# INCORPORATION OF LABELLED AMINO ACIDS INTO THE ENKEPHALINS

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## 1. Introduction

We have suggested that met$^5$-enkephalin and leu$^5$-enkephalin [1] may act as neurotransmitters in both the central and peripheral nervous systems [2,3]. The aim of this study was to develop a procedure for studying the biogenesis of the enkephalins and to test the hypothesis that these peptides are derived from larger precursors via ribosomal synthesis and subsequent proteolytic cleavage of the precursor protein.

## 2. Materials and methods

### 2.1. *Biological preparations*

For in vivo studies male, albino rats (300–350 g) were anaesthetised with ether and 100 μCi [$^3$H]tyrosine was injected intracisternally in 50 μl 0.9% sterile NaCl solution over a period of 5 min. The animals were allowed to recover consciousness and were killed at various times after the injection.

For in vitro studies 300–500 mg isolated myenteric plexus-longitudinal muscle preparation of the guinea-pig [4] was suspended in a 3 ml organ bath. This tissue was bathed in Krebs solution at 37°C and gassed with 95% $O_2$ and 5% $CO_2$. The Krebs Ringer bicarbonate solution contained 1 μg/ml each of the following L-amino acids: Ala, Arg, Asp, Cys, Glu, Gly, His, Leu, Ileu, Lys, Met, Phe, Pro, Ser, Thr, Trp and Tyr. The corresponding unlabelled amino acid was omitted when [$^3$H]Tyr, [$^3$H]Leu or [$^{35}$S]Met were added to the bathing solution. Two methods of labelling were used:

(A) Incubation with 10 μCi labelled amino acid for 1 h followed by incubation with cold amino acids for up to 12 h,
(B) Incubation with 10 μCi labelled amino acid for 4 h with fresh solution every hour followed by 3 h incubation with cold amino acids.

### 2.2. *Isolation of enkephalins*

Tissues were homogenised in ice-cold 0.1 M HCl containing 25 μg each of the enkephalins. The enkephalins were recovered from the tissue supernatant by chromatography on XAD-2 columns [2,3]. The dried eluate from the XAD-2 column was dissolved in 0.1 ml 0.2 M acetic acid and chromatographed (fig.1) on a 45 × 0.4 cm cation exchange column of HC-

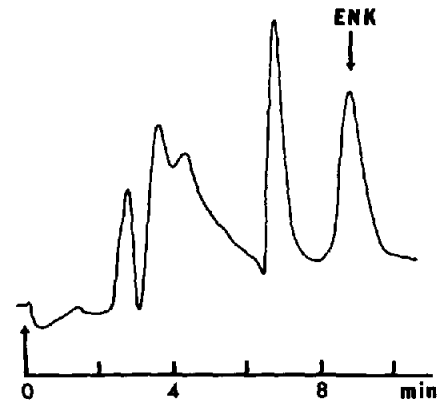


Fig.1. Chromatography of myenteric plexus extract on HC-Pellionex-SCX (45 × 0.4 cm). The column was equilibrated with 0.2 M acetic acid containing 30% (v/v) methanol. After injection of the tissue extract the solvent was changed immediately to 0.35 M ammonium acetate containing 30% methanol (v/v) at pH 8.35. Column flow rate = 0.8 ml/min, ultraviolet absorption at 280 nm, 1 cm light path with 8 μl flow cell and 0.16 AU full scale deflection.

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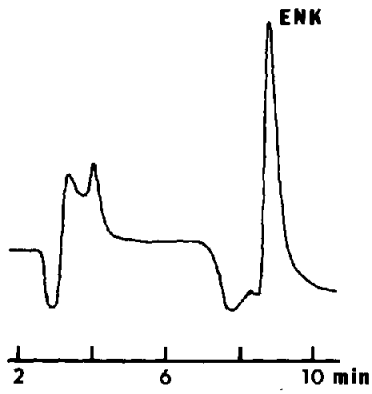


Fig.2. Chromatography of enkephalins on AE-Pellionex-SAX (100 × 0.4 cm). The column was equilibrated with 0.08 M ammonium acetate, pH 9.5 and then the enkephalin fraction from the HC-Pellionex-SCX was injected. After 1 min the eluent was changed to 0.04 M acetic acid containing 30% methanol (v/v). Column flow rate = 1 ml/min, ultraviolet absorption at 280 nm as in fig.1.

Pellionex-SCX (Whatman). The enkephalin peak was collected and further chromatographed on a 100 × 0.4 cm anion exchange column of AE-Pellionex-SAX (Whatman) as shown in fig.2. The enkephalin fraction was lyophylised, redissolved in 20 $\mu$l MeOH:$H_2O$ (80:20 v/v) and spotted on a silica gel plate (Merck 5721). The plate was developed for 10–12 cm with ethyl acetate/pyridine/water/acetic acid (100:42:25:11). The enkephalins were completely resolved in this system (fig.3). The appropriate spots were usually located for elution and liquid scintillation counting by spraying the whole plate with ninhydrin–cadmium acetate reagent; however, a marker lane was used to locate the peptides for elution when this procedure was followed by electrophoresis. The overall recovery of met- or leu-enkephalin at the thin-layer chromatographic (t.l.c.) stage was 10%.

