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Existence of a Common Precursor to ACTH and Endorphin in the Anterior and Intermediate Lobes of the Rat Pituitary

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Extracts of rat anterior and intermediate-posterior pituitary were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and assayed for immunoactive ACTH and endorphin. In both lobes the major forms of immunoactive ACTH have apparent molecular weights of 31,000 (31K), 20–21K, 14K, and 4.5K, and the major forms of immunoactive endorphin have apparent molecular weights of 31K (coincident with the peak of immunoactive ACTH), 13K (a βLPH-like peptide), and 3.5K (a β-endorphin-like peptide). However, the quantitative distribution of immunoactivity among the various forms differs greatly between the lobes. Assays using an extreme COOH-terminal ACTH antiserum indicate that the 31K ACTH/endorphin molecule in rat anterior and intermediate pituitary is similar to the pro-ACTH/endorphin molecule from mouse pituitary tumor cells. A radioimmunoassay that is specific for the NH_2-terminal non-ACTH, nonendorphin segment (referred to as 16K fragment) of the mouse pro-ACTH/endorphin molecule was used to assay extracts of rat pituitary. In addition to detecting material at 31K and 20–21K, the 16K fragment radioimmunoassay detects significant amounts of cross-reactive material with an apparent molecular weight of 16K in extracts of both lobes. This result also suggests that the structure and processing of the rat 31K ACTH/endorphin molecule is similar to that of mouse tumor cell pro-ACTH/endorphin.

Cell suspensions were prepared from the anterior and intermediate lobes of the rat pituitary and maintained in culture for a 24-h period. The isolated cells from both lobes incorporate [^{3}H]phenylalanine into immunoprecipitable ACTH- and endorphin-containing molecules. By sequential immunoprecipitation with ACTH and endorphin antisera, it is possible to demonstrate directly that a single molecule (31K ACTH/endorphin) has antigenic determinants for both ACTH and endorphin. Significant amounts of 31K ACTH/endorphin are

Abbreviations used are: βLPH, β-lipotropin; ACTH, adrenocorticotropic hormone; αMSH, α-melanotropin [N-acetyl-ACTH(1–13)-NH_2]; βMSH, β-melanotropin [βLPH(41–58)]; β-endorphin, βLPH (61–91); CLIP, ACTH(18–39); SDS, sodium dodecyl sulfate; ACTH- and endorphin-containing molecules are identified by their apparent molecular weight in the borate/acetate-buffered SDS polyacrylamide gel electrophoresis system (31K, 13K, etc.) and the antiserum used to detect them (ACTH or endorphin).

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released into the culture medium by isolated anterior lobe and intermediate lobe cells. The isolated intermediate lobe cells synthesize and secrete relatively large amounts of a β-endorphin-like molecule; the isolated anterior lobe cells secrete significant amounts of both a βLPH-like molecule and a β-endorphin-like molecule. These same quantitative differences between anterior and intermediate lobe tissue were observed in immunoassays of extracts of the separated lobes and probably reflect differences in the processing of the common precursor. The isolated anterior lobe cells can be stimulated to release increased amounts of immunoprecipitable ACTH and endorphin by incubation with a cyclic AMP analog and a phosphodiesterase inhibitor.

Key words: **ACTH, endorphin, rat pituitary, radioimmunoassay, immunoprecipitation, antibody specificity**

Studies on a mouse pituitary tumor cell line (AtT-20/D-16v) have demonstrated that a high-molecular-weight glycoprotein contains peptide segments similar in structure to both ACTH(1–39) and β-lipotropin (βLPH) [1–3]; β-endorphin, a potent morphinomimetic peptide, is identical to βLPH(61–91) [4–7]. In the mouse pituitary tumor cells this common precursor (pro-ACTH/endorphin) is processed to yield close to equimolar amounts of ACTH-containing peptides (23K ACTH; 13K ACTH; 4.5 ACTH) and endorphin-containing peptides (11.7K endorphin, a βLPH-like molecule; 3.5K endorphin, a β-endorphin-like molecule). A glycoprotein (16K fragment) that accounts for most of the remainder of the mass of the common precursor is also produced and secreted in significant amounts [8, 9]. Analysis of the structure of the pro-ACTH/endorphin molecule indicates that the ACTH-like segment is located in the middle of the precursor; the βLPH-like segment is attached to the COOH-terminal end of the ACTH-like segment and the 16K fragment is located to the NH_2-terminal side of the ACTH-like segment [41].

The existence of a common precursor to ACTH and endorphin in tissues other than the mouse pituitary tumor cells has not been directly demonstrated. Studies on the cell-free synthesis of ACTH directed by mRNA from normal rat and beef anterior pituitary suggest that normal ACTH-secreting tissues synthesize an ACTH-containing molecule similar in size to the ACTH/endorphin precursor observed in tumor tissue [10, 11]. Furthermore, extracts of mouse pituitary contain coincident peaks of ACTH and endorphin immunoactivity with a molecular weight similar to that of the tumor cell common precursor [12, 13]. In this paper we apply the techniques used to characterize ACTH and endorphin biosynthesis in the mouse pituitary tumor cells to begin to study ACTH and endorphin biosynthesis in cells of the anterior and intermediate lobes of the rat pituitary. A molecule similar to the ACTH/endorphin common precursor observed in tumor tissue is found in cells of both the anterior and intermediate lobes; however, the processing of the common percursor appears to differ greatly in the two lobes.

METHODS

Preparation of Tissue Extracts

Male rats (200–500 gm) were killed by decapitation; pituitaries were removed immediately and separated into anterior and intermediate-posterior lobes. Tissues were immediately homogenized into ice-cold 5 N acetic acid containing protease inhibitors [8] and stored at 4° over night; the supernatants were lyophilized and extracted with 1% acetic

acid containing protease inhibitors; the dilute acetic acid-soluble material was lyophilized and dissolved in sample buffer for SDS polyacrylamide gel electrophoresis [8]. When pituitaries were frozen on dry ice before extraction, significant degradation of the high-molecular-weight forms of ACTH and endorphin occurred.

