Enzymatic cleavage of pro-opiomelanocortin by anterior pituitary granules

Evidence for a chymotryptic-like enzyme

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This paper presents the results obtained when pig anterior pituitary granule lysates are incubated with rat pro-opiomelanocortin (POMC). The resultant peptides were analyzed by 3 systems of high-performance liquid chromatography. This approach, when coupled with microsequence analysis of the conversion products, allowed the unambiguous identification of a major chymotryptic-like activity (pH optimum around 8) associated with the granule lysates with a specificity directed towards selective Tyr-X and Phe-X bonds within the POMC molecule. Furthermore, these results also demonstrate that although the detected enzyme activity gives rise to products 'resembling' those expected, the characterization of the 'elusive' maturation enzyme responsible for the cleavage at pairs of basic residues remains to be critically evaluated.

Pro-opiomelanocortin *Maturation enzyme* *Specificity* *Pituitary granule* *High performance liquid chromatography* *Microsequence*

1. INTRODUCTION

Pro-opiomelanocortin (POMC), the pluripotent common precursor of ACTH, β-endorphin, MSH's and β-LPH, is synthesized in the pars distalis, pars intermedia of the pituitary and in the hypothalamus of all species studied so far [1–5]. Sequential proteolytic cleavages of POMC convert the seemingly identical precursor molecules into tissue-specific collection of maturation products of varied biological activities [2,5–9]. In the anterior lobe of the pituitary, POMC is converted into an N-terminal glycopeptide (NT) [1,5,9], a joining peptide (JP) [6], ACTH, β-LPH and to a lesser extent into γ-LPH, β-endorphin and possibly β-MSH [1,2,8]. In contrast, the major maturation products in the pars intermedia of the pituitary require a possible further maturation of the N-terminal glycopeptide [10,11], the production of α-MSH and CLIP from ACTH and the formation of γ-LPH and a collection of β-endorphin-like peptides [1–3,7,8,11]. In all cases, the sites of cleavage are represented by a pair of basic residues, most commonly Lys–Arg or Arg–Arg [1,4,5,8–11]. This signal of pairs of basic residues required for proteolytic conversion has been originally observed for β-LPH to γ-LPH conversion [12], and is now known to be common to most pro-hormone conversion processes [8,13,14].

Recent evidence has been accumulated in favor of the secretory granules as being one of the major subcellular organelles responsible for such pair of basic residues cleavage. This was reported for proinsulin [15] and for POMC [16,17]. Furthermore, post-translational modifications such as acetylation [18] and amidation [19] were also reported to occur within the secretory granules. This report presents data obtained upon incubation of POMC [1,11,20] with anterior pituitary granules and

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unambiguous characterization of the cleavage products by microsequencing.

2. MATERIALS AND METHODS

Reverse phase HPLC and molecular sieving HPLC methods used for the purification of the conversion products have been described [21,22].

The purification of de novo biosynthetically [^{3}H]Leu and [^{3}H]Phe labeled rat pars intermedia POMC and its in vitro maturation products obtained after either a 1 h or a 4 h pulse labeling was achieved as in [21,22]. The identity of the in vitro maturation products was confirmed by microsequencing [1,5,8,11].

From 100 freshly dissected pig anterior pituitary lobes, the purification of the secretory granules (fraction P3A10) was achieved by Percoll (Pharmacia) fractionation, and followed by a radioimmunoassay against β-endorphin as in [21].

For the pH study, secretory granules obtained from the Percoll fraction 10 (P3A10, see above) were osmotically lysed for 1 h at 37°C by incubation with stirring of 50 μl P3A10 (1 mg protein) to which 200 μl various buffers were added. The buffers used were 0.1 M sodium formate (pH 3.0), 0.1 M sodium acetate, pH 4.0, 4.75, 5.0, 5.25, 5.5, 6.0 and 7.0; 0.1 M NH_4HCO_3 for pH 8.0, 8.5; and 0.1 M $(NH_4)_2CO_3$ for pH 9.0. The lysed granule preparation was then added to an Eppendorf tube containing dried 20000 cpm of [^{3}H]Leu POMC [21,22] and incubation was allowed to proceed for 5 h at 37°C. The reaction was then stopped by adding acetic acid and guanidine/HCl (final conc. 1 M and 1.5 M, respectively). This was then centrifuged at 100000 × g_{av} for 1 h to remove the Percoll and membrane components and the supernate was applied to the Protein Analysis HPLC columns for molecular sieving separation.

