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COMBINATION OF ENZYMATIC AND MASS FRAGMENTOGRAPHIC ASSAYS FOR THE IDENTIFICATION AND MEASUREMENT OF [MET^5]-ENKEPHALIN

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SUMMARY

Dipeptidyl-aminopeptidase I (DAP I) was used to hydrolyze the pentapeptide-[met^5]-enkephalin into the dipeptides Tyr–Gly and Gly–Phe and methionine. The dipeptides could be derivatized and resolved by gas chromatography (GC); quantification of these dipeptides was obtained by single ion monitoring. Tissue samples were prepurified with Bio-Beads SM-2 and used as substrate for DAP I. The yield of methionine and of the two dipeptides increases with time. Since the same dipeptides are produced by [met^5]-enkephalin (ME) and [leu^5]-enkephalin, the use of the ratios of the quantities of the two dipeptides with methionine could be used for recognition. Since the only internal standard available was one with deuterated methionine, the measurement of methionine released by DAP I from the prepurified tissue could be used to measure ME content in tissues that contain a small amount of heterogeneity in the molecular forms of ME. The present GC–mass spectrometric method can be used to quantify ME in brain but not in adrenal medulla because this tissue contains a high degree of molecular heterogeneity.

INTRODUCTION

The tissue stores of [met^5]-enkephalin (ME) like those of other polypeptide hormones may include a number of structurally related polypeptides some of which can react with ME directed antisera. Since some of these ME-like peptides have biological properties different from ME it has become evident that the measure of ME content with radioimmunoassays (RIA) may not give a correct estimation of the biologically active ME-like analogue present in a given tissue. The specificity of RIA can be enhanced by associating RIA with chromatographic purification procedures. Though these procedures considerably increase the reliability of RIA they may not completely eliminate possible errors if an absolute prepurification was not performed. For instance antiserum directed toward ME fails to discriminate with sufficient specificity ME from the hexapeptide Arg–Tyr–Gly–Gly–Phe–Met which may be

present in brain, or ME from the pentapeptide [leu^5]-enkephalin (LE), which is present in every tissue where ME is stored[1].

The possibility of errors in the measurement of ME tissue content is even more relevant when peripheral neural tissues are studied because in these tissues the degree of molecular heterogeneity of the ME-like compounds is even higher than in brain tissue. For instance Yang *et al.*[2] have reported that bovine adrenal medulla contains multiple forms of ME-like peptides which cross react with ME antisera. Because of this complexity in crude tissue extracts of adrenal medulla, the RIA of ME is not reliable; similar cautionary comment sextend to the immunohistochemical studies of peripheral ME. Presently, the heterogeneity of the molecular forms of the ME-like peptides is being viewed as the starting material for the identification of ME-precursors and of specific agonists for various types of opiate receptors. In pursuing this goal, we have considered gas chromatography–mass spectrometry (GC–MS) as a potential analytical method that can measure tissue content of ME with the detailed information on the chemical structure necessary to differentiate the various ME-like compounds. An important limitation in the use of the GC–MS to assay peptides stems from their low volatility. To our knowledge none of the derivatization procedures available can render the pentapeptide ME amenable to the GC analysis. Attempts to circumvent this limitation by direct introduction of the derivatized ME into the mass spectrometer ion source[3] are hampered by the relatively low abundance of the molecular ion and of the other ions capable of giving significant structural information. Thus, this method yields an unsatisfactory sensitivity. Here we report on our experiences with an alternate approach, involving the enzymatic fragmentation of the pentapeptide ME into characteristic dipeptides by dipeptidyl-aminopeptidase I (DAP I) and the subsequent analysis by GC–MS of the dipeptides thus produced. DAP I is an exopeptidase that liberates dipeptides from the N-terminus of tri- or longer peptides[4,5]. Identification of the dipeptides produced by the action of DAP I on tissue extracts may prove useful both for the structural identification of ME in tissues and for attaining information regarding the chemical structure of ME-like peptides present in any given tissue store.

