DEMONSTRATION OF β-LIPOTROPIN ACTIVATING ENZYME IN PORCINE PITUITARY

László Gráf, Ágnes Kenessey, Ilona Berzétei and András Z. Rónai

Research Institute for Pharmaceutical Chemistry
H-1325 Budapest, P.O. Box 82, Hungary

Received August 29,1977

SUMMARY: Porcine β-lipotropin was incubated with a crude porcine pituitary homogenate, and the main cleavage products of the hormone were isolated and identified. Our results gave evidence for the enzymic cleavage of the Lys^{46}-Met^{47}, Arg^{60}-Tyr^{61}, Leu^{77}-Phe^{78}, and Lys^{79}-Asn^{80} bonds of the β-lipotropin structure. The cleavage of the Arg^{60}-Tyr^{61} peptide bond was accompanied with the concomitant release of opiate activity in the first period of incubation, provided that bacitracin was present in the incubation mixture. The enzyme was differentiated from trypsin or plasmin and appears to be a specific intracellular protease involved in the biosynthesis of pituitary endorphins.

With the exception of Leu-enkephalin all the brain (1) and pituitary endorphins (2-5) identified to date are β-lipotropin (β-LPH*) fragments containing the met-enkephalin sequence (residues 61-65 of the β-LPH structure) at their NH_2-terminus. Though the occurrence of these fragments and their complementary ones in the pituitary gland strongly suggests that β-LPH is a biological precursor of endorphins (6-8), direct biosynthetic evidence of such a relationship is still lacking. In search for β-LPH converting enzymes of the adenohypophysis, we have previously demonstrated the presence of an endopeptidase in this part of pituitary which selectively splits the Leu^{77}-Phe^{78} peptide bond of β-LPH (9). It has been proposed that this enzyme is one of those involved in the release of γ-endorphin β-LPH-(61-77) and α-endorphin β-LPH-(61-76)

* Abbreviations: β-LPH, β-lipotropin; GPI, guinea pig ileum; MVD, mouse vas deferens.

Copyright © 1977 by Academic Press, Inc.
All rights of reproduction in any form reserved.

ISSN 0006-291X

from β-LPH (9). In our present paper we provide experimental evidence for the occurrence of another endopeptidase in the adenohypophysis which generates endorphins from β-LPH by splitting the Arg^{60}-Tyr^{61} peptide bond of the hormone.

Materials and Methods

Porcine β-LPH was prepared as described previously (10). Highly purified Zn-free bacitracin was a gift of Dr. A. Kótai, L. Eötvös University, Budapest. Soy bean trypsin inhibitor was the product of Calbiochem. Bovine pancreatic trypsin inhibitor (Kunitz) was obtained from G. Richter Factory, Budapest. Porcine anterior pituitary was homogenized as described previously (9). The protein content was determined by the method of Palladin (11).

Incubation of β-LPH with the pituitary homogenate was carried out in 0.05 M ammonium acetate of pH 6.5-7.5 or in 0.05 M ammonium hydrogencarbonate of pH 8.0 at 37°C with a homogenate to β-LPH ratio of 2:1 (weight of the protein content per weight). Incubation was also performed in the presence of bacitracin (5×10^{-4} M) or soy bean or pancreatic trypsin inhibitors (the ratio of these latter inhibitors to the protein content of the homogenate was 2:1 by weight). The incubation was terminated by adding one volume of acetone to the aliquots taken. The pH of the samples was adjusted to about 6 by 1 M acetic acid, and their supernatant fractions were prepared for gel electrophoresis (12) and bioassay (13). β-LPH fragments were separated by chromatography on Bio-Gel P-6 (Bio-Rad Lab) and CM-cellulose (Whatman CM 11) columns as described previously (14). The isolated fragments were characterized by amino acid analysis of their acid hydrolysates (6 M HCl, 110°C, 24 h) in a JEOL (JLC-5AH) analyzer and by mapping their tryptic digests by two-dimensional paper electrophoresis as described previously (9). Peptides detected by ninhydrin were cut out from the paper, eluted and evaporated for amino acid analysis.

