

## OPIOID PEPTIDES: ASPECTS OF THEIR ORIGIN, RELEASE AND METABOLISM

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### SUMMARY

At the present time there is evidence for two families of related peptides which act as ligands for opiate receptor sites. The endorphin group of peptides are derived from the ACTH/LPH precursor pro-opiocortin. The enkephalins appear to be formed from a separate precursor or precursors that have yet to be fully characterized. There appear to be a number of different types of opiate receptors and this may be related to the multiplicity of peptide ligands that have so far been identified. The enkephalins and related peptides appear to have a much wider distribution than the endorphins but the latter may act as circulating hormones unlike the enkephalins. It is likely that both endorphins and enkephalins are involved in sensory modulation processes and release of these peptides has been demonstrated during brain stimulation for pain relief. The enkephalins are very rapidly inactivated by tissue proteases, the aminopeptidases appear largely responsible for the inactivation of exogenously administered enkephalins but the dipeptidyl carboxypeptidase, termed enkephalinase, may have a special inactivating function at enkephalinergic synapses.

Evidence will be presented for the biosynthesis, the release and inactivation of the enkephalins relating to the above points.

### INTRODUCTION

The opioid peptides were discovered as the result of a systematic search for an endogenous substance with opiate receptor agonist activity (Hughes, 1975; Hughes *et al.* 1975a). As an historical note it may be mentioned that Hans Kosterlitz and myself embarked on our studies on the basis of the classical pharmacological evidence for a specific opiate receptor. It has been assumed that the discovery of specific opiate membrane-binding sites led directly to the search for the endogenous ligand. In fact these studies proceeded almost in parallel although Terenius directly applied his original findings on opiate receptor binding to the search for an endogenous ligand (Terenius & Wahlström, 1975). The largest impetus and encouragement for our work stemmed from the studies of Liebeskind and his colleagues on stimulation-produced analgesia (Akil, Mayer & Liebeskind, 1972). At that particular time attention was focussed on the analgesic effects of morphine and the possibility of existence of an endogenous analgesic factor. It was clear that, extrapolating from

the myriad effects of morphine, other physiological functions might be attributed to an endogenous opiate ligand; however, no one could have predicted how complex the endogenous opiate system would become. The discovery of not one but two enkephalins (Hughes *et al.* 1975*b*) was itself a surprise, but subsequent work has revealed the existence of numerous other opioid peptide ligands, and these discoveries have been made in parallel with the recognition of multiple opiate receptor types (Lord *et al.* 1977). It now appears that opioid peptides are widely distributed phylogenetically and that the opiate receptor may have evolved into a number of differentiated receptor types for a variety of peptide ligands. Nature has skilfully evolved a system that may modulate numerous biological events using the opioid peptides as possibly hormones, neurohormones or neurotransmitters.

I wish to consider some of the problems inherent in assigning the above physiological roles to the enkephalins and other opioid peptides and in particular those relating to the biosynthesis and release of these peptides.

#### CLASSIFICATION OF OPIOID RECEPTORS AND OPIOID LIGANDS

The classical definition of opiate receptor-mediated effect is the stereospecific reversal or antagonism of the agonist-induced activity by narcotic antagonists such as naloxone. It should be shown additionally that the effect is a direct one and not mediated by the release of an endogenous opioid peptide. The biological effects of the agonist should be correlated with specific receptor binding or the displacement of labelled ligands from the opiate receptor. This definition deliberately avoids invoking an analgesic response, and although this may be used as a biological test it is not an absolute requirement for an opioid peptide. It is not within the scope of this paper to discuss the evidence for multiple forms of the opiate receptor but a brief comment is necessary for an appreciation of the problems raised by the discovery of multiple ligands. During the purification of the enkephalins we noted that the  $K_e$  for naloxone when determined against pure enkephalin was twenty times lower than that against normorphine in the mouse vas deferens (Hughes *et al.* 1975*a*). These findings were confirmed with synthetic leucine- and methionine-enkephalin and extended in a series of studies (Lord *et al.* 1976; Terenius *et al.* 1976*a*; Lord *et al.* 1977; Chang & Cuatrecasas, 1979; Robson & Kosterlitz, 1979; Kosterlitz *et al.* 1980). These studies employing parallel bioassays and radioreceptor assays have clearly defined at least three receptor types (Table 1). None of the ligands is absolutely specific for one receptor type but preferences are clearly discernible in various binding or bioassays. At the present time the mu-receptor is defined as having a preference towards morphine, dihydromorphine and  $\beta$ -endorphin as agonists and naloxone as an antagonist. The delta-receptor has a greater affinity for the enkephalins as well as  $\beta$ -endorphin but a poor affinity for naloxone. The kappa-receptor is less well defined but apparently prefers compounds of the ketocyclazocine series and only has a weak affinity for naloxone. Specific receptor-blocking agents are not available for probing these different receptor types; naloxone does have a high affinity for the mu-receptor but it is not specific in that it will interact with the delta- and kappa-receptors. Kosterlitz and his colleagues have used  $^3\text{H}$ -dihydromorphine, ( $\text{D-Ala}^2\text{-D-Leu}^5$ )-enkephalin and  $^3\text{H}$ -ethylketocyclazocine as probes for the mu-,

Table 1. *Activities of various ligands on postulated opiate receptor types*

Receptor type	Enkephalins <sup>1</sup>	B-endorphin	Morphine	Etorphine	EtKCZ <sup>2</sup>	Naloxone	Mr 2266 <sup>3</sup>
mu	++	+++	+++	+++	+	+++	+++
delta	+++	+++	+	+++	+	+	+++
kappa	+	?	+	+++	+++	+	?

