Proc. Natl. Acad. Sci. USA
Vol. 78, No. 10, pp. 6086–6090, October 1981
Biochemistry

The M_r 80,000 common forms of neurophysin and vasopressin from bovine neurohypophysis have corticotropin- and β-endorphin-like sequences and liberate by proteolysis biologically active corticotropin

(prohormones/precursors/hypothalamus/radioimmunoassay/affinity chromatography)

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Communicated by Roger Y. Stanier, June 29, 1981

ABSTRACT **We have tested the hypothesis that the high M_r forms common to both neurophysin and vasopressin detected in bovine neurohypophysis extracts (Nicolas, P., Camier, M., Lauber, M., Masse, M.-J. O., Möhring, J. & Cohen, P. (1980) *Proc. Natl. Acad. Sci. USA* 77, 2587–2591) might also contain the sequences of other known neuropeptides. The following evidence indicates that corticotropin- and β-endorphin-like sequences are associated with similar high M_r forms and are included in these M_r 80,000 molecules. During the fractionation steps of high M_r material, both corticotropin and β-endorphin immunoreactive species were found to coelute with the neurophysin and vasopressin ones, either under M_r 140,000 (in 0.1 M formic acid) or M_r 70,000–80,000 (in 6 M guanidine) elution volumes. Corticotropin immunoreactivity was found to cofocus at pIs 6.05 and 5.8 with the M_r 80,000 neurophysin-containing species. This material was submitted to affinity chromatography on purified anti-neurophysin antibodies covalently attached to Sepharose 4B. Both the corticotropin and β-endorphin immunoreactivities, together with the neurophysin and vasopressin immunoreactivities, were retained on the immunoadsorbent and codesorbed by either a drastic pH change or by selective displacement with an excess of neurophysin. Comparison of the tryptic-digest maps of either the M_r 68,000 fragment immunoprecipitated by anti-corticotropin antibodies or the M_r 68,000 fragment released after precipitation of the M_r 80,000 species by anti-neurophysin antibodies indicated large sequence homologies. Exposure of either the M_r 80,000 or 68,000 components to mild proteolytic activities resulted in the formation of lower-size fragments. The resulting corticotropin-like immunoreactive material, recovered under the elution volume of standard ^{125}I-labeled corticotropin-(1–24), was tested for its ability to activate glucocorticoid biogenesis by the amphibian interrenal tissue (adrenal) in perifusion. It was found to exhibit a noticeable activity qualitatively undistinguishable from the one of the reference human corticotropin-(1–39). The name neurohypophyseal "coenophorin" (from the Greek word for common) is proposed for this class of M_r 80,000 polypeptides that might represent the common precursor store-house for a set of neuropeptides produced in the hypothalamo-neurohypophyseal tract.**

In secreting cells, polypeptide hormones appear to be produced first as part of larger prohormone molecules. In one case, the precursor protein, named pro-opiomelanocortin, was shown to be plurifunctional—i.e., to include in its structure the aminoacid sequences of α-melanotropin, corticotropin (ACTH; adrenocorticotropic hormone) and β-lipotropin (1–4). Possible biosynthetic precursors of the neurohypophyseal protein neurophysin (M_r 10,000), with sizes ranging from M_r 17,000 to M_r 25,000, were detected in pulse–chase experiments in the rat hypothalamus (5, 6) and in the immunoprecipitable products of the cell-free translation of hypothalamic mRNA (7–9) or else were directly characterized as higher M_r immunoreactive forms (called highM_r) in the mouse hypothalamus (10–12) or bovine hypothalamo-neurohypophyseal tract (13).