Results and Discussion

As shown in Figure 1 the pH of the incubation mixture strongly influences the action of the pitutiary homogenate on β-LPH. While the conversion of β-LPH into LPH-(1-77) is practically exclusive at pH 6.5 (see ref. 9), increasing the pH causes the formation of some new electrophoretic components to become more and more prominent. To isolate and characterize these fragments, 70 mg of β-LPH was incubated with a pituitary homogenate at pH 8.0 for 16 hours. The 50% acetone supernatent fraction of the incubation mixture was fractionated on a Bio-Gel P-6 column in 0.5 M acetic (for the method see

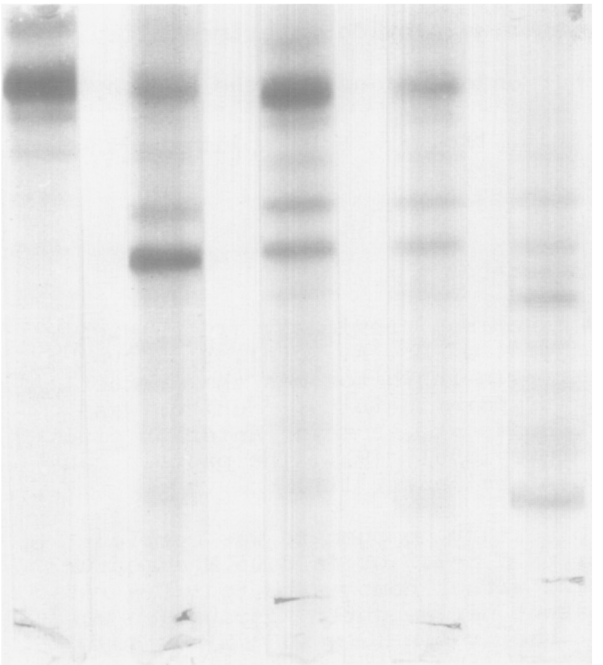

Figure 1. Effect of pH on the conversion of β-LPH by pituitary homogenate. With the exception of the control sample taken from the pH 7.0 incubation mixture and terminated at 0 h, all the samples were incubated for 16 h, and then the supernatant fractions obtained in 50% acetone at pH 6.0 were subjected to gel electrophoresis. Samples contained 200 μg β-LPH.

ref. 9). Fragments A, B, and C (Fig. 1) were eluted in a single peak from this column (the yield was 25.0 mg) and then separated by CM-cellulose chromatography, as shown in Fig. 2. For analysis of these fragments they were further purified by re-chromatography on CM-cellulose. To relate fragments A,B, and C to the structure of β-LPH, their NH_2-terminal residues (Glu for all of them) and amino acid compositions were determined, and their tryptic digests were

