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DEMONSTRATION OF *IN VIVO* SYNTHESIS OF PRO-OPIOMELANOCORTIN-, β-ENDORPHIN-, AND α-MELANOTROPIN-LIKE SPECIES IN THE ADULT RAT BRAIN[1]

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Abstract

An *in vivo* labeling technique was utilized to demonstrate the *in situ* biosynthesis of pro-opiomelanocortin (POMC)-, β-endorphin- and α-melanotropin (MSH)-like molecular species in rat brain. Unrestrained adult female rats were bilaterally cannulated in the hypothalamic arcuate nuclear region; [^{35}S]methionine was infused either over a 15-min period with sacrifice 2 hr subsequently, or at a constant rate for 6 hr prior to sacrifice. Sequential immune-affinity chromatography and several chromatographic and electrophoretic techniques were employed to detect and characterize POMC-related material in the hypothalamic arcuate nuclear region, preoptic area, and median eminence. Four molecular species containing both corticotropin (ACTH) and β-endorphin antigenic determinants within the same molecules were detected in the arcuate nuclear region and preoptic area. Two forms were similar to rat pituitary POMC with respect to apparent molecular weight (35,000 and 31,500) and [^{35}S]methionine-containing tryptic fragments (one methionine each in N-terminal glycopeptide, ACTH, and β-endorphin sequences of rat POMC). The other two forms (apparent M_r of 21,000 and 19,000) contained only the labeled tryptic fragments characteristic of ACTH and β-endorphin. The detection of the latter forms suggests that POMC in brain, unlike its post-translational processing in rat pituitary, undergoes primary cleavage between the N-terminal peptide and the ACTH sequence. Peptides physicochemically indistinguishable from authentic β-endorphin and des-acetyl α-MSH were detected in approximately equimolar amounts in all three brain regions. The ratio of POMC-like material to the α-MSH- and β-endorphin-sized peptides was highest in the arcuate nuclear region, suggesting that POMC-like proteins are synthesized in the arcuate nuclear region and are processed into the smaller molecular species during axonal transport.

ACTH- and β-lipotropin (β-LPH)-related peptides are post-translationally derived from a common precursor molecule (pro-opiomelanocortin (POMC)) in both anterior and intermediate lobe pituitary corticotrophic cells. In higher vertebrates, polypeptides resembling POMC and its derived peptides are also present in the central nervous system, as well as in several peripheral sites (*e.g.*, gastrointestinal tract, male reproductive tract, placenta, and lymphocytes) (for review see Liotta and Krieger, 1983).

Adult bovine (Liotta et al., 1979; Liotta and Krieger, 1979) and neonatal rat (Liotta et al., 1980) hypothalamic cells maintained in tissue culture incorporate radiolabeled amino acids into POMC-like material. Comparative *in vitro* biosynthetic studies demonstrated the rat hypothalamic POMC-like material to be indistinguishable from rat pituitary POMC with respect to several physicochemical criteria (Liotta et al., 1980). Recently, POMC-like mRNA has been reported to be present in the rat hypothalamus, and, to a lesser extent, in the amygdala (Civelli et al., 1982). Immunocytochemical studies utilizing antisera raised to several of the pituitary POMC-derived peptides (e.g., β-LPH, α-melanotropin (α-MSH), β-endorphin, α-, γ-endorphin, and the N-terminal fragment of POMC) have localized these immunoreactive peptides within the same neuronal cell bodies in the arcuate nuclear region of the hypothalamus and in extensive fiber pathways within hypothalamic and extrahypothalamic brain regions (Krieger et al., 1980; Pelletier et al., 1980; Liotta and Krieger, 1983).

In vitro studies have established the nature of pituitary processing of POMC and have helped to delineate the regulation of synthesis and release of POMC-derived peptides in both pituitary lobes. In view of the enor-

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mously greater morphological and organizational complexity of central nervous system neurons, such techniques, however, cannot address a number of important questions concerning the regulation of synthesis and processing of POMC-like material and axonal transport of derived products.

We now report on the *in vivo* incorporation of [^{35}S] methionine into immunoreactive POMC-related molecules in three different brain regions. The findings that the level of radiolabel incorporated was adequate to allow characterization of ^{35}S-labeled POMC-related molecules in discrete hypothalamic regions of individual rats, and that the ratio of putative precursor(s) to products varied both as a function of the hypothalamic region and of the labeling paradigm indicate that this *in vivo* labeling procedure may now be used to study the relevant physiological, biochemical, and anatomical questions raised above.

Materials and Methods

Cannulation and infusion of [^{35}S]methionine. This was carried out with slight modifications of the method described by Krause et al. (1982). Female Sprague-Dawley rats (about 250 gm) were bilaterally implanted with 30 gauge stainless steel cannulae in the posterior arcuate nuclear region (coordinates relative to bregma: A, 0.6; L, 9.0; V, 0.7 mm) using a David Kopf stereotaxic instrument. The next day, infusion of [^{35}S]methionine (New England Nuclear) was carried out using subcutaneously implanted osmotic minipumps (Alzet #2001) connected via polyethylene tubing (P-10, Dionex) to the implanted cannulae. The osmotic minipumps had been pre-incubated in a Dubnoff incubator for 12 hr at 37°C prior to implantation. Two labeling conditions were used, each in a given animal: (*a*) 1 mCi of [^{35}S]methionine (922 Ci mmol^{-1} on day of infusion) was infused (500 μCi on each side) over a 15-min period, and the animal was sacrificed 2 hr later; and (*b*) 1 mCi of [^{35}S]methionine (962 Ci mmol^{-1} on day of infusion) was continuously infused (500 μCi on each side) for a 6-hr period followed by immediate sacrifice. Animals were unrestrained during the entire infusion procedures. Sacrifice was by decapitation; the brains were then removed and immediately frozen. The median eminence (ME) was microdissected, and the arcuate nuclear region and preoptic area (POA) were punched out from frozen coronal brain sections as previously described (Advis et al., 1982).

In vitro incubation of anterior pituitary cells and neurointermediate lobe tissue in the presence of [^{35}S]methionine. To obtain ^{35}S-labeled POMC and derived peptides for simultaneous comparison with brain-synthesized material, incubation was performed for 6 hr in the presence of [^{35}S]methionine as previously described (Liotta et al., 1980).