In this laboratory, we also identified in bovine neurohypophysis extracts, in addition to the M_r 17,000–25,000 putative proneurophysin or provasopressin, immunoreactive forms of a significantly larger size (13). One class of these molecules behaves as M_r 140,000 species in acid medium (13) or as M_r 80,000 species under denaturing conditions (14, 15) and was shown neither to be aggregates nor to result from the disulfide scrambling of lower-size molecules (14). These species have both neurophysin and [8-arginine]vasopressin (AVP) antigenic determinants (13), generate by limited proteolysis both neurosecretory peptides (13), and could be shown by tryptic-digest analysis to have sequence homology with neurophysin (14). One of the main proteolytic cleavages undergone by these M_r 80,000 molecules was found to generate neurophysin-containing M_r 10,000 fragments and M_r 68,000 molecules that essentially have lost their neurophysin immunoreactivity (14). The large difference in size between the M_r 80,000 putative common precursors of neurophysin and AVP and their end products raised the question (14, 15) of whether other neurohormone sequences might be included in their covalent structure.

In this report, we show that ACTH-like and β-endorphin-like sequences are part of these M_r 80,000 molecules and are included in a M_r 68,000 fragment generated by their cleavage, with the concomittant release of $\approx M_r$ 10,000 neurophysin-containing fragments. Furthermore, we show that this ACTH immunoreactivity corresponds to a biologically active sequence that can be released by proteolysis of the larger species.

EXPERIMENTAL PROCEDURE

Bovine neurohypophysis extracts were made as described (14). Isoelectric focusing of the high M_r material was conducted in the presence of 6 M urea. For estimation of the M_r of the immunoprecipitable species, the material was first ^{125}I-labeled, then precipitated by purified anti-neurophysin or anti-ACTH

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Abbreviations: AVP, [8-arginine]vasopressin; ACTH, corticotropin (adrenocorticotropic hormone); RIA, radioimmunoassay.

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antibodies, followed by separation of the immune complexes by means of fixation to *Staphylococcus aureus* as described (14). The dissociated complexes were then analyzed for the M_r of the ^{125}I-labeled immunoprecipitated species by electrophoresis on polyacrylamide gels run in $NaDodSO_4$ and dithiothreitol. The controls were routinely carried out with nonimmune serum and by displacement with an excess of the standard unlabeled antigens (14).

Radioimmunoassays. All the fractions recovered from the molecular sieve filtration, isoelectric focusing, and affinity chromatography experiments were tested for neurophysin and AVP immunoreactivities by a radioimmunoassay procedure (RIA) with various anti-neurophysin antisera ($SIIO_4$ and 616-4 prepared in this laboratory) and one anti-AVP antiserum (16). Both RIAs were conducted as described (12, 13, 16).

For the ACTH and β-endorphin RIA, the antisera SAB 46 (final dilution, 1/175,000) and 4093 (final dilution, 1/50,000) from the Unité de Radioimmunologie (F. Dray, Institut Pasteur) were used. Freshly ^{125}I-labeled ACTH-(1–24) and β-endorphin (from camel) were used as tracers. Both RIA were conducted in 0.2 M phosphate buffer containing 3 mg of bovine serum albumin per ml, 0.15 M NaCl, and 130 units of Trasylol per ml at either pH 6 (β-endorphin) or pH 7.5 (for ACTH). After 24 hr of incubation, the antigen–antibody complexes were precipitated by a double-antibody system with anti-rabbit IgG in the presence of 6% (wt/vol) polyethylene glycol. A minimum of 20 pg of ACTH or 5 pg of β-endorphin could be detected. Less than 0.01% crossreactivity with neurophysin, β-endorphin, and AVP was observed with the ACTH RIA. The same level of crossreactivity was measured for the β-endorphin RIA with neurophysin, AVP, and ACTH. For the four RIA used, routine controls were run as in refs. 12, 13 and 16.

Affinity Chromatography by Immunoadsorption. The immunoadsorbent was prepared with antineurophysin IgG previously purified by adsorption on neurophysin-Sepharose (14, 17) and covalently crosslinked to CH-activated Sepharose 4B (Pharmacia). The adsorption/desorption steps were performed as described (Fig. 3 legend). In all experiments, the iodinated neurophysin tracer was added as internal marker. Under these conditions, ≧90% of the total neurophysin radioactivity was retained on the column, and controls indicated that ≦1.5% of the ^{125}I-labeled AVP and ≦0.1% of the β-endorphin or ACTH were adsorbed.

