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Methionine enkephalin-like immunoreactivity, substance P-like immunoreactivity and β-endorphin-like immunoreactivity *post-mortem* stability in rat pituitary

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ABSTRACT

Significant *post-mortem* changes in peptide concentration occur within the previously unstudied timeframe, *i.e.* within 1 h, for the proenkephalin A, proopiomelanoccortin, and tachykinin neuropeptidergic systems in the pituitary. These data differ from data obtained in other studies that concluded that peptides are stable for up to 72 h *post-mortem*. The *post-mortem* stability of the three neuropeptides, methionine enkephalin, substance P, and β-endorphin, was studied in the rat pituitary to test the hypothesis that significant *post-mortem* concentration changes of those three neuropeptides occur in the immediate *post-mortem* time period.

INTRODUCTION

Neuropeptide precursors are synthesized in the cell body of the neuron and are processed metabolically during their axonal transport. The resulting neuropeptides are stored within pre-synaptic vesicles; they are released, diffuse across the synaptic cleft, and bind to post-synaptic receptors, followed by rapid metabolism and as a consequence removal of the excess of neuropeptides. The corticotrophs in the anterior pituitary are one of the sites of synthesis of the proopiomelanocortin (POMC) neuropeptides [1], whereas the tachykinin and proenkephalin A sites of synthesis are not as well established. Corticotropic cells synthesize the POMC precursor, which yields the peptide products adrenocorticotropic hormone (ACTH), β-lipotropin (β-LPH), β-endorphin (BE), and α-melanocyte-stimulating hormone (α-MSH). Anatomical connections exist to other extrapituitary tissues through which the neuropeptides can be transported.

Methionine enkephalin (ME) derives from the proenkephalin A precursor molecule, and substance P (SP) from protachykinins. Proenkephalin A contains four copies of ME, one of the hexapeptide ME-Arg-Gly-Leu, one of the heptapeptide ME-Arg-Phe, and one of leucine enkephalin (LE); the LE sequence is also contained within the dynorphin precursor molecule proenkephalin

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B. Tachykinin peptides share a common C-terminal amino acid sequence, Phe-X-Gly-Leu-Met-NH_2, where X is an aliphatic or aromatic amino acid, generally either Phe or Val [2]. In mammalian tissues, two genes encode all the tachykinins. The preprotachykinin A (PPT-A) gene encodes for SP, neurokinin A (NK-A), neuropeptide K (NP-K), and neuropeptide γ (NP-γ) [3]. The PPT-B gene encodes for NK-B [4].

This present study was prompted by the analysis of neuropeptides in human anterior pituitary tissues. For example, for studies of neuropeptides in human tissues and fluids, it is important to analyze accurately the content and stability of neuropeptides contained within *port-mortem* tissues [5–16]. Although the *post-mortem* stability of neuropeptides is neither a major problem in small animal studies nor in human post-surgical studies because those tissues and fluids can be acquired immediately, stability is, however, a significant factor in human studies [16], where an appropriate period of *post-mortem* time must be considered. Many articles report the *post-mortem* stability of several neuropeptides over several days [5–7,12,15]. Those conclusions are being questioned here.

Thus, this study was designed to investigate the *post-mortem* cooling of the rat brain as a model for corresponding studies with human *post-mortem* brain samples [17]. Following reversed-phase high-performance liquid chromatographic (RP-HPLC) purification of individual neuropeptides [18], the three neuropeptide immunoreactivities were measured by radioimmunoassay (RIA).

The important new aspects of this study are the analysis of peptide content during the early, more-rapidly changing part of the cooling curve, a statistical comparison of changing neuropeptide content over the entire cooling curve, and the finding of significant changes in the neuropeptide content of three neuropeptidergic families.

EXPERIMENTAL

Chemicals

Synthetic peptides were purchased from Sigma (St. Louis, MO, USA) and tritiated peptides ($^3H_2$-[3,5-Tyr]-ME, 39.3 Ci/mmol; $^3H_4$-[2,4-Pro-(3,4)]-SP, 58.6 Ci/mmol) from Amersham (Arlington Heights, IL, USA); these were HPLC-purified before use; nembutal was from Alco Health Services (Paducah, KY, USA), acetic and formic acids from Mallinckrodt (Paris, KY, USA), triethylamine (TEA) from Pierce (Rockford, IL, USA), acetonitrile from J. T. Baker (Phillipsburg, NJ, USA), and trifluoroacetic acid (TFA) from Fisher Scientific (Fair Lawn, NJ, USA).

A Packard 1900 CA liquid scintillation analyzer (Downers Grove, IL, USA) and ScintiVerse II scintillation fluid (Fisher Scientific) were used.

Rats

Sprague–Dawley rats (Harlan Sprague–Dawley Co., Madison, WI, USA; 500 males, 50 females; 200–250 g each) were used, and appropriate NIH laboratory animal guidelines were followed.

Experimental protocol

In general, the overall experimental protocol used in this study was as follows. Animals were sacrificed by decapitation and their whole heads were incubated according to the Spokes–Koch model [17]; pituitaries were dissected and homogenized; the homogenate was centrifuged at 31 000 *g* for 30 min at 4°C; the supernatant was placed onto an octadecylsilyl (ODS) silica mini-cartridge [19]; the peptide-rich fraction was eluted and the eluate was analyzed by RP-HPLC [18]; individual neuropeptide fractions were collected; and neuropeptides were measured by RIA. Because amino acids sequences were not determined in this study, the suffix"-like immunoreactivity" is required for all peptide measurements [10].

The experimental protocol for tissue incubation was divided into two separate time regions: time = 0, and the series of times = 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 72 h. The Spokes–Koch *post-mortem* human brain time–temperature cooling curve (Fig. 1) [17] was used for incubating the decapitated rat heads. In brief, temperatures were measured in the ventral thalamus region (brain core temperature) and below the parietal cortex surface (superficial temperature)