<sup>1</sup> Enkephalins include Met<sup>5</sup>-enkephalin, Leu<sup>5</sup>-enkephalin, and D-Ala<sup>2</sup>-D-Leu<sup>5</sup>-enkephalin.

<sup>2</sup> EtKCZ = ethylketocyclazocine.

<sup>3</sup> Mr 2266 = (-)-5, 9-diethyl-2 (3-furylmethyl)-2<sup>1</sup>-hydroxy-6, 7-benzomorphan.

+++ Strong affinity, ++ moderate, + weak.

Table 2. *Amino acid sequences of opioid peptides*

Y-G-G-F-M	Methionine-enkephalin
Y-G-G-F-L	Leucine-enkephalin
Y-G-G-F-M-R-F	Adrenal peptide
Y-G-G-F-M-T-S-E-K-S-Q-T-P-L-V-T-L-F-K-N-	$\beta$ -endorphin (LPH 61-91)
A-I-V-K-N-A-H-K-K-G-Q	
Y-G-G-F-L-R-R-I-R-P-K-L-K-?	Dynorphin (pig)
Y-G-G-F-L-R-K-R- (P, G, Y <sub>1</sub> , K <sub>1</sub> R <sub>1</sub> )	$\alpha$ -neo-endorphin (cow)
Y-G-G-F-M-K	Adrenal tryptic peptide (cow)

$\alpha$ -endorphin = LPH 61-76

$\gamma$ -endorphin = LPH 61-77

#### Single letter code

A, alanine	K, lysine	T, threonine
C, cysteine	L, leucine	V, valine
D, aspartic	M, methionine	W, tryptophan
E, glutamic	N, asparagine	Y, tyrosine
F, phenylalanine	P, proline	
G, glycine	Q, glutamine	
H, histidine	R, arginine	
I, isoleucine	S, serine	

delta- and kappa-receptor-binding sites, and compounds can be classified on the basis of their efficacies in these three assays.

At the present time opiate receptors in the central nervous system can only be classified by receptor binding techniques. However, it should be noted that Martin and his colleagues originally proposed the existence of multiple receptor types from neuropharmacological observations (Martin *et al.* 1976; Gilbert & Martin, 1976). Pert & Taylor (1979) have also proposed the existence of two types of opiate receptor based on the ability of GTP to suppress opiate binding (type-A receptor) or not to affect binding (type-B receptor). The type-A receptor may be analogous to the mu-receptor and appears to be mainly localized in the striatum, mid-brain and hypothalamus. The type-B receptor appears to be more concentrated in the amygdala and frontal cortex. These A- and B-receptors might represent, by analogy with dopamine receptors, pre- and post-synaptic opiate receptors (Pert, personal communication). One other interesting observation is that there appear to be specific receptors for  $\beta$ -endorphin in the rat vas deferens that show a very low affinity for morphine and the enkephalins (Lemaire *et al.* 1978; Schultz *et al.* 1979). However, there are no other clues as to possible receptor differences between the endogenous opioid peptides other than the greater affinity of  $\beta$ -endorphin for the mu-receptor site.


*New opioid peptides*

Recent discoveries have had a profound effect on our concepts regarding the endogenous opioid peptide systems, their origins and possible functions. Table 2 illustrates the number of opioid peptides that have now been characterized in pituitary, brain and adrenal chromaffin cells. Goldstein and his colleagues first discovered the presence of endogenous opioid peptides in the pituitary (Cox *et al.* 1975). This discovery was overshadowed by the subsequent finding that the potent opioid peptide  $\beta$ -endorphin (LPH 61-91) was a major constituent of the pars intermedia. However, Goldstein and his colleagues (1979) have now partially sequenced a peptide from commercial pituitary powder that contains the leu-enkephalin sequence with a basic carboxy-terminal extension. This peptide, named dynorphin, appears to be remarkably potent in the guinea-pig ileum bioassay, being some 700 times more active than leu-enkephalin but not in receptor binding assays. Another peptide related to leu-enkephalin has been isolated in small yield from porcine hypothalami (Kangawa, Matsuo & Igarashi, 1979). This peptide, termed  $\alpha$ -neo-endorphin, also has a basic carboxy-terminal extension and has a high potency in the guinea-pig ileum. Although neither the Californian nor the Japanese peptides have been fully sequenced it appears that they represent different molecular entities. It seems from these studies that, chemically speaking, there are at least two families of opioid peptides based on met-enkephalin and leu-enkephalin respectively. It also appears that carboxy-terminal extension can lead to greatly enhanced potency of both enkephalins. In all three cases  $\beta$ -endorphin, dynorphin and  $\alpha$ -neo-endorphin have a number of strongly basic side chains. Carboxy-terminal extension employing neutral amino acids does not give this enhanced potency. We will return later to the possible significance of these findings.

Opioid peptides including met- and leu-enkephalin were also discovered in several peripheral organs including the gastro-intestinal tract and autonomic nervous system (Hughes, Kosterlitz & Smith, 1977). Immunofluorescence studies also revealed considerable amounts of enkephalin-like immunoreactivity in the adrenal medulla (Schultzberg *et al.* 1978). Udenfriend and his colleagues (Stern *et al.* 1979; Kimura *et al.* 1980) have now isolated a number of opioid peptides from adrenal medullary granules. Some of these occur naturally and others are formed after tryptic hydrolysis of larger proteins in the 5-25K molecular weight range (Lewis *et al.* 1979). These findings have considerable significance for our understanding of the biosynthesis of the enkephalins.

