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# DETERMINATION OF LEU-ENKEPHALIN DEGRADATION BY A SOLUBLE ENZYME PREPARATION FROM CALF-BRAIN STRIATUM USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

A rapid procedure for the determination of Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) and its main metabolic degradation products (Tyr, Tyr-Gly-Gly and Tyr-Gly) by reversed-phase high-performance liquid chromatography was developed. The method has good precision, the coefficient of variation determined in the range 6–20 pmole being 1.5–3% ($n = 8$), and a very low detection limit of *ca.* 10 fmole for each metabolite. An unexpectedly high percentage of Tyr-Gly production is observed after enzymatic degradation of Leu-enkephalin by a solubilized enzyme preparation of calf-brain striatum.

## INTRODUCTION

Degradation of brain enkephalins usually occurs by cleavage of peptide bonds. Several enzyme systems have been proposed, of which the Tyr and Tyr-Gly-Gly producing enzymes are the best characterized. Hydrolysis of the second peptide bond resulting in the formation of Tyr-Gly is also observed. Fig. 1 shows the three known sites of cleavage observed with brain homogenates[1].

Tyrosine is produced by a non-specific aminopeptidase present in high concentrations in plasma. From studies of its affinity for enkephalins and its localization in the brain, it was concluded that this enzyme did not have a function in the *in vivo* degradation of the enkephalins[1].

According to Schwartz *et al.*[1], the main degradation product of Leu-enkephalin *in vivo* is Tyr-Gly-Gly. This peptide is produced by a dipeptidyl-carboxy-peptidase usually called enkephalinase A. Angiotensin converting enzyme is also capable of hydrolysing the third peptide bond, but this enzyme has such a low affinity for enkephalins and the rate of hydrolysis in brain is so low that it is of no importance for the catabolism of enkephalins in brain. The Tyr-Gly producing enzyme is poorly characterized and is observed only in solubilized homogenates of brain.

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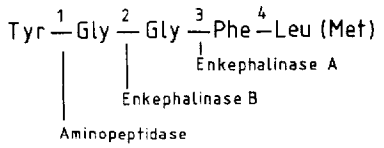


Fig. 1. Sites for Leu-enkephalin degradation by brain homogenates.

For a study of the metabolism of enkephalins, a method is required in which Tyr, Tyr-Gly-Gly and Tyr-Gly can be determined separately. The procedures described so far[2–10] include adsorption chromatography, thin-layer chromatography, (TLC) and high-performance liquid chromatography (HPLC). Tritiated enkephalins are used as a substrate.

The adsorption chromatographic method described by Vogel and Altstein[2] suffers from the inability to separate Tyr-Gly from Tyr-Gly-Gly. This method, which is very useful in the study of purified enzymes, was not inyestigated further by us. Although Tyr, Tyr-Gly and Tyr-Gly-Gly can be determined by TLC, this method is time consuming and therefore, in contrast to HPLC techniques, inconvenient for the analysis of large numbers of samples.

Because of its precision and reliability, we prefer HPLC for the routine determination of enzymatic degradation products. Unfortunately, the reported HPLC methods do not separate Tyr, Tyr-Gly-Gly and Tyr-Gly in one run[3–10]. A new method was therefore developed which was used to study the enzymatic hydrolysis of Leu-enkephalin by a soluble enzyme preparation obtained from calf-brain striatum. Apart from the expected release of Tyr and Tyr-Gly-Gly, a high level of Tyr-Gly production was observed. This finding contrasts with most literature reports, but is probably due to the superior separating power of the method used.

## EXPERIMENTAL

### *Chemicals*

[$^3$H]Leu-enkephalin (25 Ci/mmole) was obtained from New England Nuclear (Boston, MA, U.S.A.) and purified according to Vogel and Altstein[2]. Leu-enkephalin and its metabolites were purchased from Serva (Heidelberg, G.F.R.) and other chemicals, which were used without further purification, from Merck (Darmstadt, G.F.R.).