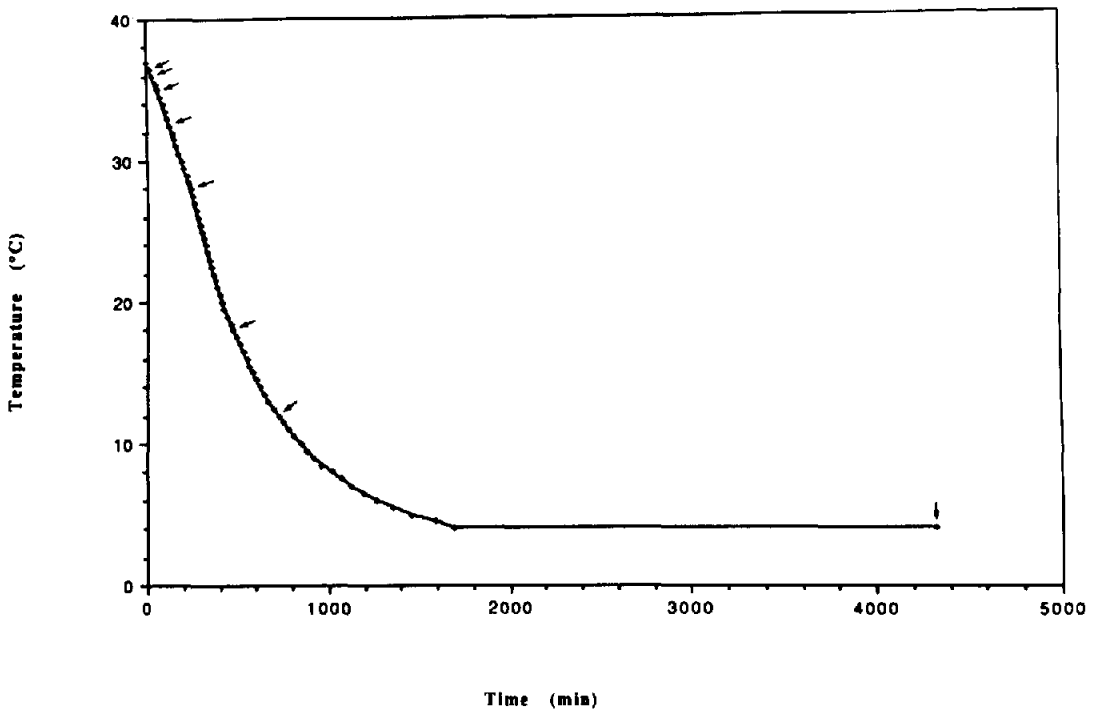

Fig. 1. *Post-mortem* time–temperature cooling curve used for incubation of rat pituitaries (Spokes and Koch, 1978). Arrows indicate the incubation times (0 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h and 72 h) used in this study. Individual dots indicate the manual temperature changes (n = 66).

in two cadavers. Body temperature at death (non-febrile) was assumed by those authors to be 37°C, and probe temperature was monitored after placing the cadaver in a 4°C mortuary refrigerator (one cadaver entered 3 h, and one 4 h after death). The resulting brain cooling curves were obtained, and were reproduced manually (see below) in this present study. For the time = 0 protocol to determine initial peptide-like immunoreactivity, 100 male and 50 female rats were used. The head was not decapitated, the rat was injected with nembutal (40 mg/kg), and the pituitary was quickly dissected, grouped into 10 pituitaries per group, collected in liquid nitrogen, and stored at −70°C.

In the second set of experiments, the whole rat head was immersed in saline and incubated for the indicated times (see the eight arrows on the curve in Fig. 1) with, for the first time, several time-points located within the initial, rapidly changing portion of the curve; the pituitary was dissected, collected into liquid nitrogen, and stored at −70°C.

For each incubation time, five sets of ten pituitaries were studied; each rat pituitary weighs approximately 8–10 mg. A saline bath (0.8%, stirred, 4 l) was used for incubation of the tissue, and the curve shown in Fig. 1 was followed, using manual temperature control of the bath *versus* time; temperature changes (n = 66) are indicated by the dots on the curve (Fig. 1). Considerable attention was focused on the accurate temperature control and on the collection of several samples during the early, rapidly changing portion of the cooling curve to define precisely the initial metabolism of the neuropeptides because other studies did not focus on that area and because we hypothesized that rapid metabolism would occur within that early *post-mortem* timeframe.

Tissue processing

The grouped pituitaries (n = 10) were weighed. The weight for the t = 0 sample was 79.0 ± 1.5 mg (n = 10). Pituitary tissues were homogenized in 1 M acetic acid (1%, w/v) and an aliquot (2%) was taken for total protein measurement. Rather than adding any enzyme inhibitors before tissue homogenization, acidification plus rapid temperature lowering stopped enzymatic processing.

Sample concentration procedure

A Sep-Pak ODS cartridge (Millipore-Waters, Bedford, MA, USA) was used to remove water-soluble endogenous substances such as proteins, saccharides, etc. from the tissue homogenate [19]. The entire supernatant from each experiment was applied to the Sep-Pak cartridge, which was previously washed with 4 ml of 0.1% TFA. The peptide-rich fraction was removed with a bolus of acetonitrile (3 ml, 100%). The eluent was evaporated, and the sample was lyophilized.

Reversed-phase high-performance liquid chromatography

Gradient HPLC [18] was performed on a Varian Model 5000 instrument (Palo Alto, CA, USA). The analytical column (Millipore-Waters; μBondapak C_{18}; 150 mm × 3.9 mm I.D.) was packed with ODS silica (particle size 10 μm). Sample was dissolved in 500 μl of the starting buffer, triethylamine formate (TEAF)–acetonitrile (40 mM TEAF, pH 3.0, 12% acetonitrile), and that entire volume was injected onto the analytical column. The gradient developed for this study was composed of the following segments of linear increases of organic modifier: 12–30% acetonitrile between 0 and 70 min; 30–100% between 70 and 80 min; and 100% between 80 and 90 min. The organic modifier was returned to its initial value (12%) within 10 min, and another 10 min were provided to re-establish UV baseline stability.

Determination of retention times for ME, SP and BE

Synthetic peptides. The retention time of each neuropeptide was determined in a separate study. The HPLC column was cleaned by gradient elution, and solutions of synthetic ME, SP and BE (1–2 μg each) were injected (n = 3 for each peptide). The following retention times were observed: 10.5 ± 0.2 min for ME; 30.0 ± 0.2 min for SP; and 46.5 ± 0.5 min for BE (see Fig. 2A).

After the injection of synthetic neuropeptides, the column was cleaned by gradient elution (n = 5) of buffer and organic modifier only. Absence of memory effects was verified [20] by collection of fractions at the appropriate retention times and by measurement of the peptide immunoreactivity co-eluting at those retention times. All values of remaining immunoreactivity were <8 fmol of peptide immunoreactivity, and thus were below the limit of detection.