It should be noted that there is still no doubt that the enkephalins are endogenous peptides and not simply generated by tissue autolysis or by tissue extraction techniques.

*Biosynthesis of opioid peptides*

It is now generally accepted that most biologically active peptides are derived from the post-translational processing of larger protein precursors. This processing involves proteolytic cleavage at selected points which may be determined by the conformation of the precursor, the degree of glycosylation (Loh & Gainer, 1978) and the existence of paired, basic amino acids adjacent to the biologically active sequence (Steiner *et al.* 1974; Steiner, 1976). The scheme outlined by Steiner (Fig )

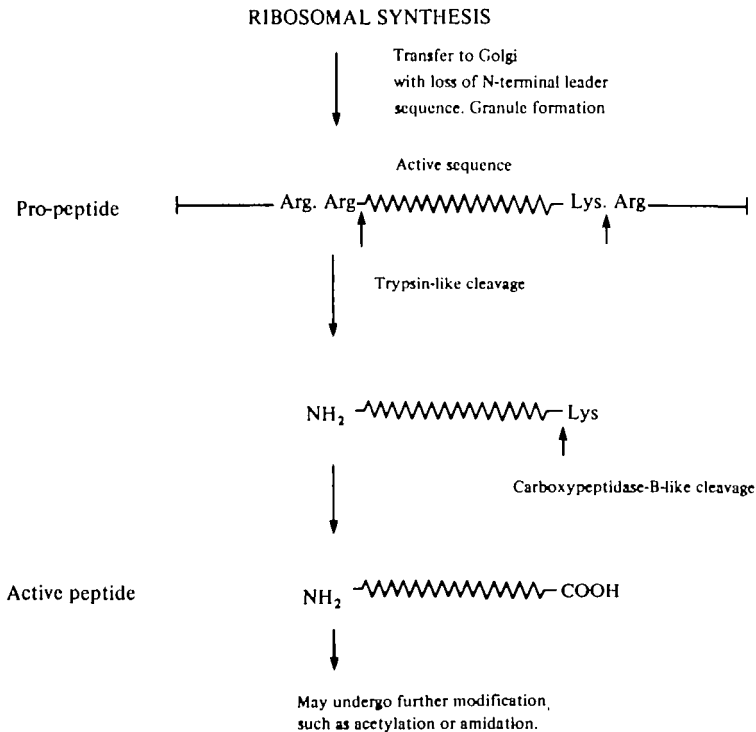


Fig. 1. Processing of precursor protein to active hormone according to Steiner (1976). The paired basic residues appear particularly sensitive to tryptic-like cleavage; in addition the specificity of the cleavage may be directed by the conformation of the pro-peptide and the degree of glycosylation. The second-stage carboxypeptidase-B-like cleavage is obviously redundant if the active sequence is C-terminal in relation to the pro-peptide.

involving tryptic-like and carboxypeptidase- $\beta$ -like cleavage is probably generally applicable for intracellularly processed peptides. Extracellularly derived peptides such as angiotensin are not derived in this way. There is now considerable evidence that the endogenous opioid peptides may be processed according to the scheme in Fig. 1. Although we originally noted the sequence homology between LPH 61-65 and methionine-enkephalin (Hughes *et al.* 1975*b*) which led to the discovery of the opiate receptor effects of  $\beta$ -endorphin (Li & Chung, 1976; Bradbury, Smyth & Snell, 1976; Li, Chung & Doneen, 1976) it is now clear that the enkephalins and  $\beta$ -endorphin have separate origins.

#### *Biosynthesis of $\beta$ -endorphin*

The biologically active peptides  $\beta$ -endorphin, corticotropin,  $\beta$ -MSH and  $\alpha$ -MSH are all formed from a common precursor of 28-41 K Daltons which has been termed pro-opiocortin (Nakanishi *et al.* 1976; Mains & Eipper, 1977; Mains, Eipper & Ling, 1977; Roberts & Herbert, 1977). The messenger RNA that codes for pro-opiocortin has been isolated from bovine pituitary intermediate lobe (Kita *et al.* 1979) and its nucleotide sequence determined from its base-pair cloned DNA insert (Nakanishi *et al.* 1979). This last achievement established the exact locations of

corticotropin, LPH and  $\beta$ -endorphin in the precursor protein. Inspection of the complete protein sequence reveals it to be composed of several repetitive units flanked by paired basic amino acids.

*In vitro* pulse chase studies with radiolabelled amino acids have confirmed the precursor product relationship between pro-opiocortin, corticotropin, MSH, LPH and  $\beta$ -endorphin (Pezalla *et al.* 1978; Seidah *et al.* 1978; Mains & Eipper, 1979; Loh, 1979). None of these studies could demonstrate the formation of methionine enkephalin from  $\beta$ -LPH. It is interesting that corticotropin is mainly stored and released from the corticotrophic cells of the anterior pituitary whereas  $\beta$ -endorphin and the melanophore-stimulating hormones are mainly concentrated in the intermediate lobe.

Evidence for differential processing of pro-opiocortin has been obtained by several groups (Loh, 1979; Zakarian & Smyth, 1979). Zakarian & Smyth isolated N-acetylated forms of  $\beta$ -endorphin and of LPH 61-87 and they have suggested that acetylation and selective proteolytic cleavage of LPH may vary with different pituitary and brain regions. Interestingly they found that LPH 61-87 is the major constituent of the rat intermediate pituitary lobe along with the acetylated forms of  $\beta$ -endorphin and LPH 61-87. The acetylated form of these opiate peptides is not active at the opiate receptor, and the significance of this finding requires further study.