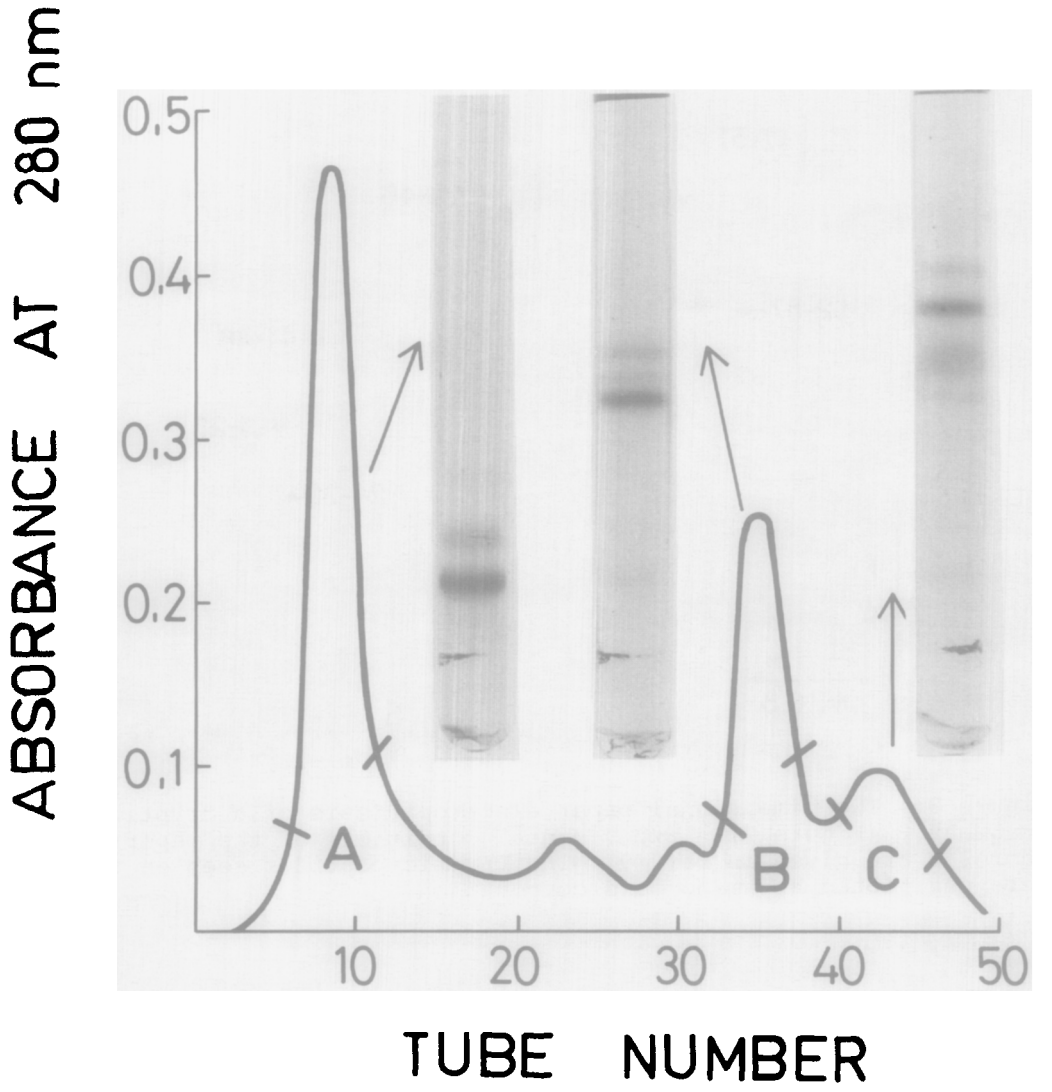

Figure 2. Separation of some β-LPH fragments on CM-cellulose. The column was equilibrated with 0.01 M ammonium acetate of pH 4.5, and the elution was performed by adding 0.1 M ammonium acetate of pH 5.3 to a 500-ml mixing flask which contained the starting buffer. Column size, 60 x 1 cm; tube volume 5 ml; flow rate, 30 ml/h. The yield of fractions A, B, and C were 9.0 mg, 4.8 mg, 2.1 mg, respectively. The gel electrophoretic patterns of these fractions are also shown in the figure.

mapped as demonstrated for fragment B in Figure 3. Based on these data the fragments could be identified as residues 1-46 (fragment A), 1-60 (fragment B) and 1-70 (fragment C) of the β-LPH structure.