Radiolabeled peptides and RIA. Two different experiments were performed to determine accurately the retention time of the endogenous neuropeptides eluted from a tissue homogenate. In one experiment (see Fig. 2B), a tritium-labeled peptide ([^{3}H]ME, [^{3}H]SP) was added to a tissue homogenate, and the neuropeptide (exogenous radiolabeled, endogenous unlabeled) was extracted and subjected to HPLC analysis. Liquid scintillation counting of the tritium-labeled peptide was used to determine the retention time of both radiolabeled and endogenous neuropeptide. In a second experiment (Fig. 2C), RIA was used to determine the retention time of the specific endogenous neuropeptide.

Two incubation samples (male rats M9 and M10) were used for the RIA measurements. Sample M9 was used for the RIA study only, and M10 was used for RIA plus the addition of [^{3}H]SP (0.2 μCi) and [^{3}H]ME (1 μCi). The purpose of using sample M9 was to demonstrate that the addition of tritiated peptides to M10 would not change the retention time. The radiolabeled

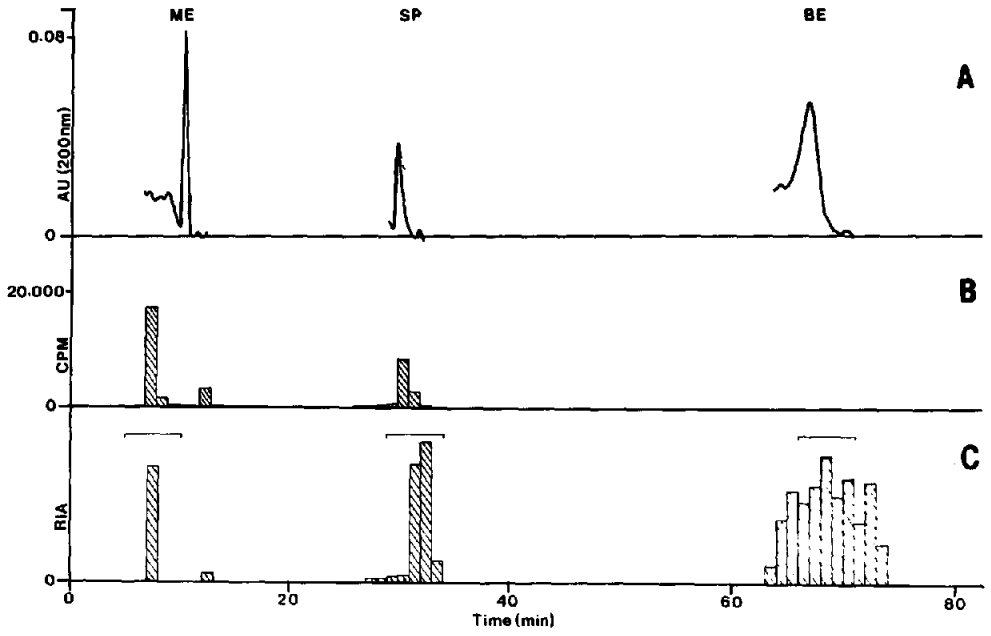

Fig. 2. RP-HPLC gradient separation of synthetic neuropeptides. (A) UV absorbance (200 nm); (B) [^{3}H]peptide (ME, SP); and (C) ME-li, SP-li, and BE-li. The horizontal brackets in C indicate those HPLC fractions that were collected for measurement of neuropeptide-li.

and RIA data that were used to locate the retention time of the endogenous neuropeptides (Fig. 2B and C) agreed closely with each other, but not with the synthetic peptide elution data (Fig. 2A). Therefore, the retention times determined in Fig. 2B and C were more appropriate for extracts from biological matrices.

For the time-course incubation studies, fractions at the different time intervals [6–10 for ME-like immunoreactivity (ME-li), 30–34 for SP-li, and 67–71 BE-li] were collected, for a total of a 5-min collection for each neuropeptide.

The 50 groups of samples (10 pituitaries in each group) were injected onto the HPLC column, followed by an extensive column rinse after each injection of a biological sample [20]. RIA performed on the post-column-cleaned fractions demonstrated column cleanliness. All values were <10 fmol peptide-li.

Recovery of tritiated peptides from pituitary tissue homogenate

Two HPLC-purified tritiated peptides, [^{3}H]ME (139 330 cpm) and [^{3}H]SP (91 312 cpm), were added to the tissue immediately before homogenization. The samples were incubated for 3 h. Following RP-HPLC separation, 43.2% of [^{3}H]ME ($n = 3$) and 55.8% of [^{3}H]SP ($n = 3$) were recovered. However, because tritiated BE was not available for this study, the RIA data were not corrected for the percentage recovery of peptide.

Commercially available RIA kits (IncStar, Stillwater, MN, USA) were used to measure ME-li, SP-li and BE-li. The cross-reactivities of the antibody in the ME kit towards synthetic peptides were 2.8% for LE, 0.10% for α-endorphin (β-LPH$_{61-77}$), and $<0.002\%$ for SP, BE, porcine dynorphin$_{1-13}$, and α-neo-endorphin. The cross-reactivities of the antibody in the SP kit toward synthetic peptides were $<0.002\%$ for ME, LE, eledoisin, and physalemin and 0.008% for BE. The cross-reactivities of the antibody in the BE kit toward synthetic peptides were 100% for human and rat BE, 5.6% for human β-LPH, and $<0.1\%$ for dynorphin, α-neo-endorphin, α-en-

dorphin, LE, ME, α-endorphin (β-LPH_{61-76}), α-endorphin (β-LPH_{61-77}), $ACTH_{1-39}$, $ACTH_{1-24}$, α-MSH, β-MSH, prolactin, luteinizing hormone, follicle-stimulating hormone, thyroid-stimulating hormone, vasopressin, and oxytocin.

Statistical analysis

The analysis of variance (ANOVA) according to the General Linear Models (GLM) procedure using SAS software (SAS Institute, Cary, NC, USA) was performed on these data to test for statistical significance. The null hypothesis (H_0) is that no significant difference exists between any sets of peptide measurements. The alternate hypothesis (H_1) states that at least two means are different. A significance level of $p = 0.05$ was used for comparison of pairs of measurements [21]. If H_1 were to be accepted, then all possible probability values for the hypothesis H_0 were evaluated.

RESULTS

Reversed-phase liquid chromatography

Fig. 3 shows a representative example of the HPLC gradient separation of the peptide-rich fraction obtained from one of the *post-mortem* rat pituitary samples (M10). The three short horizontal bars along the top of the chromatogram indicate the HPLC fractions that were collected for ME, SP and BE according to the retention times determined in Fig. 2C. Because all samples in this study were obtained from similar rat pituitaries, all of the chromatograms obtained in this study are remarkably equivalent.