MATERIALS AND METHODS

Enzymatic digestion of ME

ME (Boehringer, Mannheim, G.F.R.) was dissolved in distilled water to attain a concentration of 4 mg/ml, and this stock solution was stored at −2°. Aliquots of the stock solution containing ME 20, 100 or 400 ng were placed in conical bottom, 1-ml capacity glass vials (Supelco) evaporated to dryness and combined with 50 μl of a buffer consisting of 10 m*M* methylmorphine–CH_3COOH, pH 7.0, 1% NaCl and 2.5% mercaptoethanol[5]. The buffer also contained 1 μl (0.2 U) of DAP I in glycerol (Boehringer) per 50 μl. Control samples were added with the same quantity of inactivated boiled enzyme. After incubation at 37° for various periods of time (see Results) the samples were dried under nitrogen and derivatized for GC–MS analysis as described below.

Derivatization and GC–MS analysis

To the incubated dried samples, 50 μl of hexafluoroisopropanol (HFIP) and

50 μl of pentafluoropropionic anhydride (PFPA) (Pierce, Rockford, Ill., U.S.A.) were added. The vials were then capped and the mixture allowed to react for 30 min at 60°. After evaporating the excess reactants under nitrogen the residue was taken in ethyl acetate for GC–MS analysis. This was carried out in either a Finnigan 3200 instrument or an LKB 9000 gas chromatograph–mass spectrometer. Stationary phases employed for GC included OV-17, SE-30 and SE-54 (all at 3% on Gas-Chrom Q, 100–120 mesh). Short (1 m × 2 mm I.D.) glass columns were preferentially used. Instrument conditions for analysis were: gas, helium (30 ml/min); column temperature 200° for the dipeptides and 120° for methionine; electron energy, 70 eV.

Treatment of biological samples

Male Sprague Dawley rats (Zivic Miller) weighing 150–170 g were killed by focused microwave irradiation to the head[6]; the brains were removed and the striatum dissected out. Beef adrenal medullae, obtained from the slaughter house were kept frozen until exposed to microwave irradiation. These tissues were immediately homogenized in 4 ml 0.4 *N* acetic acid. After centrifugation at 12,000 *g* for 15 min, aliquots of 3.7 ml of the supernatant were taken and lyophilized overnight. The lyophilized samples were taken in 1 ml 10 m*M* phosphate buffer, pH 7.4, and loaded onto small adsorption chromatography columns of Bio-Beads SM-2, washed twice with 5 ml of 10 m*M* phosphate buffer, pH 7.6, then the ME was eluted with 0.9 ml of methanol. This eluate containing the enkephalins was collected in conical bottom glass vials (Supelco). Bio-Beads SM-2 adsorb strongly the enkephalins when applied in aqueous solution at neutral pH[8]. A 75–80% recovery of the ME during this step was documented using [^{3}H]ME as a monitor and an 80 ng quantity of the ME. The methanolic eluate was subsequently evaporated to dryness with a nitrogen stream and then it was enzymatically cleaved with DAP I (90 min) and successively derivatized as described above.

In the experiments set up to obtain a quantitation of the methionine produced from the tissue samples incubated with DAP I, ME-d_3 (Penninsula Labs., San Carlos, Calif., U.S.A.) was used as an internal standard. This standard contained three deuteria in the methyl group of methionine. ME-d_3 (80 ng) was added to aliquots of the supernatant, the aliquots of the same supernatant were used for parallel RIA of ME. Standard curves were constructed using 80 ng of ME-d_3 and various quantities of ME; these reference standards were run in parallel with brain tissue samples throughout the whole analytical procedure. The procedure for RIA of ME was carried out as previously described by Hong *et al.*[7].