For the processing of the high M_r forms, proteolytic activities associated with ammonium sulfate-precipitated anti-ACTH-(1–24) antiserum covalently attached to Sepharose 4B were used. This immunoadsorbent exhibits residual proteolytic activity, a fact also observed in the case of nonpurified anti-neurophysin antiserum immobilized on Sepharose (12, 13).

ACTH Bioassay. The biological potency of ACTH-like peptides was evaluated by using the perifusion system as described (18). Briefly, frog interrenal (adrenal) glands were diced, placed in Bio-Gel into a siliconized glass column, and perfused with gassed amphibian culture medium. Flow rate (18 ml/hr), pH (7.35), and temperature (24°C) were kept constant throughout the experiment. Synthetic ACTH-(1–39) (Ciba) or the generated peptides were dissolved in culture medium just before use and infused into the perifusion chamber for 20 min at 110-min intervals. A single dose of each sample and three graded doses of synthetic ACTH-(1–39) were successively infused within the same perifusion experiment. The effluent perfusate was set apart every 5 min, and corticosterone, the major glucocorticoid produced by frog adrenal tissue, was measured in each fraction by a specific RIA that gave less than 1% crossreactivity with either aldosterone, 18-hydroxycorticosterone, or 17-hydroxyprogesterone. At the doses used, neither synthetic ACTH, nor the ACTH-like peptide(s) interfered in the corticosterone assay.

Peptide Mapping. The high M_r ^{125}I-labeled samples were first immunoprecipitated by either anti-neurophysin or anti-ACTH, and the complexes were dissociated by electrophoresis

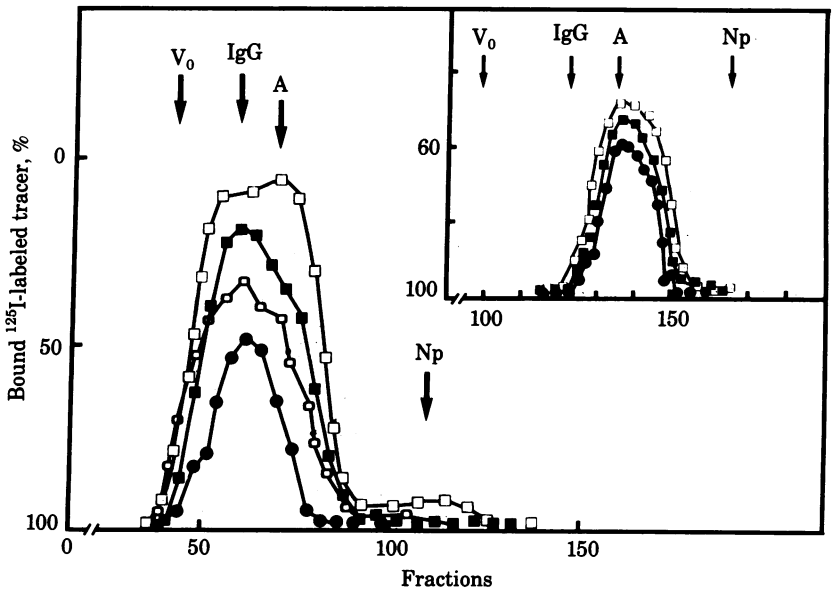

FIG. 1. Molecular sieve filtration of higher M_r forms of neurophysin, AVP, ACTH, and β-endorphin from neurohypophysis extracts. The neurophysin-like material (10 μg) recovered from Sepharose CL-4B was filtered on a Sephadex G-200 column (2.5 × 127 cm) in 0.1 M formic acid. Each fraction (4 ml) was tested for neurophysin (●), AVP (□), ACTH (■), and β-endorphin (□) immunoreactivity by using the corresponding RIA. ^{125}I-Labeled neurophysin was added as a M_r 10,000 internal marker (Np), and the column was calibrated by IgG (M_r, 140,000) and bovine serum albumin (A) (M_r, 68,000). (*Inset*) The material recovered in the exclusion volume of the Sephadex G-75 column fractionation of extracts (25 μg) was filtered on a Sepharose CL-4B column (1.6 × 120 cm) equilibrated and run in 6 M guanidine·HCl (Fluka). Each fraction (1.25 ml) was tested for neurophysin (●), ACTH (■), and β-endorphin (□) immunoreactivities by using the corresponding RIA. Results were expressed as percent of ^{125}I-labeled tracer bound by antibodies.