Tissue extraction. Tissue samples were homogenized in 250 μl of 0.9% NaCl containing 0.1 M HCl, 1 M HCOOH, and 0.01% human serum albumin. Aliquots (10 to 25 μl) of the crude homogenates were removed for determination of total tissue ^{35}S activity and ^{35}S-labeled trichloroacetic acid (TCA)-precipitable (PPT) protein as previously described (Roberts and Herbert, 1977a) prior to centrifugation at 15,000 × *g* for 10 min at room temperature. The supernatants were then lyophilized and reconstituted in 500 μl of 10 mM Tris-HCl, pH 7.5, containing 0.01% human serum albumin (recovery of ^{35}S activity >94% in all cases). Extracts were then submitted to sequential immune-affinity chromatography.

Preparation of anti-ACTH and anti-β-endorphin immune-affinity microcolumns. ACTH and β-endorphin antisera were affinity purified, and the specific immunoglobulins obtained were covalently bound to Sepharose as previously described (Liotta et al., 1978, 1979).[3]

An N-terminal-directed ACTH antiserum (BF-45) was used to prepare the anti-ACTH column. Synthetic human ACTH(1–39), ACTH(1–24), ACTH(1–13), ACTH(1–13)NH_2, des-acetyl α-MSH, α-MSH, and ACTH(1–10), and highly purified rat pituitary POMC were all bound by this column.

The anti-β-endorphin column was prepared from antiserum BF-99, which recognizes determinants in the rat β-endorphin(10–24) sequence. Camel β-endorphin (β_c) (identical sequence to rat β-endorphin), β_c-endorphin(1–26), their α-*N*-acetyl derivatives (but not α- or γ-endorphin), as well as highly purified rat POMC and rat β-LPH, were bound by this column.

Detection and initial separation of ^{35}S-labeled immunoreactive ACTH and β-endorphin. Anti-ACTH and anti-β-endorphin immune-affinity microcolumns were used in a sequential manner as previously described (Liotta et al., 1980) to obtain three classes of ^{35}S-labeled ACTH- and β-endorphin-related molecules: (*a*) molecules containing the dual antigenic determinants of ACTH and β-endorphin (designated immunoreactive ACTH/β-endorphin); (*b*) molecules containing only the ACTH determinant (designated immunoreactive ACTH); and (*c*) molecules containing only the β-endorphin determinant (designated immunoreactive β-endorphin). Briefly, each tissue extract was applied to an anti-ACTH microcolumn; the unretained effluent was then applied to an anti-β-endorphin microcolumn. The unretarded effluent from this column contained ^{35}S-labeled material unrelated to ACTH and β-endorphin and was not further analyzed; the specifically retained material represented immunoreactive β-endorphin and was eluted with an excess of synthetic β_c-endorphin. Specifically retained material was eluted from the anti-ACTH column with excess synthetic human ACTH and applied to a second anti-β-endorphin column. The unretarded effluent represented immunoreactive ACTH. The specifically retained material was eluted with an excess of synthetic β_c-endorphin; this represented molecules containing both the ACTH and β-endorphin antigenic determinants (immunoreactive ACTH/β-endorphin). In all instances, elution was performed as previously described (Liotta and Krieger, 1980), except that columns were

[3] We have found that it is essential to keep the capacity of the microaffinity columns relatively low (<25 pmol) in order to ensure efficient specific elution by the "excess ligand" technique. The use of higher capacity columns yields low, nonreproducible elution of specifically retained material and necessitates the use of a general elution method such as acid and/or dissociating reagents. These latter elution techniques also elute material nonspecifically bound to the column; in some cases, the specifically eluted radioactivity then can comprise a rather low percentage of total eluted radioactivity.

maintained at room temperature for at least 12 hr prior to a 2-day incubation at 10°C.

Sephadex G-50 superfine gel filtration. ^{35}S-Labeled material containing either the ACTH or β-endorphin antigenic determinant was chromatographed on 1.5 × 90 cm columns (eluent, 10% HCOOH containing 0.01% human serum albumin; flow rate, 6 ml hr^{-1}; fraction volume, 1.3 ml).

NaDodSo₄ Polyacrylamide Gel Electrophoresis (SDS-PAGE). [^{35}S]Methionine-labeled immunoreactive ACTH/β-endorphin (molecules containing the dual antigenic determinants of ACTH and β-endorphin) was analyzed in the discontinuous system of Laemmli (1970) as previously described (Liotta and Krieger, 1980).

Cation-exchange chromatography. SP-Sephadex cation-exchange chromatography was performed as previously described (Zakarian and Smyth, 1979), except that 0.7 × 20 cm disposable columns and a linear gradient of 0 to 1 M NaCl (100 ml mixer volume; 1-ml fractions collected) were employed.

Reverse-phase high performance liquid chromatography (HPLC). Reverse-phase HPLC was performed utilizing a Beckman Model 332 two-pump, microprocessor-controlled apparatus and either an Altex ODS column (0.46 × 25 cm) or a Waters Associates C-18 μBondapak column (0.39 × 30 cm). Both triethylammonium phosphate/CH_3CN (Rivier, 1978) and trifluoroacetic acid/CH_3CN (Bennett et al., 1980) solvent systems were employed. The actual chromatographic conditions employed are described in the appropriate figure legends. All samples were oxidized with 5% H_2O_2 at acid pH as previously described (Margioris et al., 1983). The purpose of this step was to convert all methionine residues to the sulfoxide form [Met(0)]. (The native methionine, sulfoxide, and sulfone forms of a given methionine-containing peptide exhibit different retention times (t_R) in most reverse phase HPLC systems.)

Tryptic digestion. [^{35}S]Methionine-labeled immunoreactive ACTH/β-endorphin (obtained following SDS-PAGE), immunoreactive ACTH, and immunoreactive β-endorphin (the major peaks obtained from reverse-phase HPLC) were trypsinized as previously described (Liotta et al., 1980). Two nanomoles each of synthetic human ACTH and β_c-endorphin were added to all samples prior to enzyme addition. Tryptic digestion of rat POMC yields the following methionine-containing fragments: ACTH(1–8), β-endorphin(1–9), and a doublet, or split peptide, characteristic of the N-terminal fragment of POMC (rat POMC contains three methionine residues) (Liotta et al., 1980; Roberts and Herbert, 1977b).

Paper electrophoresis. The tryptic digests of ^{35}S-labeled immunoreactive ACTH/β-endorphin were analyzed as previously described (Roberts and Herbert, 1977a). The electrophoretic system was employed since the methionine-containing N-terminal fragments of POMC are not readily resolved by the reverse phase HPLC methods employed in this study.