### *Solubilized enzyme preparation*

A crude enzyme preparation was obtained from calf-brain striata by homogenization in 15 volumes of ice-cold Tris buffer (20 m*M*, pH 7.5). After centrifugation for 30 min at 17,000 $g_{max}$ the pellet was washed by resuspension in 20–25 volumes of the Tris buffer. From the precipitate obtained after centrifugation for 30 min at 17 $kg_{max}$ the enkephalin degrading enzymes were extracted with 5–6 volumes of Tris buffer, containing 1 % (v/v) of Triton X-100. After stirring for 60 min at room temperature, the undissolved material was pelleted by centrifugation for 60 min at 100,000 $g_{max}$ Drops of solubilized enzymes with a volume of 15–20 $\mu$l were frozen and stored in liquid nitrogen.

*Enzyme assay*

A 10-ml volume of a solution containing 50 m*M* 4-(2-hydroxyethyl)-4-piperazine ethanesulphonic acid (HEPES) buffer (pH 6.85), 1 m*M* puromycin, 1 $\mu M$ captopril and 3 $\mu M$ [$^3$H]Leu-enkephalin (0.1 Ci/mmole) was incubated at 37°C for 10 min. The reaction was initiated by the addition of 0.1 mg (protein) of the solubilized enzyme preparation.

The reaction was terminated by adding 0.4-ml samples to 0.1 ml of a stop-mixture of methanol–0.85 *M* phosphate buffer (pH 1.55) (1:7), to which 5–10 $\mu M$ of either Leu-enkephalin or Tyr, Tyr-Gly-Gly and Tyr-Gly is added. After centrifugation at 10,000 $g_{max}$ for 1 min, 100 $\mu$l of the clear supernatant were analysed by HPLC.

*HPLC*

The chromatographic separations were performed on a custom-made HPLC system consisting of a Beckman Model 110 high-pressure pump, a Rheodyne syringe-loading injector fitted with a 175-$\mu$l loop and a Waters Model 440 fixed-wavelength (254 nm) detector.

The stationary phase was LiChrosorb RP-18 (Merck, 5–7 $\mu$m) and the mobile phases were aqueous phosphate buffer–methanol mixtures (buffer = 0.15 *M* disodium phosphate adjusted to pH 2.85 with perchloric acid) of the following compositions: for the separation of Tyr, Tyr-Gly-Gly and Tyr-Gly, buffer: methanol = 39:1, and for the determination of enkephalin, buffer:methanol = 1:1. Using UV detection, each metabolite fraction was collected in vials and the radioactivity measured by liquid scintillation counting. Separations were performed at ambient temperatures on 100 mm (Tyr, Tyr-Gly-Gly and Tyr-Gly) or 30 mm (Leu-enkaphalin) columns of I.D. 2.8 mm. The columns were packed by the slurry technique[11] at 500 kg/cm$^2$ using carbon tetrachloride as the dispersing medium. The flow-rate was 60 ml/h. Capacity factors ($k'$) were calculated from triplicate determinations of the retention times. Mobile phase diluted 4:1 with water was used to measure $t_0$. Column efficiencies were calculated from $N = 16\left(\frac{t_r}{t_w}\right)^2$ and $N_{eff} = N\left(\frac{k'}{k' + 1}\right)^2$ (ref. 12), ($t_0$ and $t_r$ are the elution times for an unretarded component and the eluite, respectively and $t_w$ is the peak width measured at the baseline.

## RESULTS AND DISCUSSION

*Chromatography*

The main products of enkephalin degradation are Tyr, Tyr-Gly-Gly and Tyr-Gly[1]. We therefore optimized the separation of these compounds by examining the effects of stationary phase, pH and ionic composition of the mobile phase and the concentration of modifier.

We preferred $C_{18}$-modified silica to the $C_8$ product as the former gave better results in terms of peak shape (less tailing) and column efficiency (5–10% larger values for $N$).