Cell Suspensions

The anterior and intermediate-posterior lobes of the rat pituitary were separated and cell suspensions were prepared by the modified method of Vale et al. [14–16]. Cells were preincubated for 9–18 h in 12 mm × 75 mm culture tubes in serum-free Dulbecco's modified Eagle's medium containing glucose (4.5 mg/ml), kanamycin sulfate (0.1 mg/ml), glutamine (0.6 mg/ml), bovine serum albumin (2 mg/ml), and soybean trypsin inhibitor (0.1 mg/ml). Incubation medium was made by adding 150 μM [^{3}H] phenylalanine (21.8 Ci/mmole; New England Nuclear) to culture medium lacking phenylalanine; incubation medium also contained glucose, kanamycin sulfate, glutamine, bovine serum albumin, soybean trypsin inhibitor, lima bean trypsin inhibitor (0.1 mg/ml), and aprotinin (50 KIU/ml). Double antibody immunoprecipitates of culture medium or tissue extracts were prepared as previously described [1, 8]. Affinity-purified antiserum Bertha was used to prepare ACTH immunoprecipitates and affinity-purified antiserum Melinda (raised to synthetic α-endorphin conjugated to bovine serum albumin) was used to prepare endorphin immunoprecipitates [41]. Affinity-purified antiserum Melinda was prepared as follows: synthetic α-endorphin was linked to Sepharose 4B [17]; antiserum was bound to the resin, washed, and eluted by the method of Taylor and Schimke [18]; antibody was then dialyzed into a low-ionic-strength phosphate buffer by the method of Kurtz and Feigelson [19]. Endorphin immunoprecipitates of culture medium can be prepared without using affinity-purified antiserum.

Polyacrylamide Gel Electrophoresis

[^{3}H] phenylalanine-labeled immunoprecipitates and tissue extracts were analyzed by SDS polyacrylamide gel electrophoresis using the pH 8.5 borate/acetate system described before [8]. After electrophoresis, gels were cut into 2-mm disks and prepared for liquid scintillation counting or for radioimmunoassay [8].

Radioimmunoassays

Radioimmunoassays for ACTH using antiserum Bertha (with specificity for ACTH (11–24)) and antiserum Freddie (with specificity for ACTH(25–39)) were performed as described [8, 20]. Synthetic hACTH(1–39) (from Drs W. Rittel and P.A. Desaulles, Ciba-Geigy) was used as the standard in both ACTH immunoassays; this standard agrees with our previous natural porcine ACTH standard [21]. Radioimmunoassays for endorphin using antiserum RB-100 (provided by Drs N. Ling and R. Guillemin; Guillemin, Ling, and Vargo, [22]) were performed as described [9]. Synthetic β_p-endorphin (from Dr N. Ling) was used as the immunoassay standard; the concentration of stock solutions was determined by measurement of absorbance at 275 nm. Development of a radioimmunoassay for the NH_2-terminal fragment of pro-ACTH/endorphin is described below.

Antiserum to 16K fragment. A pool of partially purified 13K ACTH prepared from spent zero serum culture medium [20] was used as antigen; this preparation contains large amounts of 16K fragment and 11.7K endorphin in addition to 13K ACTH. Antigen emulsified with complete Freund's adjuvant was used for the initial and first booster injections (separated by a 3-month interval) of a female New Zealand white rabbit (Georgie) at multiple subcutaneous sites. Two subsequent booster injections (at 1.5-month intervals)

utilized incomplete Freund's adjuvant. This crude antiserum was diluted 1:1 with 0.05 M sodium phosphate buffer (pH 7.5) and purified by passage through a series of three affinity columns: ACTH(1–24)–Sepharose 4B [8], ACTH (17–39)–Sepharose 4B [20], and α-endorphin–Sepharose 4B (see above). The flow-through serum was used for all 16K fragment radioimmunoassays.

16K Fragment radioimmunoassays. 16K fragment was purified from zero serum culture medium. In order to identify 16K fragment, AtT-20 cells were incubated in zero serum culture medium containing low specific activity [^{3}H] phenylalanine (12 mCi/mmole). Spent culture medium was concentrated by batch ion exchange chromatography on CG-50 [8, 23] and fractionated on a column of Sephadex G-75 (superfine; 55 × 1.5 cm) in 1% acetic acid; fractions from K_d = 0.43 to K_d = 0.66 were pooled, lyophilized, and applied to a column of CM-cellulose (2.5 × 0.5 cm) equilibrated with 0.01 M ammonium acetate, pH 4.8. A gradient elution protocol similar to that of Birk and Li [24] was used to elute the column. Tryptic peptides of the various protein peaks that contained neither corticotropin nor endorphin immunoactivity were examined. The fractions containing 16K fragment could then be identified by comparison to the tryptic peptides of [^{3}H] phenylalanine-labeled 23K ACTH, 13K ACTH, and 16K fragment prepared by immunoprecipitation [9, 20, 41]. Peptides related to 16K fragment elute from the ion exchange column at more than one position; the purity of one pool of material was found to be approximately 90% when determined by SDS polyacrylamide gel electrophoresis, pH 4.5 urea polyacrylamide gel electrophoresis, or gel filtration on Sephadex G-75 in 6 M guanidine HCl. The protein concentration in this pool was determined by amino acid analysis, and a stock solution (10 μg/ml) was prepared for use as a radioimmunoassay standard. 16K fragment (1 μg) was iodinated by the procedure of Redshaw and Lynch [25]. 16K fragment radioimmunoassays were performed with a 1:50,000 dilution of purified antiserum Georgie in 0.05 M sodium phosphate buffer, 2.5 mg/ml bovine serum albumin (pH 7.6); the final assay volume was 0.2 ml and incubations were carried out for 24 h at 4° with approximately 5,000 cpm ^{125}I-labeled 16K fragment per tube. A double antibody immunoprecipitation scheme utilizing goat antiserum to rabbit gamma globulin (Research Products International) was used to separate bound from free hormone. Under these conditions the assay had a midpoint of 200 pg 16K fragment (18 fmole; assuming a molecular weight of 11,200) [41]; maximum net binding was 25% of the total. The specificity of the 16K fragment radioimmunoassay was determined by measuring the ability of various synthetic peptides to compete with ^{125}I-labeled 16K fragment for binding to purified antiserum Georgie. On a weight/weight basis, hACTH(1–39), β_hMSH, β_p-endorphin, and α-endorphin compete less than 1/3,000th as well; β_hLPH competes less than 1/600th as well; purified mouse βLPH competes less than 1/100th as well.