To obtain sufficient counts for microsequence determination of the nature of the conversion products of radiolabeled POMC by secretory granule lysates, two large scale incubations were performed: 200 μl P3A10 were osmotically lysed with 800 μl 0.1 M NH_4HCO_3 (pH 8.0) for 1 h as described above. This preparation was then added to 1 × 10^6 cpm of either [^{3}H]Leu or [^{3}H]Phe HPLC-purified POMC, pre-dried on a speed vac concentrator (Savant). The incubation was performed for 8 h, at 37°C with stirring and the reaction stopped as described above. The resulting supernatant was applied on a G-75 superfine column (0.9 × 100 cm) eluted with 1 M acetic acid at 4°C, as in [21,22]. Aliquots of the resultant fractions were counted, the material under each peak was then repurified by reverse phase HPLC. Microsequencing was then performed on each HPLC-purified peak as described below.

Amino acid sequence analysis of radiolabeled peptides was performed on a Beckman 890C sequenator, using 0.3 M Quadrol in the presence of 3 mg polybrene and 2.5 mg sperm whale apomyoglobin as carrier [9,20]. The resultant thiazolinone residues at each sequenator cycle were then counted in Aquasol-2 as in [20].

3. RESULTS

3.1. *Purification of pig anterior pituitary secretory granules*

The distributions of lysosomal (β-glucuronidase activity [23]), mitochondrial (monoamine oxidase activity [24]), protein (Bio-Rad protein assay), and the RNA, DNA [25] along the subcellular fractions and the Percoll density gradient showed that the ratio of β-endorphin-like material over protein is maximal in P3A10 [21], representing a granular fraction with an apparent density of 1.084 g/ml. Some lysosomal activity was also found in this fraction but it did not exceed 1% of total lysosomal activity.

Based on the observed elution pattern on gel permeation HPLC [21] and by comparison with naturally occurring markers, it appears that the optimal specific conversion of POMC into molecules of similar size to the natural products occurs around pH 8. The quantitation of the specific conversion, defined as [cpm converted POMC]/[cpm unconverted POMC], is shown in fig.1. Although it can be seen that an important conversion does occur over pH 4–5, this conversion appears to be less specific than at pH 8 due to the formation of very small molecular weight products (eluting after α-MSH), and upon comparison of the elution position of the in vitro conversion products with that of the natural markers [21].

3.2. *Characterization of POMC conversion products at pH 8*

The preparative purification by Sephadex G-75

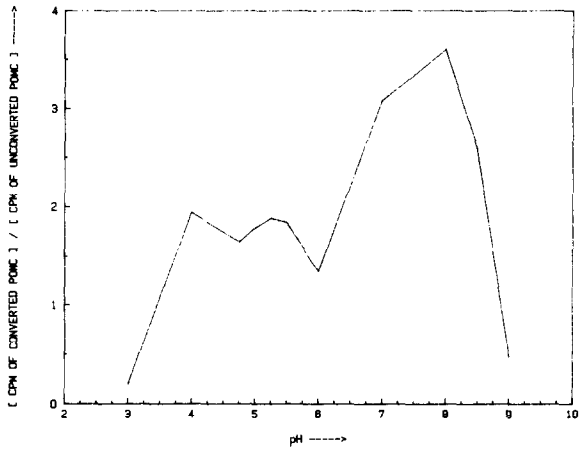

Fig.1. Calculated pH dependence of specific conversion of POMC by secretory granule lysates. The specific conversion is defined as cpm of converted POMC/CPM of unconverted POMC.