The pH of the mobile phase is important for the separation. Ionization of the COOH group causes a decrease in retention of all components between pH 2 and 4. As the capacity factors of Tyr-Gly-Gly and Tyr-Gly decrease more than that of Tyr, the separations become worse with increasing pH (see Fig. 2). A slight difference in

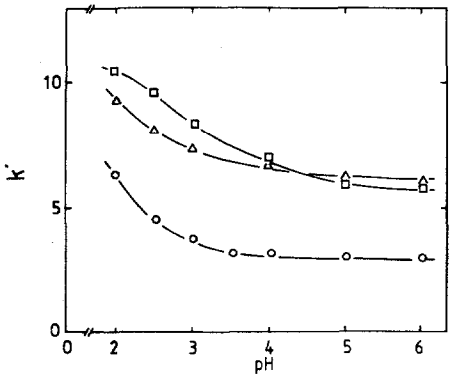


Fig. 2. Effect of pH on the capacity factors of Tyr (T), Tyr-Gly-Gly (TGG) and Tyr-Gly (TG). The $k'$ values were calculated from triplicate experiments in which 100-$\mu$l samples of stock solutions of T, TGG and TG in mobile phases were analysed separately. Conditions as in Experimental except for the mobile phases, which were prepared from 0.2 $M$ phosphoric acid solutions containing 2% (v/v) methanol. The pH was adjusted to the desired value with 25% (w/v) ammonia. O---O, T; △---△, TGG; □----□, TG.

the apparent p$K_a$ value of Tyr-Gly-Gly and Tyr-Gly is probably the reason for the reversal in the elution order of Tyr-Gly and Tyr-Gly-Gly when the pH is increased from 2 to 6. The best results for the separation of Tyr, Tyr-Gly-Gly and Tyr-Gly are observed between pH 2.5 and 3.

The concentration of phosphate, perchlorate or sodium ions hardly affects the retention times of the metabolites but perchlorate ions improve the peak symmetry and column efficiency. Increasing the concentration of perchlorate from 0 to 0.17 $M$ decreases the peak asymmetry factor from 1.6 to approximately 1.3 and increases the

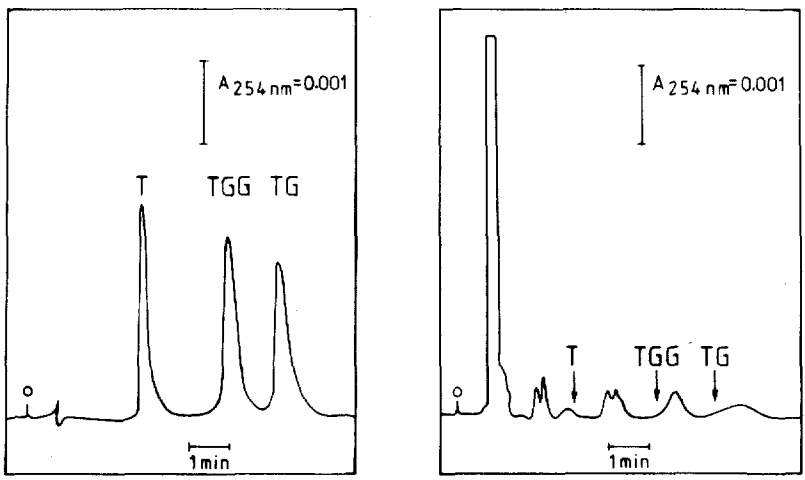


Fig. 3. Separation of T, TGG and TG; 100 $\mu$l of mobile phase containing 1 $\mu M$ T, TGG and TG analysed as described in Experimental. Abbreviations as in Fig. 2.

Fig. 4. Elution profile of denatured enzyme preparation. The enzyme was incubated at 37°C under the conditions described in Experimental. The reaction was terminated after 25 min with carrier-free stop-mixture. Abbreviations as in Fig. 2.

