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Purification to homogeneity of camel pituitary pro-opiocortin, the common precursor of opioid peptides and corticotropin

(endorphins/high-performance liquid chromatography/fluorescamine)

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ABSTRACT **Pro-opiocortin was purified from camel pituitaries by procedures including high-performance liquid chromatography. The precursor relationship of the pure protein to the opioid peptides and to corticotropin was confirmed. Partial chemical analysis consisting of amino acid analysis and tryptic peptide mapping was carried out with the aid of sensitive fluorescence detection.**

Earlier work from this laboratory has dealt with the isolation and characterization of the opioid peptides from rat pituitary (1–3). These studies indicated the presence of several peptides containing the [Met]enkephalin sequence—namely, α-endorphin (1700 daltons), β-endorphin (3500 daltons), β-lipotropin (10,000 daltons), and pro-opiocortin (*ca.* 30,000 daltons). It has also been shown that the largest precursor, pro-opiocortin, contains the corticotropin sequence (3–5). Indeed, this common precursor contains within it the sequences of many biologically active peptides including γ-lipotropin, β-melanotropin, [Met]enkephalin, the endorphins, β-lipotropin, α-melanotropin, corticotropin-inhibiting peptide, corticotropin-like-intermediate peptide, as well as corticotropin (6–9). Only about half of the primary structure of pro-opiocortin is known and it is possible that sequences in the uncharacterized region may represent other biologically important peptides.

In this report, we present the purification to homogeneity of submilligram quantities of pro-opiocortin from camel pituitaries. Microfluorometric procedures coupled with high-performance liquid chromatography were used for the isolation, as well as for tryptic peptide mapping and amino acid analysis.

MATERIALS AND METHODS

Isolation of Pro-opiocortin. Lyophilized camel pituitaries were kindly supplied by Abdul Muti-El-Adhami through Y. Birk, D. Rattner, and C. H. Li. One pituitary (200 mg) was homogenized in 4 ml of a solution containing 1 M acetic acid, 20 mM hydrochloric acid, 0.1% (vol/vol) 2-mercaptoethanol, pentachlorophenol at 1 μg/ml, phenylmethylsulfonyl fluoride at 10 μg/ml, and pepstatin at 10 μg/ml. The homogenate was centrifuged at 26,000 × *g* for 30 min. The precipitate was extracted with an additional 4 ml of the same acid solution and again centrifuged. The combined supernatant solutions (7 ml) were clarified by centrifugation at 200,000 × *g* for 1 hr, concentrated to 4 ml under reduced pressure, and fractionated on a Sephadex G-100 (superfine) column (1.5 × 90 cm) equilibrated with 1 M acetic acid/20 mM HCl/0.01% (vol/vol) thiodiglycol. As indicated in the figure legends, aliquots of each fraction were lyophilized and digested with 40 μg of trypsin treated with tosylphenylalanyl chloromethyl ketone (TPCK) in 200 μl of 50 mM Tris·HCl buffer (pH 8.5) at 37°C for 16 hr. Opioid activity was measured with a radioreceptor binding assay (10). The highest molecular weight species yielding opioid activity after trypsin digestion was evaporated under vacuum to reduce the volume to 4 ml. It was then applied to a Sephadex G-75 (superfine) column (1.5 × 90 cm) equilibrated with the same buffer as the Sephadex G-100 column and fractions were assayed as described above. The pro-opiocortin-containing fractions from the Sephadex G-75 column were subjected to high-performance liquid chromatography (HPLC) on a reverse-phase column (EM Lichrosorb RP-8, 4.6 × 250 mm, Scientific Products, Edison, NJ). The column was eluted with a 2-hr linear gradient of 0–40% (vol/vol) 1-propanol in 0.5 M formic acid/0.14 M pyridine (pH 3.0) at a flow rate of 30 ml/hr. The column effluent was monitored by an automated fluorescence-detection system (11). Aliquots from fractions were assayed for opioid activity as above. The fraction showing the highest specific activity was rechromatographed on the same reverse-phase column, using a 4-hr gradient of 1-propanol at a flow rate of 15 ml/hr.

Electrophoresis. Lyophilized samples were dissolved in 20 μl of 10 mM sodium phosphate (pH 7.0), containing 1% sodium dodecyl sulfate ($NaDodSO_4$), 2% 2-mercaptoethanol, 10% (vol/vol) glycerol, and 0.01% bromphenol blue. They were heated at 100°C for 3 min and then applied to 10% polyacrylamide gels prepared in $NaDodSO_4$ and Tris/glycine buffer at pH 8.3 (12). Protein calibration markers were obtained from Pharmacia. Samples of pro-opiocortin were run alone on gels to assess purity or run in combination with the markers for molecular weight determination.