RESULTS

Forms of ACTH and Endorphin in Rat Pituitary

Radioimmunoassays for ACTH and endorphin can be used to obtain a static picture of the forms of ACTH- and endorphin-containing molecules present in tissue extracts. If the biosynthetic pathways for ACTH and endorphin in normal rat pituitary tissue are similar to those described in mouse pituitary tumor cells, rat pituitary extracts should have a collection of ACTH- and endorphin-containing molecules similar to those found in mouse pituitary tumor cell extracts. There are two sources of ACTH and endorphin in rat

pituitary. Essentially all of the cells in the intermediate lobe contain ACTH (or related peptides such as αMSH and CLIP) and endorphin (or related peptides such as βMSH, βLPH), and a small percentage of the cells in the anterior lobe contain ACTH and endorphin [26–29]. Since there is abundant evidence indicating that the physiologic control of hormone release from the two lobes differs [29–32], the anterior and intermediate lobes of the pituitary were investigated separately.

Extracts of the anterior and intermediate-posterior lobes of the rat pituitary were fractionated by SDS polyacrylamide gel electrophoresis and analyzed with an antiserum that recognizes the ACTH(11–24) region (antiserum Bertha) and an antiserum that recognizes the β-endorphin(20–27) region (antiserum RB-100) (Fig 1). Rat anterior pituitary extracts contain peaks of immunoactive endorphin at 31K, 13K, and 3.5K; the two smaller endorphins would correspond to βLPH and β-endorphin, respectively (Fig 1A). The peak of endorphin immunoactivity at 31K is coincident with a peak of ACTH immunoactivity; this observation is consistent with the existence of a common precursor to ACTH and endorphin in rat tissue as well as in mouse tumor tissue [1]. Additional peaks of immunoactive ACTH are observed at 21K, 14K, and 4.5K. Qualitatively these are the same forms of ACTH observed in mouse anterior pituitary and in mouse tumor cells [12, 13]; however, compared to mouse tissue, rat anterior pituitary is relatively enriched in 4.5K ACTH. With the antisera used, extracts of rat anterior pituitary contain close to equimolar amounts of immunoactive endorphin and immunoactive ACTH. Based on this static picture, we would anticipate that the pathway for ACTH and endorphin biosynthesis in rat anterior pituitary is similar to that described in the mouse pituitary tumor cells.

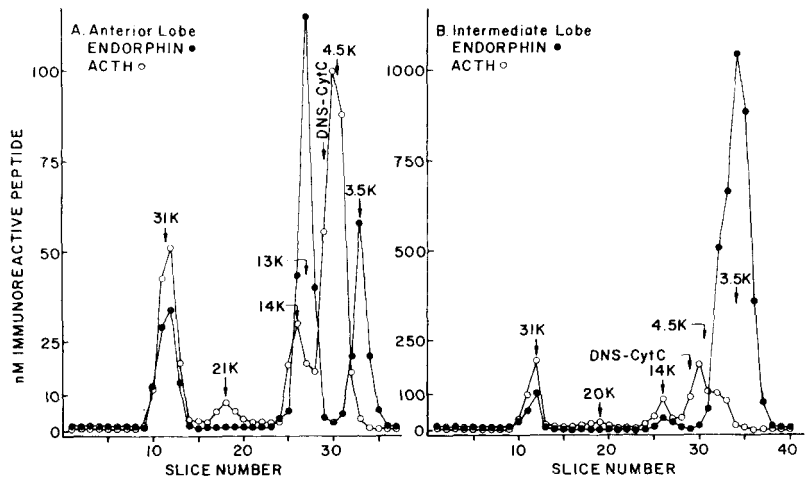

Fig 1. Rat pituitary; tissue extracts. The pituitary glands of two adult male rats (500 gm) were separated into anterior (A) and intermediate-posterior (B) lobes and extracted as described in Methods. Extracts (representing 0.27 anterior lobes and 1.62 intermediate-posterior lobes) were fractionated by SDS polyacrylamide gel electrophoresis; gels were sliced, eluted, and immunoassayed for ACTH (ACTH (11–24) antiserum Bertha, ○) and for endorphin (antiserum RB-100, •); recovery of immunoactivity was 98 ± 15%. Dansylated cytochrome C (DNS-Cyt C) was included in each gel as an internal standard; the apparent molecular weights of the immunoactive peaks are indicated. The results of a separate complete analysis of the forms of ACTH and endorphin in the separated lobes of the pituitary from a single male rat were identical (± 10%) to those shown here.

Using the same antisera, the distribution of activity among the forms of ACTH and endorphin in the intermediate-posterior lobe is qualitatively similar but quantitatively very different (Fig 1B). There are coincident peaks of ACTH and endorphin immunoactivity at 31K; this observation is consistent with the existence of a common precursor for ACTH and endorphin in the intermediate lobe. In the intermediate-posterior lobe extracts, approximately 93% of the endorphin immunoactivity migrates at the position of β-endorphin; a βLPH-like molecule (14K) is detectable, but accounts for only 3% of the endorphin immunoactivity. The ACTH immunoassay detects the same set of molecules observed in the anterior lobe extracts but the relative amounts differ; in addition, the total amount of immunoactive ACTH detected is less than 30% of the total amount of immunoactive endorphin detected. Antiserum Bertha detects the ACTH(11–24) region and does not detect NH_2-terminal or COOH-terminal pieces of ACTH [eg, ACTH(1–13) or ACTH(22–39)]; αMSH [N-acetyl-ACTH(1–13)NH_2] and CLIP [ACTH(18–39)] are thought to be the major ACTH(1–39)-related products in the intermediate lobe [33–36]. As indicated below, by using an ACTH antiserum with different specificity, one can detect an amount of ACTH-related material that is approximately equimolar to the amount of endorphin-related material present in intermediate-posterior lobe extracts. ACTH and endorphin biosynthesis in the two lobes may involve a similar common precursor, but the posttranslational processing of this precursor appears to differ greatly in these two tissues.

Antibody Specificity

The profile of immunoactive ACTH present in tissue extracts varies greatly depending on the specificity of the antiserum used. The binding of ^{125}I-labeled ACTH(1–39) to antiserum Freddie can be fully competed by ACTH(1–39), ACTH(25–39), and ACTH(34–39) [there is no competition by ACTH(1–24)]; this antiserum will be referred to as an extreme COOH-terminal ACTH antiserum. In anterior pituitary extracts (Fig 2A), the extreme COOH-terminal ACTH antiserum and the ACTH(11–24) antiserum (Bertha) detect approximately the same amount of the two smaller forms of ACTH. The extreme COOH-terminal ACTH antiserum detects immunoactive material at 21K but does not detect any material at the position of 31K ACTH/endorphin. In intermediate-posterior lobe extracts (Fig 2B) the extreme COOH-terminal ACTH antiserum still fails to detect any 31K ACTH/endorphin. In addition, there is a quantitative difference between the amount of lower-molecular-weight immunoactive material detected by the two ACTH antisera; the extreme COOH-terminal ACTH antiserum detects an amount of ACTH that is roughly equimolar to the amount of immunoactive endorphin detected (compare Figs 1B and 2B). The different patterns seen with the two ACTH antisera in intermediate-posterior lobe extracts presumably reflect cleavage of ACTH to αMSH and CLIP [33–36]; αMSH and CLIP are not found in the anterior pituitary and there is much less of a discrepancy between the two assays when looking at anterior pituitary extracts.

