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REVIEW ARTICLE

Distribution of β-endorphin-related peptides in rat pituitary and brain

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β-Endorphin, the most potent known naturally occurring analgesic agent, is found in pituitary and brain in company with a series of structurally and biosynthetically related peptides that are essentially devoid of opiate activity. In studies of β-endorphin it is important to discriminate between the active and inactive forms of the peptide. This review describes the use of chemical and immunological methods for localizing the peptides in the tissues, extracting and resolving the peptides by chromatography, and determining the concentrations of the peptides by radioimmunoassay. These approaches have allowed the distribution of β-endorphin and its related peptides to be assigned unequivocally in regions of rat pituitary and brain. It has been found that the multifunctional corticotropin–endorphin prohormone can undergo processing by different mechanisms in different tissues, permitting the intrinsic activities of its fragments to be expressed selectively. The different processing patterns can be attributed to the existence of highly specific enzymes, characteristic of individual cells, which regulate the formation of this potent opiate.

Introduction

β-Endorphin is the *C*-terminal fragment of a 31 kDa prohormone which is biosynthesized in pituitary and brain. This 31-residue peptide was first isolated from pig pituitary, when it was found to occur in company with a series of peptides that form contiguous or overlapping sections of the endorphin prohormone (Bradbury *et al.*, 1975; Smyth *et al.*, 1978). One of the fragments isolated with β-endorphin was lipotropin, a 91-residue peptide that had been identified previously (Li *et al.*, 1965) and whose *C*-terminal sequence was seen to be identical to the sequence of β-endorphin; because of the structural relationship of the two peptides, β-endorphin was initially named the 'C-fragment' of lipotropin (Bradbury *et al.*, 1976). A second group of peptides isolated from pig pituitary included corticotropin (corticotropin 1–39) and its constituent fragments, α-melanotropin (corticotropin 1–13) and CLIP (the corticotropin-like intermediary peptide, corticotropin 18–39); later it was shown that β-endorphin and corticotropin are derived from the same biosynthetic precursor, the 31 kDa corticotropin–endorphin prohormone (Fig. 1; Roberts & Herbert, 1977; Mains *et al.*, 1977; Nakanishi *et al.*, 1979).

In a different field, concurrent studies were providing evidence for the existence of peptides with opiate activity (Terenius & Wahlström, 1974; Cox *et al.*, 1975; Hughes, 1975; Pasternak & Snyder, 1975); these studies culminated in the isolation of two pentapeptides, methionine enkephalin (*a*) and leucine enkephalin (*b*), which exhibited opiate properties *in vitro* (Hughes *et al.*, 1975).

(*a*) Tyr-Gly-Gly-Phe-Met
(*b*) Tyr-Gly-Gly-Phe-Leu

When the structures of the enkephalins were established, it became clear that the sequence of methionine enkephalin was the same as the sequence at the *N*-terminus of lipotropin C-fragment and by implication the possibility arose that the C-fragment, like the enkephalins, might possess opiate activity. Investigation of the biological activity of the 31-residue peptide then revealed that it possessed opiate properties to an exceptional degree, both *in vitro* (Bradbury *et al.*, 1976) and *in vivo* (Loh *et al.*, 1976; Feldberg & Smyth, 1976, 1977) and remarkably the C-fragment, though isolated from the pituitary, was found to exhibit its morphine-like effects in the brain. In view of the similarity of its properties to those of morphine, the general term endorphin (endogenous morphine), which had earlier been proposed by E. J. Simon for peptides with opiate activity (Goldstein, 1976), seemed especially apposite when applied to lipotropin C-fragment and

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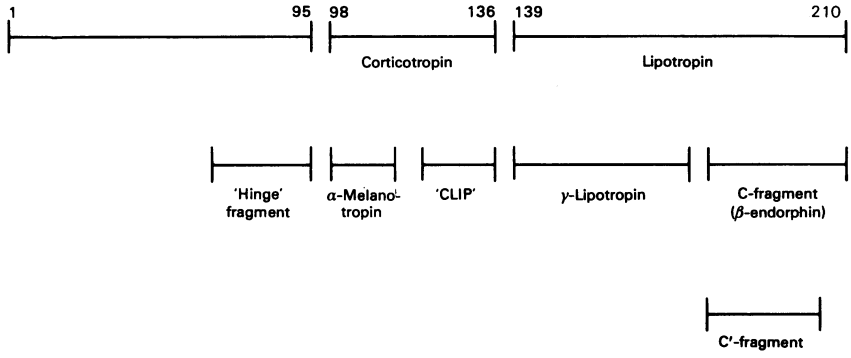
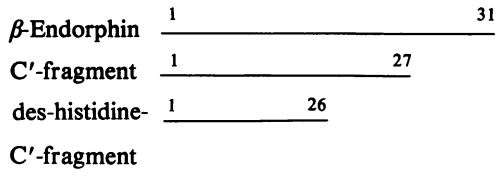


Fig. 1. *Schematic representation of naturally occurring fragments of the 31 kDa prohormone of corticotropin and β-endorphin*

The numbers represent residue positions in the sequence of the rat molecule (according to Drouin & Goodman, 1980). Each of the peptides is flanked by pairs of basic residues in the prohormone sequence. Several of the prohormone fragments exist in both acetylated and unacetylated forms.

the name '*β*-endorphin' has become generally accepted.

After the demonstration that *β*-endorphin was endogenous in pig pituitary, it was shown that the peptide occurs in a variety of mammalian species, including camel (Li & Chung, 1976), ox (Li *et al.*, 1977), sheep (Seidah *et al.*, 1977) and man (Dragon *et al.*, 1977). It is notable that the amino acid sequence is strongly conserved, the only differences being at position 23 in pig *β*-endorphin where valine replaces isoleucine and at positions 27 and 31 in human *β*-endorphin where tyrosine and glutamic acid replace histidine and glutamine (Fig. 2). Such a strong conservation of structure in diverse species would indicate that the full sequence of *β*-endorphin may be important for its opiate properties; in this context it is of interest that a peptide that contains most of the sequence, the C'-fragment of lipotropin (*β*-endorphin residues 1–27), also occurs in the pituitary (Smyth *et al.*, 1978) but exhibits no more than weak analgesic properties; the potency of the C'-fragment is nearly three orders of magnitude less than that of *β*-endorphin (Deakin *et al.*, 1980). In addition, further work on the isolation of *β*-endorphin-related peptides has recently revealed the existence of another 'endorphin', present in pig pituitary in similar quantity to *β*-endorphin and the C'-fragment; this peptide (*β*-endorphin 1–26) lacks the *C*-terminal histidine of the C'-fragment and, like the C'-fragment, has negligible analgesic properties (Smyth *et al.*, 1982).