Amino Acid Analysis. Analyses were performed at the picomole level on an instrument using fluorescamine (13). Samples were lyophilized in glass ampules and hydrolyzed at 110°C for 22 or 48 hr in 200 μl of constant-boiling hydrochloric acid containing 0.1% thioglycolic acid. Under these hydrolysis conditions cystine was quantitatively converted to cysteine. Tryptophan was determined by hydrolyzing in the presence of 4% (vol/vol) thioglycolic acid. Pro-opiocortin was reduced and carboxymethylated for confirmation of the cysteine content. Pro-opiocortin (3.6 μg) was dissolved in 50 μl of 1% sodium bicarbonate containing 0.1% sodium dodecyl sulfate and 5 μmol of 2-mercaptoethanol and incubated at 37°C for 1 hr. After reductive cleavage of disulfide bonds, 20 μl of monoiodoacetic acid (4.8 μmol) solution (pH 8.0) was added and the solution was incubated at 37°C for 3 hr. To stop the reaction, 20 μl of 2-mercaptoethanol was added and the carboxymethylated protein was lyophilized and hydrolyzed at 110°C for 24 hr in 200 μl of constant-boiling hydrochloric acid containing 0.1% thioglycolic acid. For sugar-amine analysis, samples of pro-

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Abbreviations: HPLC, high-performance liquid chromatography; TPCK, tosylphenylalanyl chloromethyl ketone; $NaDodSO_4$, sodium dodecyl sulfate.

opiocortin were hydrolyzed in 200 μl of 4 M hydrochloric acid for 4 hr at 104°C. Control hydrolysates of glucosamine with bovine serum albumin indicated minimal sugar-amine destruction under these hydrolysis conditions.

Peptide Mapping. Pro-opiocortin (8.5 μg) was digested with 0.2 μg of TPCK-trypsin for 16 hr at 37°C in 1% sodium bicarbonate, pH 8.0. The digest was lyophilized, redissolved in the starting buffer, and chromatographed on a reverse-phase column (Lichrosorb RP-18, Altex Scientific, Berkeley, CA), using a 2-hr gradient of 0–20% 1-propanol at a flow rate of 15 ml/hr. The column effluent was monitored automatically and collected fractions were tested for opioid activity.

Affinity Chromatography. Antiserum to corticotropin coupled to Sepharose and synthetic human corticotropin were kindly supplied by D. Krieger and A. Liotta. A column (bed volume 250 μl) of Sepharose-bound corticotropin antiserum was prepared in a plastic syringe. The column was washed before use with 5 ml of buffer A (0.5 M KCl/5 mM EDTA/0.1% Triton X-100), followed by 5 ml of buffer B (6 M guanidine·HCl), and equilibrated with 10 ml of buffer C (50 mM Tris·HCl, pH 7.5, containing bovine serum albumin at 1 mg/ml). Pure pro-opiocortin (6 μg) was dissolved in 0.2 ml of buffer C and applied to the column at room temperature (flow rate 2 ml/hr). The column was washed with about 10 column vol of buffer C. Specifically bound material was eluted with 2 ml of buffer C containing corticotropin (10 μg/ml). Each fraction (250 μl) was adjusted to pH 8.5 with 1 M Tris, digested with 40 μg of TPCK-treated trypsin, and assayed as above.

RESULTS

Isolation of Pro-opiocortin. The first chromatographic step for the purification of camel pro-opiocortin was on Sephadex G-100 (Fig. 1). The high molecular weight region contained two peaks that showed opioid activity after digestion with trypsin, corresponding in molecular weights to pro-opiocortin (*ca.* 30,000) and β-lipotropin (10,000). Rechromatography on Sephadex G-75 (not shown) was used to complete the separation of pro-opiocortin from β-lipotropin and other proteins of similar size. Reverse-phase chromatography resolved several major protein peaks, one of which corresponded with the opioid activity (Fig. 2). The fraction having the highest specific activity (fraction 25 in Fig. 2) was rechromatographed under similar conditions, except for the collection of smaller fractions and the use of a lower flow rate (Fig. 3). A single symmetrical peak was obtained. The fraction with the highest specific activity (fraction 50 in Fig. 3) was found to be homogeneous by polyacrylamide gel electrophoresis in $NaDodSO_4$ (Fig. 4). About 5 nmol of pure pro-opiocortin was isolated from the extract of one camel pituitary by this procedure. This represented a 77-fold purification from the initial acid extract with a 15% overall yield (Table 1). Five other camel pituitaries were carried through the same procedure. Yields as high as 50% were obtained with these pituitaries by rechromatographing less pure fractions. Homogeneous pro-opiocortin from the first pituitary was used for the analytical studies, but the results were confirmed with later preparations.