When mouse pituitary tumor cell culture medium is analyzed by the same techniques, it resembles anterior lobe extracts more than intermediate lobe extracts (Fig 2C). The two ACTH antisera detect similar amounts of the two smaller forms of ACTH; the extreme COOH-terminal ACTH antiserum detects approximately 45% as much 22K ACTH as the ACTH(11–24) antiserum and less than 2% as much pro-ACTH/endorphin as the ACTH(11–24) antiserum. Even with the high concentrations of antibody utilized during immunoprecipitation, the extreme COOH-terminal ACTH antiserum fails to precipitate pro-ACTH/endorphin. Identical aliquots of [^{3}H]tryptophan-labeled AtT-20 culture medium were

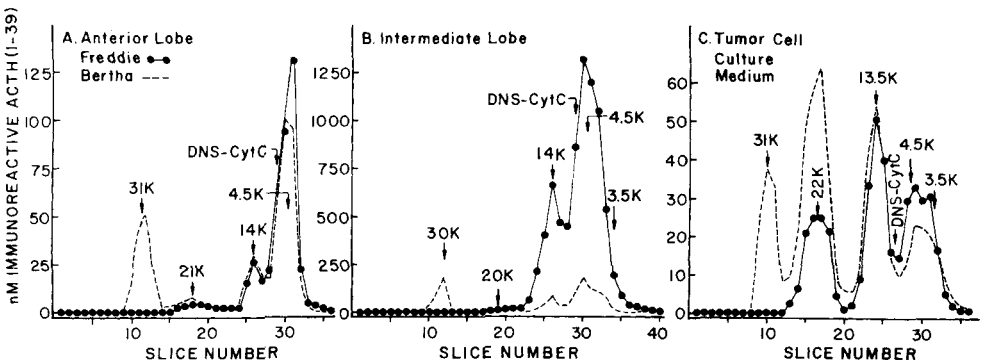

Fig 2. Antibody specificity – immunoassay. The anterior (A) and intermediate-posterior (B) extract gels analyzed in Figure 1 were assayed with an antiserum specific for the extreme COOH-terminal region of ACTH (antiserum Freddie, •). The results with the ACTH(11–24) antiserum (antiserum Bertha) are redrawn for comparison (- - -). Spent tumor cell culture medium (C) was concentrated by chromatography on CG-50 and fractionated by SDS polyacrylamide gel electrophoresis; individual slices were eluted and assayed with the ACTH(11–24) antiserum (- - -) and the extreme COOH-terminal ACTH antiserum (•).

immunoprecipitated with the extreme COOH-terminal ACTH antiserum and the ACTH (11–24) antiserum (Fig 3). The results of the immunoprecipitation are similar to those of the immunoassay; no pro-ACTH/endorphin is detected in the immunoprecipitate prepared with the extreme COOH-terminal ACTH antiserum and the amount of 22K ACTH present is only 60% of the amount present in an immunoprecipitate prepared with the ACTH (11–24) antiserum; the amount of 13K ACTH present is the same in the two immunoprecipitates.

Attempts to analyze the biosynthesis of ACTH using an antiserum with specificity similar to that of the extreme COOH-terminal ACTH antiserum would have failed to detect the early stages of the biosynthetic pathway. The ACTH(11–24) antiserum used in these studies is capable of immunoprecipitating the initial gene product [3, 13]. Studies on the cell-free synthesis of ACTH using mRNA from normal pituitary and from pituitary tumor cells indicate that the primary gene product has a molecular weight similar to that of pro-ACTH/endorphin [3, 10, 11, 13]. During short incubations of the mouse pituitary tumor cells with labeled amino acids, the pro-ACTH/endorphin molecule labels linearly from approximately time zero [8, 9].

Immunologic Probes for the Structure of Rat ACTH/Endorphin

Immunologic techniques were used to compare rat 31K ACTH/endorphin to the pro-ACTH/endorphin described in mouse pituitary tumor cells. The extreme COOH-terminal ACTH antiserum fails to detect a 31K ACTH/endorphin in the mouse tumor cells or in rat anterior or intermediate pituitary (Figs 2, 3). Our knowledge of the structure of the common precursor from mouse tumor tissue [41] provides an explanation for the inability of the extreme COOH-terminal ACTH antiserum to detect the common precursor; the βLPH-like sequence in the common precursor is located at the COOH-terminal side of the ACTH-like sequence and blocks the antigenic site recognized by the extreme COOH-terminal ACTH antiserum. The smaller forms of ACTH in the mouse tumor cells are

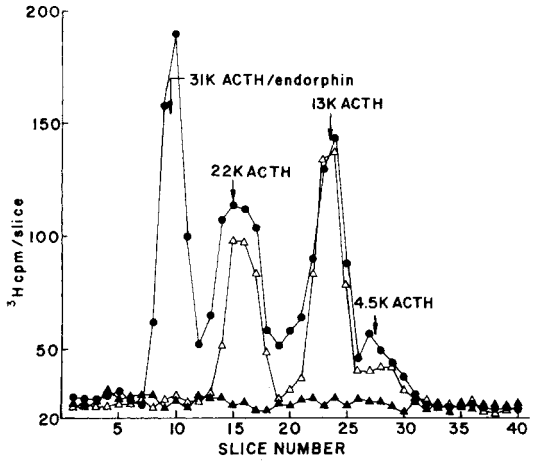

Fig 3. Antibody specificity – immunoprecipitation. A microwell of AtT-20/D-16v cells was incubated with 40 μM L-[G-^{3}H]-tryptophan (7.0 Ci/mmole; Amersham-Searle) for 8 h. The culture medium was split into three equal aliquots; immunoprecipitates were prepared using (•) affinity-purified ACTH (11–24) antiserum (Bertha) [4] or affinity purified extreme COOH-terminal ACTH antiserum (Freddie) [14] in the absence (△) or presence (▲) of excess synthetic hACTH(17–39). Immunoprecipitates were solubilized and analyzed on SDS polyacrylamide gels.

detected by the extreme COOH-terminal ACTH antiserum because the βLPH-like peptide extension has been removed. The fact that the same differential reactivity toward extreme COOH-terminal ACTH antisera is observed with rat pituitary extracts (Fig 2) suggests that the only form of rat ACTH in which the extreme COOH-terminal ACTH antigenic site is completely hidden is 31K ACTH/endorphin.