on polyacrylamide gels run under reducing and denaturing conditions (14). The relevant ^{125}I-labeled material was eluted from the gels and then digested by trypsin (14). The generated fragments were analyzed by autoradiography after two-dimensional separation by chromatography, and then by electrophoresis on thin-layer cellulose plates (14). Alternatively they were fractionated by high-pressure liquid chromatography on a SP 8000 apparatus (Spectra Physics, Santa Clara, CA) with different elution systems.

RESULTS

Extracts of bovine neurohypophysis were submitted to successive fractionations. The immunoreactive material containing high M_r neurophysin (M_r, ≧60,000) was recovered in the exclusion volume of the Sephadex G-75 column (13, 14) and was further analyzed after filtration on a Sepharose CL-4B column both equilibrated and run in 6 M guanidine (Fig. 1 *Inset*). Because AVP immunoreactivity could not be measured in the presence of guanidine by our RIA procedure, each of the eluted fractions was assayed first for its reactivity toward anti-ACTH, -β-endorphin and -neurophysin antibodies. The three immunoreactive peaks were found to coelute under a volume corresponding approximately to M_r 70,000 (Fig. 1 *Inset*).

In contrast, when filtered subsequently on Sephadex G-200 in 0.1 M formic acid, this material was eluted under a broad peak as M_r 140,000 species (Fig. 1). This change from apparent M_r 70,000 to M_r 140,000 was found to be reversible and to affect similarly the neurophysin, AVP, ACTH, and β-endorphin-like materials. This peculiar behavior still remains unexplained. It may either reflect dimerization of the molecule or else be due to an anomalous behavior related to the globular proteins used as standards for calibration of the column (Fig. 1). It should be pointed out that, when labeled with ^{125}I and immunoprecipitated with anti-neurophysin antibodies, this material, after disulfide reduction, appeared to migrate essentially as a M_r 80,000 band on NaDodSO$_4$/polyacrylamide electrophoretic gels (14). Taken together, these data indicate the presence in the extracts

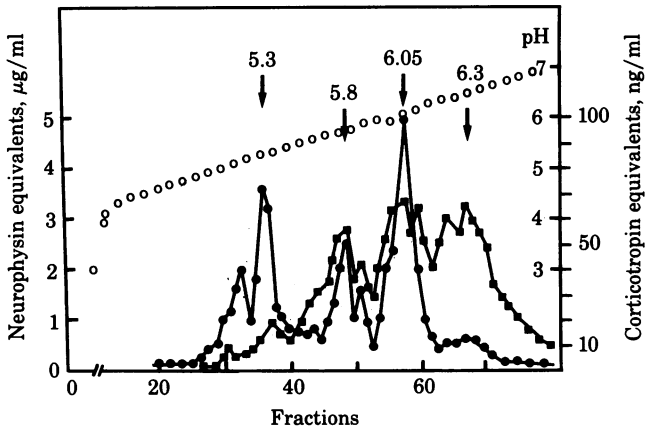

FIG. 2. Isoelectric focusing analysis in 6 M urea of the high M_r material. Isoelectric focusing was run on 25 μg of high M_r material recovered from the G-200 fractionation step (Fig. 1). The pH gradient (○) was from pH 4–7 in 6 M recrytallized urea on a 110-ml LKB column (1 W; 48 hr). ^{125}I-Labeled neurophysin (M_r, 10,000) was added as internal marker and focused with a pI = 5.3 (arrow); other arrows indicate the M_r 80,000 fractions (pIs 5.8 and 6.05) and the M_r 68,000 fraction (pI 6.3). Each fraction (1 ml) was tested for its immunoreactivity toward anti-neurophysin (●) or anti-ACTH (■) antibodies on 50-μl and 10-μl aliquots, respectively. The M_r of the immunoprecipitable material was determined as described. After the corresponding fractions (arrows) had been filtered on a Sephadex G-25 column (0.9 × 20 cm) to remove sucrose, urea, and Ampholines, the material was labeled with ^{125}I and submitted to further analysis (14).