Even though several UV-absorbing peaks may be collected within an individual HPLC fraction (see, for example, the ME and SP fractions), RIA provides an increased level of detection sensitivity (but not molecular specificity) [10–14,22,23] towards each neuropeptide contained in that fraction. The level of UV detection sensitivity is

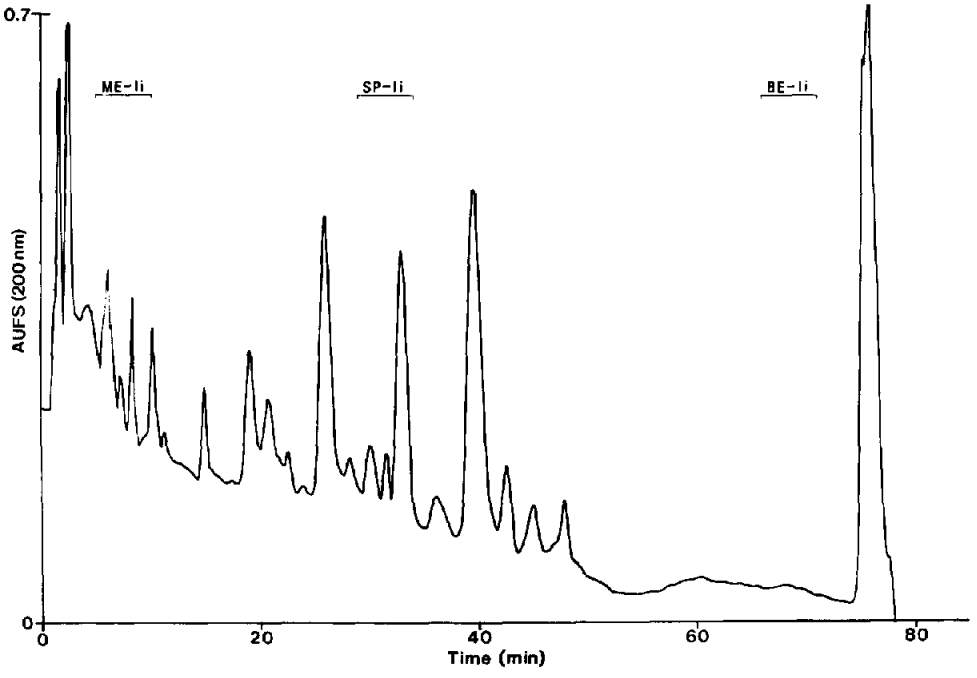

Fig. 3. RP-HPLC gradient separation of the peptide-rich fraction obtained from rat pituitary *post-mortem* sample M10. The three indicated fractions were collected for analysis.

TABLE I

ME-li, SP-li, AND BE-li MEASUREMENTS *VERSUS POST-MORTEM* INCUBATION TIME

Values are averages ± S.E.M. (n = 5).

Time (min)	ME-li[a]	SP-li[a]	BE-li[b]
0	124 ± 8	126 ± 10	148 ± 11
15	667 ± 154	160 ± 18	221 ± 26
30	363 ± 89	155 ± 10	229 ± 9
60	280 ± 83	145 ± 10	166 ± 6
120	437 ± 23	133 ± 15	136 ± 3
240	547 ± 52	103 ± 22	138 ± 4
480	329 ± 19	132 ± 25	152 ± 8
720	105 ± 29	101 ± 5	152 ± 11
4320	128 ± 44	85 ± 7	90 ± 18

[a] fmol neuropeptide-li/mg protein.
[b] pmol neuropeptide-li/mg protein.

usually in the pico- to nanomole range, and RIA sensitivity in the femtomole range.

Individual neuropeptide-li measurements

Table I summarizes the individual analytical measurements of ME-li, SP-li and BE-li *versus post-mortem* incubation time. The ME-li and SP-li data are given as average ± S.E.M. fmol (10^{-15} mol)/mg protein and BE as pmol neuropeptide-li/mg protein (n = 5). The suffix (-li) is required in these data because no animo acid sequences have been determined [10,11]. ANOVA demonstrated that significant between-group differences occur in all three neuropeptide-li measurements ($p < 0.0001$ for BE-li and ME-li, $p < 0.05$ for SP-li). Table II contains the list of significant differences found ($p < 0.05$). The male *versus* female difference was significant for BE-li ($p < 0.0001$).

Time-course profiles of neuropeptide-li measurements

The individual neuropeptide-li data are plotted over a short (0–300 min; Figs. 4A, 5A and 6A) and a long (0–4320 min; Figs. 4B, 5B and 6B) time-frame; the shorter range demonstrates clearly the early, rapid changes that occur in neuropeptide-li concentrations. Each data point plots average ± S.E.M. (n = 5). This report is the first

TABLE II

LIST OF TIMES AT WHICH SIGNIFICANT DIFFERENCES FOR INDIVIDUAL CONCENTRATIONS OF NEUROPEPTIDE-li WERE FOUND *VERSUS* TIME 1

p = 0.05 is significant.

	Time 1 (min)	Time 2 (min)
ME-li[a]	0	15, 30, 120, 240, 480
	15	0, 30, 60, 120, 480, 720, 4320
	30	0, 15, 240, 720, 4320
	60	15, 120, 240
	120	0, 15, 60, 720, 4320
	240	0, 30, 60, 480, 720, 4320
	480	0, 15, 240, 720, 4320
	720	15, 30, 120, 240, 480
	4320	15, 30, 120, 240, 480
SP-li[a]	0	4320
	15	240, 720, 4320
	30	240, 720, 4320
	60	240, 720, 4320
	120	4320
	240	15, 30, 60
	480	4320
	720	15, 30, 60
	4320	0, 15, 30, 60, 120, 480
BE-li[b]	0	15, 30, 4320
	15	0, 60, 120, 240, 480, 720, 4320
	30	0, 60, 120, 240, 480, 720, 4320
	60	15, 30, 4320
	120	15, 30, 4320
	240	15, 30, 4320
	480	15, 30, 4320
	720	15, 30, 4320
	4320	0, 15, 30, 60, 120, 240, 480, 720

[a] Male *versus* female not significant at time = 0.
[b] Male *versus* female significant at time = 0.

description of the changes observed during the shorter time period, whereas the data from the long-term observations correspond to previous studies.