Since the mouse tumor cell pro-ACTH/endorphin molecule serves as a biosynthetic precursor to molecules that possess extreme COOH-terminal ACTH immunoactivity, it should be possible to expose this antigenic site by treatment of pro-ACTH/endorphin with the proper combination of endo- and/or exopeptidases. As shown in Figure 4, brief treatment of mouse tumor cell pro-ACTH/endorphin with α-chymotrypsin reveals a COOH-terminal ACTH antigenic determinant at the same time that it destroys the ACTH(11–24) antigenic determinant (there are several chymotryptic cleavage sites within the ACTH (11–24) segment). In a similar experiment using a 20 times lower concentration of α-chymotrypsin (weight ratio 1:1,000), the extreme COOH-terminal ACTH antigenic site was fully exposed within 10 min without destruction of the ACTH(11–24) site (not shown). Treatment of mouse tumor cell pro-ACTH/endorphin with cyanogen bromide or trypsin does not reveal the COOH-terminal ACTH antigenic determinant. If the structure of the 31K ACTH/endorphin molecule in rat anterior and intermediate pituitary is similar to that of mouse tumor cell pro-ACTH/endorphin, treatment of rat 31K ACTH/endorphin with α-chymotrypsin should generate extreme COOH-terminal ACTH immunoactivity. Rat anterior and intermediate-posterior lobe extracts were fractionated by SDS polyacrylamide gel electrophoresis and fractions containing 31K ACTH/endorphin were localized by radioimmunoassay. These fractions were each treated with α-chymotrypsin (as described in Fig 4 except with a 20 times lower concentration of α-chymotrypsin; weight ratio 1:1,000) and assayed for ACTH(11–24) and extreme COOH-terminal ACTH immunoactivity; treatment with α-chymotrypsin for 10 min increased the COOH-terminal ACTH immunoactivity to 60–70% of the ACTH(11–24) immunoactivity. These results suggest that the

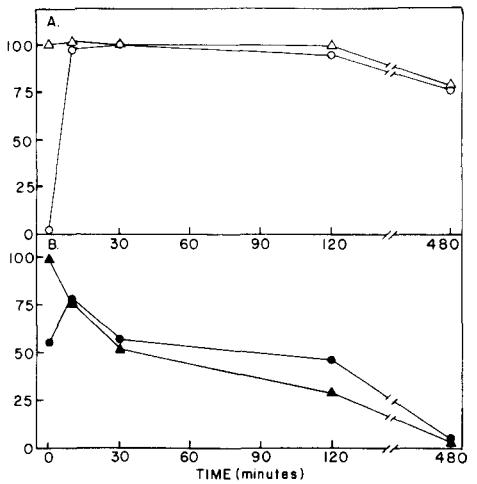

Fig 4. Treatment with α-chymotrypsin. Samples of pACTH(1–39) (10 μg) and partially purified pro-ACTH/endorphin (1.6 μg pACTH(1–39) equivalents, determined with the ACTH(11–24) antiserum) were dissolved in 100 μl 0.2 M NH_4HCO_3, 1 mg/ml bovine serum albumin. Chymotrypsin (2 μg; Worthington CDS, 61 units/mg) was added and samples were incubated at 37°. Aliquots (10 μl) were removed at the times indicated, diluted fivefold with 0.03 M sodium phosphate and 2 mg/ml bovine serum albumin (pH 7.4), treated with phenylmethylsulfonyl fluoride (final concentration 0.3 mg/ml), and stored frozen until assayed. A) Results obtained when each sample was immunoassayed with the extreme COOH-terminal ACTH antiserum: pACTH(1–39) (△); pro-ACTH/endorphin (○). B) Results obtained when each sample was immunoassayed with the ACTH(11–24) antiserum: pACTH(1–39) (▲); pro-ACTH/endorphin (●). Immunoassay of a blank tube (containing bovine serum albumin and α-chymotrypsin, but no ACTH) indicated that there was no detectable effect on either immunoassay. The pACTH(1–39) immunoactivity at time zero was taken as 100%; the maximum immunoactivity of the pro-ACTH/endorphin sample was taken as 100%.

structure of the rat 31K ACTH/endorphin molecules is similar to that of mouse pro-ACTH/endorphin in the COOH-terminal region of the ACTH-like segment.

The structure of the mouse pro-ACTH/endorphin molecule is almost entirely accounted for by its ACTH-like segment, its βLPH-like segment, and the glycoprotein segment that extends from the NH_2-terminal end of the ACTH-like segment. This glycoprotein (referred to as 16K fragment) has no known function as yet, but significant amounts of it are secreted intact by the mouse pituitary tumor cells [9, 41]. If the rat pituitary 31K ACTH/endorphin molecule is similar in structure to the mouse tumor cell pro-ACTH/endorphin molecule, one might expect to be able to detect a peptide similar to 16K fragment in rat pituitary extracts. A radioimmunoassay for mouse tumor cell 16K fragment was developed and used to assay rat pituitary extracts and mouse tumor cell culture medium (Fig 5). Tumor cell medium contains a large amount of 16K fragment immunoactivity with an apparent molecular weight of 16,000. As expected from structural studies, the 16K fragment assay also detects pro-ACTH/endorphin and 23K ACTH; the relative reactivity of these molecules appears to be low. Extracts of both rat anterior and intermediate lobe contain a major peak of immunoactive material at about 16K. In both cases the 16K fragment immunoassay detects the 31K molecule and the 21K or 20K molecules. The intermediate lobe extracts contain some cross-reactive material with an apparent molecular weight less than 16K; the identity of this material is not known. The amount of