of high M_r forms of both β-endorphin and ACTH, which are not the result of aggregation of lower-size molecules and which exhibit a chromatographic behavior comparable to that of the high M_r neurophysin/AVP species previously described (13–15).

Analysis of this high M_r material by isoelectric focusing in 6 M urea indicated the distribution of neurophysin-like species under three main peaks corresponding to pIs 5.3, 5.8, and 6.05, respectively. Each of the fractions corresponding to the top of these respective peaks were analyzed for the M_r of their immunoreactive species after ^{125}I-labeling and NaDodSO$_4$/polyacrylamide gel electrophoresis of the immunoprecipitated material. Alternatively, this was evaluated by means of molecular-sieve filtration on a calibrated Sephadex G-150 column (13). The pI 5.3 material appeared to comprise a M_r ≃10,000 material, possibly neurophysin II, generated by proteolysis of the high M_r material. (Bovine neurophysin II pI is 5.3 in 6 M urea, whereas it decreases to 4.7 in the absence of this denaturing agent.) Both pI 5.8 and 6.05 species behaved like M_r 80,000 molecules on the gels (not shown) after precipitation by anti-neurophysin. ACTH immunoreactivity was found to cofocus under the 5.8 and 6.05 pI peaks (Fig. 2), but these species were found to be weakly immunoprecipitable by anti-ACTH antibodies. This may reflect the poor accessibility of the ACTH antigen (in the high M_r molecules) to the high-affinity antibody binding sites. AVP and β-endorphin immunoreactivities also were detected under those two peaks (not shown).

Besides these species showing both ACTH/β-endorphin and neurophysin/AVP immunoreactivities, the bulk of the ACTH-like material was found to focus in the 6.2–6.3 pI range under peaks showing only residual neurophysin immunoreactivity (Fig. 2). The M_r of this material was evaluated to be 68,000 after electrophoretic analysis of the material immunoprecipitated by