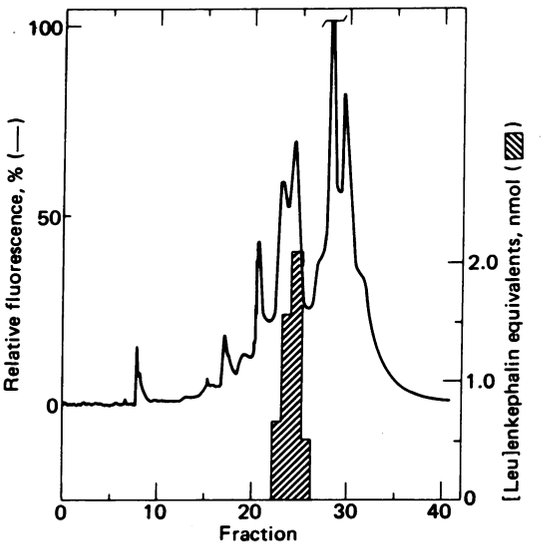

FIG. 2. HPLC of the pro-opiocortin peak fractions from the Sephadex G-75 column on a Lichrosorb RP-8 column (4.6 × 250 mm) at 25°C. The column was eluted at 30 ml/hr with 0.5 M formic acid/0.14 M pyridine, using a gradient of 1-propanol; 0% (3 min), 0–12% (9 min), 12–40% (90 min), and 40% (18 min). A portion (2%) of the column effluent was diverted to the fluorescamine monitoring system. Aliquots (10 μl) of each fraction (1.5 ml) were taken for digestion with trypsin and the receptor binding assay.

Evidence of Homogeneity. The product was judged to be homogeneous by the appearance of a single symmetrical peak

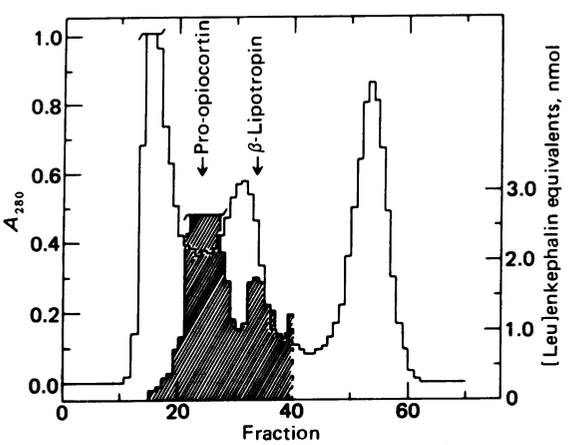

FIG. 1. Filtration of an acid extract of a single camel pituitary (200 mg) on Sephadex G-100 (superfine) column at 25°C. The fractions collected were 2.5 ml. Aliquots of fractions 11–40 (45 μl) were digested with trypsin and the digests were assayed for opioid activity (hatched area, right ordinate). Fractions 21–30 were combined, concentrated under reduced pressure, and purified further on a Sephadex G-75 (superfine) column (1.5 × 90 cm).

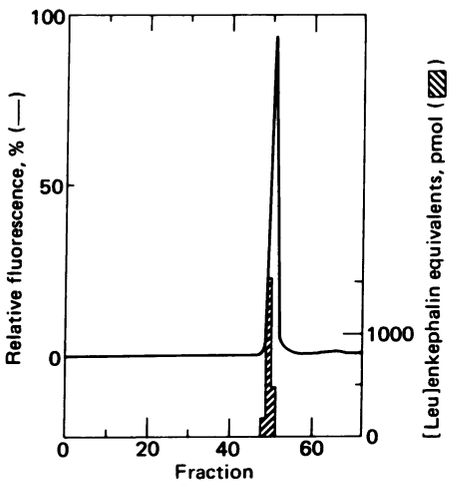

FIG. 3. Rechromatography of fraction 25 from the RP-8 column (see Fig. 2). The HPLC column was eluted under similar conditions except the flow rate was 15 ml/hr, a 4-hr gradient was used, and smaller fractions were collected. A portion (6%) of the column effluent was diverted to the fluorescamine monitoring system. Aliquots (20 μl) of each fraction (0.75 ml) were taken for the receptor binding assay.