Electrophoresis at pH 2 was carried out on 20 cm lengths of silica gel impregnated glass fibre sheets (Gelman Instrument Co.). A constant current of 30 mA (450–500 V) was applied for 1.5 h.

### 2.3. *Isotopes*

L-[2,3,5,6-$^3$H]Tyrosine (80 Ci/mmol), L-[4,5-$^3$H]-leucine (20 Ci/mmol) and L-[$^{35}$S]methionine (800–1200 Ci/mmol) were purchased from the Radiochemical Centre, Amersham.

## 3. Results and discussion

### 3.1. *Radiochemical purity*

Individual radioactive bands corresponding to met$^5$-enkephalin and leu$^5$-enkephalin were observed on t.l.c. of brain and myenteric plexus extracts after labelling with [$^3$H]tyrosine (fig.3). Incubation with

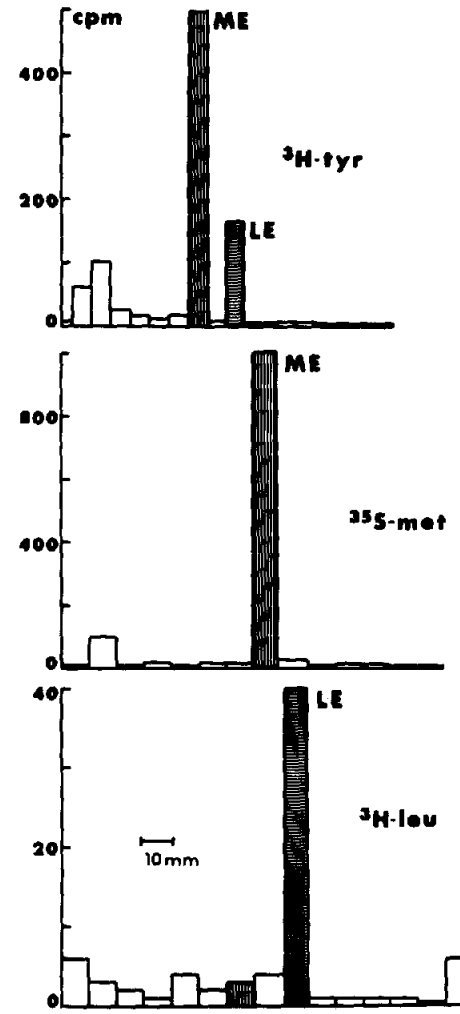


Fig.3. Thin-layer chromatographic separation of enkephalins. The peptides were located by ninhydrin, 5 mm or 7 mm bands of the plate were eluted with 1 ml of methanol/water (1:1, v/v) and the radioactivity determined in a liquid scintillation counter. The patterns of activity were obtained with tissues incubated with [$^{35}$S]methionine, [$^3$H]tyrosine and [$^3$H]leucine for 4 h. The actual cpm less background counts (12 cpm) are shown.

[$^3$H]leucine resulted in the appearance of radioactivity only in the leu$^5$-enkephalin position whilst incubation with [$^{35}$S]methionine selectively labelled the material corresponding to met$^5$-enkephalin (fig.3).

The peptides isolated by t.l.c. were adjudged radiochemically homogenous since electrophoresis showed that >95% of the radioactivity migrated as one band corresponding to the enkephalins. Further, there was no change in the specific activity of the t.l.c. peptides when subjected to electrophoresis.

### 3.2. *Rate of incorporation into myenteric plexus*

Incorporation of [$^3$H]tyrosine into the two enkephalins showed two distinct phases (fig.4). There was an initial lag phase of 1–2 h after which incorporation followed a linear time course for up to 12 h. The rate of incorporation into met$^5$-enkephalin was much faster than that for leu$^5$-enkephalin and this may correlate with our observation [2,3] that there is a three-times greater concentration of met$^5$-enkephalin in this tissue than leu$^5$-enkephalin.

### 3.3. *Effect of inhibiting protein synthesis*

Puromycin (0.1 mM) and cycloheximide (0.1 mM) caused a significant inhibition of [$^3$H]tyrosine incorporation into tissue protein and the enkephalins (table 1). These effects did not appear to be of a