superfine of [^{3}H]Leu-labeled conversion products obtained at pH 8.0 is shown in fig.2 (upper panel). This allowed the separation of 7 peaks (denoted I_L to VII_L). The material under each peak was pooled and repurified by reverse phase HPLC in HFBA/*n*-propanol system [21] as shown in the lower panel of fig.2: the elution position of the natural marker peptides of similar size is also shown (fig.2). The material under each peak which appeared to coelute with the rat marker peptides, was subsequently repurified in the TFA/*n*-propanol system [21]. In this system, apparent coelution with the rat marker similarly sized molecules also occurred, thereby providing the source of peptides used for microsequence determination.

From fig.2, it can be seen that incubation of rat POMC with pig anterior pituitary granule lysates at pH 8, results in conversion products eluting close to the position of in vitro rat POMC maturation products. These include the NT-ACTH, NT-JP, β-LPH and γ-LPH marker peptides (fig.2, middle and lower panels, peaks 2, 3, 5, 6, 8). However, no peak appeared to coelute with β-endorphin. Moreover, it can be noticed that peak 5 elutes slightly later than authentic rat β-LPH marker (fig.2, panel IV_L).

An identical approach was also used for the purification of [^{3}H]Phe-labeled POMC conversion products, and gave similar results (not shown).

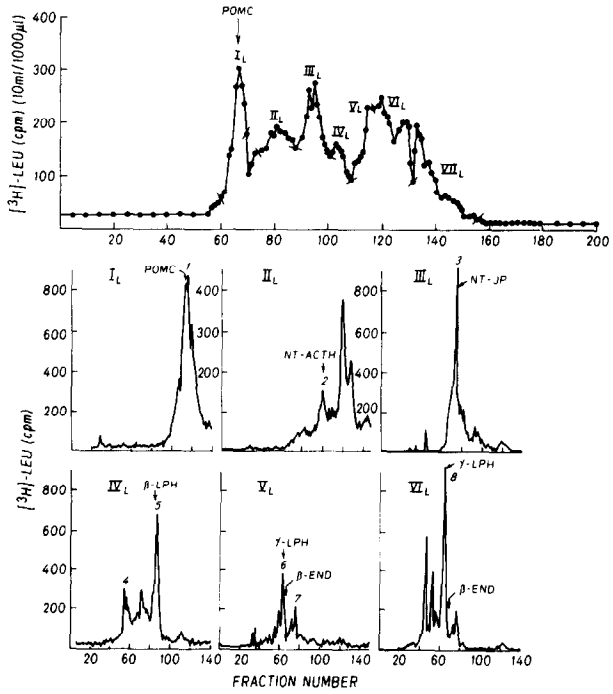

Fig.2. Upper panel: Preparative Sephadex G-75 superfine chromatography of cleavage products obtained following incubation of 10^6 cpm of [^{3}H]Leu POMC with P3A10 at pH 8.0 (section 2). The 7 peaks resolved are denoted I_L–VII_L, where the subscript L denotes [^{3}H]Leu-labeled peptides. The fraction size is 1 ml, and an aliquot of 10 µl was counted. Lower panel: The repurification of peaks I_L–VII_L by reverse-phase HPLC using a 5 µM ODS column eluted using the HFBA/*n*-propanol system [21]. The elution positions of rat marker peptides are shown here for comparative purposes. The material under peaks 1–8 was repurified using the same column by reverse-phase HPLC using the TFA/*n*-propanol system [21], before microsequence determination. The size of each fraction is 0.5 ml and an aliquot of 25 µl was counted.

This provided [^{3}H]Phe-labeled conversion products for further confirmation of the microsequence identification of the nature of peptides 4, 6 and 7 (fig.2, table 1).