In the ME-li data (Fig. 4A), local concentration maxima occur at 15 and 240 min, and a minimum at 60 min (see also Table I). In the SP-li data (Fig. 5A), a maximum occurs at 15 min and a minimum at 240 min; and in the BE-li data (Fig. 6A), local maxima occur at 30 min and at 480 and 720 min, and a minimum at 120 min. The three plots of the longer incubation time indicate

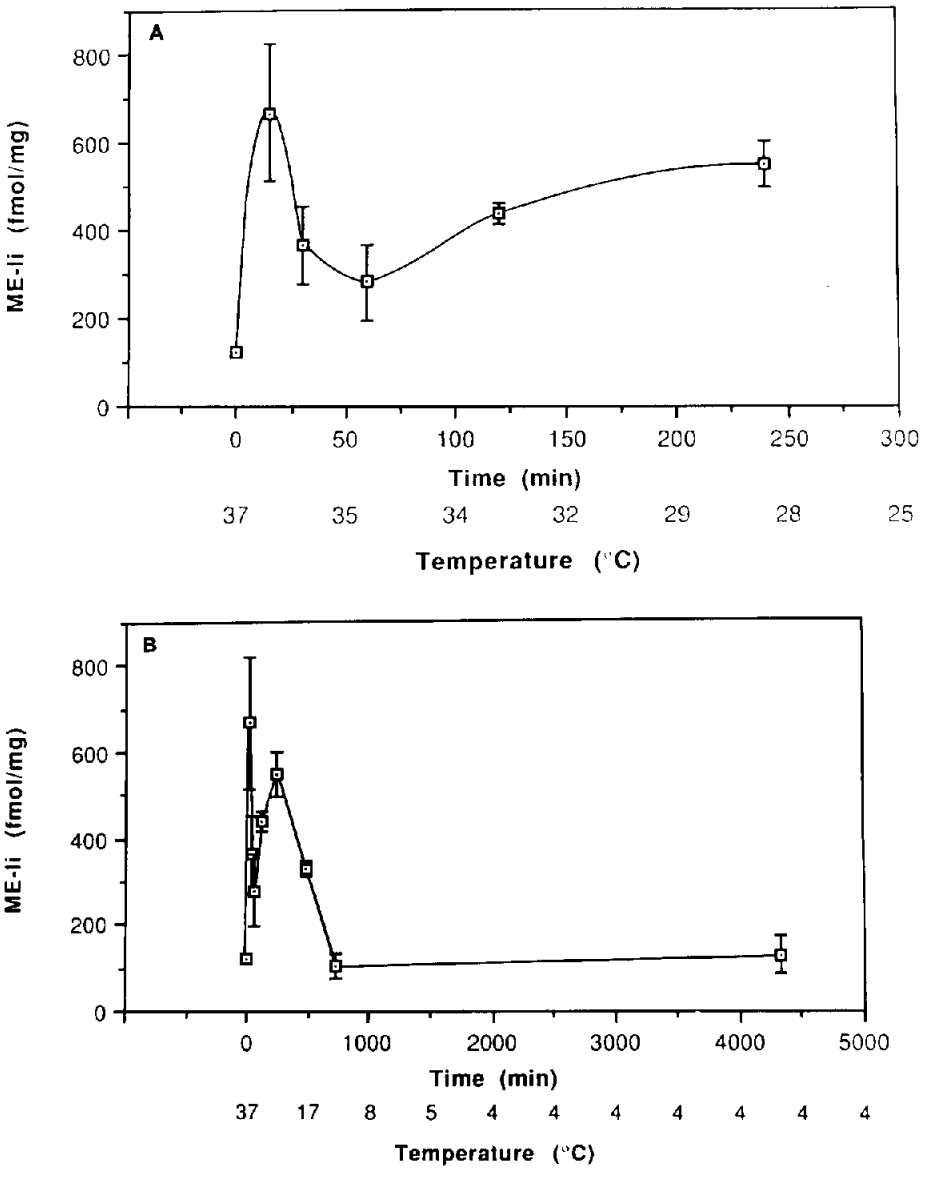

Fig. 4. Plot of ME-li *versus* time: (A) 0–300 min; (B) 0–4320 min. ME-li (fmol ME-li/mg protein) was measured at the indicated times and temperatures.

rather gentle, long-term increases (ME-li) and decreases (SP-li and BE-li). These long-term data are similar to those data published by others [5–7,12,15].

DISCUSSION

Significant and rapid changes of the proenkephalin A, tachykinin, and POMC neuropeptide-li contents in the pituitary are reported for the first time to occur within 1 h *post-mortem* (see Figs. 4–6 and Tables I and II). Relatively rapid increases and decreases were found for those peptides. The Spokes–Koch model for brain-cooling was used to study *post-mortem* stability in the rat pituitary of the neuropeptides ME-li, SP-li, and BE-li. The tabulated (Tables I and II) and plotted (Figs. 4–6) data demonstrate clearly that signif-