At the present time there is no agreement as to the physiological role of the opioid peptides in the pituitary. They are released under conditions which release corticotropin, but the blood levels are not considered sufficient to affect sensory perception under these conditions (Guillemin *et al.* 1977). However, neurones with immunoreactivity to  $\beta$ -endorphin and LPH are present in brain (Bloom *et al.* 1978; Watson *et al.* 1977) and there is evidence for the release of  $\beta$ -endorphin during analgesia evoked by electrical stimulation of the peri-aqueductal grey matter as well as release of enkephalin-like material.

There is much to be learned about the endorphin system and its relationship to the other biologically active peptides derived from pro-opiocortin. It is tempting to assume that such a potent molecule present in such high amounts in the pituitary must have an important physiological role. This question will not be resolved in a simple fashion, thus administration of pharmacological doses of  $\beta$ -endorphin is unlikely to yield much information as to possible physiological effects. Much more precise measurements of the blood and CSF levels of  $\beta$ -endorphin and of LPH 61-87 are required. It will then be necessary to study the effects of these opioid peptides in the physiological rather than the pharmacological dose range. It is patently absurd to draw conclusions from studies which have employed doses of  $\beta$ -endorphin that represent more than the total amount of this peptide found in the whole pituitary gland and more than ten times that found in the brain.

### *Biosynthesis of enkephalins*

Initial studies in our laboratories provided support for the concept that the enkephalins were derived from the processing of ribosomally synthesized proteins. Thus in both isolated slices of striatum and in the guinea-pig myenteric plexus (Sosa *et al.* 1977; Hughes, Kosterlitz & McKnight, 1978; McKnight, Hughes &

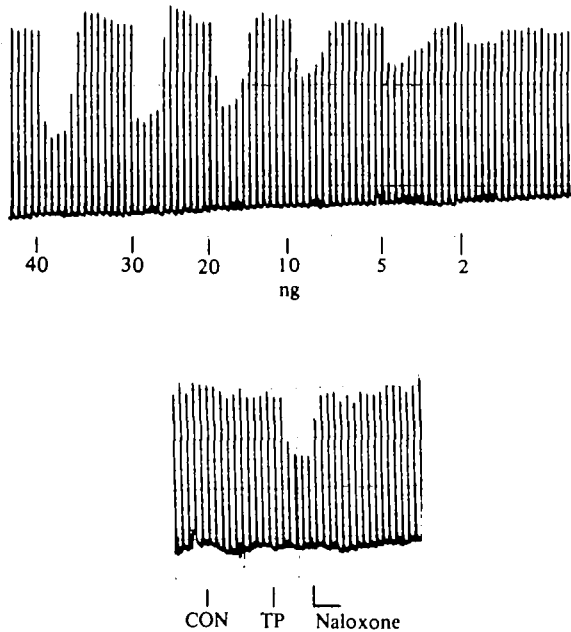


Fig. 2. Bioassay of opioid peptides on mouse vas deferens. The upper panel shows dose responses evoked by graded amounts of leucine-enkephalin (40–2 ng) injected into the 1 ml-organ bath. The lower panel shows the inhibition evoked by a tryptic digest of guinea-pig striatal protein. The protein was obtained by homogenizing the tissue in 5 ml/g of 1 M acetic acid (adjusted to pH 1.9 with HCl), the supernatant was applied to a  $100 \times 2.5$  cm column of Sephadex G-100 and each 5 ml fraction emerging from the column was dried and incubated for 2 h with trypsin (10  $\mu$ g/ml in 50 mM Tris-HCl pH 8.4 + 1 mM  $\text{CaCl}_2$ ). This particular fraction emerged with an apparent molecular weight of 70 K. Note that the inhibition evoked by the tryptic peptide (TP) was completely reversed by naloxone, and this constitutes the test for opioid activity. A control sample (CON) in which the trypsin was boiled before incubation had no opioid activity. The vas deferens was stimulated electrically with double square-wave pulses (0.4 ms and 40 V) every 10 s.

Kosterlitz, 1979) we demonstrated that labelled amino acids are incorporated into the enkephalins and that this incorporation could be blocked by cycloheximide or puromycin. Further, the time course of incorporation indicated a lag period of 1–2 h, which was consistent with a process involving ribosomal synthesis followed by proteolytic cleavage to the final products. It was also noted that the protein synthesis inhibitors were only effective when given during or prior to the lag phase.