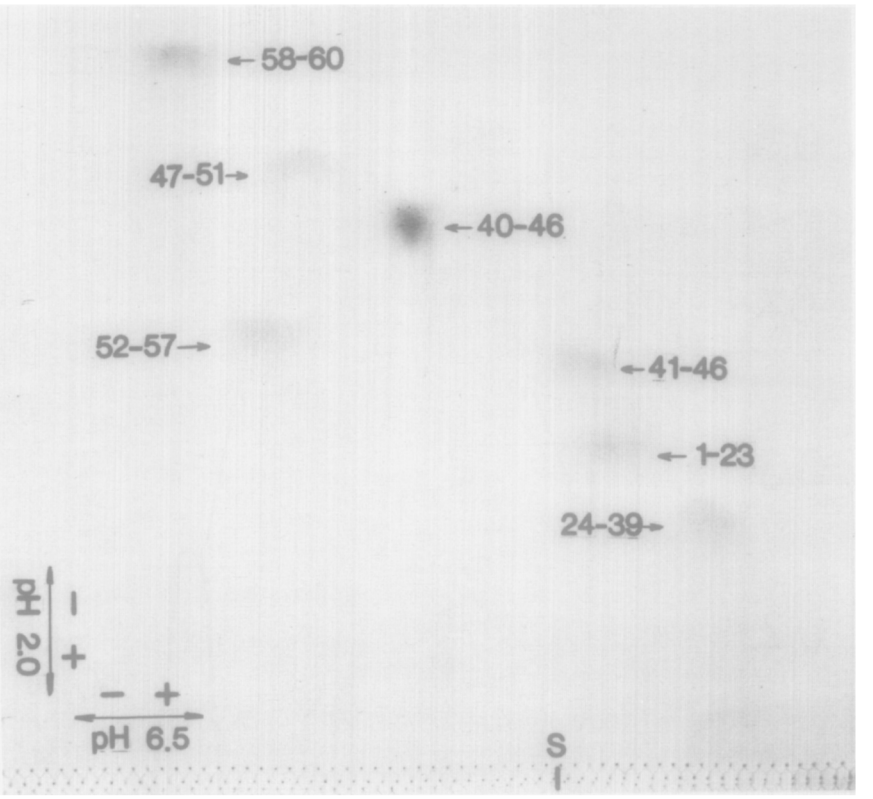

Figure 3. Two-dimensional paper electrophoresis of a tryptic digest fragment B (Figure 2). Experimental conditions of the paper electrophoresis are given in ref. 9. The tryptic peptides seen on the map were positioned by amino acid composition.

From the structure of the above fragments it appears that all of them were formed by the action of trypsin-like enzyme(s) on β-LPH. The formation of LPH-(1-60) is of particular interest, since it can be regarded as an indirect evidence for the release of its complementary fragment(s) with opiate agonist activity. The appearance of such fragments, however, could not be detected in the 16-hour incubation mixture by peptide analytical or *in vitro* biological methods (13).

In view of some recent data on the metabolic instability of enkephalins and endorphins (15) it was reasonable to assume that these newly formed β-LPH fragments may have undergone subsequent degradation