3.3. *Microsequence identification of POMC conversion products*

Table 1 and fig.3 summarize the [^{3}H]Leu and [^{3}H]Phe microsequence data of the peptides under peaks 1–8 defined in fig.2. The [^{3}H]Leu amino acid sequence of peptides 1, 2 and 3 clearly showed

Table 1

Microsequence results of [^{3}H]Leu- and [^{3}H]Phe-labeled peptides 1–8 (see fig.4, middle and lower panels)

Peak no.	Sequence positions		Peptide starts from POMC residue no.	Probable identification	Probable cleavage site
	[^{3}H]Leu	[^{3}H]Phe			
1	3,11,17,18		1	POMC (1–209)	
2	3,11,17,18		1	POMC (1–136)	136 ↓ 137 Phe–Lys
3	3,11,17,18		1	POMC (1–99)	99 ↓ 100 Tyr–Ser
4		5,10	52	POMC (52–)	52 ↓ 52 Tyr–Val
5	4,12,16		137	POMC (137–209)	136 ↓ 137 Phe–Lys
6		5	100 (or) 164	POMC (100–136) or (164–)	99 ↓ 100 Tyr–Ser or 163 ↓ 164 Tyr–Arg
7		14	169	POMC (169–209)	168 ↓ 169 Phe–Arg
8	4,12,16		137	POMC (137–168)	136 ↓ 137 Phe–Lys

The identification of the peptides and the deduction of the cleavage site is also shown, based on the nucleotide [27] and protein [20,26,28] sequences of rat POMC and related peptides. The numbers in parentheses represent the residue positions along the proposed protein sequence [27]. No Leu could be detected during the first 20 cycles in the sequence of peptides 4, 6 and 7. For peptides 4 and 6, the (52–) and (164–) mean that no carboxy-terminal identification could be deduced, although these peptides start at residues 52 and 164 of POMC, respectively

that they contain the NH_2-terminal segment of POMC, since leucine appears at residues 3, 11, 17 and 18 [26–28] (fig.3). These results taken together with the app. M_r-values on Sephadex G-75 superfine (fig.2, upper panel), and their elution position on reverse phase HPLC in either HFBA/*n*-propanol or TFA/*n*-propanol of these peptides, allowed their identification as authentic unconverted POMC for peak 1 (residues 1–209 of POMC, fig.3); NT-ACTH for peak 2 (POMC residues 1–136); and NT-JP-like for peak 3 (POMC residues 1–99).

Table 1 also shows that peptides 5 and 8 contain leucine at residues 4, 12 and 16. Based on the known leucine sequence of rat β-LPH, γ-LPH and POMC [20,26–28], it is clear that these two peptides contain the NH_2-terminal sequence of either β-LPH or γ-LPH plus two residues (since the natural form of these peptides contain leucine at positions 2, 10 and 14; fig.3). From the reported DNA sequence of rat POMC [27] and the elution position of these peptides on either HPLC or Sephadex G-75, it can be inferred that peak 5 represents Lys–Arg–β-LPH (POMC residues 137–209; see fig.3). Using the above arguments, added to the fact that no β-endorphin-like material was observed, peak 8 was identified as a Lys–Arg–γ-LPH related peptide. Both of these peptides imply a Phe↓Lys cleavage of POMC to produce them (cleavage between residues 136–137 of POMC; fig.3). Furthermore, the finding of phenylalanine at position 14 in peak 7 (table 1) suggests that this peptide could start either from position 168 or from position 183 of POMC (fig.3), both indicating a Phe↓X cleavage. However, the absence of leucine in the first 20 residues (present at positions 14 and 17 of β-endorphin) clearly points out the position 169 in POMC as the most probable starting position for peptide 7. This assertion is reinforced by the elution of this peptide