Additionally, it has been shown that the three peptides can occur in an α,*N*-acetyl form (Smyth *et al.*, 1979) and the acetylated peptides are inert as opiates (Deakin *et al.*, 1980). Thus there appear to be six naturally occurring peptides related to *β*-endorphin, each one derived as a fragment of the *C*-terminal region of the corticotropin–endorphin prohormone and each one occurring in the pituitary in substantial quantity. It would seem significant that the biosynthetic formation of these peptides involves proteolytic cleavage of the 31 kDa prohormone at sites containing consecutive basic residues, a common mechanism for prohormone activation (Steiner *et al.*, 1974) and suggestive of possible physiological function. However, of the *β*-endorphin-related peptides, *β*-endorphin alone possesses potent analgesic properties.

In an attempt to understand the physiological role of *β*-endorphin, studies have been carried out on the distribution of 'endorphins' in the pituitary and brain of a number of species. In general the peptides occur at low concentrations, which makes isolation and analysis difficult, but they can be detected and measured readily with the aid of highly sensitive immunological techniques. The use of antibodies to lipotropin and *β*-endorphin has been particularly valuable for localizing '*β*-endorphin immunoreactivity' by immunohistochemical methods (Watson *et al.*, 1977; Bloom *et al.*, 1978; Zakarian & Smyth, 1979) as well as for radioimmunoassay of tissue extracts, but since the antibodies used would react with the entire range of lipotropin *C*-terminal fragment distinction cannot be made between the different peptides. Because of this difficulty, immunological methods have been combined with chemical methods for resolution of the immunoactive components and with this approach it has been possible to identify and determine the indi-

Pig	1	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Val-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln 31
Bovine (camel, sheep)	1	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln 31
Rat	1	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Val-His-Lys-Lys-Gly-Gln 31
Human	1	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu 31

Fig. 2. *Comparison of the amino acid sequence of β-endorphin in different species*

vidual peptides. In recent experiments, methods have been used which allow the peptides to be extracted from the tissues in good yield (Scott & Lowry, 1974; Rossier *et al.*, 1977; Smyth *et al.*, 1977) and chromatographic procedures have been developed to resolve the six β-endorphin-related peptides from each other, even at the fmol level (Smyth *et al.*, 1981). It should be mentioned that basic, hydrophobic peptides adsorb readily to glass and to column supports and separation of the β-endorphin related peptides can present difficulty. The problems have been overcome, however, by the use of dissociating solvents at all stages of extraction, gel-filtration and ion-exchange chromatography (Smyth & Zakarian, 1980).

Localization of β-endorphin-related peptides in pituitary by immunohistochemistry

Several studies have been reported on the distribution of 'β-endorphin immunoreactivity' in rat pituitary using the method of immunofluorescence and antibodies with widely differing specificities have been employed. It is of interest that the patterns of immunofluorescence obtained were essentially identical, despite the use of the different antibodies. In the first studies, the antibodies were raised against human lipotropin (Pelletier *et al.*, 1977; Watson *et al.*, 1977) but later experiments were carried out with antibodies to β-endorphin. The specificity of the lipotropin antibodies was principally for the *N*-terminal region of lipotropin which differs extensively across species, rat lipotropin, for example, having 22 amino acid residues fewer than bovine lipotropin (Nakanishi *et al.*, 1979; Drouin & Goodman, 1980). This disadvantage is avoided by using antibodies that have specificity for the *C*-terminal region of the peptide chain (β-endorphin), as this region is strongly conserved, and antibodies with the required specificity have been obtained by using preparations of synthetic human β-endorphin (Deftos & Catherwood, 1980), synthetic pig β-endorphin (Bloom *et al.*, 1978) or β-endorphin isolated from pig pituitary (Zakarian & Smyth, 1979). These antibodies cross-react with rat β-endorphin, which differs from bovine β-endorphin only at position 26 where alanine is replaced by valine (Drouin & Goodman, 1980).

In immunohistochemical studies of rat pituitary, with antibodies to lipotropin or β-endorphin, it has been shown that only some of the cells are reactive in the anterior lobe whereas all the cells are reactive in the pars intermedia; no fluorescence has been seen in rat posterior pituitary (see Plate 1). Furthermore, fluorescence studies using antibodies raised against corticotropin have indicated that this peptide is distributed similarly; indeed the immunoreactive

corticotropin and β-endorphin appear to be elaborated in the same cell (Pelletier *et al.*, 1977). As lipotropin, β-endorphin and corticotropin are derived from a common precursor, the presence of these peptides in the same cells is not surprising. However, in contradistinction, other evidence obtained by immunofluorescence has indicated a dissociation between cells that produce β-endorphin and cells that produce corticotropin (Deftos & Catherwood, 1980; Wilkes *et al.*, 1980). Since rat anterior pituitary contains more corticotropin than 'β-endorphin' (Jackson & Lowry, 1980), it would seem that the two peptides are not always formed concomitantly. Histochemical studies employing highly specific antibodies will be necessary to support this important conclusion.

It should be emphasized that the precise nature of the immunoreactive peptides is not demonstrated by the fluorescence technique. In general, the antibodies available exhibit cross reactivity to peptides that have overlapping sequences, whether the peptides occur independently of their precursors or whether they occur within the precursor structure. Thus antibodies raised against corticotropin can react not only with corticotropin (1–39) but also with the corticoprotin–endorphin prohormone and with CLIP (corticotropin 18–39) and antibodies to β-endorphin generally react with all peptides that contain the *C*-terminal determinants of lipotropin (Guillemin *et al.*, 1977; Hollt *et al.*, 1978; Zakarian & Smyth, 1979), which includes the range of β-endorphin-related peptides described. Nevertheless the method of studying peptide distribution by immunofluorescence remains invaluable for identifying the individual cells in the pituitary and the specific pathways in the brain where biosynthesis and processing of the peptide prohormone may take place.