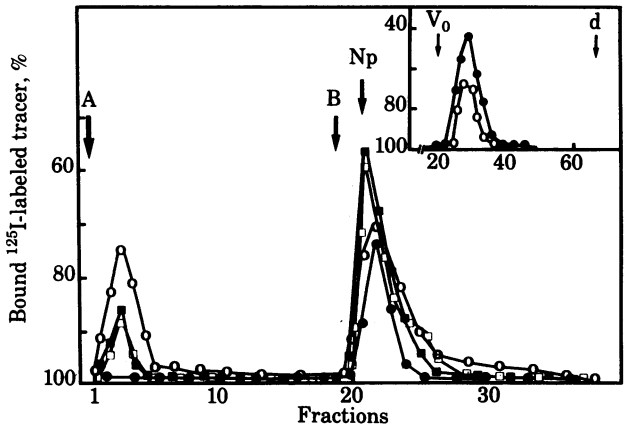

FIG. 3. Affinity chromatography: immunoadsorption of the high M_r form of neurophysin, AVP, ACTH, and β-endorphin. Step A (arrow): High M_r components were adsorbed together with ^{125}I-labeled neurophysin on the affinity column (2 ml) for 30 min at 4°C in 0.1 M sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl, 1 mg of pepstatine per liter and 130 kallikrein-inhibitor units of aprotinin per ml. The complex was washed by 10 column volumes with the same buffer solution at 4°C. Step B (arrow): Desorption was done by 1 M acetic acid. An aliquot of each 1-ml fraction was lyophilized and then tested by RIA for neurophysin (●), AVP (○), ACTH (■), and β-endorphin (□) immunoreactivities. ^{125}I-Labeled neurophysin (Np) was 95% desorbed by the 1 M acetic acid step (arrow). Less than 0.5% of standard ^{125}I-labeled AVP, ACTH, and β-endorphin was retained on the column. The recovery yield of immunoreactive material was ≧75%. (*Inset*) Elution profile of neurophysin (●) and AVP (○) immunoreactivities coadsorbed on the affinity column and subsequently applied on a Sephadex G-200 column under the same experimental conditions as in Fig. 1. d, Elution volume of ^{125}I neurophysin. Results were expressed as percent of ^{125}I-labeled tracer bound by antibodies.

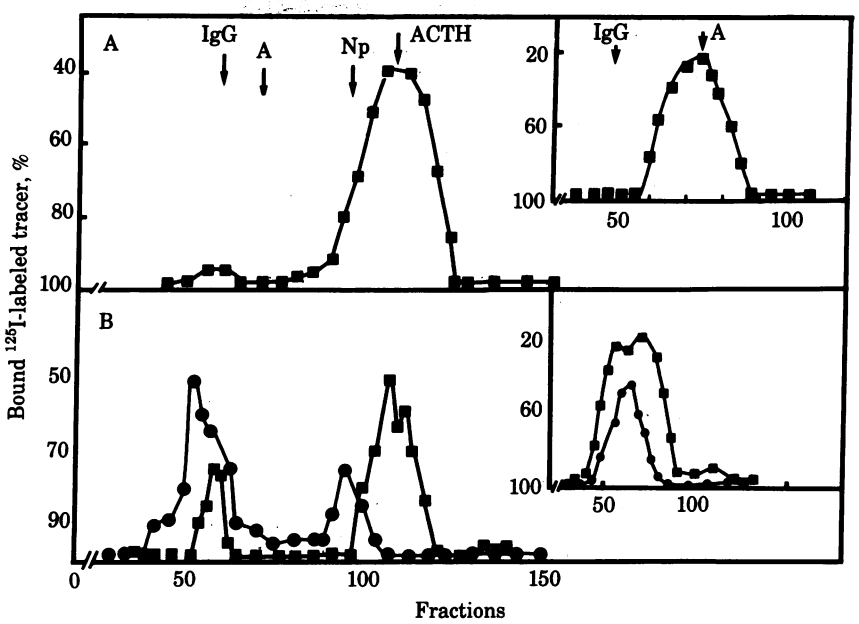

FIG. 4. Processing of the M_r 68,000 and 80,000 forms. (*A*) Filtration on a Sephadex G-200 column (1.2 × 90 cm) of the compounds generated after exposure of the M_r 68,000 material of *Inset A* to anti-ACTH antiserum immobilized on Sepharose 4B. Markers (arrows): IgG, M_r 140,000; bovine serum albumin (A), M_r 68,000; neurohypophysin (Np), M_r 10,000; ACTH, ^{125}I-labeled ACTH-(1–24). The reaction was for 15 hr in 0.1 M Na phosphate buffer (pH 7.9) in the absence of protease inhibitors and at 22°C. At the end of the reaction, the products were eluted with 3 vol of phosphate buffer and then 5 vol of 1 M acetic acid, lyophilized, and applied to the G-200 column. The ACTH RIA was determined on each fraction. (*Inset A*) Molecular sieve filtration on a Sephadex G-200 column (1.2 × 90 cm) of the starting material corresponding to the pI 6.3, M_r 68,000 species recovered from the isoelectric focusing (Fig. 2). (*B*) Same filtration as in *A* but with the material recovered after exposure of the M_r 80,000 form of *Inset B* to the anti-ACTH antiserum immobilized on Sepharose 4B under the same conditions. (*Inset B*) Molecular sieve filtration on a Sephadex G-200 column of the starting material under the same conditions as in *Inset A*. Neurophysin (●) and ACTH (■) immunoreactivities were determined on aliquots of each 1-ml fraction. ^{125}I-Labeled neurophysin and ^{125}I-labeled ACTH were added as internal markers. A control was made by two successive cycles of washing of the anti-ACTH/Sepharose 4B column with as much as 10 vol of the reaction and elution buffers, respectively. They were both lyophilized and ACTH was evaluated by RIA. Less than 1% of the quantity of immunoreactive ACTH generated in the processing experiments was released by this treatment of the column. Results were expressed as percent of ^{125}I-labeled tracer bound by antibodies.