RESULTS

When ME is the substrate for DAP I, an amino acid and two dipeptides are formed: Tyr–Gly, Gly–Phe and methionine. The structures of these compounds were verified by recording the mass spectra of their HFIP–PFP derivatives as shown in Fig. 1. Neither methionine nor the two dipeptides, Tyr–Gly and Gly–Phe could be detected when the samples of ME were incubated with heat inactivated DAP I. Fig. 2 shows the activity of DAP I on ME, as a function of incubation time using ME as the substrate, the enzyme activity is expressed as the ratio of Gly–Phe/Tyr–Gly formed. In the conditions used in these experiments (see legend to Fig. 2), the enzymatic

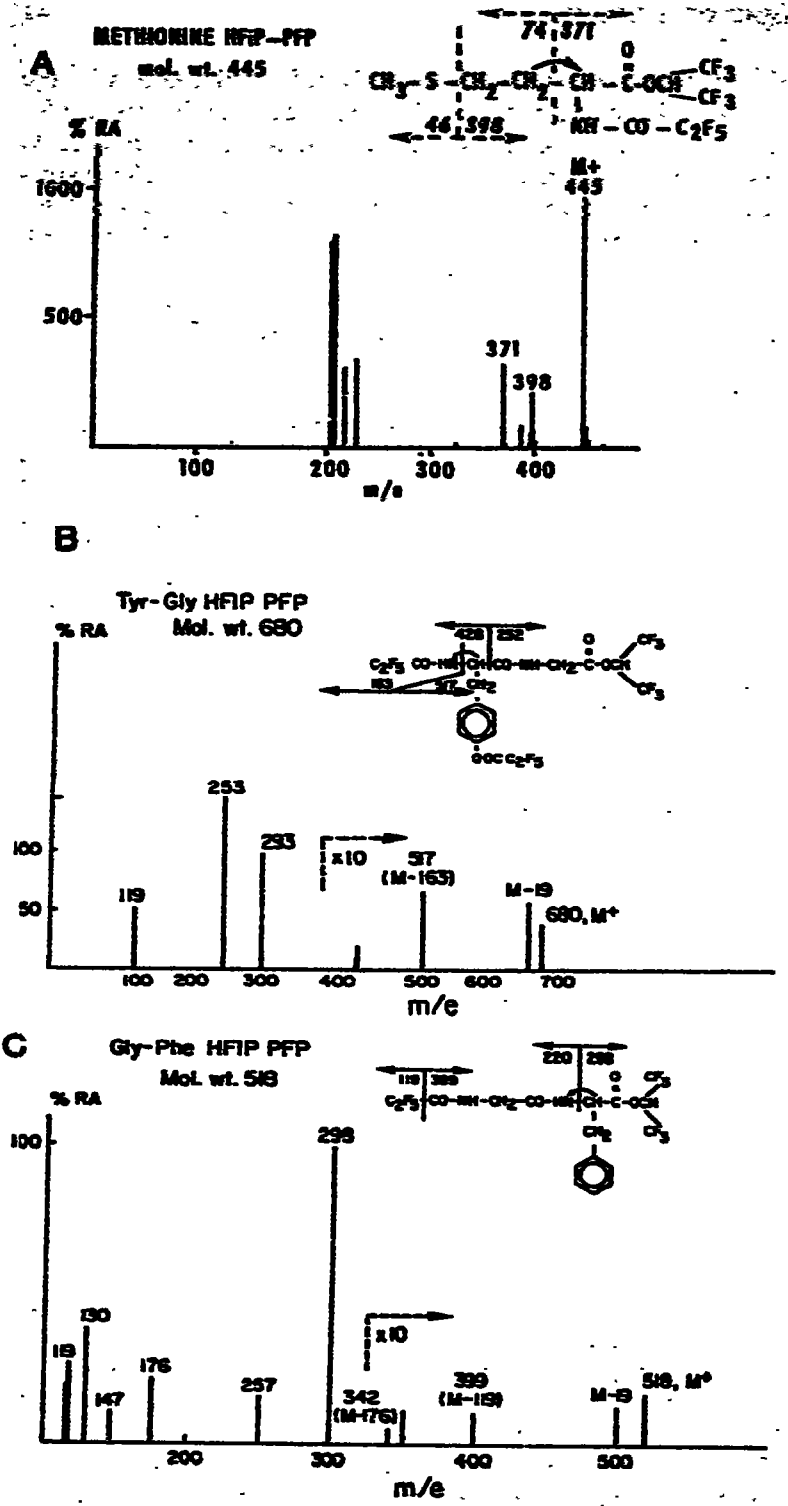

Fig. 1. Mass spectra of the HFIP–PFP derivation of Tyr–Gly (B), Gly–Phe (C) and Met (A), as obtained in a quadropolar instrument (Finnigan 3200) at 70 eV.