Identification and distribution of β-endorphin-related peptides in the pituitary by extraction, chromatography and radioimmunoassay

To distinguish between the many related peptides that exhibit immunofluorescence with antibodies to β-endorphin, a different approach has been used which involves extraction of the immunoreactive material from the tissues and gel filtration to separate the group of β-endorphin related peptides from lipotropin. For this purpose, a wide variety of experimental conditions has been employed in order to achieve good resolution with high yield (Table 1). This diversity is probably a reflection of the difficulties that can be experienced in the chromatography of basic, hydrophobic peptides, especially when small quantities are involved. It appears that acidic solvents such as 50% acetic acid, 1% formic acid or 0.1 M-hydrochloric acid are the most satisfactory eluents but problems associated with non-specific adsorption still arise. Among the alternatives 50% acetic acid, a volatile solvent with strong dissociating properties, has been found to be exceptionally suitable for the gel filtration of β-endorphin. This eluent has the additional advantage in that further chromatography can be carried out in the same medium, which eliminates the need for removal of solvent prior to the next step. To resolve the β-endorphin-related peptides from each other, ion-exchange chromatography (Smyth & Zakarian, 1980) and high pressure liquid chromatography (Loeber *et al.*, 1979) have been applied successfully. After chromatography, the amounts of the individual peptides and their elution positions can be determined by radioimmunoassay in the knowledge that the antibodies are reacting with defined substances.

In this laboratory the chromatographic methods that have been developed for separating the six β-endorphin related peptides make extensive use of 50% acetic acid, not only during gel filtration (resolving on the basis of molecular size) but during ion-exchange chromatography (resolving by difference in electrical charge). The peptides, located by radioimmunoassay with an antibody to β-endorphin, are identified by comparison of their elution positions with the positions of radiolabelled marker peptides added in trace quantities to the tissue extracts (Smyth & Zakarian, 1980). In studies carried out in the rat, β-endorphin-related peptides of bovine origin (Massey & Smyth, 1980) have proved useful, since the sequence of β-endorphin is similar in the two species (Nakanishi *et al.*, 1979; Drouin & Goodman, 1980). Further confirmation of the identity of the endogenous peptides can be obtained by comparison of their chromatographic behaviour with the behaviour of the corresponding bovine reference peptides on h.p.l.c.. The β-endorphin antibody used for radioimmunoassay in these studies (Zakarian & Smyth, 1979), like all antibodies raised against β-endorphin, has specificity for the *C*-terminal region of lipotropin and consequently exhibits affinity for the C-fragment (β-endorphin residues 1–31), the C′-fragment (residues 1–27) and the des-histidine-C′-fragment (residues 1–26). The antibody also recognizes the α,*N*-acetyl forms of the peptides, with affinity equal to that for the parent peptides, and in addition it reacts with lipotropin and the 31 kDa prohormone; it does not react with methionine enkephalin, leucine enkephalin, α-endorphin (β-endorphin 1–16), γ-endorphin (β-endorphin 1–17), corticotropin, α-melanotropin or CLIP. Thus the multiple specificities exhibited by antibodies to β-endorphin, which is a drawback in the identification of the peptides by the method of immunofluorescence, or in the direct assay of tissue extracts,

Table 1. *Chromatographic systems used for gel filtration of β-endorphin and lipotropin extracted from mammalian tissue*

Column	Dimensions (cm)	Eluant	Reference
Bio-Gel A-1.5m	0.8 × 50	6 M-Guanidine hydrochloride/0.2% bovine serum albumin	Roberts & Herbert (1977)
Bio-Gel P-4	1.5 × 97	100 mM-Ammonium acetate	Sabol (1978)
Bio-Gel P-6	1.0 × 11	50 mM-Ammonium formate	Ross *et al.* (1977)
Bio-Gel P-60	0.7 × 49	50 mM-Phosphate (pH 7.0)/0.5% human serum albumin/0.4% 2-mercaptoethanol	Nakao *et al.* (1980)
Bio-Gel P-60	0.7 × 45	4 M-Guanidine hydrochloride	Rossier *et al.* (1977)
Bio-Gel P-60	0.9 × 45	20 mM-Phosphate/150 mM-NaCl/10 mM-EDTA/0.1% gelatin/0.05% bovine serum albumin	Wilkes *et al.* (1980)
Sephadex G-50	1.0 × 50	100 mM-Acetic acid	Matsukara *et al.* (1978)
Sephadex G-50	1.0 × 50	100 mM-Hydrochloric acid	Liotta *et al.* (1978)
Sephadex G-50	3.6 × 200	200 mM-Acetic acid	Lissitsky *et al.* (1978)
Sephadex G-50	1.5 × 100	1% Formic acid	Jackson & Lowry (1980)
Sephadex G-75	1.0 × 50	0.5% Human serum albumin/150 mM-NaCl	Krieger *et al.* (1977)
Sephadex G-75	1.6 × 40	1 M-Acetic acid	Rubinstein *et al.* (1977)
Sephadex G-75	2.5 × 70	50% Acetic acid/0.25% formic acid	Zakarian & Smyth (1979)
Sephadex G-75	1.0 × 57	10% Formic acid/0.1% bovine serum albumin/0.1% 2-mercaptoethanol	Eipper & Mains (1981)
Sephadex G-100	1.5 × 90	100 mM-Acetic acid	Yoshimi *et al.* (1978)

becomes a positive advantage in studies where the peptides are first resolved by chromatography.

There is now general agreement that gel filtration of the peptides extracted from rat anterior pituitary (for references see Table 1) leads to the separation of two fractions, the first emerging in a position close to pig lipotropin and the second emerging with β-endorphin; there appears to be approximately an equal amount of immunoreactive peptide in each fraction. To establish the identity of the immunoreactive peptides in the 'β-endorphin' fraction, further purification is required to separate the peptides that differ in respect of their net charge. When this procedure is applied, it is found that the β-endorphin fraction from the anterior pituitary contains essentially a single immunoreactive peptide which chromatographs similarly to the radioiodinated bovine β-endorphin (Fig. 3*a*), in agreement with the known elution characteristics of the unlabelled versus labelled peptides (Smyth *et al.*, 1981; Zakarian & Smyth, 1982). Further identification of the principal immunoreactive peptide in the anterior pituitary has been obtained by subsequent fractionation on h.p.l.c., which demonstrates that the rat β-endorphin behaves very similarly to bovine β-endorphin.

In addition to the β-endorphin in the anterior pituitary there is evidence for the presence of small amounts of other immunoreactive peptides which correspond to endorphins found in the pars intermedia, However, the principal *C*-terminal fragments of the 31 kDa prohormone in the anterior pituitary are clearly lipotropin and β-endorphin. Lipotropin is the major peptide while β-endorphin in its opiate active form accounts for most of the β-endorphin-size peptides in this region.