Results

Incorporation of ^{35}S activity into immunoreactive ACTH- and β-endorphin-related material and its distribution into immunoreactive classes. Table I shows the distribution of ^{35}S-labeled immunoreactive ACTH and β-endorphin among three immunoreactive classes obtained from the sequential immune-affinity chromatographic procedure (see "Materials and Methods" for explanation).[4] The ratio of ^{35}S-labeled immunoreactive ACTH/β-endorphin to ^{35}S-labeled immunoreactive ACTH or β-endorphin was significantly higher in the arcuate nuclear region than in the POA; only monodeterminant material was detectable in ME. Since (as noted above) rat pituitary α-MSH and β-endorphin each contain one methionine residue, it is of interest that roughly equal quantities of ^{35}S activity are present in immunoreactive β-endorphin and immunoreactive ACTH in each region.

Characterization of ^{35}S-labeled immunoreactive ACTH/β-endorphin

NaDodSO₄ polyacrylamide gel electrophoresis. Figure 1 depicts the SDS-PAGE profile obtained for the arcuate nuclear region from the 6-hr steady labeling experiment. Approximately 75% of the ^{35}S activity is partially resolved into two peaks of apparent M_r of 35,000 and 31,500; this is the apparent molecular weight range that pituitary POMC exhibited in the comparative *in vitro* pituitary labeling experiment (data not shown; see "Materials and Methods"). Approximately 25% of the ^{35}S activity, however, was present in two partially separated peaks of apparent M_r of 21,000 and 19,000. Similar results were obtained for the 15-min labeled arcuate nucleus extract and for the 6-hr POA sample (15-min POA sample was not analyzed due to insufficient ^{35}S activity). We were not able to detect material of M_r = 21,000 or 19,000 in the *in vitro* 6-hr labeling profiles of either anterior or intermediate pituitary cells (data not shown).

Tryptic mapping of ^{35}S-labeled immunoreactive ACTH/β-endorphin. The regions of M_r = 35,000 and 31,500 and M_r = 21,000 and 19,000 from the above SDS-PAGE analysis were separately pooled, trypsinized, and the digest analyzed by paper electrophoresis (Fig. 2*A*). The pool of the regions of M_r = 35,000 and 31,500 yielded the characteristic profile that has been demonstrated for pituitary POMC (see "Materials and Methods," Tryptic Digestion). The ^{35}S activity in the tryptic profile of the material of M_r = 21,000 and 19,000 was distributed solely in the β-endorphin(1–9)- and ACTH(1–8)-like peaks; N-terminal-like material was absent (Fig. 2*B*). This was not due to procedural losses of such material, since 91% of the ^{35}S activity spotted on the paper was recovered.

Sephadex G-50 characterization of ^{35}S-labeled immunoreactive ACTH and ^{35}S-labeled β-endorphin. ^{35}S-Labeled material extracted from the arcuate nuclear region and from the POA (6-hr labeling protocol) containing either the N-terminal ACTH or β-endorphin antigenic

[4] Both the anterior and neurointermediate pituitary lobes of these rats were also similarly analyzed. No specific ^{35}S-labeled immunoreactive material was detectable in the anterior lobes at either time period. Specific ^{35}S activity (126 dpm) was detected in the neurointermediate lobe extract from the 15-min labeling experiment. These data rule out the possibility that [^{35}S]methionine was incorporated in the pituitary into specific POMC-related peptides which were subsequently released and taken up by brain tissue. Furthermore, at both labeling times, the largest amount of specifically labeled material was found in the arcuate nuclear region, the site of [^{35}S]methionine infusion.

TABLE I

Distribution of ^{35}S activity in discrete hypothalamic regions of rat brain following in vivo [^{35}S]methionine infusion

	Duration of [^{35}S]Methionine Infusion	Brain Region		
		Arcuate Nuclear Region	Preoptic Area	Median Eminence
Total tissue ^{35}S,[a] dpm[b]	15 min	8.8×10^7	6.7×10^6	3.2×10^5
	6 hr	8.1×10^7	7.2×10^6	9.0×10^5
TCA PPT ^{35}S,[a] dpm	15 min	5.7×10^7	4.5×10^6	7.1×10^4
	6 hr	5.2×10^7	5.4×10^6	1.7×10^5
Percentage of specific ^{35}S/TCA PPT ^{35}S[a]	15 min	0.010%	0.037%	0.32%
	6 hr	0.018%	0.047%	0.31%
^{35}S-immunoreactive ACTH/β-endorphin,[a] dpm	15 min	2627	222	<25
	6 hr	5522	743	<25
^{35}S-immunoreactive β-endorphin,[a] dpm	15 min	1407	820	101
	6 hr	2091	622	283
^{35}S-immunoreactive ACTH[a] dpm	15 min	1115	641	123
	6 hr	1883	508	248

[a] For description of labeling paradigms and description of detection of all forms of radiolabeled material, "Materials and Methods." ^{35}S-immunoreactive ACTH/β-endorphin, ^{35}S-immunoreactive β-endorphin, and ^{35}S-immunoreactive ACTH represent the three major species of ^{35}S-labeled immunoreactive β-endorphin- and ACTH-related material as defined under "Materials and Methods." Specific ^{35}S activity comprises all ^{35}S-labeled immunoreactive ACTH and β-endorphin.

[b] dpm: ^{35}S activity expressed as disintegrations per minute as of the day of infusion.