The evidence supporting a precursor relationship between pro-opiocortin, lipotropin and  $\beta$ -endorphin does not apply to methionine- or leucine-enkephalin. Thus there is immunochemical and immunohistochemical evidence that the enkephalins and the endorphins are present in separate neuronal systems (Rossier *et al.* 1977; Bloom *et al.* 1978; Watson *et al.* 1978). In particular there are areas such as the striatum and the substantia gelatinosa of the spinal cord which are rich in enkephalins but do not apparently contain significant amounts of pro-opiocortin, LPH or  $\beta$ -endorphin. In the case of leucine-enkephalin there is direct chemical evidence in

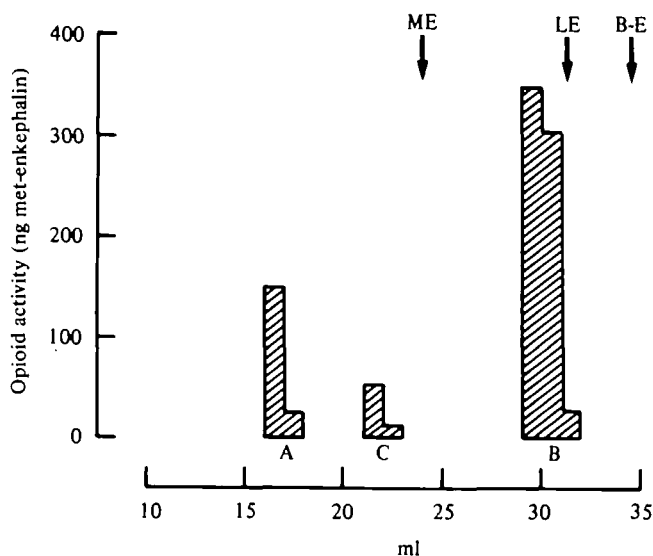


Fig. 3. High-pressure liquid chromatographic analysis of opioid peptides generated by trypsin. Guinea-pig striata were homogenized in 1 M acetic acid (see legend to Fig. 5) and the supernatant chromatographed on a 100 × 2.5 cm Sephadex G-100 column in 1 M acetic acid at 4 °C. Individual fractions were freeze-dried and incubated with trypsin (10 µg/ml) for 2 h. Opioid peptide activity (assayed on mouse vas deferens) was generated from protein peaks corresponding to  $\geq 70$  K, 36 K and 16 K molecular weights. The opioid peptides were injected on to a Waters Bondapak-C18 column (0.78 × 30 cm) and eluted with a concave gradient from 10:90 (vol:vol) to 40:60 propan-1-ol:5% acetic acid at 1 ml/min. The graph shows the elution positions of the opioid peptides generated from the 70 K protein; similar patterns were obtained with tryptic digests of the 36 K and 16 K proteins. The elution positions of these peptides clearly differ from that of known opioid peptides indicated at the top of the graph; ME = met-enkephalin, LE = leu-enkephalin, BE =  $\beta$ -endorphin. Other fragments of  $\beta$ -endorphin elute between LE and BE.

that not only has it proved impossible to demonstrate a leucine 65 analogue of LPH but, as mentioned above, larger carboxy-terminal extended forms of this peptide have been isolated. It remains to be seen whether  $\alpha$ -neo-endorphin and dynorphin have a physiological secretory role, but the presence of the basic lysine and arginine molecules indicates that they could act as precursors to leucine-enkephalin in accord with Steiner's scheme. However, the most direct evidence for the separate precursor concept has come from studies on the putative precursors themselves.

Lewis *et al.* (1978) first obtained evidence that larger molecular weight forms of the enkephalins existed in bovine striata. These proteins could be distinguished from pro-opiocortin chromatographically, and most importantly could be shown to be inactive as opiate ligands until treated with trypsin. Furthermore, the active products of the tryptic digest could be distinguished from tryptic fragments of  $\beta$ -endorphin such as LPH 61-69.

We obtained similar results with protein extracts from guinea-pig, rat and mouse striatum (Beaumont *et al.* 1979). When these tissues are homogenized and the supernatant chromatographed on a Sephadex G-100 column several peaks of activity can be detected when the effluent is freeze-dried, incubated with trypsin and then bioassayed on the mouse vas deferens (Fig. 2). No such activity can be detected when



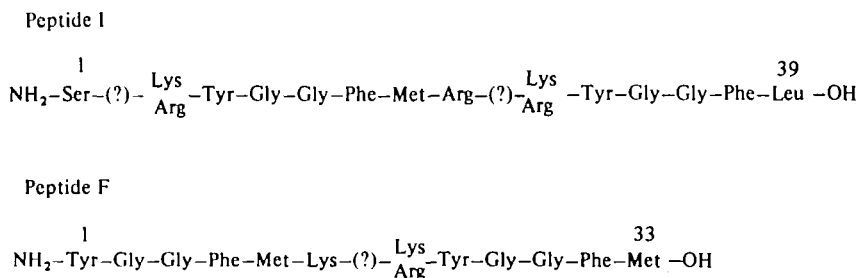


Fig. 4. Partial sequence of peptides isolated from adrenal chromaffin granules (Kimura *et al.* 1980).

the boiled enzyme is used as a control. Initial experiments indicated that the apparent molecular weights of these proteins were  $\sim 70000$ ,  $36000$  and  $16000$ . Electrophoretic analysis using SDS-gel slabs indicates that the high molecular weight component is not a polymer of the lower molecular weight forms, but it may contain several proteins of molecular weight  $\geq 80000$ .

Chromatographic analysis of our tryptic digests shows several important points. First, each separate protein peak described above yields a similar pattern of opioid peptides after tryptic digestion as adjudged by HPLC analysis (Fig. 3). Secondly, these opioid peptides are demonstrably different in their chromatographic behaviour in several HPLC and TLC systems from either the enkephalins or LPH 61–9. The separation of these tryptic peptides from LPH 61–69 is crucial since the latter peptide is the major opioid product resulting from the sequential tryptic hydrolysis of LPH under our conditions. These results and those of Stern *et al.* (1979) provide strong evidence for the separate nature of the enkephalin and endorphin precursors. Further evidence has now been obtained by Udenfriend's laboratory and by our group that the proteins in adrenal medulla and in brain are indeed enkephalin precursors.