Experiments involving gel filtration of the peptides extracted from rat pars intermedia plus posterior pituitary have shown an entirely different pattern. In this region of pituitary there are relatively small amounts of lipotropin and 31 kDa prohormone but large quantities of immunoreactive peptide with the approximate molecular size of β-endorphin (Eipper & Mains, 1981; Smyth & Zakarian, 1980). On ion-exchange chromatography, the β-endorphin-containing fraction has been found to be resolved into six components (Smyth *et al.*, 1981; Zakarian & Smyth, 1982*b*) and it is of particular interest that the biologically active form of β-endorphin is relatively minor, representing less than 10% of the total immunoreactive peptides. Since the extracted peptides do not give rise to artifactual products during isolation, it can be concluded that the different components resolved by ion-exchange chromatography represent endogenous peptides (see Fig. 4). Of the six peptides that have been resolved, four have been identified as α,*N*-acetyl C′-fragment, C′-fragment, α,*N*-acetyl β-endorphin and β-endorphin (Peaks II, IV, V and VI, Fig. 3*b*), and the behaviour of these peptides on h.p.l.c. has supported their assignments. The remaining peptides (Peaks I and III) correspond in their chromatographic behaviour to two endorphins recently isolated from pig pituitary, des-histidine-α,*N*-acetyl C′-fragment and des-histidine-C′-fragment (Smyth *et al.*, 1982).

The results identifying the pars intermedia peptides suggest that the intense lipotropin fluorescence that has been observed in the pars intermedia

(Watson *et al.*, 1977) is unlikely to be due to the presence of lipotropin but should be attributed to peptides the size of β-endorphin. β-Endorphin itself, however, accounts for no more than a small fraction of those peptides. The major β-endorphin-related peptides in rat pars intermedia are the α,*N*-acetyl derivatives of des-histidine-C′-fragment, C′-fragment and β-endorphin, substances devoid of opiate properties.

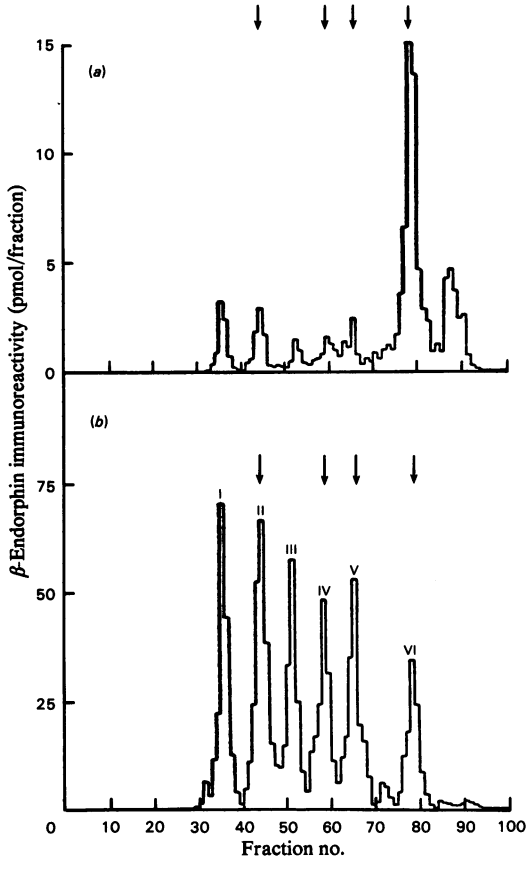


Fig. 3. *Ion-exchange chromatography of β-endorphin-related peptides extracted from the anterior pituitary (a) and the pars intermedia plus posterior pituitary (b) of the rat*

The 'β-endorphin' fraction obtained by gel filtration was chromatographed on a column (60 × 0.6 cm) of SP Sephadex C-25 (pyridinium form) and chromatography was performed in 50% acetic acid/0.25% formic acid, with a linear gradient from 0 to 1 M-pyridine, mixer volume 100 ml. Aliquots of the eluted fractions (1.5 ml) were dried *in vacuo* and immunoassayed with an antiserum to β-endorphin. The elution positions of radiolabelled reference peptides (α,*N*-acetyl-C′-fragment, C′-fragment, α,*N*-acetyl-β-endorphin and β-endorphin respectively) are indicated from left to right by the arrows; des-histidine-α,*N*-acetyl C′-fragment (Peak I) and des-histidine-C′-fragment (Peak III) chromatographed in positions not indicated by marker peptides.

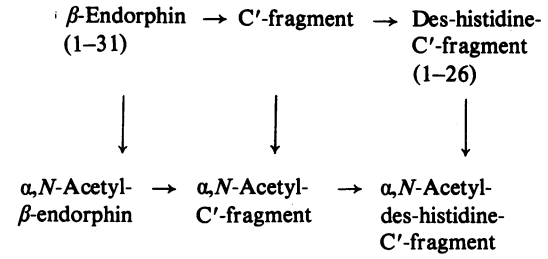


Fig. 4. *Secondary processing of β-endorphin in rat pars intermedia*

Resolution of the β-endorphin-related peptides by h.p.l.c. applied directly after gel filtration has proved to be less useful than when it is applied after the ion-exchange-chromatographic step, since it has proved difficult to resolve the C′-fragment from acetyl β-endorphin. Furthermore, on h.p.l.c. the sulphoxide forms of the peptides, which seem to be generated during isolation, are resolved from the parent peptides and this adds to the apparent complexity of the elution patterns. In contrast, the sulphoxides chromatograph together with the parent peptides during gel filtration and ion-exchange chromatography (Smyth *et al.*, 1981).

The studies on β-endorphin distribution in rat pituitary have been extended to a number of other species, including mouse, cat, guinea pig, pig, armadillo, baboon and human, selected on the basis of evolutionary differences that might have physiological significance. In nearly all cases, a similar pattern of distribution of the β-endorphin related peptides exists, with lipotropin and β-endorphin predominating in the anterior pituitary and the α,*N*-acetyl forms of the C′-fragment and β-endorphin in the pars intermedia. Minor variations do occur, however, in the degree of acetylation of the pars intermedia peptides, the acetylation being more pronounced in the mouse and rat than in the pig and baboon. A more striking difference is seen in human pituitary, which lacks a pars intermedia (Purves & Bassett, 1963; Osamura & Watanabe, 1978); it contains β-endorphin and a little C′-fragment but essentially no acetylated peptides. The pars intermedia of the armadillo, curiously, appears to elaborate only the biologically potent form of β-endorphin. Finally, the des-histidine-C′-fragment does not seem to be formed in the human, baboon or armadillo (D. G. Smyth & S. Zakarian, unpublished work).