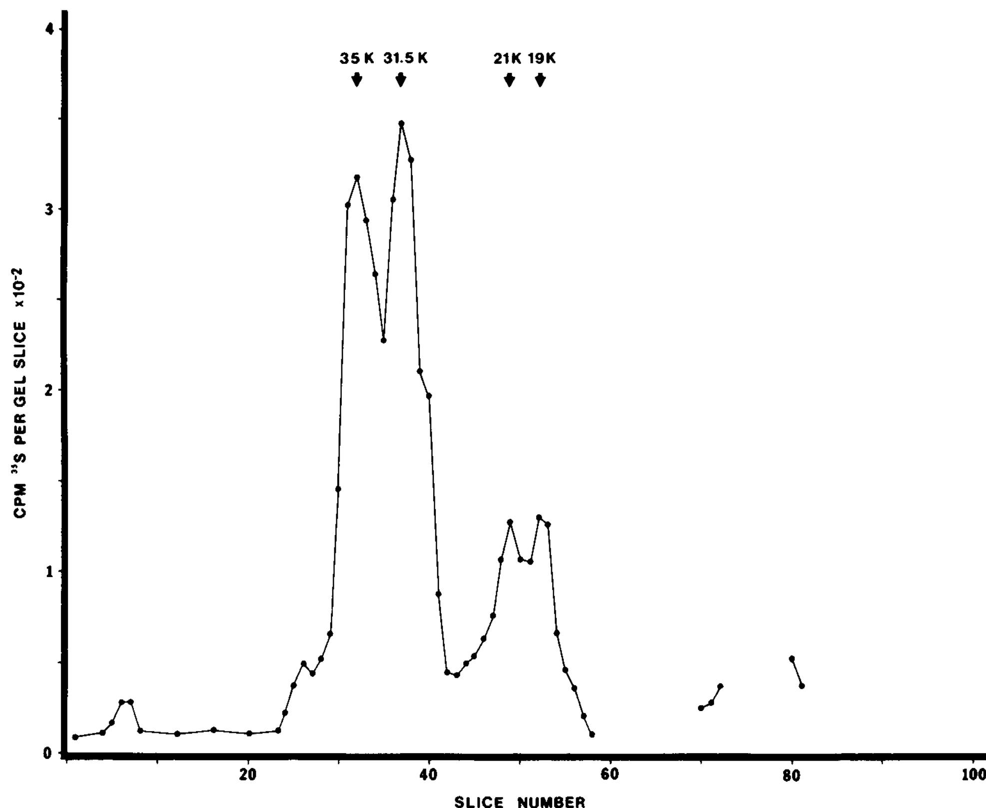

Figure 1. $NaDodSO_4$/polyacrylamide gel electrophoresis. Immunoreactive ACTH/β-endorphin derived from the hypothalamic arcuate nuclear region tissue (6-hr steady labeling experiment) was subjected to disc electrophoresis. The majority of the radioactivity was partially resolved into four molecular species of apparent M_r = 35,000, 31,500, 21,000, and 19,000, as indicated by the *arrows* (K = 1000 daltons). Pharmacia low molecular weight calibration proteins were run on separate gels in the same electrophoretic run. Migration positions of rat β-LPH, synthetic human ACTH, and β_c-endorphin were at slice numbers 62, 72, and 77, respectively. Background activity (17 cpm) was subtracted from the activity of all gel slices.

determinant were individually analyzed by gel filtration on columns of Sephadex G-50 superfine. In all cases, the majority (76 to 82%) of the immunoreactive β-endorphin eluted with the K_{av} of synthetic β_c-endorphin, and the majority (71 to 80%) of the immunoreactive ACTH eluted with the K_{av} of synthetic α-MSH. Figure 3 depicts the profiles obtained for the arcuate nuclear area 6-hr steady labeling experiment. Low amounts of ^{35}S-labeled immunoreactive β-endorphin also eluted at positions corresponding to the void volume, K_{av} of rat pituitary β-

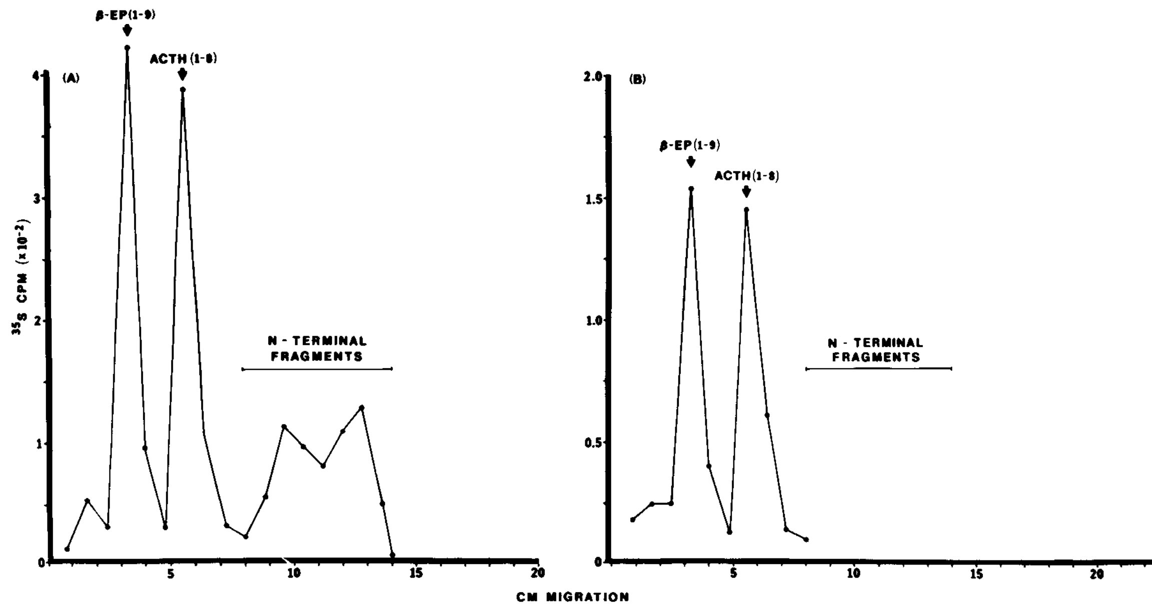

Figure 2. Paper electrophoretic analysis (pH 6.5) of tryptic peptides derived from ^{35}S-labeled immunoreactive ACTH/β-endorphin. *A*, The methionine-containing tryptic fragments of the M_r = 35,000 and 31,500 ^{35}S-labeled immunoreactive species derived from the SDS-PAGE analysis (Fig. 1, slices 29 to 42). *B*, Tryptic fragments of the M_r = 21,000 and 19,000 species (Fig. 1, slices 46 to 55). Background ^{35}S activity was subtracted in all instances. *Arrows* indicate migration positions of synthetic marker peptides, the horizontal bar that of the N-terminal methionine-containing tryptic fragments derived from rat pituitary POMC.

LPH, the K_{av} of β_c-endorphin(1–27), and the K_{av} of α-endorphin (as noted under "Materials and Methods," the anti-β-endorphin columns do not bind α- or γ-endorphin; this material may, therefore, represent C-terminal fragments of β-endorphin) (Fig. 3*A*). Low levels of ^{35}S-labeled immunoreactive ACTH were also detected at positions eluting before the α-endorphin marker (Fig. 3*B*). Because of the insufficient amounts of ^{35}S activity present in them, these minor peaks could not be further analyzed. A similar distribution was obtained for the 15-min labeled tissue, except that an even greater amount of the total activity eluted with the K_{av} of α-MSH and β-endorphin (about 82% and 80%, respectively).