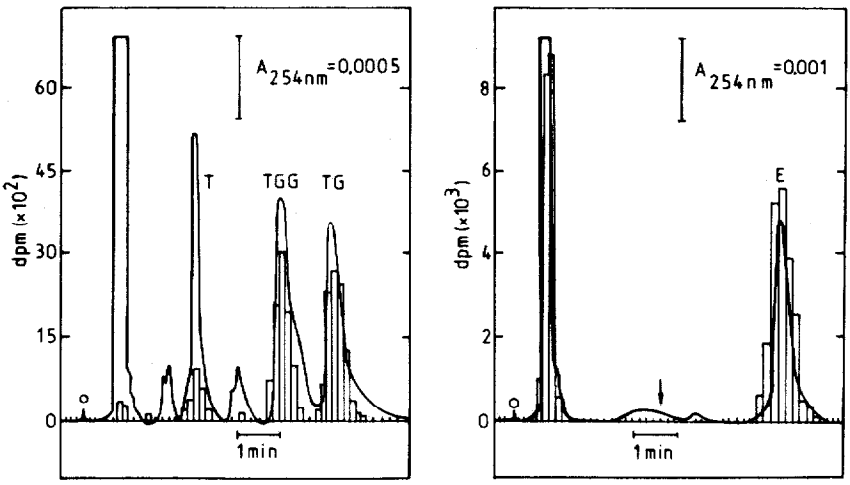


Fig. 5. Separation of radioactive T, TGG and TG after enzymatic hydrolysis of [$^3$H]Leu-enkephalin. Solid line, 254-nm absorbance; histograms, radioactivity measured in 5-drop fractions. Incubation time 25 min. Other conditions as described in Experimental. Abbreviations as in Fig. 2.

Fig. 6. Separation of Leu-enkephalin (E). Conditions as in Fig. 5 except that the mobile phase consisted of a 1:1 mixture of methanol and phosphate buffer (see Experimental). Arrow indicates the elution time for Tyr-Gly-Gly-Phe.

resolution, $R_s$ (*cf.*, ref. 12), from 1.2 to approximately 2. To prevent changes in the pH during the analysis a relatively high concentration of phosphate buffer of 0.15 *M* is used. The concentration of methanol can be varied between 1 and 6% (v/v) without affecting the quality of the separation. The most important effect of increasing the organic modifier concentration is the decrease in the retention time. When biological samples are analysed, the concentration of methanol should not be greater than 3–4% because otherwise tissue components will co-elute with Tyr.

Optimal conditions with respect to resolution and time of analysis were obtained with a mobile phase consisting of 0.15 *M* phosphate ($H_2PO_4^-$ + $HPO_4^{2-}$), 0.3 *M* $Na^+$, 0.17 *M* $ClO_4^-$ and 0.6 *M* methanol [2.5% (v/v)] adjusted to pH 2.85.

Figs. 3–6 summarize the results of the chromatographic separations.

Fig. 3 shows the analysis of 100 $\mu$l of a test mixture of 1 $\mu M$ Tyr, Tyr-Gly-Gly and Tyr-Gly dissolved in the mobile phase. All three components have baseline separation; $R_s$ = 4.6 for Tyr and Tyr-Gly-Gly and 2.1 for Tyr-Gly-Gly and Tyr-Gly and the peak asymmetry factors are 1.3–1.4 for the three compounds. The column efficiency, *N*, is approximately 13,000 per metre determined with Tyr-Gly as the test substance. $N_{eff}$ is 7800 for Tyr, 8700 for Tyr-Gly-Gly and 11,300 for Tyr-Gly (calculated per metre).

Fig. 4 shows the elution pattern of the supernatant of the enzyme preparation after denaturation at pH 2.85. The bulk of the 254-nm absorbing material elutes unretarded, followed by about seven minor peaks between 2 and 7 min. Tyr, Tyr-Gly-Gly and Tyr-Gly elute approximately at the positions indicated by the arrows. The overlap of these peaks with the tissue components makes the determination of concentrations of Tyr, Tyr-Gly-Gly and Tyr-Gly below 1 $\mu M$ impossible when UV

detection at 254 nm is used. Electrochemical detection[13] did not improve the limit of detection, as the relatively crude enzyme preparation produces too high blank signals to allow the reliable determination of low concentrations of metabolite. We used therefore tritiated Leu-enkephalin with a high specific activity as a substrate. The detection of the labelled metabolites is possible when unlabelled carriers are added just prior to the HPLC analysis.