Although the processing patterns are largely preserved in the different species, the ratio of β-endorphin immunoreactivity in the two regions of pituitary varies widely. For example, in the rat the concentration of β-endorphin-related peptides in the pars intermedia is approximately 100 times greater than the concentration in the anterior pituitary, in the pig and cat the concentrations are about equal in the two regions, in the baboon the ratio is inverted at 1:5 and in the armadillo the ratio is about 1:20. The pars intermedia region of human pituitary is normally insignificant. These findings suggest that whilst the processing pattern characteristic of each region of pituitary is likely to be of physiological significance, the relative amounts of the predominant β-endorphin related peptides in the two regions may determine the functional importance of a particular region in a given species.

The formation and storage of 'endorphins' that are inactive as opiates is an interesting finding. It should not be excluded that the acetylated peptides and the C'-fragment may have a non-opiate activity which is awaiting discovery and the neuroleptic properties that have been reported for des-tyrosine γ-endorphin (β-endorphin 2–17; De Wied *et al.*, 1978) represents such a possibility. On the other hand, while β-endorphin is inactivated by *N*-acetylation, α-melanotropin (another fragment of the corticotropin–endorphin prohormone) is activated; and this acetylated peptide (Lowry & Scott, 1975) is elaborated in the same region of pituitary as the acetylated endorphins. The acetylation reaction thus appears to offer a selective mechanism for expressing the different activities associated with different sequences in the 31kDa prohormone. It may be mentioned that whilst α-melanotropin is important for colour adaptation to environment in amphibians and fish, its role in mammals is obscure. It remains to be seen whether the primary purpose of the acetylation of β-endorphin is to generate a novel biological activity or whether the purpose is to negate its potent opiate properties.

The formation of β-endorphin seems to involve the action of a processing enzyme with specificity for the Lys-Arg-Tyr sequence at positions 39, 40 and 41 in rat lipotropin (positions 159–161 of the rat prohormone) while the formation of the C'-fragment requires an additional cleavage at the Lys-Lys-Gly residues at positions 68, 69 and 70 of lipotropin (188–190 of the rat prohormone). Since the anterior pituitary contains β-endorphin but very little C'-fragment it would appear that different enzymes are involved in the formation of the two peptides; experiments on the isolation and purification of prohormone activating enzymes in bovine pituitary have lent support to this conclusion (Austen & Smyth, 1979).

The existence of processing enzymes that act at specific paired basic residue sites but have in addition more demanding specificities with respect to the surrounding sequences has a precedent in enzymes that are involved in complement fixation (Kerr, 1979). Such specific enzymes can produce different fragments by processing at different sites. With respect to the 31kDa prohormone, the nature of the fragments formed in different cells would appear to depend on the specificity of the relevant processing enzymes. In the anterior pituitary, the evidence points to the existence of certain cells that contain corticotropin but no β-endorphin and other cells that contain both peptides (Watkins *et al.*, 1981). In the pars intermedia, fluorescence studies indicate that all the cells appear to elaborate both β-endorphin and its fragments and corticotropin together with its fragments, suggesting that in this region there is no distinction between processing mechanisms that lead to the formation of different biologically active segments of the 31kDa prohormone.

The existence of two additional forms of the C'-fragment (β-endorphin 1–26 and its α,*N*-acetyl derivative) deserves comment. Since the des-histidine derivatives lack only the *C*-terminal amino acid of the C'-fragment (histidine) it seems likely that they are formed biosynthetically by the action of a carboxypeptidase B-like enzyme that has specificity for basic residues. In keeping with this possibility, human and baboon β-endorphin, which possess tyrosine in place of histidine, do not appear to form analogous derivatives. The removal of *C*-terminal lysine and arginine, of course, is a well recognized activity of prohormone processing enzymes (Steiner *et al.*, 1974) but attention has not been focused on the removal of *C*-terminal histidine. As the C'-fragment survives in substantial quantity in the pituitary gland, it would appear that the histidine cleavage must take place comparatively slowly. Consequently the relative amounts of the C'-fragment versus its des-histidine derivative (with their acetylated counterparts) may provide an index of the rate of turnover of the C'-fragment *in vivo*.

It is interesting that the potentiality for producing biologically active β-endorphin in the anterior pituitary is not fully utilized, since most of the prohormone molecules there do not undergo proteolytic processing beyond the point of producing lipotropin. In the majority of cells in this tissue it seems likely that corticotropin is the major end product. In other cells of the anterior pituitary, however, β-endorphin appears to be formed and stored together with corticotropin, possibly for the purpose of co-ordinated function.

At the present time no distinctive peripheral effects have been reported for intravenously administered β-endorphin and the physiological role of

the pituitary peptide is not known. Nevertheless, studies on the distribution of β-endorphin related peptides in the pituitary have revealed that alternative processing mechanisms exist for fragmentation of the multifunctional prohormone, mechanisms that generate different biological activities in different tissues. It may be of more immediate significance that differential mechanisms for activation of the 31kDa prohormone have also been found to exist in the brain, where the potent opiate properties of β-endorphin seem likely to be expressed.

Localization by immunohistochemistry and study of the distribution of β-endorphin-related peptides in brain

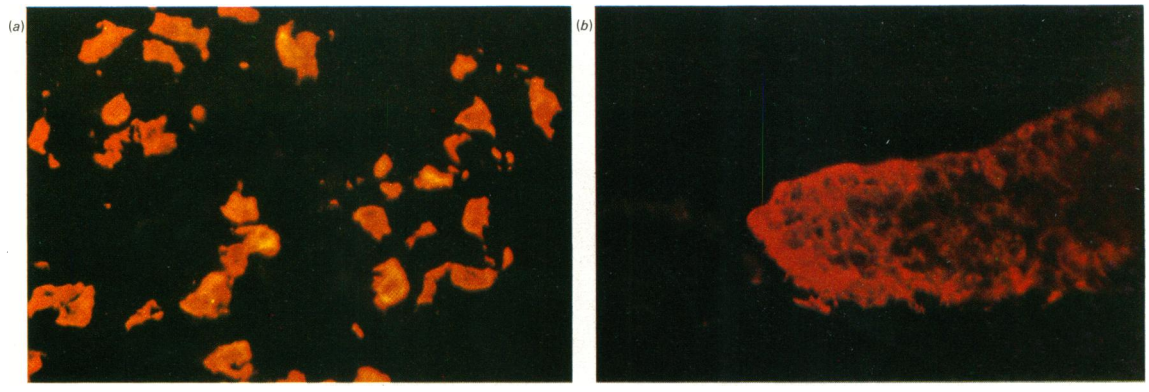
The regions of rat brain where the corticotropin–endorphin prohormone and its fragments occur have been identified by immunofluorescence, using antibodies raised against lipotropin (Watson *et al.*, 1977) or β-endorphin (Bloom *et al.*, 1978; Zakarian & Smyth, 1979). The principal areas where fluorescence is seen are the hypothalamus, thalamus–midbrain, amygdala, hippocampus and brain stem; in addition, dense fluorescence has been observed in the region of the dorsal colliculae (Zakarian & Smyth, 1980; Smyth & Zakarian, 1981). However, the main concentrations of 'β-endorphin immunoreactive' peptides are in the hypothalamus where cell bodies are confined to the arcuate nucleus, median eminence and the ventromedial border of the third ventricle, and axons and terminals occur in abundance along the walls of the third ventricle (Plates 2 and 3). From the hypothalamus long beaded axons extend dorsally and laterally, the fluorescence becoming less dense in the thalamus, and terminals are present at more distant locations in the amygdala, colliculae and hippocampus. Light staining is seen in the form of fibres and terminals in the periaqueductal grey region where the fluorescence is confined to the ventral side of the aqueduct. No fluorescence is seen in the cerebral cortex, cerebellum or spinal cord.