Further characterization of ^{35}S-labeled immunoreactive β-endorphin in arcuate and preoptic areas

Cation-exchange chromatography. Fractions comprising the major peak of immunoreactive β-endorphin-sized material on Sephadex G-50 chromatography of these areas were pooled and analyzed on SP-Sephadex C-25 columns (pooling of material derived from the two brain regions was necessary to ensure sufficient activity for subsequent sequential characterization). Figure 4 depicts the profile of the β-endorphin-sized material after 6 hr of labeling. Approximately 78% of the ^{35}S activity eluted at the position of synthetic β_c-endorphin; two minor peaks eluted at the position of synthetic β_c-endorphin(1–27) (about 9%) and [Arg^0]-β_c-endorphin (about 13%) (we have recently identified [Arg^0]-β-endorphin as a minor β-endorphin species in human anterior pituitary tissue). Analysis of β-endorphin-sized material from the 15-min labeling procedure yielded a major peak at the position of β_c-endorphin (about 82%) (data not shown). Insufficient amounts of material prevented further characterization of the minor peaks.

Reverse-phase high performance liquid chromatography and tryptic mapping. Material eluting at the position of β_c-endorphin in the cation-exchange chromatograms (15-min and 6-hr labeling protocols) was desalted (on Sep-Pak C-18 cartridges), oxidized with 5% H_2O_2, and analyzed by reverse-phase HPLC. More than 90% of the injected ^{35}S-labeled material eluted as a discrete peak exhibiting the retention time (t_R) of [Met(0)]-β_c-endorphin (Fig. 5).

This material was trypsinized and analyzed by reverse-phase HPLC. Approximately 92% of the injected activity eluted with the t_R of [Met(0)]-β_c-endorphin(1–9) (Fig. 6).

Further characterization of α-MSH-sized ^{35}S-labeled immunoreactive ACTH

Reverse-phase HPLC and tryptic mapping. Two pools (15-min and 6-hr labeling) of ^{35}S-labeled α-MSH-sized immunoreactive ACTH were prepared by combining material obtained from the arcuate nuclear area and POA Sephadex G-50 gel filtration chromatograms. Each pool was sequentially run in two different reverse-phase HPLC systems, after oxidation of samples with H_2O_2. In each instance, greater than 88% of the activity recovered after the first HPLC run (total recovery of injected material about 90%) eluted in a sharp peak, at the t_R of [Met(0)]-des-acetyl α-MSH. The fractions comprising this peak were pooled and analyzed in the second reverse-phase HPLC system. In both instance (15-min and 6-hr labeling sample pools), greater than 94% of the injected material (overall column recoveries about 93%) again eluted with the t_R of [Met(0)]-des-acetyl α-MSH (Fig. 7

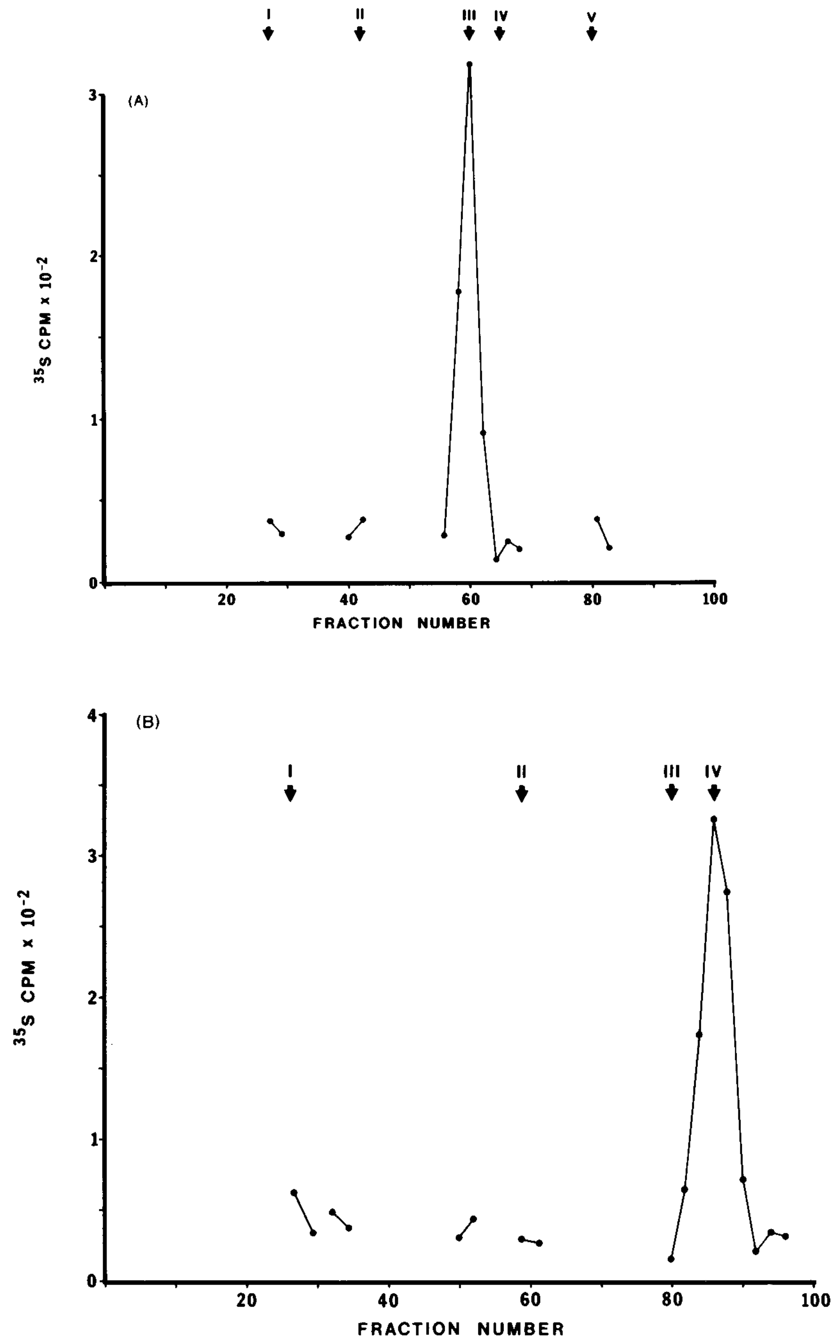

Figure 3. Sephadex G-50 (superfine) gel filtration of monodeterminant ^{35}S-labeled immunoreactive β-endorphin (*A*) and ACTH (*B*) derived from the arcuate nuclear region (6-hr steady labeling). The *arrows* indicate the void volume (*I*) and the elution positions of marker peptides. *A*, *II*: rat β-LPH; *III*: β_c-endorphin(1–27); *V*: α-endorphin. *B*, *II*: human ACTH(1–39); *III*: α-endorphin; *IV*: α-MSH.

illustrates the second HPLC profile obtained for the 6-hr labeled sample; see figure legend for chromatographic details).