**Adrenal medulla.** The identification of the heptapeptide tyr-gly-gly-phe-met-arg-phe (Stern *et al.* 1979) was the first indication of the existence of methionine-enkephalin sequences distinct from LPH. Lewis *et al.* (1979) had previously described the existence of peptides in the adrenal medulla that yielded opiate receptor activity after tryptic digestion. There also appears to be little doubt that the enkephalins are both stored and released from the adrenal medulla (Schultzberg *et al.* 1978; Yang *et al.* 1979, 1980; Viveros *et al.* 1979; Kumakura *et al.* 1980). Kimura *et al.* (1980) have now purified a number of peptides contained in adrenal chromaffin granules that yield opioid peptides on trypsin treatment or possess intrinsic opiate activities. Two of these peptides have been partially sequenced (Fig. 4). These peptides designated F and I have molecular weights of  $3800$  and  $4700$  respectively and appear to contain more than one enkephalin sequence, thus peptide F yielded both free enkephalin and lys<sub>6</sub>-Met<sub>6</sub>-enkephalin on trypsin digestion whilst peptide I yielded free leucine-enkephalin and Arg<sub>6</sub>-Met<sub>6</sub>-enkephalin after trypsin treatment.

**Brain.** We have yet to complete a sequence analysis of the tryptic peptides arising from the striatal protein extracts. However, molecular weight estimations indicate that our two major tryptic peptides (A and B) are fractionally larger than that of enkephalin. Further, treatment of these two peptides with cyanogen bromide causes

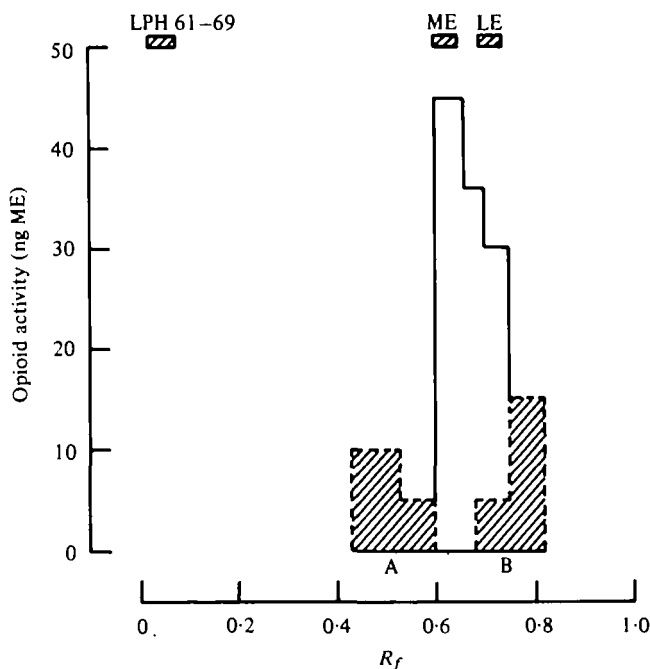


Fig. 5. Thin-layer chromatography of opioid peptides derived from pro-enkephalin. The protein fraction (molecular weight  $> 5000$ ) from 0.2 g of guinea-pig striatum was obtained by homogenizing fresh tissue in 1 ml of 1 M acetic acid (adjusted to pH 1.9 with HCl) at 4 °C; after centrifugation the supernatant was passed through a Sephadex G-25 column and the void volume was collected and lyophilized. This fraction did not contain any intrinsic opiate activity. The protein was incubated with trypsin (10  $\mu$ g/ml in 50 ml Tris-HCl pH 8.4 + 1 mM-CaCl<sub>2</sub>) at 37 °C for 2 h. After boiling for 10 min one half of the fraction was further incubated with carboxypeptidase-B for 2 h. The peptide products of both the trypsin and the trypsin + carboxypeptidase incubations were absorbed on 100 mg of Poropak-Q, and after a water wash the absorbed products were eluted with 8 ml of ethanol. The ethanol eluates were dried, redissolved in 50  $\mu$ l of methanol and spotted on silica gel plates which were developed with ethyl acetate/pyridine/water/acetic acid (50/20/11/6). Opioid activity was assayed on the mouse vas deferens after drying the plate and eluting 5 mm bands with ethanol.

Trypsin digestion alone (shaded columns) gave two areas of activity only slightly overlapping the enkephalin marker positions. Incubation with trypsin and then carboxypeptidase-B (open columns) yielded considerably more activity, that largely co-migrated to the enkephalin marker positions. Thus the tryptic peptides are distinct from LPH 61-69 and the enkephalins but are converted to enkephalin-like peptides by carboxypeptidase-B. Recovery of peptides in these experiments was only 20-25 %; the total amount of enkephalin-like activity generated was estimated at approximately 5  $\mu$ g/g tissue.

a greater than 80% loss of the biological activity of peptide A, with no loss of activity with peptide B. This suggests the presence of methionine in peptide A. Initial experiments indicated that one of our tryptic peptides eluted from the reverse phase HPLC column in the same position as the lysine-extended form of leucine-enkephalin. Confirmation of this was obtained by incubating the tryptic peptides with carboxypeptidase-B. This had two effects: first the opiate activity was increased between 5- and 10-fold, and secondly the products of this digestion had identical  $R_f$  values on TLC and elution times on HPLC as authentic methionine- and leucine-enkephalin (Fig. 5). Our results therefore indicate that the products of the tryptic digest are (Arg<sub>6</sub> or Lys<sub>6</sub>)-Leu<sub>5</sub>-enkephalin (Peak B) and (Arg<sub>6</sub> or Lys<sub>6</sub>)-Met<sub>5</sub>-enkephalin.