Table 1. Purification of camel pituitary pro-opiocortin

Step	Total activity,* nmol	Total protein, mg	Specific activity, nmol/mg	Purification factor	Yield, %
1. Acid extraction	(43.6)†	83.2	0.20‡	1.0	
2. Sephadex G-100 chromatography	16.3	11.1	1.47	7.4	100
3. Sephadex G-75 chromatography	13.5	6.4	2.11	10.6	83
4. RP-8 HPLC	5.1	0.40	12.8	64	31
5. 2nd RP-8 HPLC	2.5	0.17	15.3	77	15

* These values show the opioid activity in [Leu]enkephalin equivalents after trypsin digestion. It has been found that 1 pmol of [Leu]enkephalin activity corresponds to 2.2 pmol of trypsin-digested pro-opiocortin, β-lipotropin, or β-endorphin (10).

† Total activity as nmol [Leu]enkephalin equivalents, determined on fractions from the Sephadex G-100 column: pro-opiocortin, 16.3; β-lipotropin, 9.3; endorphins, 15.7; and enkephalins, 2.3.

‡ Calculated from 16.3 nmol/83.2 mg.

on a HPLC column (Fig. 3) and a single sharp band on an electrophoretic gel (Fig. 4), as well as by obtaining the theoretical value for the specific activity of the product. With regard to this last criterion, because 1 mg of pro-opiocortin is equivalent to about 30 nmol, digestion with trypsin should release 30 nmol of the active nonapeptide β-lipotropin-(61–69) or 14 nmol of [Leu]enkephalin equivalents (see Table 1). The actual specific activities determined for pure pro-opiocortin ranged from 14 to 15 nmol equivalents per mg of protein; the value shown in Table 1 is 15.3.

Analysis of Pro-opiocortin. Amino acid analysis was carried out on hydrolysates prepared under several different conditions in order to obtain the most accurate values. Although electrophoresis indicated a molecular weight of 33,000 (Fig. 4), the composition was arbitrarily adjusted to reflect a molecular weight of 28,000, corresponding to nine phenylalanine residues. This was done because pro-opiocortin has been reported to be a glycoprotein, the protein portion of which has this lower molecular weight (14, 15). Characteristic features are the low isoleucine and high glutamate (plus glutamine) content (Table 2). The two isoleucine residues are accounted for in the β-endorphin sequence. Camel β-melanotropin and corticotropin (C. H. Li, personal communication) each contain a single tryptophan residue, accounting for the two residues of tryptophan found in pro-opiocortin. There are also eight methionine and three cysteine residues. Cysteine analysis of hydrolysates prepared in the presence of thioglycolic acid (without carboxymethylation) indicated a value of five residues. The lower value found by carboxymethylation is believed to be more accurate. The absence of cysteine and the low methionine content (two to three residues) in β-lipotropin and corticotropin from several species indicate that these residues are concentrated in the (as yet) uncharacterized portion of pro-opiocortin. Although glucosamine has been reported to be a constituent of the pro-opiocortin in mouse pituitary tumor cells (14, 15), at this point we have been unable to detect glucosamine or galactosamine in 4-hr hydrolysates of camel pro-opiocortin. Injection of as much as 6 μg of pro-opiocortin hydrolysate onto the analyzer column revealed less than one-third of a residue of glucosamine or galactosamine with respect to phenylalanine.

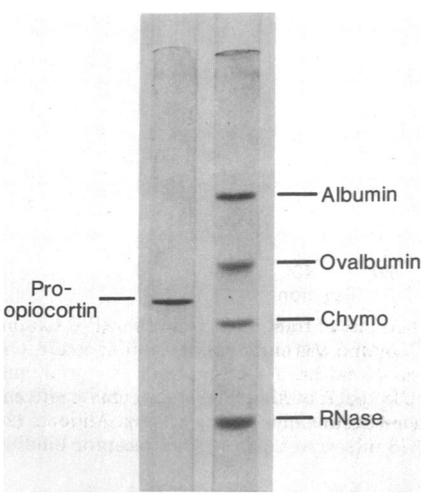

FIG. 4. NaDodSO$_4$/polyacrylamide gel electrophoresis of fraction 50 from the RP-8 column (see Fig. 3). An aliquot of pro-opiocortin (2 μg) was applied and electrophoresis was carried out in Tris/glycine/NaDodSO$_4$ (pH 8.3). The marker proteins used were bovine serum albumin (68,000 daltons), ovalbumin (43,000 daltons), chymotrypsinogen (Chymo) (25,000 daltons), and ribonuclease (RNase) (13,700 daltons).