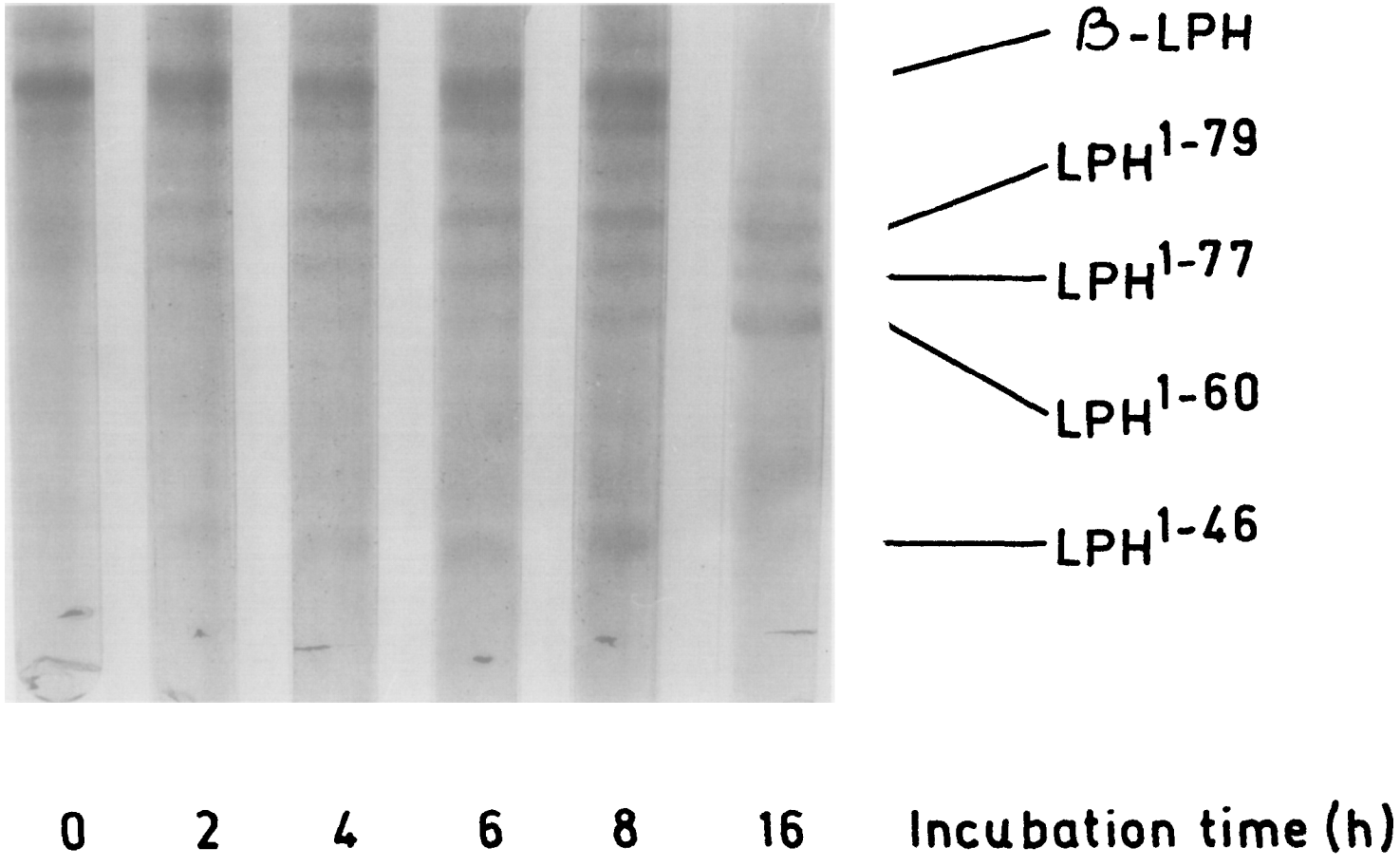

Figure 4. Time-course of the degradation of β-LPH by pituitary homogenate plus soy bean trypsin inhibitor as followed by gel electrophoresis. Samples contained 100 µg of β-LPH.

by some enzymes of the pituitary homogenate. To check this assumption the incubation of β-LPH with the pituitary homogenate was carried out at pH 8.0 in the absence or presence of bacitracin, a potent inhibitor of the biodegradation of several peptides (16,17), and the conversion of β-LPH was followed by gel electrophoresis and by testing the released opiate activity in two in vitro systems (13). Time-course of the digestion revealed a successive generation of LPH-(1-46), LPH-(1-60), LPH-(1-79) and some further so far unknown fragments from β-LPH (Fig. 4), and that bacitracin has no effect on this electrophoretically detectable enzymic process (not demonstrated) Table 1 presents the opiate agonist activities in the same incubation mixtures determined in longitudinal muscle strip of guinea pig ileum (GPI) and mouse vas deferens (MVD). As can be seen in the Table, β-LPH intself had a slight opiate activity, presumably due to the presence of some contaminating opioid peptide(s) in our preparation. This activity was however pregressively increased upon the incubation of β-LPH with the homogenate in the absence as well as in the presence of bacitracin for 8 hours. It is of particular interest that the incubation mixtures prepared in the presence of bacitracin were significantly more potent than the corresponding ones which did not contain bacitracin. Since the LPH-(1-60) releasing enzyme was not affected by bacitracin at all, this increment of the active peptide(s) can clearly be related to the selective inhibition of endorphin inactivating enzyme(s) by this antibiotic.

The high MVD/GPI potency ratios of the incubation mixtures (Table 1 indicate the presence of shorter opioid peptide(s) than LPH-(61-91) (13,18). This latter is the only β-LPH fragment which is equipotent in GPI and MVD (13,18). Provided that the biological activities of the bacitracin containing incubation mixtures are accounted for by "short" β-LPH fragment(s), approximately 10-15% "activation" of

Table 1

Release of Opiate Agonist Activity* from β-LPH Upon Its Incubation with Pituitary Homogenate

Duration of incubation in hours	Opioid Activity in NME** (nM) on GPI		MVD	
	Control	+ Bacitracin	Control	+ Bacitracin
0	16.0 ± 8.3	16.0 ± 8.2	356.0 ± 107.4	250.7 ± 107.0
2	11.3 ± 4.1	21.6 ± 7.3	293.3 ± 49.0	384.0 ± 90.0
4	23.2 ± 12.7	33.0 ± 16.5	522.7 ± 119.0	670.7 ± 223.6
6	20.7 ± 10.4	50.3 ± 5.8†	595.7 ± 194.0	1100.0 ± 110.0
8	28.3 ± 14.3	70.0 ± 17.0†	725.0 ± 280.0	1246.7 ± 342.6†
16	14.2 ± 7.2	43.3 ± 1.2†	177.3 ± 14.6	860.0 ± 200.3†

* Mean ± SE (n=3).

** NME (normorphine equivalent): The opiate agonist activity liberated from 10^{-6} M β-LPH was characterized by equiactive doses of normorphine expressed in nM. The specificity of activities was checked by 100-400 nM naloxone or naltrexone. ID_{50} values for normorphine in these series of experiments (i.e. from April 25, 1977 till June 10, 1977) were as high as 129.1 ± 16.9 nM (n=8) in GPI and 378.6 ± 49.3 nM (n=8) in MVD.

