exchange and reverse phase columns. Two major opioid active peaks were detected at the final purification step, consistent with the presence of β -EP and β -EP-(1-27) (the acetylated peptides are not active in the opioid receptor assay that these authors used for detection). The first peak was shown to have the same amino acid composition and tryptic map as synthetic β_c -EP. The authors, rightfully, did not attribute significance to the second peak, since they were concerned that extensive autolysis may have occurred in their starting tissue (frozen whole pituitaries were obtained from a commercial source). In addition to cited reports of Smyth et al. (1978, 1979), Lissitsky et al. (1978) also have reported that the major β -EP-like species in extracts of freshly prepared bovine NIL tissue eluted slightly after the authentic β -EP marker peptide following Sephadex G-50 gel filtration on a large (3.6×200 cm) column.

In contrast to NIL tissue, gel filtration of freshly prepared anterior pituitary extracts revealed that IR- β -EP, eluting with a K_{av} similar to authentic β_c -EP, comprised ~20% of the IR-(β -LPH + β -EP) content. Approximately 90% of this total IR- β -EP migrated with the synthetic camel β -EP standard in all chromatographic and electrophoretic systems and thus is physicochemically similar to it. A similar result was obtained for the anterior pituitary cell labeling experiments at the same times used for NIL, with the predominant labeled species again behaving like the authentic β -EP molecule.

In an earlier study (Liotta et al., 1978), we reported that, following gel filtration of freshly prepared rat anterior pituitary extracts, IR material eluting with a K_{av} similar to β -EP comprised only 2 to 5% of the total IR- β -EP.⁷ At that time, we postulated that this β -EP-like

component may have been formed secondary to rapid postmortem autolysis. However, subsequent to this study, we have observed that this component comprises 5 to 32% of the total IR- β -EP content of anterior pituitary extracts (Krieger et al., 1980; this study; A. S. Liotta, H. Yamaguchi, and D. T. Krieger, unpublished data).⁸ Reports from other laboratories since 1977 reveal variability and no consensus regarding the relative amount of β -LPH and β -EP present in anterior pituitary. It is therefore possible that the relative amounts of β -LPH and β -EP in rat anterior lobe are not fixed.

The basis for the discrepancy between the present findings and those of Zakarian and Smyth (1979), who reported that more than 50% of rat anterior pituitary IR- β -EP represented the β -EP-(1-27) forms, is not immediately evident. However, consistent with our findings are our recent (unpublished) observations which demonstrate that, when rat pituitary homogenates are used as a source of acetylating activity and incubated with [³H]acetylcoenzyme A in the presence of synthetic camel β -EP, the NIL homogenate, but not the anterior lobe homogenate, can incorporate considerable [³H]acetate at the α -NH₂ group of tyrosine (α -N-[³H]acetyl tyrosine identified by paper chromatography and HPLC after chymotryptic digestion of extracted β -EP).

Our current findings present additional distinguishing characteristics, over those previously noted, with respect to the differential processing of (the) precursor ACTH/ β -LPH molecule(s) in the two rat pituitary lobes. They also indicate that all of these forms will have to be characterized in future studies investigating intermediate lobe function.

Note added in proof. Subsequent to submission of this paper for publication, Smyth and Zakarian (1980) reported that β -endorphin is not processed further in rat anterior lobe. They determined that their earlier anterior pituitary extracts (Zakarian and Smyth, 1979) were contaminated with some neurointermediate lobe tissue.

Additionally, we have obtained the synthetic α -N-acetyl derivatives of β -endorphin and β -endorphin-(1-27); peaks II and IV, respectively, migrated with these marker peptides in all separation systems used.

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⁷ The objective of that study was to investigate whether different types of extraction procedures would alter the IR- β -LPH/IR- β -EP molar ratio observed in such extracts.

⁸ To ascertain if any of these results were biased by methodological artifacts, we re-analyzed aliquots of most of these anterior pituitary extracts (including the control pools from the 1978 study) as well as the corresponding NIL extracts (we routinely lyophilize and store aliquots of all of the tissue extracts that we prepare); within the limits of interassay variation, we were able to reproduce the gel profiles originally obtained. In all instances save one, the IR-ACTH to total IR- β -EP molar ratio approximated 1 (0.87 to 1.1) and >81% of total IR- β -EP eluted at the position of the authentic β_c -EP molecule in the ion exchange chromatographic system.

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