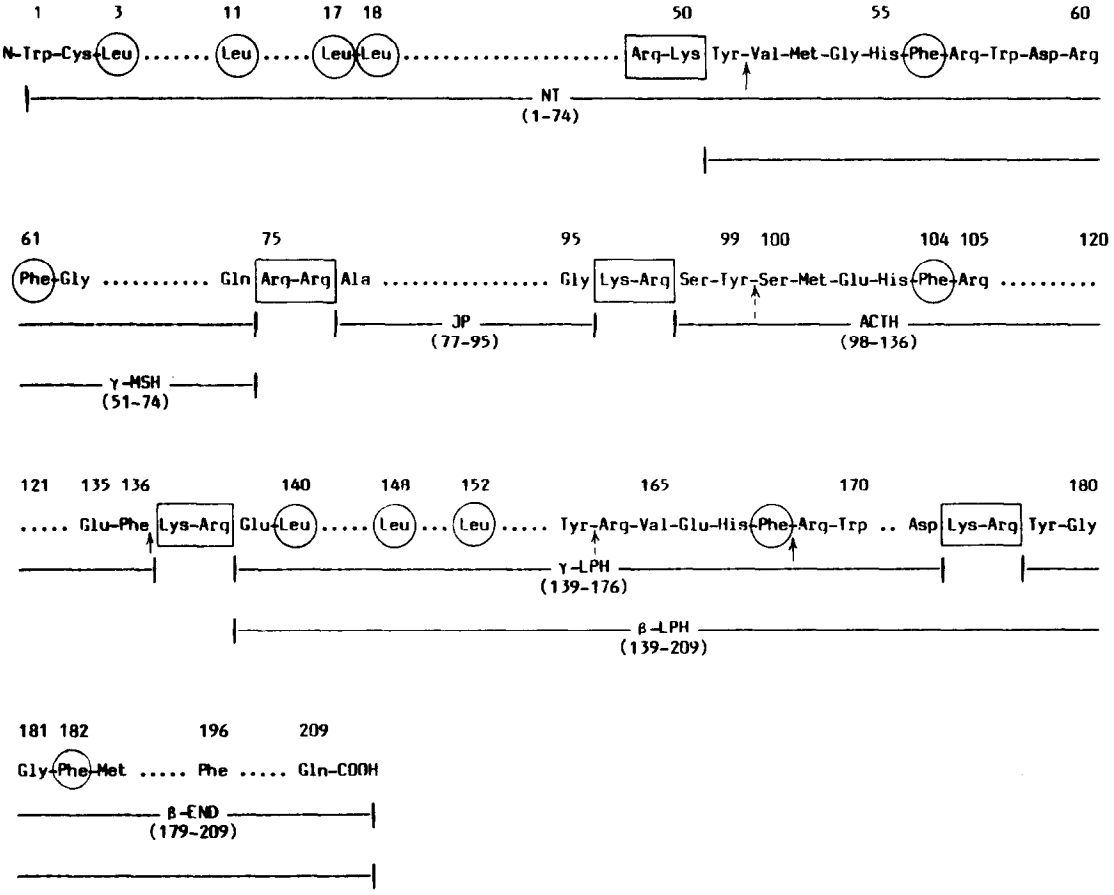

Fig.3. Schematic representation of rat POMC sequence [27] and the peptide bonds cleaved at pH 8.0 by pig secretory granule lysates. The pairs of basic residues expected to be cleaved in vivo are boxed in rectangles. The circles represent the leucine and phenylalanine residues positioned by microsequence of peptides 1–8 (fig.2, table 1). The full arrows represent the unambiguously deduced cleavage sites based on the microsequence results. The dashed arrows represent the two possible cleavage sites inferred from the sequence of [^{3}H]Phe-labeled peptide 6 (section 3, table 1). The definition of the 74, 19, 39, 38 and 71 residues N-terminal glycopeptide (NT), joining peptide (JP), adrenocorticotropin (ACTH), γ-lipotropin (γ-LPH), β-endorphin (β-END) and β-lipotropin (β-LPH), respectively, is depicted below the POMC sequence.

in peak V_L on Sephadex G-75, thus indicating an M_r slightly greater than that of β-endorphin. Taken together, it is proposed that peak 8 is a shorter Lys–Arg–γ-LPH-like peptide ending at residue 168 (i.e., POMC residues 137–168), while peak 7 represents an N-terminally extended β-endorphin-like peptide starting at residue 169 (i.e., POMC residue 169–209). This result implies a Phe$\downarrow$Arg cleavage site (between residues 168–169 of POMC).