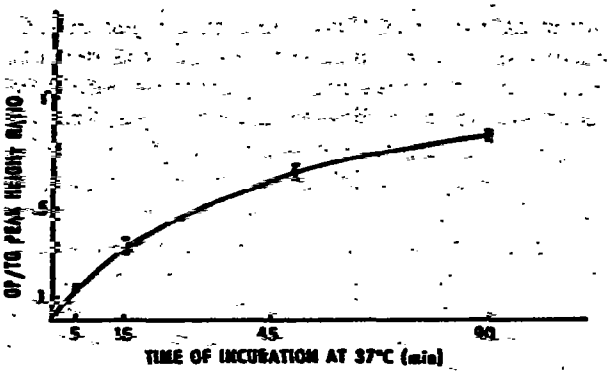

Fig. 2. Time course of the production of various fragments of ME after incubation with DAP I. Buffer: 10 m*M* methylmorpholine–CH_3COOH, pH 7, 1% NaCl, 2.5% mercaptoethanol. 0.2 U enzyme in 50 µl buffer/sample of 0.5 nmol. Each point represents mean of 5 determinations.

reaction reached a plateau in about 20 min. At this time point, the ratio Gly–Phe/Tyr–Gly was 0.85 ± 0.020, when an equimolar mixture of Gly–Phe and Tyr–Gly was analyzed in parallel, this ratio was found to be 0.90 ± 0.040 (not shown in Fig. 2). It should be noted that in Fig. 2, as well as in Table I, Gly–Phe and Tyr–Gly are expressed as peak heights of the characteristic fragments of their mass spectra (*m/e* 298 for Gly–Phe, *m/e* 293 for Tyr–Gly), and not as GC peak heights. Similarly, the values of methionine given in Table I correspond to the peak height of the molecular ion, *m/e* 445. GC separation of Tyr–Gly and Gly–Phe was readily achieved using the stationary phases SE-54 and SE-30 (column efficiencies, 15 and 16 theoretical plates per cm, respectively); by contrast, the stationary phase OV-17 provided only partial resolution

TABLE I

QUANTITATIVE RELATIONSHIPS OF TYR–GLY, GLY–PHE AND MET FORMED BY DAP I INCUBATED WITH SYNTHETIC ME AND TISSUE EXTRACTS

Analysis was carried out by mass fragmentography as described under Materials and methods. Mass fragments used to calculate ratios were: *m/e* 293 for Tyr–Gly, *m/e* 298 for Gly–Phe; and *m/e* 445 for Met.

Peptide	*Synthetic ME (100 ng), n = 8*	*Caudate extract (8 mg protein), n = 6*	*Caudate extract (8 mg protein) + 100 ng ME, n = 8*	*Adrenal medulla extract (10 mg protein), n = 8*
Gly–Phe/Tyr–Gly peak height ratio	0.88 ± 0.02	0.91 ± 0.03	0.87 ± 0.03	$1.3 \pm 0.2^{*}$
Tyr–Gly/Met peak height ratio	1.45 ± 0.2	1.53 ± 0.2	1.50 ± 0.4	$4.8 \pm 0.6^{**}$
Gly–Phe peak height (mm)	44 ± 4	51 ± 4	87 ± 10	—
Tyr–Gly peak height (mm)	50 ± 4	56 ± 3	100 ± 12	—
Met peak height (mm)	34 ± 5	37 ± 3	67 ± 5	—

$^{*}P < 0.01$.
$^{**}P < 0.001$.