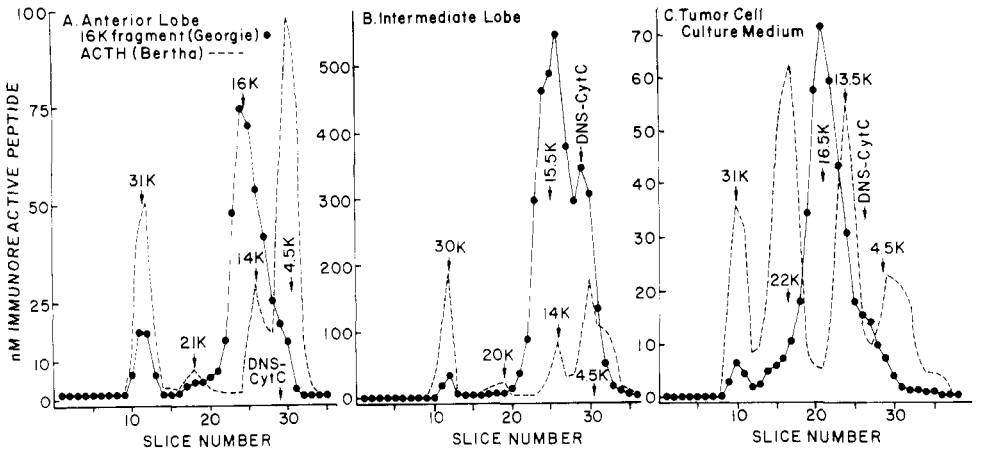

Fig 5. 16K Fragment immunoassay. The SDS polyacrylamide gels of rat anterior and intermediate-posterior lobe extracts described in Figures 1 and 2 were also immunoassayed for 16K fragment activity (antiserum Georgie, •); the ACTH(11–24) immunoassay data are redrawn for comparison (antiserum Bertha, - - -). The SDS gel of mouse pituitary tumor cell culture medium analyzed in Figure 2 was also assayed for 16K fragment immunoactivity. Recovery of 16K fragment immunoactivity was 86 ± 5%.

16K fragment immunoactivity in moles found in rat intermediate-posterior lobe extracts is similar to the amount of extreme COOH-terminal ACTH immunoactivity and the amount of endorphin immunoactivity. These results suggest that the basic scheme for biosynthesis of ACTH and endorphin in both lobes of the rat pituitary is qualitatively similar to that described in mouse tumor cells.

Rat Pituitary Cell Suspensions

In order to study the kinetics of ACTH and endorphin biosynthesis, it is necessary to be able to introduce radioactively labeled precursors into an actively metabolizing tissue. We have chosen to begin our studies on ACTH and endorphin biosynthesis in normal rat pituitary tissue by using isolated cell suspensions after a brief preincubation period in culture medium to allow recovery from the isolation procedure. The studies of Hopkins and Farquhar [37] and Vale and associates [14–16] have demonstrated the problems associated with use of chunks of pituitary tissue or acute preparations of dispersed single cells. Long-term pituitary cultures offer an interesting system for the study of ACTH and endorphin biosynthesis, but (for the present) we wanted to avoid the interesting problems associated with dedifferentiation usually seen in long-term pituitary cultures [14, 15, 38, 39].

Cell suspensions were prepared essentially by the procedure of Vale and colleagues [14–16]. In order to test the viability of the isolated cells following 24 h of incubation in culture medium, cell extracts were compared to extracts of the intact separated lobes of the pituitary (Fig 6). Extracts of isolated anterior pituitary cells were examined by immunoassay for ACTH and endorphin (Fig 6A). The distribution of ACTH and endorphin immunoactivity among the various molecular forms is essentially the same in the cell suspensions as in the intact anterior pituitary (compare Figs 6A and 1A). The isolated intermediate lobe cells were studied by incubation with [^{3}H]phenylalanine during the

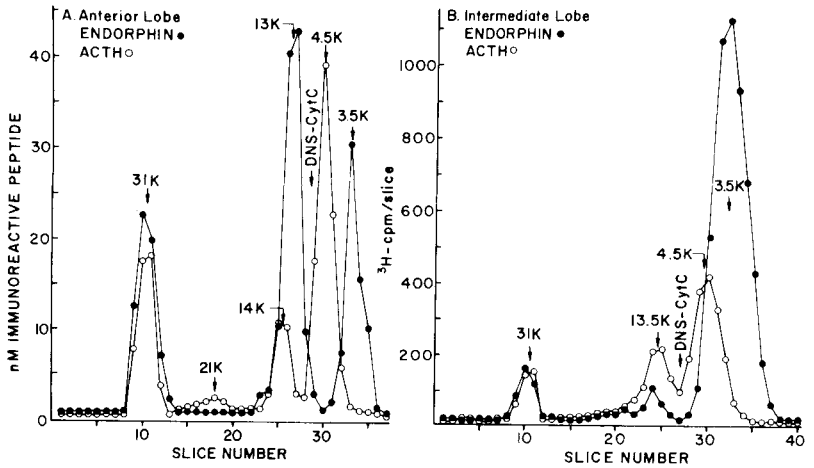

Fig 6. Rat pituitary cell suspensions. A) Anterior pituitaries from two male rats (450 gm) were used to prepare cell suspensions (Methods). The yield was 0.7×10^6 cells/pituitary; by radioimmunoassay this represented about 20% of the ACTH and endorphin present in a male rat anterior pituitary. The cells were incubated for 24 h (see Methods) and 25% of the cell suspension was extracted and analyzed by SDS polyacrylamide gel electrophoresis (Methods); gel slices were eluted and assayed with endorphin antiserum (RB-100, •) and ACTH(11–24) antiserum (Bertha, ○). B) Intermediate-posterior pituitaries from two male rats (200 gm) were used to prepare suspensions of intermediate lobe cells. The yield was about 5×10^4 cells/pituitary. The cells were incubated for 9 h in nonradioactive medium, and then for 15 h in 150 μl of medium containing [^{3}H] phenylalanine. The cells were than extracted and analyzed by immunoprecipitation with endorphin antiserum (Melinda, •) or ACTH(11–24) antiserum (Bertha, ○). The samples shown correspond to 0.6% of the cell suspension obtained from the two intermediate lobes. Addition of the appropriate synthetic peptide (ACTH(1–24) or β-endorphin) results in elimination of all of the peaks from each immunoprecipitate.

last 15 h of their 24-h incubation period and preparation of ACTH and endorphin immunoprecipitates of equivalent aliquots of cell extract. The distribution of material among the various forms of ACTH and endorphin is similar to that observed in the intact intermediate lobe extracts (Fig 1B); there is a relatively large amount of β-endorphin-like material and a relatively small amount of βLPH-like material. The intermediate lobe extracts show a quantitative lack of ACTH-immunoprecipitable material compared to the amount of material observed in an endorphin immunoprecipitate. The differences between anterior lobe tissue and intermediate lobe tissue appear to be preserved in short-term culture.