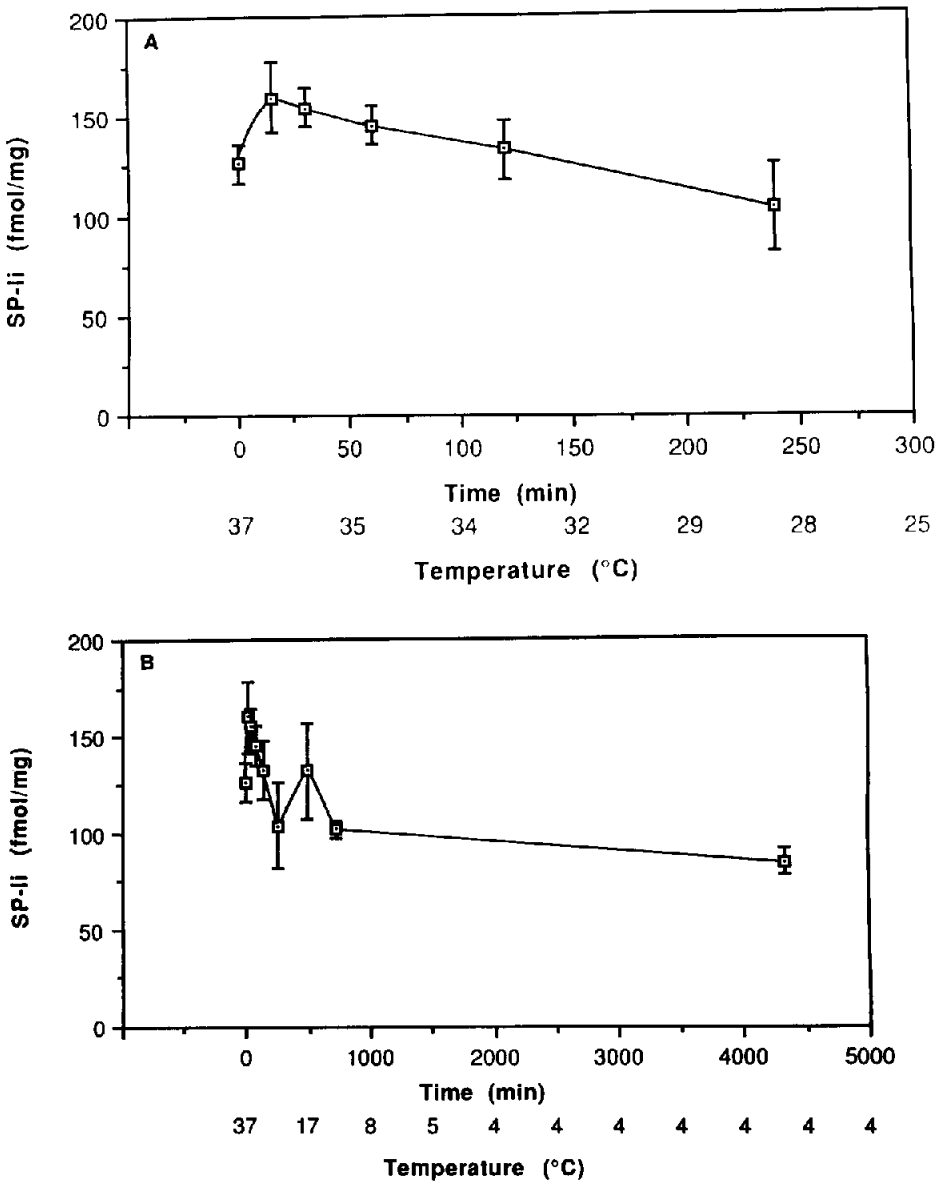

Fig. 5. Plot of SP-li *versus* time: (A) 0–300 min; (B) 0–4320 min. SP-li (fmol SP-li/mg protein) was measured at the indicated times and temperatures.

icant changes occur in the neuropeptide-li measured in these *post-mortem* pituitary tissue samples, especially in the earlier, previously uninvestigated time period (< 1 h). For example, the ME-li data (Fig. 4A and B) demonstrate four separate regions where a statistically significant change in ME-li occurs: a rapid increase from 0 to 15 min, a rapid decrease from 15 to 60 min, a slow increase from 60 to 240 min, and a slow decrease from 240 to 720 min. Thus, it is evident that various portions of proenkephalin A within the neuropeptidergic system continue to undergo *post-mortem* metabolism (*de novo* synthesis, processing of higher molecular mass intermediate precursors, degradation). For example, rapid synthesis of ME (measured as ME-li) continues for about 0–15 min, presumably due to a continuing metabolic excision of ME from proenkepha-

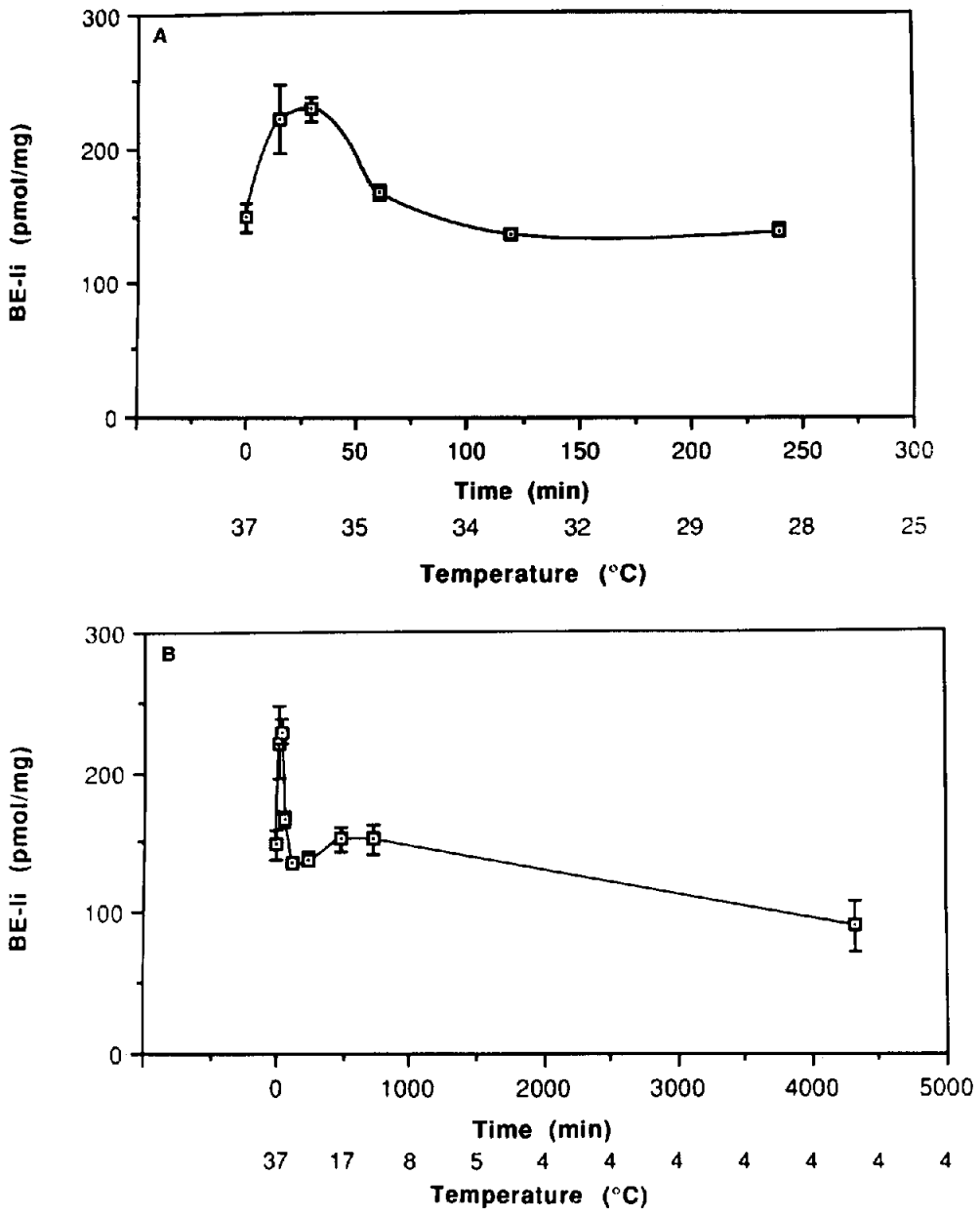

Fig. 6. Plot of BE-li *versus* time: (A) 0–300 min; (B) 0–4320 min. BE-li (fmol BE-li/mg protein) was measured at the indicated times and temperatures.

lin A and from variously sized ME-containing precursors [23,24]. From 15 to 60 min, degradation occurs, presumably from the action of various aminopeptidases, enkephalinase, dipeptidylcarboxypeptidases. etc.