for these dipeptides ($R < 0.5$ for a column attaining 16 theoretical plates per cm). In any of these phases, the derivative of methionine exhibits a retention time considerably shorter than that of Tyr–Gly or Gly–Phe. Thus, temperature programming would be needed in order to monitor the amino acid and the two dipeptides in a single chromatographic run. However, the high temperature required caused a high degree of dipeptide decomposition during the chromatography which was unacceptable; therefore, it was found more convenient to perform the analysis of methionine and that of the dipeptides in two separate chromatographic runs. At any rate, extensive column preconditioning and the use of sylanized glass wool plugs was necessary to attain a reasonable stability of the dipeptide derivatives in the GC system, in contrast, the methionine derivative exhibited less susceptibility to degradation and/or adsorption. Typical sensitivity limits were about 8 ng for the dipeptides and 1 ng for methionine.

Biological samples analyzed for the presence of ME were in every instance submitted to partial prepurification by adsorption chromatography on Bio-Beads SM-2. Tissue samples partially purified as described under Materials and methods were subsequently incubated with DAP I and Gly–Phe, Tyr–Gly and methionine were detected by mass fragmentography (Table II). The following ions were recorded: *m/e* 445, 398 and 371 for methionine; *m/e* 293 and 253 for Tyr–Gly; *m/e* 298 and 257 for Gly–Phe. Table II shows that the ratios of these ions measured at the appropriate retention times were the same when either synthetic methionine, Tyr–Gly or Gly–Phe, or the product yielded by an incubation mixture of DAP I with synthetic ME or tissue extracts were analyzed by GC–MS. Mass fragmentographic analysis of brain extract processed in parallel with the aforementioned samples but incubated with heat inactivated DAP I failed to detect either methionine, Tyr–Gly or Gly–Phe. Similar results were obtained with adrenal medulla extracts. Whenever ME-d_5 was added to the samples, the corresponding tritiated methionine was detected by monitoring its molecular ion at *m/e* 448.

Table I, upper section, lists the ratios of the characteristic ions of Tyr–Gly/methionine generated by DAP I incubated with synthetic ME (100 ng), extracts

TABLE II

IDENTIFICATION OF METHIONINE, TYR–GLY AND GLY–PHE BY MEASURING THE RATIOS OF SELECTED IONS PRODUCED BY GC–MS OF THE HEXAFLUOROISOPROPYL–PENTAFLUOROPROPIONYL DERIVATIVES OF THE DIPEPTIDES

All analyses were performed in 3% SE-30, 100–120 mesh, See Materials and methods for other details.

Compound	*Ions monitored*[*]	*Ion ratios*		
		Authentic compound	*ME-Enkephalin incubated with DAP I*	*Tissue extract incubated with DAP I*
Methionine	445; 398	3.0 ± 0.05[**]	2.8 ± 0.2[**]	3.0 ± 0.2[**]
	371	4.1 ± 0.05[***]	4.3 ± 0.2[***]	4.3 ± 0.2[***]
Tyr–Gly	293; 253	1.2 ± 0.05[‡]	1.3 ± 0.06[‡]	1.2 ± 0.06[‡]
Gly–Phe	298; 257	6.8 ± 0.4[‡‡]	6.3 ± 0.8[‡‡]	6.0 ± 0.7[‡‡]

[*] Ions are monitored with reference to their *m/e* values.
[**] *m/e* 445/371.
[***] *m/e* 445/398.
[‡] *m/e* 253/293.
[‡‡] *m/e* 298/257.

of either adrenal or striatal tissue samples (8 mg protein). Data obtained with striatal extracts added with 100 ng of synthetic ME are also shown; it should be noted that a striatal sample of 8 mg protein will contain about 100 ng of ME when analyzed by RIA[7] (Table III). We found that the ion ratio of Gly–Phe/Tyr–Gly and Tyr–Gly/methionine that are characteristic of ME can be generated by samples of striatum, but not by samples of bovine adrenal medulla which have been processed as described under Materials and methods. In fact, in adrenal medulla samples, the abundance of Gly–Phe or Tyr–Gly was several fold higher than that of methionine which could be expected as a result of ME cleavage. When the average peak height of the individual compounds, rather than the compound ratios, were compared (Table I, lower section), a good correlation was found for the three substances detected in synthetic ME samples *vs.* the non-added striatal extracts; moreover, extracts added with 100 ng of ME yielded Gly–Phe, Tyr–Gly and methionine peak heights roughly twice as high.