In general, immunofluorescence reveals a highly organized neuronal network commencing with the hypothalamic cell bodies, ramifying through long axons and ending in bundles of terminals at a number of defined locations. Detailed mapping of the immunoreactive peptides by fluorescence, however, will require further investigations with the aid of more specific antibodies, in combination with lesioning experiments that can provide evidence for the transport of β-endorphin between the cell bodies and terminals. Meanwhile, to define the nature of the immunoreactive material visualized by fluorescence, the peptides have been extracted from dissected regions of rat brain and evidence obtained for their chemical nature.

Studies involving gel filtration of β-endorphin related peptides present in regions of rat brain have shown that lipotropin represents no more than a minor component (Rossier *et al.*, 1977; Zakarian & Smyth, 1979): the majority of the peptides have the approximate molecular size of β-endorphin. On ion-exchange chromatography two distinctive patterns can be discerned, the first characteristic of the hypothalamus, mid brain and amygdala (Fig. 5*a*) and the second of the hippocampus, colliculae and brain stem (Fig. 5*b*). The hypothalamus contains predominantly the biologically active form of β-endorphin and similarly the midbrain and amygdala contain β-endorphin as the principal peptide though it is accompanied there by des-histidine C′-fragment; in these regions there is a negligible quantity of the *N*-acetylated peptides. In contrast the hippocampus, colliculae and brain stem contain mainly the α,*N*-acetyl form of the C′-fragment together with its des-histidine derivative; the NH_2 forms of the peptides are much less prominent (Zakarian & Smyth, 1982*b*).

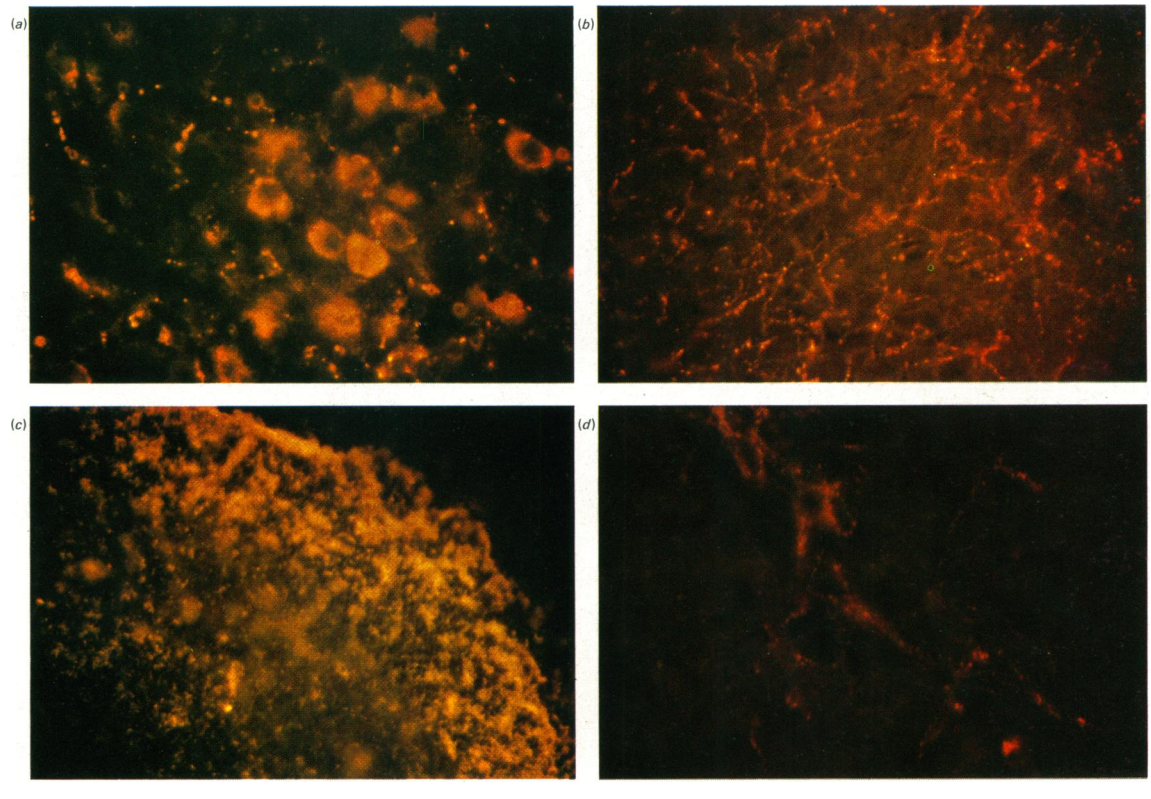
Significance of the alternative mechanisms for processing of β-endorphin

The experimental findings point to the existence of two processing mechanisms in rat brain, corresponding to the mechanisms found to exist in the regions of the pituitary. In one, characteristic of the anterior pituitary and the hypothalamus, β-endorphin is elaborated in its analgesically active form. The formation of the potent peptide seems to involve the action of an enzyme that cleaves between lysyl-arginine and tyrosine, but it does not involve the action of an enzyme with specificity for lysyl-lysylglycine necessary for production of the C′-fragment. This demonstrates again that different enzymes are involved in the formation of β-endorphin and the C′-fragment. In the amygdala, the des-histidine form of C′-fragment is also a substantial component, which implies that both the Lys-Arg-Tyr and the Lys-Lys-Gly processing enzymes, as well as the carboxypeptidase B-like enzyme necessary for removal of *C*-terminal histidine, are prominent in that tissue. In contrast, the pattern of the second processing mechanism resembles that seen in the pars intermedia of the pituitary. Its most striking feature is that the peptides produced in the largest quantity are derivatives of the C′-fragment and to a large degree they are present in the acetylated form. Thus in the hippocampus, colliculae and brain stem, the principal 'endorphins' are inactive as opiates and only a small amount of the biologically active form of β-endorphin remains.