The slower increase in synthesis from 60 to 240 min seems to be somewhat surprising, yet may arise from a second wave of metabolism of precursors, possibly from those precursors that required a longer time to travel the entire length of the axon and that were located more proximal to the cell body at the time of death. This last feature could only be observed owing to our experimental design of keeping the pituitary within the decapitated head during incubation in a bath.

In addition to the statistically significant changes found for the tachykinin system (SP), a maximum of SP-li occurs at 15 min and a minimum at 240 min. Those data probably indicate a rapid (t = 0–15 min) increase in the *post-mortem* synthesis of the tachykinin neuropeptide, SP. A similar *post-mortem* profile of SP-li, although displaced later in time, was found in a study of the mouse brain maintained at 28°C [15].

The POMC neuropeptide, BE, data demonstrate three regions of statistically significant changes in BE-li concentration, a rapid increase in synthesis (0–30 min), and two lower rates of degradation (30–120 and 720–4320 min).

These collective data also indicate the relative expression of proenkephalin A, protachykinin, and POMC, as represented by the three neuropeptides ME, SP, and BE measured in this study as ME-li, SP-li, and BE-li, respectively. For example, the ME-li concentration maximizes at 15 min to 667 ± 154 fmol ME-li/mg protein (average ± S.E.M., n = 5), SP-li at 15 min to 160 ± 18 fmol SP-li/mg protein, and BE-li to 30 min at 229 ± 9 pmol BE-li/mg protein. Assuming that the three measured peptide-li represent only the three corresponding neuropeptides, the total pituitary content of each neuropeptide equals the sum of the individual measurements under the corresponding amount *versus* time curve. On the other hand, at the three curve maxima, the processing of the proenkephalin A, tachykinin, and POMC precursors to the three neuropeptides studied is represented by the molar ratio 4.2:1:1400. It is important to realize that it is possible that the ratio among other intermediate peptides within these three neuropeptide systems would display a different ratio at different timepoints.

These conclusions differ from other studies that conclude that neuropeptides are stable for up to 72 h. For example, the *post-mortem* stability of NK-A-li was confirmed for at least 48 h after death, even when brains were left at room temperature [5]. After 48 h, the NK-A-li decreased to a value of approximately 50%. The authors speculated that the reason for that stability might be due to the fact that neuropeptides are packaged in vesicles, and would therefore be protected against cytoplasmic peptidase activity. *Post-mortem* stability of SP-li showed a >30% loss at 4 and 28°C [15]. The authors state that the effect of the different temperatures on the *post-mortem* content of the peptide is unexpected because lysis in *post-mortem* tissue would be expected to cause a gradual degradation of peptides, and that the degradation should be slower at lower temperatures. They also state that it is theoretically possible that a more immunoreactive fragment of SP was generated during the process of degradation, and that this fragment might be more apparent at the higher temperature. ME and SP were both studied in *post-mortem* human brain, and those two neuropeptides showed no significant change in contents over a 72-h period [12]. Another study applied chromatographic characterization of neuropeptides in *post-mortem* human brain studies [8].

The *post-mortem* stability of neuropeptide Y-li was studied in a rat model simulating human autopsy conditions. No significant differences were found in the content within the cortex, hippocampus, and striatum at several time points (0, 1, 4, 8, 12, and 24 h). As indicated above, several workers suggest *post-mortem* stability for several neuropeptides. In a review [6], it was stated that many studies show no correlation of peptide concentration with *post-mortem* delay. Numerous peptides such as SP, somatostatin, cholecystokinin$_{1-8}$ (CCK$_{1-8}$), ME, thyroid-releasing hormone (TRH), growth hormone-releasing hormone (GRH), neurotensin, vasoactive intestinal peptide (VIP), and neuropeptide Y were all shown to be stable over time periods up to 72 h *post-mortem*.

Microdialysis revealed a *post-mortem* increase in the release of opioid peptides, following neck vessel severance, in the rat globus pallidus/ventral pallidum [25].

Therefore, the data in this manuscript do not agree with the conclusions of many of the previous publications. However, the differences are relatively easy to rationalize. One explanation for that difference is that in our study more samples were taken during the initial rapid temperature

decrease of the cooling curve and therefore, a greater level of accuracy in monitoring the neuropeptide metabolism. For example, as indicated above for SP, another paper indicated some decrease, but that timeframe was shifted to later times compared to Figs. 4–6 [15].

The obvious limitation to the present study is, for example, that HPLC plus RIA cannot determine the amino acid sequence of a peptide [10,11], and thus molecular specificity has not been optimized. However, our other experiments aim towards that direction [10,11,13,14,22].

These present experiments demonstrate multiple synthetic and degradation processing of three neuropeptides within 0–12 h in a rigorously calibrated experiment. The SP-li and ME-li measurements indicate a stability of those neuropeptides between 12 and 72 h, whereas BE decreases (rate of degradation > rate of synthesis) approximately 40% between 12 and 72 h. Thirdly, the neuropeptide-li studied in this set of experiments was HPLC-purified, and thus a higher level of confidence is conveyed by these experiments that only one neuropeptide may have been measured.

It is significant that, in experiments of less than 12 h incubation, it was easy to dissect the pituitary away from the surrounding tissue. However, the 72-h experiments indicated tissue degradation because the tissue was softer, morphological changes had started, and it was more difficult to separate the meninges from the soft pituitary mass.

The *post-mortem* stability data of neuropeptides reported in this paper are of fundamental importance for our studies on the qualitative and quantitative analysis of neuropeptides in human pituitary tumors [9–11,13,14,22]. Thus, the question addressed in this present study was the accurate definition of the *post-mortem* stability of neuropeptides contained in tissues because many enzymatic and chemical systems continue to function. For example, even though death has occurred, synthesis and processing of intermediate-sized precursors can continue within the cell body–axon–synapse axis, and thus degradation of the neuropeptides can proceed. With time, of course, the latter reactions will dominate. Although some studies have suggested *post-mortem* stability up to 72 h of the total immunoactivities of several peptides [5–7,12,15], the limitations of those studies are the lack of any HPLC purification of individual peptides, the possible cross-reactivity of the antibodies used to measure peptide-like total immunoreactivity, and insufficient data accumulated within the immediate *post-mortem* time where more rapid metabolic (synthetic and degradation) changes can occur.