*The significance of enkephalin in precursors*

There seems little doubt from the foregoing section that the enkephalins and  $\beta$ -endorphin have separate biosynthetic pathways. It may well prove that separate genes for the enkephalins and endorphins evolved from a common ancestor. In this respect it is worth noting that separate leu-enkephalin and  $\beta$ -endorphin immunoreactive cell bodies have been localized in the nervous system of *Lumbricus terrestris* (Alumets *et al.* 1979). Comparative studies of this type may yield useful information as to the evolution of the opioid peptide systems which seem to be of a much more primitive nature than hitherto realized.

The greatest significance of studies on the enkephalin precursors (pro-enkephalins) relates to the information that can be derived from these studies on the physiological roles of the enkephalins and other associated peptides. If the enkephalins are of physiological significance either as neurotransmitter or neurohormones then changes in enkephalin nerve activity should be reflected in changes in the turnover of these peptides. Changes in brain enkephalin content have been noted during stress (Madden *et al.* 1977; Rossier *et al.* 1978) and neuroleptic drug treatment (Hong *et al.* 1978). However, a true measurement of turnover requires accurate estimates of product and precursor pool sizes and a knowledge of the relationships governing precursor processing, enkephalin release and metabolism. This goal has yet to be achieved, but the identification of the pro-enkephalins brings us nearer to that goal. At the present time it appears that pro-enkephalin is processed by tryptic-like and carboxypeptidase-B-like enzymes. The brain precursor(s) are of a high molecular weight, but at present we do not know whether leu-enkephalin and met-enkephalin are derived from the same molecule, although our data tend to support this view. It is also uncertain what the relationship is between the brain precursors and those found in the adrenal gland. The latter proteins are of much smaller size, 25K compared with 100K in the brain, but the large brain precursor may be cleaved to form intermediate molecules of 38K and 16K.

According to the work of Udenfriend's group cited above it is also likely that there are multiple copies of the enkephalin sequences within the same precursor molecule which contains the repetitive core MSH sequence (his-phe-arg-trp). If there are repetitive met-enkephalin and leu-enkephalin peptide sequences within a single molecule then it is possible that differential processing in different brain areas could lead to varying ratios of the enkephalins as final products and also possibly to the production of other opioid peptides based on the enkephalin sequence. Thus differential processing could explain why one finds equimolar amounts of the enkephalins in the cerebral cortex but eight times more met-enkephalin than leu-enkephalin in the hypothalamus (Hughes *et al.* 1977). Alternatively, these varying ratios could reflect the existence of multiple precursors containing variable ratios of the enkephalins, but this seems unlikely.

Incorporation of labelled amino acids into the enkephalins tends to be rather slow and inconsistent *in vivo* (Sosa *et al.* 1977; Yang & Costa, 1979). This probably does not reflect a slow rate of synthesis or turnover but rather the existence of large stores of pro-enkephalin. Our studies indicate that in every brain region the total amount of pro-enkephalin is some five to ten times greater than that of the enkephalins. Thus in the mouse striatum we find endogenous enkephalin levels of 800 ng/g

tissue whilst the stores of pro-enkephalin amount to some 10  $\mu\text{g/g}$  tissue. Initial studies also indicate that the ratio of pro-met-enkephalin to pro-leu-enkephalin may be considerably lower (2:1) than the ratio of the final products (5:1). This may possibly indicate a higher rate of turnover of leu-enkephalin compared to met-enkephalin, and preliminary pulse-labelling studies support this view. These points are being further investigated, since these results have a direct bearing on the respective roles of these two very similar peptides.

Identification and sequence analysis of pro-enkephalin should also help to resolve the question of whether opioid peptides other than enkephalins, which are known to be present in the brain (Beaumont & Hughes, 1979), are biosynthetic intermediates, trace products of cleavage or stored active products with a physiological function. It may be argued that biosynthetic intermediates should not be biologically active, particularly since they may be released from cells along with the final products. This argument is difficult to resist and has particular relevance to molecules such as  $\alpha$ -neo-enkephalin and dynorphin. This problem is only likely to be finally settled when we possess the full chemical structure of these peptides and their relationship to pro-enkephalin.

#### *Release of enkephalins*

The release of a biologically active compound from nerve terminals constitutes strong evidence for a physiological role for that compound if release occurs under physiological conditions of nerve stimulation and can be shown to be a calcium-dependent process. It is also necessary to show that the compound is not a metabolite formed after release from the nerve ending. Studies on the release of the enkephalins have shown that these criteria are at least partly fulfilled both with *in vitro* and *in vivo* studies. Thus the enkephalins can be released from brain synaptosome preparations or from brain slices by depolarizing stimuli such as veratridine or potassium (Henderson, Hughes & Kosterlitz, 1978; Iversen *et al.* 1978; Osborne, Holtt & Hertz, 1978). We showed that both met-enkephalin and leu-enkephalin are released from the striatum in the same ratio that they are present in the tissue stores (Henderson *et al.* 1978). Bayon *et al.* (1978) also showed that both enkephalins were released by potassium from slices of rat pallidus. However, they concluded that met-enkephalin was more rapidly degraded than leu-enkephalin and that the ratio of ME/LE was greater in the perfusate than in the tissue stores.