EXPLANATION OF PLATE 1

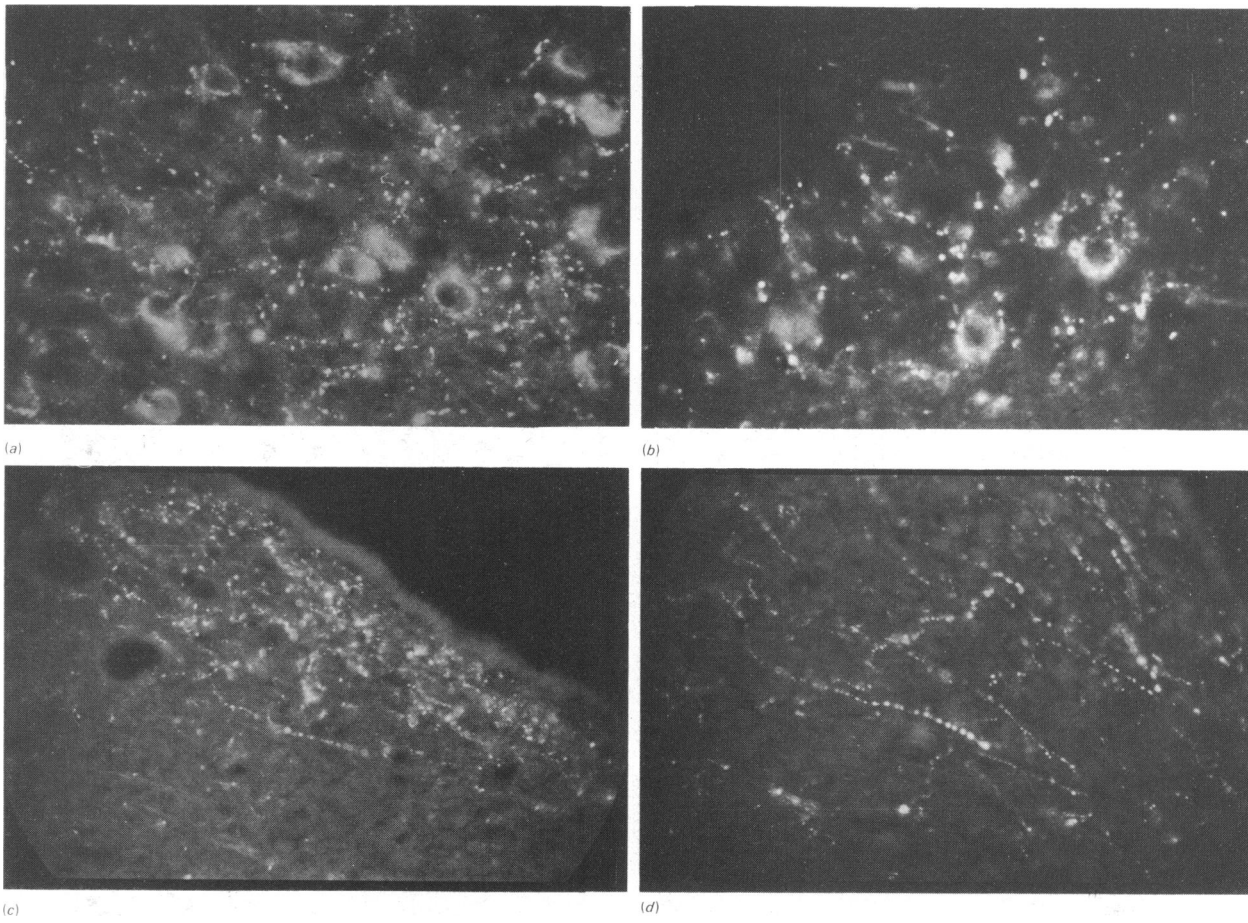
Localization of peptides related to β-endorphin in rat anterior pituitary (a) and pars intermedia (b) by immunofluorescence (Zakarian & Smyth, 1979)



EXPLANATION OF PLATE 2

Localization of peptides related to β-endorphin in regions of rat brain by immunofluorescence

Serial sections of perfused and fixed brain from Lewis rats were stained with antibody to β-endorphin (Zakarian & Smyth, 1979, 1980; Smyth & Zakarian, 1982). In (*a*) β-endorphin-synthesizing cell bodies and beaded fibres are seen in the median eminence and arcuate nucleus; cell bodies with dark unstained nuclei are surrounded by positively stained cytoplasm. (*b*) shows numerous beaded axons typical of the region of the thalamus and mid brain. In (*c*) dense fibres and terminals in the nucleus amygdaloidus and (*d*) relatively sparse but highly organized fibres and axons throughout the brain stem are seen.



EXPLANATION OF PLATE 3

Different forms of β-endorphin fluorescence seen in rat hypothalamus

Serial sections of perfused and fixed tissue from Lewis rats were stained with antibody to β-endorphin. In (*a*) cell bodies synthesizing β-endorphin and β-endorphin containing fibres in the region of the arcuate nucleus are seen. (*b*) shows cell bodies and fibres ventromedial to the third ventricle. In (*c*) dense fluorescence in the form of axons and fibres along the entire length of the wall of the third ventricle, and in (*d*) long beaded axons extending dorsolaterally from the ventricle toward the thalamus are seen (Zakarian & Smyth, 1979, 1908).