Further Characterization. Camel pro-opiocortin was subjected to digestion with trypsin and the fragments were separated by HPLC and monitored fluorometrically. The pattern is presented in Fig. 5. Collected fractions were tested for opioid activity, which was found at only one position in the chromatogram (indicated by the arrow). This position corresponded to the elution position of synthetic Tyr-Gly-Gly-Phe-

Table 2. Amino acid composition of pro-opiocortin

	Number of residues*		
	A	B	Integer value
CM-Cys	2.6	—	3†
Asp	16.3	15.9	16
Thr	8.8	7.9	10‡
Ser	12.9	10.0	15‡
Glu	38.0	36.9	38
Pro	14.3	14.8	15
Gly	27.4	26.3	27
Ala	17.8	17.2	18
Val	5.6	5.7	6
Met	8.0	8.2	8
Ile	2.3	2.2	2
Leu	24.3	24.3	24
Tyr	10.9	10.8	11
Phe	9.0	9.0	9
His	4.0	3.8	4
Lys	18.7	18.6	19
Trp	1.9	—	2
Arg	17.4	17.6	18
		Total	245

Column A, 22-hr hydrolysates; column B, 48-hr hydrolysates; 150–200 pmol was used.

* Phenylalanine was arbitrarily set at nine residues. Values are the averages of replicates of three independent determinations; average deviations were typically within 3%. Tryptophan analysis was based on duplicate determinations.

† Cysteine analysis of hydrolysates without carboxymethylation indicated 5.1 (22 hr) and 4.9 (48 hr) residues.

‡ These values were extrapolated from the results obtained after hydrolysis for 22 and 48 hr.

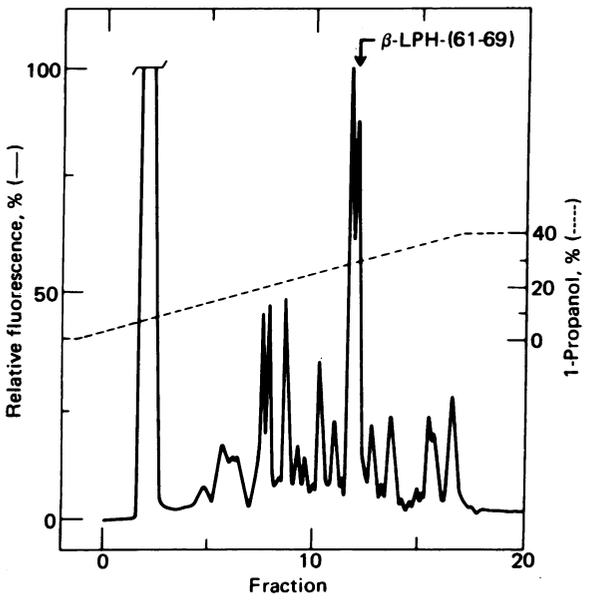

FIG. 5. HPLC of tryptic fragments of pro-opiocortin. Trypsin-treated pro-opiocortin (8.5 μg) was applied to a Lichrosorb RP-18 column (4.6 × 250 mm) at 25°C. The column was eluted at 15 ml/hr with 0.5 M formic acid/0.35 M pyridine (pH 4.0) using a linear gradient of 1-propanol from 0 to 20% for 120 min. A portion (24%) of the column effluent was diverted to the fluorescamine monitoring system. Each fraction (1.5 ml) was lyophilized and aliquots were used for the receptor binding assay. The elution position of the β-lipotropin (β-LPH) fragment consisting of residues 61–69 is noted.

Met-Thr-Ser-Glu-Lys, the opioid peptide produced on digestion of β-lipotropin with trypsin. This is in agreement with previous results on rat pro-opiocortin (3). Camel pro-opiocortin, like rat pro-opiocortin (3), was found to be retained on a column of Sepharose-bound corticotropin antiserum and was eluted by the addition of an excess of synthetic corticotropin. Specific binding was confirmed by adding an excess of synthetic corticotropin to the pro-opiocortin solution prior to its application to the affinity column. In this case the pro-opiocortin was not retained. These results confirm the common precursor relationship of pro-opiocortin to the opioid peptides and to corticotropin in the camel pituitary.