The advantages of the present rat pituitary study are: (1) the ready availability of rats and the easy temperature control of *post-mortem* incubation; (2) it is an appropriate reference for human *post-mortem* pituitary (the study of *post-mortem* stability of neuropeptides in human pituitaries is impossible generally within the initial few hours, but it is possible to incubate the rat pituitary as it remains within the decapitated head, surrounding tissues, and connections); and (3) human and rat pituitaries are similar because both contain similar biologic mechanisms and biochemical pathways. Also, HPLC purification preceded measurement of neuropeptide immunoreactivity to minimize cross-reactivity problems. The disadvantage for using the rat pituitary is that species differences exist in the rat *versus* human pituitary (for example, the human lacks the neurointermediate lobe).

It is important to rationalize the metabolic basis for the rapid changes of the amounts of the three neuropeptides observed in these studies. For example, the ME-li (Fig. 4) and the BE-li (Fig. 6) increased and then decreased before reaching an area of stability. These two neuropeptides are cleaved directly from their respective precursors by the action of a trypsin-like activity followed by a carboxypeptidase B-like activity [26]. On the other hand, SP-li decreased then increased before reaching an area of stability. It is possible to rationalize that difference for the tachykinin peptide by remembering that the peptidylglycine-amidating monooxygenase (PAM) enzyme system required for the synthesis of SP from its precursor uses a different type of peptide processing, wherein trypsin-like activity produces the N-terminus of SP, and another enzyme ami-

dates the C-terminal portion of SP. That more complicated carboxamide synthesis system requires cupric ions (Cu^{2+}), ascorbate, and molecular oxygen. Incidentally, the ascorbate concentration in the pituitary is very high [27].

These experimental data reject our null hypothesis for the proenkephalin A, tachykinin, and POMC neuropeptides. Thus, the alternative hypothesis is accepted for the proenkephalin A, tachykinin, and POMC neuropeptidergic systems in the rat pituitary; namely, significant changes in ME-li, SP-li, and BE-li, respectively, do occur in the early, rapidly changing, portion of the *post-mortem* cooling curve.

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REFERENCES

1 S. L. Asa, E. Horvath, K. Kovacs and C. Ezrin, in W. D. Odell and D. H. Nelson (Editors), *Pituitary Tumors*, Futura, Mt. Kisco, NY, 1984, p. 71.
2 J. E. Maggio, *Ann. Rev. Neurosci.*, 11 (1988) 13.
3 J. E. Krause, M. R. McDonald and Y. Takeda, *BioEssays*, 10 (1989) 62.
4 H. Kotani, M. Hashimaru, H. Nawa and S. Nakanishi, *Proc. Natl. Acad. Sci. U.S.A.*, 83 (1986) 7074.
5 H. Arai, P. C. Emson and L. H. Carrasco, *Ann. Neurol.*, 22 (1987) 587.
6 M. F. Beal and J. B. Martin, *Ann. Neurol.*, 20 (1986) 547.
7 M. F. Beal, M. F. Mazurek, L. J. Lorenz, G. K. Chattha, D. W. Ellison and J. B. Martin, *Neurosci. Lett.*, 64 (1986) 69.
8 J. A. Biggins, J. R. McDermott, A. I. Smith, J. M. Candy, R. H. Perry, D. E. Bateman and J. A. Edwardson, *J. Neurol. Sci.*, 66 (1984) 47.
9 D. M. Desiderio, R. C. Cezayirli, G. Fridland, J. T. Robertson and H. Sacks, *Life Sci.*, 37 (1985) 1823.
10 D. M. Desiderio (Editor), *Mass Spectrometry of Peptides*, CRC Press, Boca Raton, FL, 1991.
11 D. M. Desiderio (Editor), *Mass Spectrometry: Clinical and Biomedical Applications*, Vol. 1, Plenum Press, New York, 1993.
12 P. C. Emson, A. Arregui, V. Clement-Jones, B. E. B. Sandberg and M. Rossor, *Brain Res.*, 199 (1980) 147.
13 J. J. Kusmierz, R. Sumrada and D. M. Desiderio, *Anal. Chem.*, 62 (1990) 2395.
14 J. Kusmierz, C. Dass, J. T. Robertson and D. M. Desiderio, *Int. J. Mass Spectrom. Ion Proc.*, 111 (1991) 247.
15 G. P. McGregor and S. R. Bloom, *Life Sci.*, 32 (1983) 655.
16 E. K. Perry and R. H. Perry, *Life Sci.*, 33 (1983) 1733.
17 E. G. S. Spokes and D. J. Koch, *J. Neurochem.*, 31 (1978) 381.
18 G. Fridland and D. M. Desiderio, *J. Chromatogr.*, 379 (1986) 251.
19 T. Higa and D. M. Desiderio, *Int. J. Pept. Prot. Res.*, 33 (1989) 250.
20 S. Harajiri, G. Wood and D. M. Desiderio, *J. Chromatogr.*, 575 (1992) 213.
21 R. R. Sokal and F. J. Roth, *Biometry: The Principles and Practice of Statistics in Biological Research*, W. H. Freeman, New York, 2nd ed., 1981, p. 859.
22 D. M. Desiderio, J. J. Kusmierz, X. Zhu, C. Dass, D. Hilton, J. T. Robertson and H. S. Sacks, *Biomed. Mass Spectrom.*, 22 (1993) 89.
23 J. L. Lovelace, *Ph.D. Thesis*, University of Tennessee, Memphis, TN, 1991.
24 F. S. Tanzer, E. Tolun, G. H. Fridland, C. Dass, J. Killmar, P. W. Tinsley and D. M. Desiderio, *Int. J. Pept. Prot. Res.*, 32 (1988) 117.
25 N. T. Maidment, B. Siddall, V. D. Rudolph and C. D. Evans, *J. Neurochem.*, 56 (1991) 1980.
26 R. E. Mains, C. C. Glembotski and B. A. Eipper, *Endocrinology*, 114 (1984) 1522.
27 V. May and B. A. Eipper, *J. Biol. Chem.*, 260 (1985) 16224.