This peak of activity was trypsinized, oxidized with H_2O_2, and run in the same HPLC system employed for analysis of the tryptic fragments of the immunoreactive β-endorphin material. Approximately 86% of the injected material was recovered from the column (for both samples); all of these materials eluted in a single discrete peak at t_R = [Met(0)]ACTH(1–8). No activity was detected at the position of [Met(0)]-α-*N*-acetyl ACTH(1–8), the tryptic fragment generated from authentic α-MSH (Fig. 8).

Characterization of immunoreactive β-endorphin and ACTH in ME area

Because of the low amounts of ^{35}S-labeled immunoreactive β-endorphin and ACTH present in the ME extracts, these samples were analyzed directly by reverse-phase HPLC after sample oxidation. In both cases, ap-

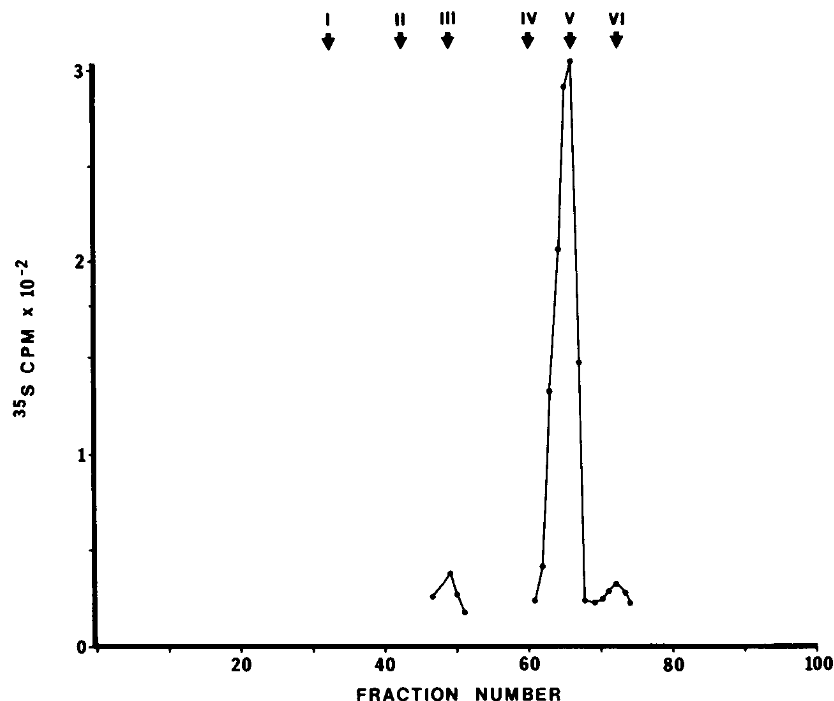

Figure 4. SP Sephadex C-25 cation-exchange chromatography of ^{35}S-labeled β-endorphin-sized material: profile of pool of material obtained from Sephadex G-50 gel filtration of arcuate nuclear region (see Fig. 3) and preoptic areas that eluted with the K_{av} of β_c-endorphin. The *arrows* indicate: *I*, α-*N*-acetyl β_c-endorphin(1–26); *II*, α-*N*-acetyl β_c-endorphin(1–27); *III*, β_c-endorphin(1–27); *IV*, α-*N*-acetyl β_c-endorphin(1–31); *V*, β_c-endorphin(1–31); *VI*, the theoretical elution position of [Arg^0]-β_c-endorphin.

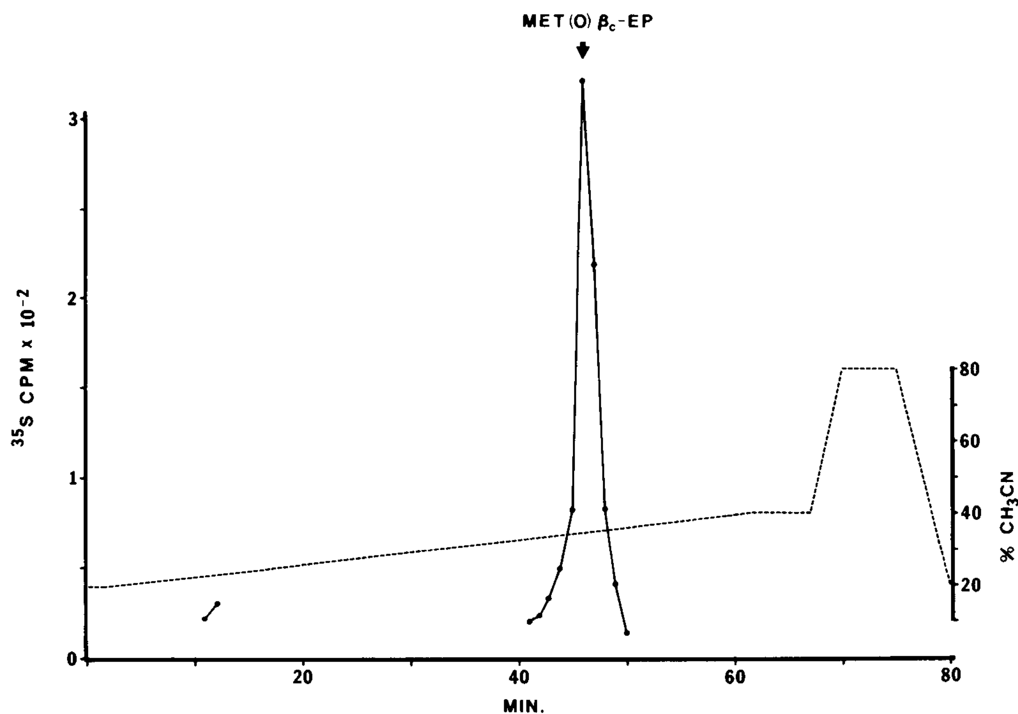

Figure 5. Reverse-phase HPLC analysis of β-endorphin-like material. A pool (15-min and 6-hr labeling protocols) of ^{35}S-labeled immunoreactive β-endorphin, derived from the cation-exchange chromatograms (see Fig. 5) of the arcuate nuclear region and POA, was oxidized with H_2O_2 (see "Materials and Methods"). Elution was performed with a gradient of CH_3CN (indicated by *dashed lines*); trifluoroacetic acid was kept at a constant concentration of 0.01 M. A C-18 μBondapak column was employed. Flow rate was 0.75 ml min^{-1}; 1-min fractions were collected. The majority of the activity eluted at the position of [Met(0)]-β_c-endorphin (indicated by *arrow*).

proximately 84% of recovered immunoreactive β-endorphin eluted at t_R = [Met(0)]-β_c-endorphin, and approximately 78% of immunoreactive ACTH eluted at t_R [Met(0)]des-acetyl α-MSH (total recovery from columns about 91%) (data not shown).