TABLE III

COMPARISON OF THE QUANTITATIVE ANALYSIS OF ME IN RAT CAUDATE NUCLEUS CARRIED OUT WITH RIA OR WITH GC–MS MEASUREMENTS OF METHIONINE FORMED FOLLOWING STRIATAL INCUBATION WITH DAP I

Group	*RIA (ng/mg protein)*	*GC–MS (ng/mg protein)*
Untreated ($n = 14$)	12 ± 0.80	13 ± 1.3
Treated**		
Chronic LiCl ($n = 11$)	20 ± 1.3*	23 ± 1.1*
Chronic saline ($n = 10$)	10 ± 0.60	13 ± 0.80

* $P < 0.001$ when compared to chronic saline.

** Rats received daily injections of LiCl (5 mequiv./kg; i.p.) or saline for 6 days and were sacrificed 24 h after the last injection.

The data reported in Table I are uncorrected for procedural losses since no internal standards were used in these experiments. Subsequently, the availability of ME labeled with deuterated methionine made it possible to perform an accurate quantitation of the liberated methionine when the tissue extracts were incubated with DAP I. This methionine may then be expressed in terms of ME equivalents. Using this approach, we calculated the levels of ME in the rat striatum; parallel RIA of aliquots of the same samples were run for comparison purposes. As shown in Table III, there was a remarkable agreement between the data obtained with the two assays. Table III also shows that after chronic treatment with LiCl, both the RIA and the assay of methionine produced from the tissue extracts treated with DAP I detect a similar increase in the striatal ME content.

DISCUSSION

The purpose of this work was to evaluate the possibility of quantifying and measuring by GC–MS the ME which is present in biological samples. The approach used was that previously proposed by Krutzsch and Pisano[5] and consists of combining GC–MS with specific polypeptide cleaving by DAP I. DAP I was chosen because it releases dipeptides sequentially from N-terminus[4,5]. These peptides were

measured specifically with GC–MS as we had hoped that with this approach, we could obtain information on the structure of the polypeptide that is analyzed. Since DAP I cleaves ME starting from the N-terminal dipeptide, the ratio Gly–Phe/Tyr–Gly increased with time (Fig. 2). In 90 min DAP I cleaves both peptide bonds by about 100%; in fact, the Gly–Phe/Tyr–Gly ratio in samples of ME incubated with DAP I is similar to that obtained from an equimolar mixture of synthetic dipeptides. These findings suggest that the tripeptide Gly–Phe-methionine is a substrate for DAP I; this was noteworthy because not all of the tripeptides were substrates for DAP I[4]. Although we have not studied the complete time course of the action of DAP I on LE, we did establish that after 90 min of incubation, the Gly–Phe/Tyr–Gly ratio for this pentapeptide was not significantly different from that found with ME. Thus, it may be tentatively concluded that the tripeptides Gly–Phe–methionine and Gly–Phe–Leu are both suitable as substrates for DAP I. Indeed, this indicates that the kinetics of ME and LE degradation by DAP I is quite similar. Since tissue stores containing ME contain also LE, the precise assessment of the amount of ME present crucially depends on the capability to measure the dipeptides and the methionine produced by DAP I after ME is separated from LE.