Fig. 5 shows the result of such an experiment: 3 $\mu M$ [$^3$H]Leu-enkephalin (0.1 Ci/mmole) was incubated with a crude striatal enzyme preparation at 37°C. After 20 min the reaction was terminated by the addition of stop-medium and, after centrifugation at 10,000 $g_{max}$, 100 $\mu$l of the clear supernatant were analysed. The solid line represents the 254-nm absorbance and the bars the radioactivity, measured in approximately 150-$\mu$l samples (5 drops). As can be seen, the distribution of the radioactivity virtually coincides with the Tyr, Tyr-Gly-Gly and Tyr-Gly peaks. The small differences are due to UV-absorbing tissue components.

Under the conditions used no other peaks are observed after 7 min and therefore about eight samples can be analysed per hour. Leu-enkephalin and Triton X-100 concentrate on the column. It is therefore necessary to flush the column after 40–60 runs with 70% methanol in pH 2.85 buffer. This reactivation is most benificial to Tyr-Gly-Gly and Tyr-Gly separation. With this precaution several hundred chromatograms can be made with the same column without impairing its properties.

As Leu-enkephalin is much more hydrophobic than Tyr, Tyr-Gly-Gly or Tyr-Gly, higher concentrations of modifier are needed for its elution. It was not necessary to change the pH. Good results in terms of baseline stability and time of analysis were obtained with a 1:1 mixture of methanol and phosphate buffer (pH 2.85) (*cf.*, Experimental).

Fig. 6 shows the chromatogram of a sample taken after enzymatic degradation of Leu-enkephalin. One nmole of Leu-enkephalin was added to the stop-medium and radioactivity was measured in 150-$\mu$l samples.

Tyr, Tyr-Gly-Gly and Tyr-Gly elute unretarded with most tissue components. A broad band with low absorbance elutes after about 3.5 min. Again the radioactivity and the absorbance of Leu-enkephalin elute at the same time. It is interesting that no radioactivity is observed in samples eluting between 1 and 6 min. This is an indication that no Tyr-Gly-Gly-Phe is formed. This compound will elute at approximately 3.8 min (arrow in Fig. 6). Tyr-Gly-Gly-Phe formation would be due to hydrolysis of the fourth peptide bond of the enkephalins.

As no UV-absorbing materials elutes after about 8 min, approximately seven samples per hour can be analysed.

*Quantitative aspects of the method*

To investigate the method further, we measured the linearity of the calibration graphs. The peak heights were measured as a function of the amount of unlabelled Tyr, Tyr-Gly-Gly, Tyr-Gly or Leu-enkephalin injected. Over the range 0.2–10 nmole good linearity for all compounds was observed: $r = 0.999$ ($n = 6$–8). Similar results were obtained for the dependence of the radioactivity eluted on the amounts of labelled Leu-enkephalin (0.1 Ci/mmole) injected. Good linearity was observed in the range 10–500 pmole ($r = 0.997$, $n = 10$).

We were also interested in the linearity of the response at very low concen-

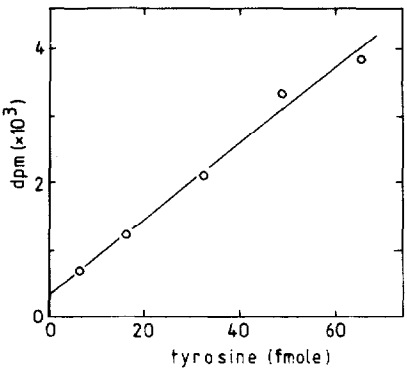


Fig. 7. Calibration line for [$^3$H]tyrosine. Carrier free Leu-enkephalin (12 n$M$) (25 Ci/mmole) was incubated with solubilized calf-striatal enzyme (final concentration 10 $\mu$g protein/ml) at 37°C. After 25 min the reaction was terminated as described in Experimental. The clear supernatant obtained after centrifugation at 10,000 $g_{max}$ was diluted with mobile phase + carrier and 100 $\mu$l of each dilution (1:10; 1:4; 1:2; 3:4) as well as the undiluted supernatant were analysed by HPLC. The concentration of tyrosine was calculated from the ratio of sample radioactivity to input radioactivity.