Discussion

The present data demonstrate the *in vivo* biosynthesis of POMC-like material and related peptides by adult rat hypothalamus. They additionally suggest differences in processing of brain and pituitary POMC-like material, both with regard to initial cleavage sites and covalent modification (i.e., acetylation). In both pituitary lobes, the first major cleavage of POMC (M_r = 31,500 and 35,000) is between the ACTH and β-lipotropin segments of the molecule, yielding β-LPH and what has been termed the "23K ACTH" biosynthetic intermediate (comprising the N-terminal segment covalently linked to the ACTH sequence). In the present studies, the material of M_r = 35,000 and 31,500 was physicochemically indistinguishable from rat anterior or intermediate pituitary lobe POMC (synthesized *in vitro* in the presence of [^{35}S]methionine). However, the material of M_r = 21,000 and 19,000 (about 25% of the total ^{35}S-labeled immunoreactive ACTH/β-endorphin; about 33% on a molar basis) lacked the methionine-containing tryptic fragments characteristic of the N-terminal glycopeptide of pituitary POMC. This latter form may, therefore, represent POMC molecules that underwent a primary cleavage which removed all or part of the N-terminal fragment, indicative of an altered order of processing of POMC-like material in at least a subset of arcuate nucleus neurons.[5]

The majority of the monodeterminant molecular species represented α-MSH- and β-endorphin-sized peptides, consistent with other reports that these are the

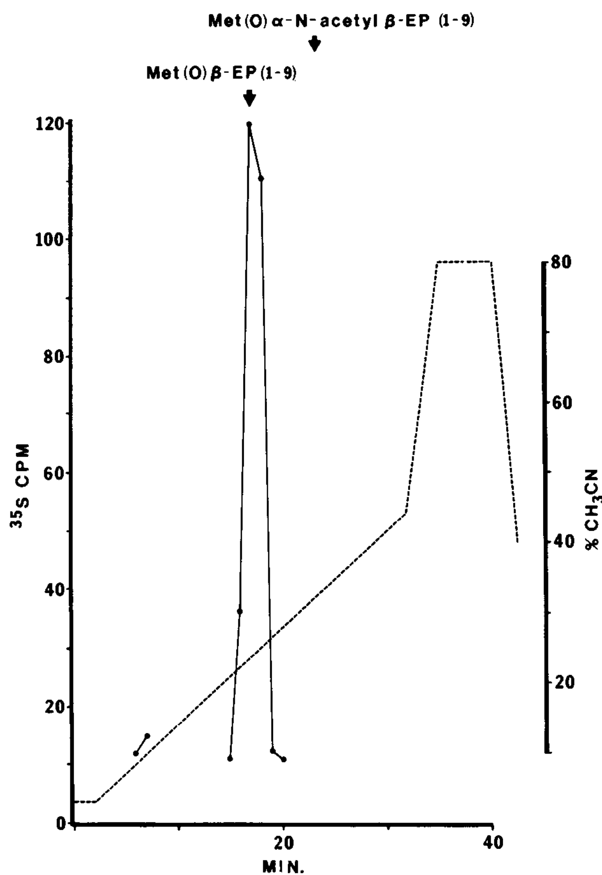

Figure 6. Reverse-phase HPLC analysis of tryptic digests of β_c-endorphin-like peptide. ^{35}S-labeled β-endorphin that eluted with the t_R of [Met(0)]-β_c-endorphin in the HPLC analysis depicted in Figure 5 was trypsinized, oxidized with H_2O_2, and analyzed by reverse-phase HPLC, on a C-18 μBondapak column, using the 0.01 M trifluoroacetic acid/CH_3CN solvent system. The gradient of CH_3CN is indicated by *dashed lines*. The elution positions of synthetic markers are indicated. Flow rate was 0.75 ml min^{-1}; 1-min fractions were collected.

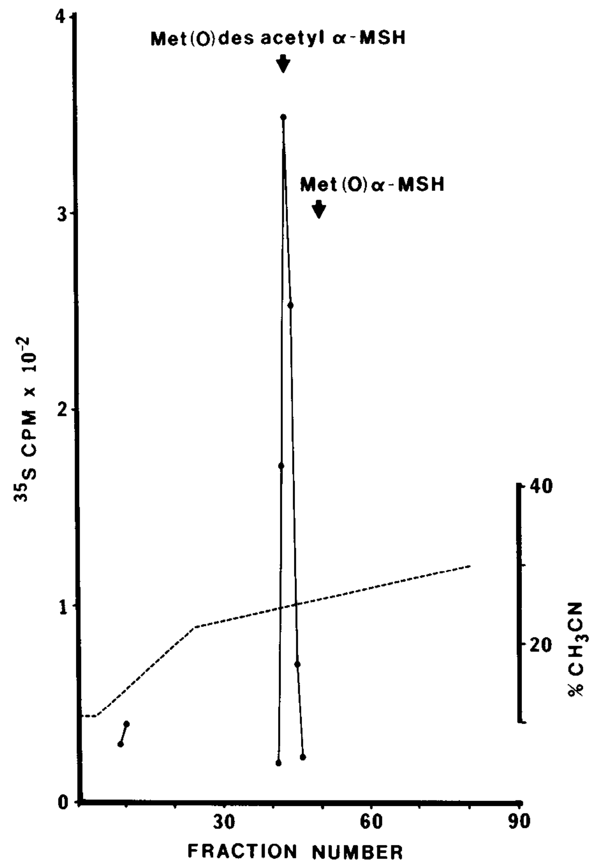

Figure 7. Reverse-phase HPLC of α-MSH-sized immunoreactive ACTH. Reanalysis of fractions with the t_R of [Met(0)]-des-acetyl α-MSH derived from HPLC of pools of arcuate nuclear region and POA material, 6-hr steady labeling experiment (see the text for details). Fractions comprising this peak were pooled, dried, reoxidized, and reanalyzed by reverse-phase HPLC using the triethylammonium phosphate/CH_3CN solvent system described by Rivier (1978), using an Altex ODS column. The CH_3CN gradient is depicted by the *dashed line*. Flow rate was 0.75 ml min^{-1}; 1-min fractions were collected. The elution positions of synthetic [Met(0)]-des-acetyl α-MSH and authentic α-MSH are indicated by the *arrows*.