anti-ACTH antibodies. It has been shown that a M_r 68,000 material was generated by proteolytic cleavage of the M_r 80,000 molecules as shown by analysis of tryptic digests (14). Also, comparison of the peptide maps generated by trypsinolysis of the M_r 68,000 material derived from the M_r 80,000 molecule (14) or directly precipitated by anti-ACTH antiserum from pI 6.2–6.3 material revealed large sequence homologies (not shown). Therefore, it can be concluded that the 80,000 neurophysin/AVP species focusing under pIs 6.05 and 5.8 exhibit both ACTH and β-endorphin immunoreactivities and can generate by proteolysis M_r 68,000 fragments that have lost essentially their neurophysin immunoreactivity.

Further evidence that the high M_r neurophysin/AVP molecules have both ACTH and β-endorphin determinants was provided by affinity chromatography experiments. It was found that the M_r 80,000 material, when applied to previously purified anti-neurophysin IgG covalently immobilized on CH-Sepharose 4B, was quantitatively adsorbed. The ACTH/β-endorphin immunoreactivities were desorbed together with the neurophysin/AVP species by a drastic pH change (Fig. 3). The M_r of the desorbed material was further checked by filtration on Sephadex G-200 (Fig. 3 *Inset*). Alternatively, this experiment was run at first by desorption of weakly attached species in 1 M NaCl containing bovine serum albumin (0.5 mg/ml), followed by displacement of the immunospecifically bound material by competition with a 100-fold excess of neurophysin. Again, high M_r ACTH- and β-endorphin-like material was desorbed by this immunocompetition on the anti-neurophysin antibody binding sites (results not shown). These observations indicate that the high M_r molecules bound on purified anti-neurophysin antibodies by means of their neurophysin determinants have both ACTH and β-endorphin antigenic determinants.

That their ACTH immunoreactivity corresponds to a genuine ACTH sequence was demonstrated by the following experiments. The pI 6.3, M_r 68,000, ACTH-like material was exposed to proteolytic activities associated with anti-ACTH antiserum covalently attached to CH-Sepharose 4B. Analysis of the material on a Sephadex G-200 column before and after digestion indicated the transformation of the M_r 68,000 material into lower-size ACTH-like fragments recovered under the elution volume of standard ^{125}I-labeled ACTH (Fig. 4*A*).

The biological activity of this low-size material was evaluated by means of the perifusion model (18), which provides both qualitative and quantitative indications on the corticotropin-like peptides. The results obtained indicated that the kinetic parameters of the response of the amphibian adrenal tissue either to the synthetic ACTH-(1–39) standard or to the material generated by processing of the M_r 68,000 species were strictly alike. The lag period between the onset of the adrenal response was 20 min, whereas the maximum amplitude of corticosterone output occurred 50 min after the onset of the infusion. The duration of the effect (50 min) was constant for the three concentrations tested. From these results it appears that the dynamics of the effects triggered by either synthetic human ACTH-(1–39) or the ACTH-like peptides generated in the processing experiment on corticosterone production by the adrenal are undistinguishable. Quantitatively, it was found that the processing of 8 ng of ACTH-like immunoreactivity (equivalents) of the M_r 68,000 material generated a total of 17 ng of ACTH-like immunoreactivity, which showed a total biological potency equivalent to 14

ng of reference ACTH. Hence, the generated fragments show a larger immunoreactivity than the starting material, a fact consistently observed when a small-size antigen is unmasked from larger-size molecules. These fragments behaved like 70% active corticotropin molecules.