trations of metabolite. We therefore incubated 12 n$M$ carrier-free Leu-enkephalin (25 Ci/mmole) with striatal enzyme. From the mixture obtained after 20 min at 37°C five dilutions were prepared with concentrations of Tyr, Tyr-Gly-Gly and Tyr-Gly ranging from 0.2 to 10 p$M$. The results for the tyrosine analysis are given in Fig. 7. The relationship between the amount of radioactivity in the eluate ($x$) and the amount of Tyr injected ($y$) is linear over the range from 0 to 70 fmole (eqn. 1).

$$y = 55.5\,x + 352.1\ (r = 0.995,\ n = 5) \tag{1}$$

Similar results were obtained for Tyr-Gly-Gly and Tyr-Gly (eqns. 2 and 3, respectively):

$$y = 55.5\,x + 396.2\ (r = 0.9997,\ n = 5) \tag{2}$$

$$y = 55.5\,x + 256.0\ (r = 0.9995,\ n = 5) \tag{3}$$

The similarity of the slopes is not unexpected, as it represents the specific activity of the metabolites. The relatively large amount of radioactivity observed at zero concentration is caused by radioactive impurities of unknown nature coeluting with Tyr, Tyr-Gly-Gly and Tyr-Gly. These impurities, which were also observed by Vogel and Altstein[2], can be partly removed by chromatography on $C_{18}$-modified silica. A small amount, however, remains even after re-chromatography. The concentration of impurities in freshly purified preparations is fortunately so low that it hardly interferes with the measurements. Small aliquots of the enkephalin stock solutions were therefore purified regularly, as the amount of impurity slowly increases on ageing of the preparation.

Eqns. 1–3 can also be used to calculate the detection limit of the method. The detection limit is defined as the amount of labelled metabolite that will provide a signal-to-noise ratio of 3. With this definition the detection limits for Tyr, Tyr-Gly-

TABLE I

PRECISION OF THE METHOD

Means, standard deviations (S.D.) (pmol) and coefficients of variation (C.V.) (%) for single chromatography and complete assay; $n = 8$ in all instances.

| *Metabolite* | *RP-HPLC* | | | *Complete assay* | | |
|---|---|---|---|---|---|---|
| | *Mean* | *S.D.* | *C.V.* | *Mean* | *S.D.* | *C.V.* |
| Tyr | 5.40 | 0.15 | 2.78 | 6.12 | 0.19 | 3.12 |
| Tyr-Gly-Gly | 18.85 | 0.31 | 1.63 | 19.34 | 0.49 | 2.65 |
| Tyr-Gly | 19.05 | 0.27 | 1.50 | 20.06 | 0.46 | 2.30 |

Gly and Tyr-Gly, calculated from eqns. 1–3, are 9.6, 10.6 and 6.9 fmole, respectively. This detection limit is approximately three orders of magnitude smaller than that obtained with direct HPLC methods using unlabelled Leu-enkephalin (P. van Krimpen, unpublished observations).

The precision of the method was determined by analysing a mixture of Tyr, Tyr-Gly-Gly and Tyr-Gly obtained by enzymatic degradation of 3 $\mu M$ Leu-enkephalin (0.1 Ci/mmole) incubated with 10 $\mu$g/ml of crude striatal calf-brain enzymes at 37°C for 25 min. Eight samples of the same supernatant and eight samples from eight supernatants were analysed for their metabolite content. The results are summarized in Table I.

*Enzymology*

Fig. 8 shows the time course for Leu-enkephalin degradation by a crude striatal enzyme preparation. Tyr is continuously produced although its rate diminishes gradually. The initial rates of Tyr-Gly and Tyr-Gly-Gly production exceed that of Tyr production, but these rates decrease rapidly and after 30 and 60 min, respectively, a decrease in concentration is observed.