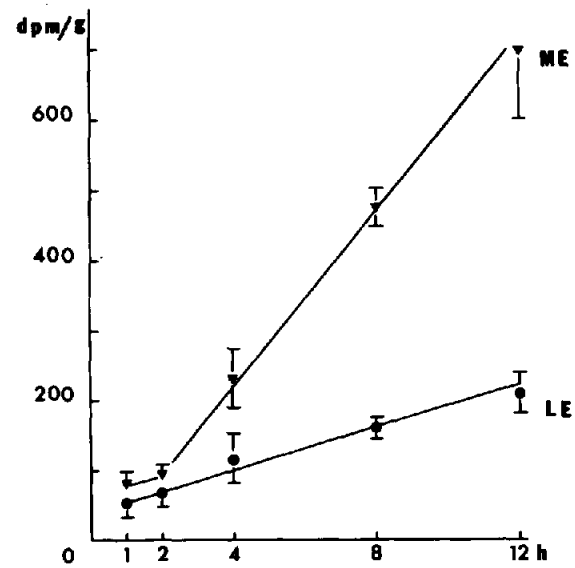


Fig.4. Time course of [$^3$H]tyrosine incorporation into enkephalins of the myenteric plexus. The tissues were labelled by incubation with 10 μCi tyrosine for 1 h followed by incubation with cold amino acids for up to 12 h (method A). Linear regression analysis was carried out on the 2–12 h points, this gave correlation coefficients of 0.999 and 0.987 and slopes of 60.3 and 12.8 for met$^5$-enkephalin and leu$^5$-enkephalin, respectively. No correction has been made for the recovery of the peptides which averaged 10%.

Table 1
Effect of puromycin and cycloheximide on [$^3$H]tyrosine incorporation into the myenteric plexus

| | | [$^3$H]Tyrosine incorporated into: | | |
|---|---|---|---|---|
| | | Met$^5$-enkephalin (dpm.g$^{-1}$) | Leu$^5$-enkephalin (dpm.g$^{-1}$) | Protein (dpm × 10$^{-6}$).g$^{-1}$ |
| (A) | Control | 3752 ± 240 | 1424 ± 64 | 24.6 ± 2.4 |
| | Puromycin | 312 ± 56 | 296 ± 128 | 1.5 ± 0.3 |
| (B) | Control | 2888 ± 312 | 1436 ± 192 | 20.2 ± 2.7 |
| | Cycloheximide | 544 ± 160 | 197 ± 72 | 2.1 ± 0.1 |
| (C) | Control | 1360 ± 188 | 740 ± 132 | 18.7 ± 1.4 |
| | Puromycin | 1312 ± 160 | 560 ± 48 | 14.8 ± 1.3 |

Paired experiments with 3 tissues/group. In (A) and (B) the tissues were incubated for 4 h with a total of 80 μCi [$^3$H]tyrosine followed by 2 h without label; puromycin and cycloheximide (0.1 mM each) were present throughout the expt. In (C) the tissues were incubated for 2 h with a total of 40 μCi of [$^3$H]tyrosine and then for a further 4 h in the presence of puromycin (0.1 mM) or just with cold amino acids (control). The incorporation of [$^3$H]tyrosine into protein was determined after 5% trichloroacetic acid precipitation of the original tissue pellet

Table 2
[$^3$H]Tyrosine incorporation into rat brain enkephalins

| Hours after injection | (*n*) | Met$^5$-enkephalin (dpm.g$^{-1}$) | Leu$^5$-enkephalin (dpm.g$^{-1}$) | Protein (dpm × 10$^{-6}$).g$^{-1}$ |
|---|---|---|---|---|
| 1 | (2) | 54 | 12 | 3.45 |
| 2 | (5) | 53 ± 18 | 13 ± 3.5 | 3.90 ± 0.47 |
| 4 | (7) | 57 ± 13 | 22 ± 5 | 1.73 ± 0.65 |
| 8 | (6) | 49 ± 10 | 18 ± 5 | 3.26 ± 0.33 |
| 16 | (3) | 55 ± 16 | 15 ± 5 | 3.14 ± 0.42 |
| 32 | (3) | 38 ± 16 | 13 ± 5 | 1.72 ± 0.39 |
| 48 | (4) | 45 ± 8 | 20 ± 2 | 2.95 ± 0.37 |
| 96 | (3) | 28 ± 1 | 13 ± 2 | 2.03 ± 0.10 |

Rats were injected intracisternally with 50 μCi [$^3$H]tyrosine as in Materials and methods. The incorporation into protein was determined after 5% trichloroacetic acid precipitation. The incorporation into enkephalin is not corrected for recovery (mean = 10%). *n* = number of experiments. Means and standard errors are shown for each time point except 1 h where only the mean is shown

general neurotoxic nature since addition of puromycin after incubation with [$^3$H]tyrosine did not affect the subsequent appearance of labelled enkephalins (table 2) and nerve-induced contractions of the myenteric plexus were unaffected by these concentrations of cyclohexamide and puromycin.

The isolated myenteric plexus preparation is likely to prove an excellent system for studying enkephalinergic mechanisms. Our results are compatible with the hypothesis that the enkephalins are derived from ribosomally synthesized protein precursors. We also conclude that stores of both met$^5$- and leu$^5$-enkephalin in the myenteric plexus are produced locally and are not derived from pituitary or brain endorphins.

### 3.4. *Incorporation into rat brain enkephalins*

Preliminary experiments with intracisternally administered [$^3$H]tyrosine showed incorporation of labelled tyrosine into both enkephalins (table 2). The degree of labelling was small and rather variable; however, the time course was consistent with the labelling of a precursor store that turns over rather slowly. Attempts are now being made to refine this approach so as to allow a more accurate assessment of the biogenesis and turnover of enkephalin in vivo; these estimations should greatly aid our understanding of the physiological roles of these peptides.

## Acknowledgements

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## References

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