A similar result was obtained when the M_r 80,000 species was processed by the same technique. In that case, only part of the starting material was digested (Fig. 4*B*), generating about 30 ng of ACTH-like product which was found to exhibit a biological activity corresponding to 25 ng of reference ACTH.

DISCUSSION

The present data unequivocally show immunoreactive ACTH and β-endorphin associated with protein species of a much larger size than the well-described M_r 35,000 pro-opiomelanocortin (1–4). Their behavior indicates that they are neither aggregates nor the result of disulfide scrambling. ACTH has been detected in the hypothalamus by several authors (19–21); more recently, enkephalins were revealed at the synaptic end, in rat neurohypophysis, of ocytocin and vasopressin neurones originating in the hypothalamic magnocellular nuclei (22). The exact physiological significance of such findings remains rather speculative at this time.

In biosynthetic precursors, the association of ACTH with β-endorphin, whose NH_2-terminal pentapeptide sequence represents [Met]enkephalin (4), appears to be a feature common to all the systems of ACTH biosynthesis so far described. In contrast, the evidence that both molecules may be included, together with neurophysin and AVP, in the M_r 80,000 neurohypophyseal polypeptide is an entirely unexpected observation. The data presented here do not allow us to state whether the entire amino acid sequences of both β-endorphin and ACTH are responsible for the immunoreactivities detected in the high M_r form characterized.

Structure–activity correlations have delineated the essential role of the NH_2-terminal sequence of ACTH-(1–24), which appears sufficient for triggering at least 70% of the maximum biological activity. Therefore, the liberation of biologically active ACTH fragments by proteolysis of the M_r 80,000 precursor is consistent with the idea that the essential structural features of the amino acid sequence of natural ACTH-(1–39) are included in this molecule. Peptide-map analysis of tryptic digests in our laboratory provide strong support for this conclusion, and the extensive analysis will be published elsewhere. Consequently, it is rather tempting to speculate that the M_r 80,000 polypeptides are the primary product of hypothalamic gene translation for neurohormones biosynthesis. They may constitute a hypothalamo-neurohypophyseal precursor form common to both neurophysin/AVP and ACTH/β-endorphin pairs. Two M_r 80,000 dalton species with pIs of 5.8 and 6.05, respectively, could be detected (ref. 14; this work). They may reflect a charge microheterogeneity of the same molecule. The alternative—that they could represent two distinct M_r 80,000 precursors for neurophysins I and II, respectively, hence producing two possibly distinct M_r 68,000 fragments— remains open.

Although immunoreactive ocytocin was measured in the crude M_r 80,000 material (unpublished data) the present data do not permit us to exclude the thought that this hormone may be part of a distinct M_r 80,000 precursor from the one of AVP. Therefore, we propose to name this M_r 80,000 type of molecule neurohypophyseal "coenophorin,"[§] representing the largest composite prohormone(s) so far described in the literature. We anticipate that other molecules of this type, carrying various sets of neuropeptide sequences, might well be characterized in this and other regions of the brain (e.g., ref. 23); such molecules also should be termed coenophorins, this name being preceded by the name of the brain region where they were discovered.

[§] From the Greek κοινος = common.

We thank Profs. C. H. Li, J. Porath, and M. Chrétien for stimulating discussions and for the gifts of hormone samples and C. Rougeot (Unité de Radioimmunologie Analytique, Institut Pasteur) for her continuous assistance with the immunological techniques. This work was supported in part by funds from the Université Pierre et Marie Curie, the Centre National de la Recherche Scientifique (Equipes de Recherches Associées 693 et 891), the Programme Interdisciplinaire de Recherche sur les Bases Scientifiques des Médicaments, and the Délégation à la Recherche Scientifique et Technique (Contract 79-7-0788). The help of the Departement de Biologie du Commissariat à l'Energie Atomique, Saclay, for the supply of radiochemicals and a doctorate fellowship to M.L. is appreciated. P.B. was supported by a long-term fellowship from the European Molecular Biology Organization.

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