† Comparing the opiate activities of paired samples with or without bacitracin the differences are significant ($p<0,05$) at 6, 8, and 16 hours incubation times in GPI and at 8 and 16 hours in MVD.

β-LPH may be estimated in the first 8-hour period of incubation. The relative intensity of the LPH-(1-60) band in the gel electrophoretic pattern of the 8-hour sample (Fig. 4) suggests similar extent of cleavage at the Arg^{60}-Tyr^{61} peptide bond. As is evident from the low biological potency of the 16-hour incubation mixture, in the second period the inactivating enzymic processes became preponderant.

While pancreatic trypsin inhibitor selectively inhibited the formation of LPH-(1-60) and LPH-(1-79) from β-LPH, soy bean trypsin inhibitor did not affect the conversion pattern shown in Fig. 4. This observation together with our finding related to the subcellular

localization of β-LPH activating enzyme in the secretory granule fraction (19), differentiate this enzyme from trypsin and plasmin. In this context it is also worth mentioning that β-LPH activating enzyme could be detected only in pars distalis and pars intermedia of the pituitary gland, and not in pars nervosa and other brain areas (20). This specific regional location of the enzyme lends further support to our assumption that it may have a physiologically important role in the intracellular "activation" of β-LPH and probably in the enzymic conversion of other pituitary hormones, too.

Acknowledgement: The authors are grateful to Prof. C. H. Li, University of California, San Francisco, for helpful discussion and criticism in the preparation of the manusctipt.

References

1. Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A., and Morris, H. R. (1975) Nature, 258, 577-579.
2. Guillemin, R., Ling, N., and Burgus, R. (1976) C. R. Hebd. Séanc. Acad. Sci. Paris, D282, 783-785.
3. Li, C. H. and Chung, D. (1976) Proc. Natl. Acad. Sci. USA 73 1145-1148.
4. Bradbury, A. F., Smyth, D. G., Snell, C. R., Birdsall, N.J.M., and Hulme, E. C. (1976) Nature 260, 793-795.
5. Gráf, L., Székely, J. I., Rónai, A. Z., Dunai-Kovács, Zs., and Bajusz, S. (1976) Nature 263, 240-242.
6. Gráf, L., Rónai, A. Z., Bajusz, S., Cseh, G., and Székely, J. I. (1976) FEBS Lett. 64, 181-184.
7. Bradbury, A. F., Smyth, D. G., and Snell, C, R. (1976) Biochem. Biophys. Res. Commun. 69, 950-956.
8. Crine, P., Benjannet, S., Seidah, N. G., Lis, M., and Chretien, M (1977) Proc. Natl. Acad. Sci. USA 74, 1403-1406.
9. Gráf, L. and Kenessey, A. (1976) FEBS Lett. 69, 255-260.
10. Gráf, L. and Cseh, G. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. 3, 175-177.
11. Palladin, A. V. (1961) Regional Neurochemistry, pp. 8-12, Pergamon Press, Oxford.
12. Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404-427.
13. Rónai, A. Z., Gráf, L., Székely, J. I., Dunai-Kóvacs, Zs., and Bajusz, S. (1977) FEBS Lett. 74, 182-184.
14. Gráf, L. (1976) Acta Biochim. Biophys. Acad. Sci. Hung. 11, 267-277.

15. Marks, N., Grynbaum, A. and Neidle, A. (1977) Biochem. Biophys. Res. Commun. 74, 1552-1559.
16. Desbuquois, B., Krug, F., and Cuatrecasas, P. (1974) Biochim. Biophys. Acta 343, 101-120.
17. Miller, R. J., Chang, K. J., and Cuatrecasas, P. (1977) Biochem. Biophys. Res. Commun. 74, 1311-1317.
18. Lord, J.A.H., Waterfield, A. A., Hughes, J., and Kosterlitz, H. W. (1977) Nature 267, 495-499.
19. Gráf, L., Cseh, G., Barát, E., Rónai, A. Z., Székely, J. I. , Kenessey, A., and Bajusz, S. (1977) Ann. N.Y. Acad. Sci. in press.
20. Kenessey, A., Gráf, L.,and Palkovits, M. (1977) Brain. Res. Bull. in press.