When partially purified tissue extracts are used as a substrate for DAP I, GC–MS analysis can identify Gly–Phe, Tyr–Gly and methionine and the content of these compounds can be measured. Since they could not be detected in tissue extracts purified and treated with heat inactivated enzyme, one can safely assume that they are the result of the catalytic activity of DAP I on peptides present in the tissue extracts. While free methionine or Tyr–Gly are eliminated from the tissue extracts by adsorption chromatography[8], the Gly–Phe and ME are adsorbed onto the Bio-Beads resin and are eluted with methanol from the resin[8]. Since Gly–Phe was absent in the tissue extract treated with heat inactivated enzyme it might be inferred that the tissue extracts we studied, are devoid of free Gly–Phe. Because of these problems it became important to evaluate to what extent the Gly–Phe, Tyr–Gly and methionine we detected after incubation with DAP I originates from ME or from other peptides present in the tissue extracts. A guiding principle in this evaluation is to measure the specific ratios of Gly–Phe/Tyr–Gly and Tyr–Gly/methionine which are characteristic for ME. It should be recalled that in the conditions used in the present experiments, the ratio Tyr–Gly/methionine could not be determined in the same GC–MS run due to the problems of derivatization and separation of methionine from the dipeptides by one column. A similar difficulty does not apply to the measurement of Gly–Phe/Tyr–Gly ratios. The problems inherent to the measurement of the Tyr–Gly/methionine ratios may be overcome in part by the use of deuterated ME as an internal standard; ideally, the first glycine as well as methionine should be deuterated. Unfortunately, such a standard was not available to us at this time but we used ME with deuterated methionine. The use of this internal standard reduces the resolution power of our method but it still allows for some important conclusions.

The data reported in Table I strongly suggest that in striatum, ME alone is the main source of Gly–Phe, Tyr–Gly and methionine we detected by GC–MS after DAP I incubation. In fact, both the dipeptide ratio and Tyr–Gly/methionine ratio coincide with those obtained from synthetic ME; neither this dipeptide nor methionine is detected in the purified tissue extracts. Moreover, these ratios were not modified when ME was added to the tissue extract. At the present time, however, we cannot

exclude a slight contribution of striatal LE to the striatal dipeptide content calculated in this paper.

The measurements performed in the extracts from adrenal medulla (Table I) indicate a heavy contribution to both Gly–Phe and Tyr–Gly by some peptide(s) other than ME. This is in agreement with the finding[1] that the adrenal medulla contains a variety of molecular forms of ME-like peptides in significant amounts. These peptides cross react with the ME antibody, and hence they must share with ME some degree of structural similarity, of which the presence of Tyr–Gly and Gly–Phe might be a part. Thus, in order to specifically identify ME through the detection of Tyr–Gly and Gly–Phe, a more thorough prepurification of adrenal medulla samples seems necessary.

The measurement of methionine gives a great deal of reliability to the quantitative information obtained in the present experiments. In fact, any peptide that yields free methionine after DAP I digestion, must have the following structural characteristics: (1) methionine is present in the carboxy terminal, but not in the amino terminal; (2) the polypeptide consists of an odd number of aminoacids; (3) the polypeptide size is relatively small; otherwise the enzymatic reaction may be terminated before amounts of methionine are liberated in any significant amount.

Since in brain extracts the molecular heterogeneity of ME-like peptides is less than in adrenal medulla, in this tissue, it is possible to perform accurate estimations of ME content with the GC–MS with a simple prepurification. Therefore, we have verified whether the tissue concentrations of ME are accurately estimated by measuring methionine liberated from tissue extract by DAP I. Since this approach involved an exact quantitation of methionine, ME-d_3 was used as the internal standard (see Materials and methods). This compound behaves exactly like endogenous ME throughout the entire analytical procedure but in the final GC–MS analysis where the deuterated methionine originated from ME-d_3 is differentiated from the methionine originated from endogenous ME and, therefore, may serve as an internal standard. Our results (Table III) show that in our extracts from rat brain the amount of methionine produced after 90 min of incubation with DAP I closely reflect the levels of ME as measured by RIA, both in normal conditions and under the influence of chronic LiCl treatment. It must be said that up to now GC–MS measurements of ME is less sensitive than RIA. However, this is only the beginning, it is clear that further work is necessary to improve the sensitivity of the GC–MS to measure tissue content of ME.

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