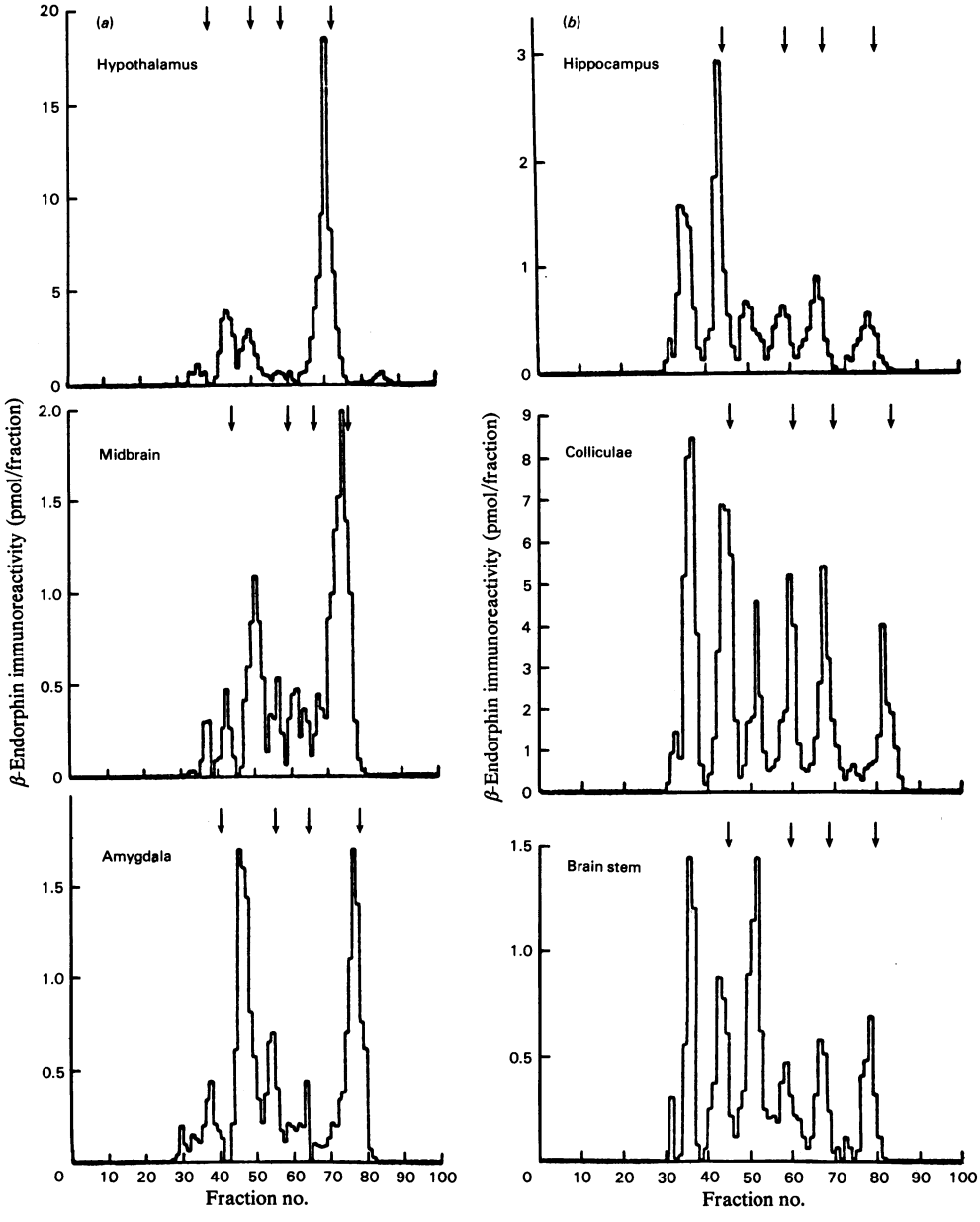


Fig. 5. *Ion-exchange chromatography of β-endorphin-related peptides present in regions of rat brain*
The column conditions were as in Fig. 3 except that fractions were 1.6 ml. The elution positions of the ^{125}I-labelled reference peptides (α,*N*-acetyl-C′-fragment, C′-fragment, α,*N*-acetyl-β-endorphin and β-endorphin respectively) are indicated from left to right by the arrows. The des-histidine-α,*N*-acetyl C′-fragment and the des-histidine-C′-fragment do not elute in positions indicated by the reference peptides. Note that the hypothalamus, mid-brain and amygdala exhibit one pattern of processing (*a*) while the hippocampus, colliculae and brain stem exhibit another (*b*). Each elution pattern represents material obtained from 30 rats.

It would seem reasonable to conclude that the first processing mechanism is responsible for activation of the 31 kDa prohormone to allow expression of the potent opiate properties of β-endorphin, while the second mechanism represents a specific inactivating process (with respect to opiate activity). In this

regard it would be of interest to correlate the distribution of the acetylated endorphins with the distribution of α-melanotropin, since these peptides may be formed by similar mechanisms but have different functions (Lichtensteiger & Linehart, 1977). It is of special interest that recent studies by immunofluorescence have provided evidence for the presence in rat brain of two cell types that contain immunoreactive α-melanotropin (Watson & Akil, 1979). In one, α-melanotropin is accompanied by fragments of the corticotropin–endorphin prohormone, but in the other the α-melanotropin appears to be formed independently. These observations seem to point to the existence of more than one prohormone for α-melanotropin, which is an interesting possibility. Furthermore, fluorescence studies have indicated that in the hypothalamus every cell that contains β-endorphin also contains immunoreactive corticotropin (Watson *et al.*, 1978); these cells are clearly different from the cells that produce α-melanotropin independently of β-endorphin. Consequently, studies comparing the distribution of the acetylated endorphins with the distribution of α-melanotropin may have to take into account the existence of a new peptide which has α-melanotropin-like immunoreactivity but differs from α-melanotropin structurally.

An intriguing question of physiological significance is whether the prohormone processing mechanisms are sensitive to environmental stimuli. For example when neuronal transport is inhibited, do alterations occur in the nature and amounts of the peptides stored in the terminals? Similarly, to what extent is the pattern of β-endorphin related peptides influenced by factors such as age, gender and stress? It should be recognized that regulation of processing may take place at the level of the gene, or by the action of endogenous inhibitors of the processing enzymes, or by control mechanisms that function through the fusion of compartments or granules containing the enzymes with compartments containing the sequestered peptide substrates. Such control mechanisms are open to investigation.

In conclusion, the knowledge gained by studies on the distribution of the β-endorphin family of peptides in tissues defined by gross dissection provides a guide line for the complex processing mechanisms that exist in pituitary and brain. Future studies will need to concentrate on obtaining a knowledge of the identity of the peptides stored in individual cells in the pituitary and in individual nuclei in the brain. For this purpose more specific antibodies obtained by hybridization may prove valuable, in conjunction with experiments involving cloning of cells that synthesize endorphins. Certainly the availability of the range of β-endorphin-related peptides that occur in the tissues, together with the methodology that has been developed over the last few years for studying fmol quantities of the endogenous peptides, should assist in resolving the many questions and lead toward an understanding of the role of β-endorphin in brain function.

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