Opioid peptides also appear to be released into the spinal cerebrospinal fluid during electrical stimulation of the periventricular medial thalamic region in human subjects (Akil *et al.* 1978a). We have recently extended these observations by HPLC analysis of the opioid peptides found in such samples. In two patients we were able to show that both met-enkephalin and leu-enkephalin are present, together with other as yet unidentified opioid peptides not related to  $\beta$ -endorphin. However, in a separate study it has been shown that  $\beta$ -endorphin-like immunoreactivity increases in ventricular CSF during analgesic electrical stimulation (Akil *et al.* 1978b; Hosobuchi *et al.* 1979). These studies indicate that a complex mixture of opioid peptides may be released in response to brain stimulation, and further studies are required

elucidate the origin and nature of these peptides. In addition to the above findings Terenius *et al.* (1976b) and Terenius (1978) have investigated the relationship of opioids found in the CSF to various pathological states and to electro-acupuncture. His findings also indicate an increase in endogenous opioid levels during electro-acupuncture, but these opioids have yet to be characterized, although they appear to be distinct from the enkephalins and  $\beta$ -endorphin.

The release of enkephalin-like material has also been observed from the perfused dog adrenal gland (Viveros *et al.* 1979). It appears that enkephalins are stored and secreted from the chromaffin vesicles that store and release the catecholamines. Thus stimuli which lead to release of the catecholamines result in a parallel release of enkephalins. The enkephalins or related peptides are also present in the cholinergic nerves innervating the adreno-medullary cells (Hokfelt *et al.* 1980) and evidence has been presented for an enkephalinergic inhibitory modulation of nicotinic receptors in chromaffin cells (Kumakura *et al.* 1980). It appears that the enkephalins may have multiple roles at these peripheral sites; release from the splanchnic nerves may modulate cholinergic activation of chromaffin cell secretion, whilst the subsequent release of enkephalins from the chromaffin cells may further modulate catecholamine secretion. There is also the possibility of an extra-adrenal role for the circulatory enkephalins and enkephalin-like peptides that are secreted from the adrenals.

No discussion of enkephalin release could be complete without reference to the metabolism and inactivation of these peptides. Initial studies indicated that the major route of enkephalin inactivation involved removal of the N-terminal tyrosine by various soluble and membrane-bound aminopeptidases in brain and other tissues (Hambrook *et al.* 1976). Subsequent work indicated that rat brain synaptic membranes also contain a dipeptidyl carboxypeptidase which hydrolyses the 3- to 4-peptide bond in enkephalin (Malfroy *et al.* 1978). This enzyme, termed enkephalinase, may be of physiological significance for several reasons. First, it has been shown that the tyr-gly-gly metabolite of  $^3\text{H}$  leu-enkephalin is generated after intraventricular injection of the parent peptides (Craves *et al.* 1978). Secondly, we have noted that certain dipeptides such as tyr-tyr, phe-leu and leu-leu can prevent the inactivation of enkephalin released by depolarization of striatal slices, and that dipeptides seem to act by inhibiting a dipeptidyl carboxypeptidase (Malfroy & Hughes, in preparation). In other preliminary experiments we have also found evidence that tyr-gly-gly is formed from endogenously released enkephalin.

Gorenstein & Snyder (1979) have recently reported the solubilization and partial purification of three brain membrane-bound enkephalin-hydrolysing enzymes. One appears to be an aminopeptidase which releases free tyrosine, another corresponds to the enzyme described by Malfroy *et al.* (1978), whilst the third appears to be a dipeptidyl-aminopeptidase which releases the tyr-gly fragment from enkephalin. At present it is difficult to reach any conclusions about the relative role of these membrane-bound enzymes in the physiological disposition of the enkephalins. It is not unrealistic to suppose that the rapid inactivation of neuronally released enkephalin may occur through the action of such enzymes. However, it remains to be seen whether these enzymes are specific for the enkephalins in terms of substrate specificity and in their distribution relative to that of enkephalinergic neurones.

## CONCLUDING REMARKS

The endogenous opioid peptides appear to be a family of expanding peptides which are still far from being chemically and biologically classified. At present we can conclude that the lipotropin-related peptides and the enkephalins form separate systems in terms of their distribution and origin, but they may nevertheless have overlapping functions. Although there is now strong evidence that the enkephalins may act as neurotransmitters or neurohormones in that they are released from chromaffin cells and from neurones by a calcium-dependent process, it is also possible that their release is accompanied by other opioid peptides. All the opioid peptides that have been fully or partially characterized have similar effects, although there are differences in potency depending on what test system is employed. The absence of really specific opiate receptor-blocking agents is proving a handicap in assessing the roles of these peptides.

The opioid peptides, like other peptide families, all share a common amino acid sequence which is essential for receptor recognition. It is likely therefore that all opiate receptors contain a common site recognizing the sequence tyr-gly-gly-phe-, and that variations in receptor specificity reflect the absence or presence of subsidiary binding sites.

It also appears that at some receptor sites the presence of basic side chains in the opioid ligand can lead to enhanced activity as with dynorphin or  $\alpha$ -neo-endorphin. Since the enkephalins appear to be formed from precursors containing one or more paired basic residues at critical cleavage points the question arises as to whether peptides such as dynorphin arose from a specific modification of the enkephalin-processing mechanism or vice versa. Certainly molecular adaptation allowing the formation of multiple ligands from the same precursor opens out many intriguing possibilities. These questions will only be answered when we have a full knowledge of the biosynthetic mechanisms leading to the formation of the opioid peptides.

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