The sequential immunoprecipitation techniques used to study the common precursor in mouse pituitary tumor cells [1] were used to demonstrate directly that the 31K ACTH and 31K endorphin peaks detected in both anterior and intermediate lobe tissue actually represent a single molecule containing both antigenic determinants (ie, a 31K ACTH/endorphin). Equal aliquots of culture medium from a 15-h incubation of intermediate lobe cells with [^{3}H] phenylalanine were immunoprecipitated with ACTH or endorphin antiserum (Fig 7). The expected forms of ACTH and endorphin were observed. When the supernatant from an ACTH immunoprecipitate was subsequently immunoprecipitated with endorphin antiserum, only the two smaller endorphin peaks were present; the 31K endorphin molecule had been removed by the prior incubation with ACTH antiserum. Similar experiments on culture medium from a 15-h incubation of anterior lobe cells with [^{3}H] phenylalanine

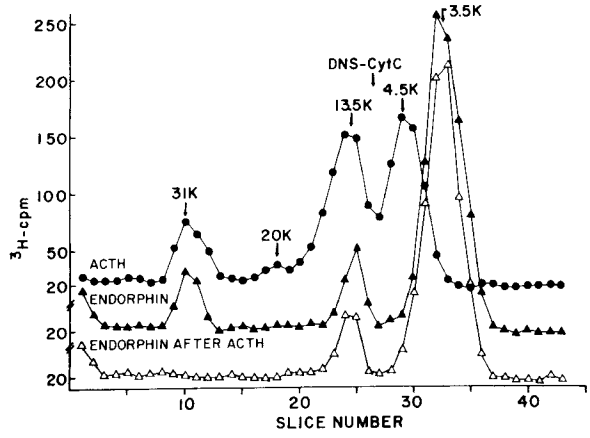

Fig 7. Culture medium from intermediate lobe cells: immunoprecipitation. Intermediate lobe cells were prepared and incubated as described in Figure 6B; 40μl aliquots of medium were immunoprecipitated with ACTH(11–24) antiserum (●) or endorphin antiserum (▲). The supernatant from the ACTH immunoprecipitate was immunoprecipitated with endorphin antiserum (endorphin after ACTH, △). The samples were analyzed by SDS polyacrylamide gel electrophoresis. Addition of the appropriate synthetic peptide resulted in elimination of all of the peaks from each immunoprecipitate.

indicate that a single 31K molecule contains antigenic determinants for both ACTH and endorphin (Fig 8).

Both the anterior lobe and intermediate lobe cells appear to release significant amounts of 31K ACTH/endorphin into the culture medium (Figs 7 and 8); the release of high-molecular-weight forms of ACTH and endorphin may not be restricted to tumor cells. Note also that the anterior and intermediate lobe cells release a different distribution of molecules; the major form of endorphin released from intermediate lobe cells is the size of β-endorphin and relatively little βLPH-like material is released. In anterior lobe medium there are significant amounts of both βLPH-like material and β-endorphin-like material. Based on immunoprecipitation with antiserum Georgie, both lobes secrete significant amounts of material that appears to be similar to 16K fragment (not shown).

The isolated anterior lobe cells are capable of responding to stimulation by secretagogues [16]. Incubation with a cyclic AMP derivative plus a phosphodiesterase inhibitor for the entire 15-h incubation period stimulates release of endorphin-precipitable material (Fig 9) and ACTH-precipitable material (not shown). Release of the β-endorphin- and βLPH-like material is stimulated 15- to 30-fold by this treatment; release of the 13-14K and 4.5K forms of ACTH is also stimulated 15- to 30-fold, but release of 31K ACTH/endorphin is only stimulated 2-fold.

DISCUSSION

The AtT-20/D-16v mouse pituitary tumor cell line has proven to be a useful system in which to study ACTH and endorphin synthesis and secretion [1–3, 8, 9, 12, 13, 20, 21]. The forms of immunoactive ACTH and endorphin observed in extracts of rat anterior and intermediate-posterior pituitary are similar to the forms of ACTH and endorphin observed in the mouse pituitary tumor cells (Figs 1, 2). The experiments reported here

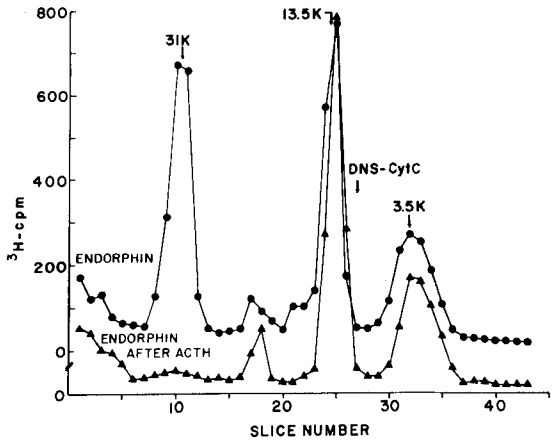

Fig 8. Culture medium from anterior lobe cells: immunoprecipitation. Anterior pituitary cell suspensions were prepared from two male rats (200 gm) and incubated in nonradioactive medium for 9 h. Cells were then placed into 225 μl of medium containing 150 μM [^{3}H] phenylalanine, 1 mM 8-bromoadenosine 3′, 5′-cyclic monophosphoric acid (Sigma; see Vale and Rivier [16]), and 0.25 mM isobutyl-3-methylxanthine (Sigma; see Vale and Rivier [16]). A 40-μl aliquot of the medium was immunoprecipitated with endorphin antiserum (•), and another 40 μl was immunoprecipated with ACTH(11–24) antiserum to remove ACTH-containing molecules; the supernatant of the ACTH immunoprecipitate was then immunoprecipitated with endorphin antiserum (endorphin after ACTH, ▲). The samples were analyzed by SDS polyacrylamide gel electrophoresis. The labeled protein in slices 17–19 is not competed out of the immunoprecipitate by the addition of excess synthetic β-endorphin, and is therefore not an endorphin-containing molecule; the peaks at 31K, 13.5K, and 3.5K are competed out of the immunoprecipitate by excess synthetic β-endorphin.