Finally, two other minor peptides were identified (table 1), one of them (peptide 4) as a γ-MSH-like peptide produced via a Tyr$\downarrow$Val cleavage, thereby starting at residue 52 of the POMC sequence, and the second (peptide 6) either as a shorter ACTH-like peptide starting at residue 100 (POMC residues 100–136), or a β-MSH-like fragment starting at residue 164. Nevertheless, both possibilities clearly indicate a Tyr$\downarrow$X cleavage site, either a Tyr$\downarrow$Ser or a Tyr$\downarrow$Arg cleavage, respectively.

4. DISCUSSION

Pulse and pulse–chase experiments together with subcellular fractionation techniques have shown that the cleavage of precursor molecules at pairs of basic residues seems to be localized within the secretory granules (maturation processes reviewed in [13]). However, very little information pertaining to the nature of the enzyme(s) involved in such an important cellular processing step is yet available. Two schools of thought have been brought forward. On the one hand, results obtained with pro-insulin and pro-glucagon [15,29], and with POMC [16,17], have shown that the enzyme involved is an arginyl thiol protease-like with an acidic pH optimum around pH 5. On the other hand, data obtained on secretory granules preparations using β-LPH as a substrate, also have shown that the enzyme involved is possibly a serine protease with a basic pH optimum around pH 8 [30–32]. Although an intragranular pH of 5–6 has been reported [33], this does not necessarily mean that the pH optimum for the maturation enzyme should be coincident.

In this report, it was shown that a pH study by molecular sieving HPLC of the conversion activity observed in porcine anterior pituitary granule lysates using rat POMC as a substrate (pH 8.0) was optimal for cleavage into products similarly sized to known maturation products (fig.1) [21]. At pH 5, although some conversion does occur, yet it does not seem to yield similarly sized products as natural markers in this chromatographic system using denaturing eluants [21].

The major type of cleavage observed at pH 8.0 is shown to be directed towards either Phe–X or Tyr–X bonds. This suggests a chymotryptic-like proteolytic activity. Hence, the major enzymatic activity one observes with secretory granule preparations at pH 8.0 is not that of the sought maturation enzyme(s), since pairs of basic residues cleavage did not occur in significant amounts. Although electron microscopy of fraction P3A10 showed an enrichment in granules (not shown), contamination by other organelles is not unexpected. Indeed, due to the presence of some lysosomes in P3A10 ($<1\%$), it cannot be excluded that the proteolytic activity observed at pH 8 could be the result of a highly active lysosomal contaminant.

These results clearly point out the pitfalls and limitations of subcellular fractionation for obtaining sufficiently pure secretory granules. This is especially more pronounced when an enzymatic activity is sought which could well be masked by another major contaminating activity; e.g., the chymotryptic-like enzyme reported here.

In conclusion, the approach used for conversion product characterization coupled with a further purification of the granule lysates preparation should lead to a more realistic procedure for the systematic search of pro-hormone maturation enzyme(s). Furthermore, the results reported in the literature concerning the nature of the converting enzyme present in secretory granules preparations should be seriously and critically evaluated in the light of the present results, and the products carefully checked by microsequencing.

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REFERENCES

[1] Chrétien, M., Benjannet, S., Gossard, F., Gianoulakis, C., Crine, P., Lis, M. and Seidah, N.G. (1979) Can. J. Biochem. 57, 1111–1121.
[2] Eipper, B.A. and Mains, R.E. (1980) Endocrine Rev. 1, 1–27.
[3] Krieger, D.T., Liotta, A.S., Brownstein, M.J. and Zimmerman, E.A. (1981) Rec. Prog. Horm. Res. 36, 277–344.
[4] Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A.C.Y., Cohen, S.N. and Numa, S. (1979) Nature 278, 423–427.
[5] Seidah, N.G. and Chrétien, M. (1981) Proc. Natl. Acad. Sci. USA 78, 4236–4240.
[6] Seidah, N.G., Rochemont, J., Hamelin, J., Benjannet, S. and Chrétien, M. (1981) Biochem. Biophys. Res. Commun. 102, 710–716.
[7] Mains, R.E. and Eipper, B.A. (1980) J. Biol. Chem. 256, 5683–5688.
[8] Chrétien, M. and Seidah, N.G. (1981) Mol. Cell. Biochem. 34, 101–127.
[9] Seidah, N.G., Rochemont, J., Hamelin, J., Lis, M. and Chrétien, M. (1981) J. Biol. Chem. 256, 7977–7984.