DISCUSSION

Camel pituitaries were chosen for these studies because they were found to contain a high concentration of pro-opiocortin (*ca.* 20 nmol/g). This level is comparable to that found in rat pituitary (3), but is more than an order of magnitude higher than levels measured in beef, sheep, and pig pituitaries. These differences may relate to a combination of environmental and genetic factors. Furthermore, the pro-opiocortin content of camel pituitaries was twice the β-lipotropin content. Although a series of pro-opiocortin-like proteins, ranging in molecular weight from 28,500 to 34,000, has been reported to be present in mouse pituitary tumor cells (14, 15), in the camel we detected only a single form, with an apparent molecular weight of 33,000. The reported heterogeneity, which has been attributed to differences in glycosylation of the protein, has led to speculation about the possibility of various postribosomal modifications. However, this heterogeneity may be a characteristic of the tumor cell lines in culture.

One reason for isolating and sequencing pro-opiocortin is to determine the presence of other biologically active sequences within the molecule by using purely chemical means. There are two approaches to be taken. One is to look for the characteristic Lys-Arg sequence that precedes β-endorphin in the β-lipotropin structure and is the pair of residues separating β-lipotropin from corticotropin in the pro-opiocortin structure (16). The Lys-Arg sequence is known to be the cleavage site for generation of active peptides such as insulin (17, 18). The second way is to look for homologous sequences in pro-opiocortin isolated from different species. The evolutionary conservation of structure is well demonstrated in the interspecies identities of both β-endorphin and corticotropin.

The combination of HPLC and sensitive fluorescent detection systems for proteins, peptides, and amino acids has allowed us to isolate and partially characterize this important protein from relatively few pituitary glands. Sufficient amounts of camel pro-opiocortin have been prepared for complete chemical characterization, including sequencing. Availability of pure pro-opiocortin will also make it possible to search for the proteases that convert it into its active components and for factors that regulate this biosynthetic pathway.

Note Added in Proof. We recently detected another form of pro-opiocortin (*ca.* 35,000 daltons) by gel electrophoresis in $NaDodSO_4$ in fraction 23 of the RP-8 column (Fig. 2). This minor component (*ca.* 5% in the first camel pituitary) had the same specific activity as the major component. In a later preparation of five combined pituitaries this higher molecular weight component represented about 40% of the total activity. The two components were resolved on a Spherisorb-CN HPLC column (Laboratory Data Control, Riviera Beach, FL).

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1. Rubinstein, M., Stein, S., Gerber, L. D. & Udenfriend, S. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3052–3055.
2. Rubinstein, M., Stein, S. & Undenfriend, S. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4969–4972.
3. Rubinstein, M., Stein, S. & Udenfriend, S. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 669–671.
4. Mains, R. E., Eipper, B. A. & Ling, N. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3014–3018.
5. Roberts, J. L. & Herbert, E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4826–4830.
6. Li, C. H. & Chung, D. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1145–1148.
7. Ling, N., Burgus, R. & Guillemin, R. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3942–3946.
8. Scott, P., Ratcliff, J. G., Rees, L. H., Landon, J., Bennett, H. P. J., Lowry, P. J. & McMartin, C. (1973) *Nature (London) New Biol.* **244**, 65–67.
9. Li, C. H., Chung, D., Yamashiro, D. & Lee, C. Y. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4306–4309.
10. Gerber L. D., Stein, S., Rubinstein, M., Wideman, J. & Udenfriend, S. (1978) *Brain Res.* **151**, 117–126.
11. Böhlen, P., Stein, S., Stone, J. & Udenfriend, S. (1975) *Anal. Biochem.* **67**, 438–445.
12. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412.
13. Stein, S., Böhlen, P., Stone, J., Dairman, W. & Udenfriend, S. (1973) *Arch. Biochem. Biophys.* **155**, 203–212.
14. Eipper, B. A. & Mains, R. E. (1978) *J. Biol. Chem.* **253**, 5732–5744.
15. Roberts, J. L., Phillips, M. P., Rosa, P. A. & Herbert, E. (1978) *Biochemistry* **17**, 3609–3618.
16. Nakanishi, S., Inoue, A., Kita, T., Numa, S., Chang, A. C. Y., Cohen, S. N., Nunberg, J. & Schimke, R. T. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 6021–6025.
17. Dayhoff, M. O. (1972) *Atlas of Protein Sequence and Structure* (National Biomedical Research Foundation, Washington, DC), Vol. 5.
18. Dayhoff, M. O. (1976) *Atlas of Protein Sequence and Structure* (National Biomedical Research Foundation, Washington, DC), Vol. 5, Suppl. 2.