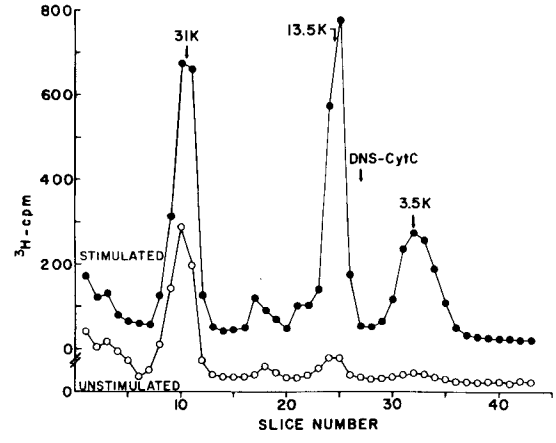

Fig 9. Culture medium from anterior lobe cells incubated with secretagogues: immunoprecipitation. Anterior pituitary cells were prepared and incubated as described in Figure 8, with ("stimulated," •; redrawn from Fig 8) and without ("unstimulated," ○) the addition of cyclic nucleotide plus phosphodiesterase inhibitor. Equal aliquots (40 μl) of the medium were immunoprecipitated with endorphin antiserum and analyzed by SDS polyacrylamide gel electrophoresis. Inclusion of the secretagogues stimulated total protein synthesis 20–25%.

suggest that the biosynthetic pathway previously delineated in mouse tumor cells is utilized by normal cells of both the anterior and intermediate lobes of the rat pituitary. The existence of a single molecule containing antigenic determinants for both ACTH and endorphin (31K ACTH/endorphin) was demonstrated directly by sequential immunoprecipitation with ACTH and endorphin antisera (Figs 7, 8). Several pieces of evidence indicate that the structure of the 31K ACTH/endorphin molecule in rat pituitary is similar to the structure of the mouse tumor cell pro-ACTH/endorphin molecule. First, the smaller ACTH- and endorphin-containing molecules in the rat pituitary are similar in size to those in the mouse tumor cells (Figs 1, 2). Second, use of an extreme COOH-terminal ACTH antiserum indicates that the COOH-terminus of the ACTH-like segment is inaccessible in both mouse tumor cell pro-ACTH/endorphin and in rat pituitary 31K ACTH/endorphin (Figs 2, 3); brief treatment with α-chymotrypsin exposes this antigenic site in both mouse and rat molecules (Fig 4). In the mouse tumor cell pro-ACTH/endorphin molecule, the βLPH-like segment extends from the COOH-terminus of the ACTH-like segment [41] and presumably masks the extreme COOH-terminal ACTH antigenic site. Third, the structure of the mouse tumor cell pro-ACTH/endorphin precursor is largely accounted for by the sum of three product peptides: the ACTH-like segment, the βLPH–like segment, and the 16K fragment (which comprises the NH_2-terminal region of the common precursor). Three similar peptide pieces can be detected in extracts of rat anterior and intermediate-posterior pituitary (Figs 1, 5).

Although these studies suggest that ACTH and endorphin biosynthesis in both lobes of the rat pituitary begins with synthesis of a common precursor, it is clear that the subsequent processing of the common precursor differs greatly in the two lobes. Intermediate lobe cells contain and secrete large amounts of a β-endorphin-like peptide; anterior lobe cells contain and secrete relatively more of a βLPH-like peptide (Figs 1, 6–9). Beef intermediate lobe cells have also been reported to contain large amounts of newly synthesized β-endorphin and relatively little βLPH [40]. Anterior lobe extracts contain approximately equimolar amounts of activity when assayed with antisera to ACTH(11–24), the extreme COOH-terminal of ACTH, endorphin, and 16K fragment (Figs 1, 2, 5). Intermediate lobe extracts contain close to equimolar amounts of activity when assayed with antisera to the extreme COOH-terminal of ACTH, endorphin, or 16K fragment; however, there is significantly less ACTH(11–24) immunoactivity present. This loss of ACTH(11–24) immunoactivity presumably reflects proteolytic cleavage in this region to form αMSH and CLIP [33–36]. Thus data reporting dissimilar distributions of immunoactive ACTH and endorphin in various tissues (eg, different regions of the brain) do not necessarily provide evidence for or against the hypothesis that endorphin and ACTH biosynthesis proceeds via a common precursor similar to that described in mouse pituitary tumor tissue.

The extreme COOH-terminal ACTH antiserum is useful as a probe for the structure of the larger ACTH-containing molecules and for differences in posttranslational processing between anterior and intermediate lobe tissue. However, its inability to detect 31K ACTH/endorphin limits the usefulness of this antiserum in studies of the early stages of biosynthesis. Antisera with specificity for the NH_2- or COOH-terminal regions of small peptides may often have difficulty detecting precursor molecules; the use of antisera with specificity for internal sequences should minimize this potential problem.

The short-term cultures of isolated cells prepared from rat anterior and intermediate pituitary exhibit the differences in ACTH and endorphin synthesis and metabolism that would be expected from examination of the tissue extracts (Figs 1, 6). Although the direct pulse-chase experiments on ACTH and endorphin biosynthesis remain to be done, it is

anticipated that the 31K ACTH/endorphin molecule will serve as a common precursor in both tissues; presumably the complement of processing enzymes differs in the two tissues.

The mouse tumor cells secrete substantial amounts of pro-ACTH/endorphin; similarly, the 31K ACTH/endorphin molecule appears in culture medium from both anterior and intermediate lobe cells (Figs 7–9). At least for the anterior lobe cells the appearance of 31K ACTH/endorphin in culture medium is not simply the result of cell lysis. After a 15-h incubation with labeled phenylalanine, the two smaller forms of ACTH and endorphin are the major forms of labeled ACTH and endorphin in both the stimulated and unstimulated cells. However, 31K ACTH/endorphin is the predominant form of labeled ACTH or endorphin in the culture medium from unstimulated cells (Fig 9); upon incubation with a cyclic AMP analog and a phosphodiesterase inhibitor the release of the smaller forms of labeled ACTH and endorphin is preferentially stimulated. If the results in Figure 9 reflect the normal physiology of the anterior pituitary gland, significant amounts of 31K ACTH/endorphin may be secreted in vivo.

The two populations of ACTH and endorphin-secreting cells provide an excellent opportunity to investigate the factors responsible for the differences between ACTH/endorphin cells in the anterior lobe and the intermediate lobe; the effects of glucocorticoids, neurotransmitters, and corticotropin-releasing factor(s) on the two cell populations can be compared.

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