[10] Browne, C.A., Bennett, H.J.P. and Solomon, S. (1981) Biochem. Biophys. Res. Commun. 100, 336–343.
[11] Crine, P., Seidah, N.G., Jeannotte, L. and Chrétien, M. (1980) Can. J. Biochem. 58, 1318–1322.
[12] Chrétien, M. and Li, C.H. (1967) Can. J. Biochem. 45, 1163–1174.
[13] Lazure, C., Seidah, N.G., Pélaprat, D. and Chrétien, M. (1983) Can. J. Biochem., in press.
[14] Steiner, D.F., Quinn, P.S., Chan, S.J., Marsh, J. and Tager, H.S. (1980) Ann. NY Acad. Sci. 343, 1–16.
[15] Fletcher, D.J., Quigley, J.P., Bauer, G.E. and Noe, B.D. (1981) J. Cell. Biol. 90, 312–322.
[16] Loh, Y.P. and Chang, T.L. (1982) FEBS Lett. 137, 57–62.
[17] Chang, T.L., Gainer, H., Russell, J.T. and Loh, Y.P. (1982) Endocrinology 111, 1607–1614.
[18] Glembotski, C.C. (1982) J. Biol. Chem. 257, 10493–10500.
[19] Bradbury, A.F., Finnie, M.D.A. and Smyth, D.G. (1982) Nature 298, 686–688.
[20] Seidah, N.G., Gianoulakis, C., Routhier, R. and Chrétien, M. (1978) Proc. Natl. Acad. Sci. USA 75, 3153–3157.
[21] Seidah, N.G., Pélaprat, D., Rochemont, J., Lambelin, P., Dennis, M., Chan, J.S.D., Hamelin, J., Lazure, C. and Chrétien, M. (1983) J. Chromatogr., in press.
[22] Lazure, C., Dennis, M., Rochemont, J., Seidah, N.G. and Chrétien, M. (1982) Analyt. Biochem. 125, 406–414.
[23] Russell, J.T. (1981) Analyt. Biochem. 113, 229–238.
[24] Wurtman, R.J. and Axelrod, J. (1963) Biochem. Pharm. 12, 1439–1440.
[25] Prasad, A.S., Dumouchelle, E., Konick, D. and Oberleas, D. (1972) J. Lab. Clin. Med. 80, 598–602.
[26] Gianoulakis, C., Seidah, N.G., Routhier, R. and Chrétien, M. (1979) J. Biol. Chem. 254, 11903–11906.
[27] Drouin, J. and Goodman, M. (1980) Nature 288, 610–613.
[28] Gossard, F., Seidah, N.G., Crine, P., Routhier, R. and Chrétien, M. (1980) Biochem. Biophys. Res. Commun. 92, 1042–1051.
[29] Docherty, K., Carroll, R.J. and Steiner, D.F. (1982) Proc. Natl. Acad. Sci. USA 79, 4613–4617.
[30] Austen, B.M. and Smyth, D.G. (1979) in: Molecular Endocrinology, vol.I (MacIntyre, I. and Szelke, M. eds) pp.99–110, Elsevier, Amsterdam, New York.
[31] Graf, L. and Kenessey, A. (1981) in: Hormonal Proteins and Peptides (Li, C.H. ed) vol.10, pp.35–63, Academic Press, New York.
[32] Graf, L., Kennesey, A., Berzetei, I. and Ronai, A.X. (1977) Biochem. Biophys. Res. Commun. 78, 1114–1123.
[33] Scherman, D. and Nordmann, J.J. (1982) Proc. Natl. Acad. Sci. USA 79, 476–479.