The decrease of in Tyr-Gly-Gly and Tyr-Gly concentration is most probably due to further degradation by non-specific aminopeptidases that hydrolyse Tyr-Gly-Gly and Tyr-Gly and contribute to the formation of Tyr. This may explain why Tyr

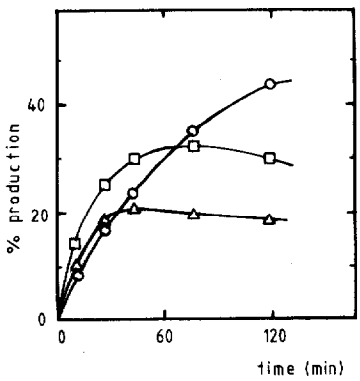


Fig. 8. Time course for the production of T, TGG and TG from Leu-enkephalin. Percentage production is calculated from the ratio of sample radioactivity to input radioactivity. Conditions as described in Experimental. ○---○, T; △---△, TGG; □---□, TG. Abbreviations as in Fig. 2.

production continues even after 120 min, although then nearly all of the enkephalin has been hydrolysed. The assumption of non-specific degradation of Tyr-Gly and Tyr-Gly-Gly[14] is supported by the fact that when Leu-enkephalin is incubated with the enzyme for 7 h only small amounts (3%) of Tyr-Gly-Gly can be detected in the reaction medium.

From the samples taken between 0 and 120 min, the amount of non-metabolized Leu-enkephalin was also determined. There is a linear relationship between the amount of Leu-enkephalin degraded ($y$) and the sum of Tyr, Tyr-Gly-Gly and Tyr-Gly produced ($x$):$y = 1.08\ x - 1.84$ ($r = 0.990$; $n = 10$). The slope of 1.08 indicates that the solubilized enzyme preparation degrades Leu-enkephalin solely in Tyr, Tyr-Gly-Gly and Tyr-Gly. The liberation of tyrosine is due to non-specific aminopeptidase(s) (see Fig. 1). These soluble enzymes are present in high concentrations in homogenates of whole brain and are probably captured within the membraneous structure of the precipitate used as the enzyme source. This may be concluded from the observation that extensive washing with buffer could not remove the Tyr-producing activity. The activity of the aminopeptidase is inhibited by the addition of puromycin[15]. However, complete inhibition of Tyr production was never observed. Our finding that after prolonged incubation of Leu-enkephalin with the enzyme preparation nearly all Tyr-Gly-Gly has disappeared from the samples is an indication that, apart from tyrosine-releasing enzyme, low concentrations of other aminopeptidases are present.

Tyr and Tyr-Gly-Gly are produced by membrane-bound enzymes, as even repeated washing (four times) did not remove this activity from the precipitate. Tyr-Gly-Gly is probably produced by enkephalinase A (*cf.*, Fig. 1), as sufficient captopril was added to inactivate the angiotensin converting enzyme completely[16].

The high percentage of TG production at low substrate concentration observed in Figs. 5 and 8 was unexpected, as it is commonly reported that *in vivo* enkephalins are converted mainly into Tyr-Gly-Gly + Phe-Leu or Phe-Met, respectively[1].

It is possible that solubilization by Triton X-100 unmasks the dipeptidyl-aminopeptidase activity (enkephalinase B). This agrees with the observations of Gorenstein and Snyder[17] and of Hazato *et al.*[18], who found Tyr-Gly production in fractions obtained from DEAE-cellulose chromatography of Triton X-100-solubilized brain homogenates. However, preliminary experiments in which particle-bound enzymes from both rat and calf-brain striatum were incubated with 1–3 $\mu M$ substrates also showed appreciable amounts of Tyr-Gly produced. There are no data in the literature to confirm these observations, which may be due to previous inadequate separation of Tyr-Gly from Tyr-Gly-Gly, which causes Tyr-Gly production to be overlooked. Whether enkephalinase B is also important for the *in vivo* degradation of enkephalin is not yet known, but the fact that even at 1 $\mu M$ substrate concentration this enzyme competes effectively with the Tyr-Gly-Gly producing enzyme is an intriguing observation and needs further investigation.

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