[5] Such a molecule has been described in AtT20 mouse pituitary cell culture medium as representing about 1% of the mass of total immunoreactive ACTH or β-endorphin (Eipper and Mains, 1978).

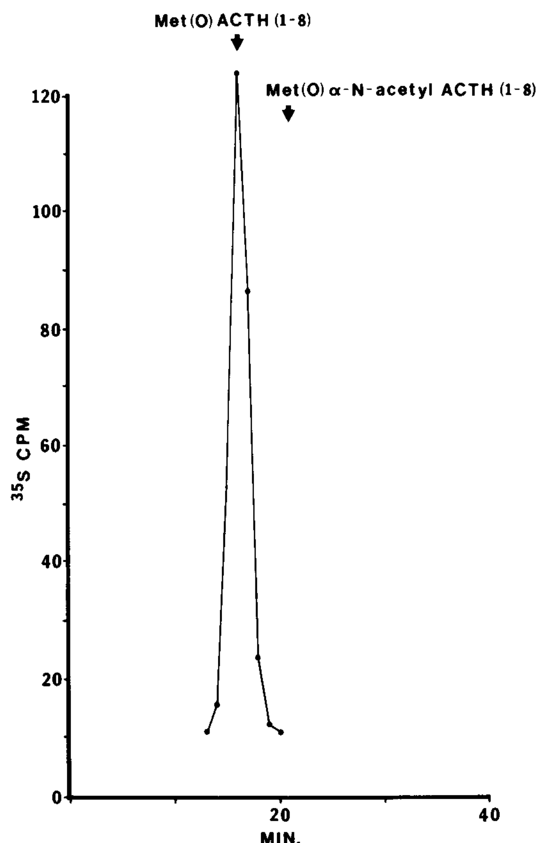

Figure 8. Reverse-phase HPLC analysis of tryptic digest of des-acetyl α-MSH-like material. The sole peak of ^{35}S activity which eluted at the t_R of [Met(0)]-des-acetyl α-MSH, in the HPLC analysis depicted in Figure 7, was trypsinized, oxidized with H_2O_2, and analyzed in the HPLC system described in Figure 6. The elution positions of synthetic marker peptides are indicated.

major ACTH- and β-LPH-related peptides present in brain extracts (Rossier et al., 1977; Gramsch et al., 1980; Krieger et al., 1980; Wilkes et al., 1980; Watson and Akil, 1981; Liotta and Krieger, 1983) and suggesting that such peptides are end products of the post-translational processing of the POMC-like molecule. Such processing is similar to what has been reported for intermediate lobe processing of POMC. In the intermediate lobe, the majority of the β-endorphin- and α-MSH-sized molecules are acetylated. However, the α-MSH- and β-endorphin-sized peptides detected in the present experiments were physicochemically similar to des-acetyl α-MSH and authentic rat pituitary β-endorphin, respectively.

There is no consensus with respect to the presence or extent of α-*N*-acetylation of these peptides in brain (Zakarian and Smyth, 1979; Loh et al., 1980; Pelletier et al., 1980; Weber et al., 1981; Smith et al., 1982). If acetylation occurred in these *in vivo* experiments with the approximate time course and to a similar extent as observed in rat pituitary intermediate lobe cells in culture (Eipper and Mains, 1981; Liotta et al., 1981; Glembotski, 1982), we should easily have been able to detect such modified peptides. (Approximately 76% of α-MSH and approximately 33% of β-endorphin-sized peptides are α-*N*-acetylated after 6-hr steady-state labeling of pituitary intermediate lobe cells (unpublished results).) Therefore, we are in agreement with those studies reporting that little or no acetylation of these peptides occurs in brain (Weber et al., 1981; Evans et al., 1982; Smith et al., 1982), but not with studies which suggest that such acetylation occurs in this tissue. Such lack of acetylation may well modify the physiological role of these peptides (O'Donohue et al., 1982; Zakarian and Smyth, 1982). If acetylated peptides are indeed synthesized in brain, the present results suggest that the cells of origin exist outside the arcuate nuclear area.

The presence of roughly equimolar ratios of α-MSH- and β-endorphin-sized moieties under both labeling paradigms in all three brain regions studied is consistent with the premise that these peptides are post-translationally derived from POMC-like material synthesized in the hypothalamus (one copy of ACTH and β-LPH per pituitary POMC molecule). The evidently higher ratio of immunoreactive ACTH/β-endorphin to α-MSH- or β-endorphin-sized peptides in the arcuate nuclear area than in POA, and the presence of only α-MSH- and β-endorphin-sized peptides in the ME extracts are consistent with the hypothesis that POMC-like material was synthesized by perikarya in the arcuate nuclear region of the hypothalamus, with post-translational processing to α-MSH- and β-endorphin-sized peptides occurring during axonal transport to the POA and ME. Our finding that ratios of immunoreactive ACTH/β-endorphin to α-MSH- or β-endorphin-sized peptides in POA were lower following the 15-min labeling procedure, compared to the 6-hr labeling studies, is presumably due to the fact that the 15-min labeling procedure more closely resembled a 15-min pulse/2-hr chase paradigm (see "Materials and Methods").

The *in situ* labeling approach should permit the study of the expression of the POMC gene in the central nervous system. It overcomes the major disadvantage of *in vitro* systems, which cannot reproduce the *in situ* projection and network formation pathways which are essential characteristics of neural systems. It can be used to explore the possibility that diverse regulatory mechanisms can influence the nature of POMC processing along its diverse trajectories in the neural axis, so as to allow for the distribution of processed peptides differentially to the various terminal fields. Additionally, the acceptable level of radiolabel incorporation and the ability to obtain discrete anatomical samples from individual animals offer further promise for elucidation of the regulatory mechanisms of POMC production by central neurons.

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