Biosynthesis of Adrenocorticotropic Hormone in Mouse Pituitary Tumor Cells*

(Received for publication, February 20, 1976)

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A double antibody immunoprecipitation technique using affinity-purified adrenocorticotropic hormone (ACTH) antiserum was employed to investigate the biosynthesis of ACTH in a mouse pituitary tumor cell line. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of cell extracts resolved four forms of ACTH with apparent molecular weights of 4,500, 13,000, 23,000, and 31,000. These four forms of ACTH can be detected by radioimmunooassay of cell extracts or by immunoprecipitation of cell extracts following incubation of cultures in [3H]tryptophan, [3H]lysine, or [3H]tyrosine. The double antibody immunoprecipitation scheme developed is specific, quantitative, and reproducible. ACTH biosynthesis was examined in both steady and pulse-labeling experiments using [3H]tyrosine or [3H]lysine. The results of these experiments are consistent with the proposal that M, = 31,000 ACTH is the biosynthetic precursor for all three smaller forms of ACTH and that M, = 23,000 ACTH is a biosynthetic intermediate. Both M, = 13,000 ACTH and M, = 4,500 ACTH are derived from the intracellular processing of M, = 31,000 ACTH.

High molecular weight forms of adrenocorticotropic hormone have been observed in pituitary extracts and pituitary tumor extracts from several different species (1-9). The ACTH- secreting mouse pituitary tumor cell line, AtT-20/D-16v, contains several high molecular weight forms of ACTH (1) and provides a convenient experimental system in which to investigate the function of these forms in the biosynthesis of ACTH. Tumor cells were incubated with 3H-amino-acids and a double antibody immunoprecipitation scheme was used to separate 3H-labeled ACTH from other 3H-labeled cell proteins.

METHODS

Production and Purification of Antibodies—AtT-20/D-16v cells were incubated in medium containing no serum for consecutive 24-hour periods (1). The incubation medium was desalted by dialysis against 10% (v/v) acetic acid (at 4°C), lyophilized, dissolved in sterile saline (0.9% NaCl solution), and mixed with complete Freund's adjuvant (Grand Island Biological Co.). A female New Zealand white rabbit was injected with this mixture, containing an amount of extract equivalent to approximately 150 pg of RIA-ACTH (ACTH activity determined by radioimmunoassay) in the thigh muscles; three booster injections were given at 2- to 3-month intervals by multiple subcutaneous injections. Radioimmunoassays were performed as described previously (1), except that antiserum Bertha (at a final dilution of 1:10,000 to 1:15,000) was used in this work. Antiserum Bertha reacts on an equimolar basis with α(1-39) and synthetic α(1-24) (kindly provided by Organon). Synthetic α(1-24) and α(11-24) are at least 104 times more potent at inhibiting binding of 35I-α(1-39) to this antiserum than α(1-10), α-melanocyte-stimulating hormone, α(10-18)NH2, or α(5-38) (fragments of ACTH were provided by Dr. W. Rittel, CIBA-GEIGY Ltd., Basel). With antiserum Bertha, M, = 31,000 ACTH gives competition curves that are slightly steeper than the competition curves for α(1-39) and the other three forms of tumor cell ACTH; the apparent RIA-ACTH (Bertha) value for M, = 31,000 ACTH is determined at the midpoint of the assay.

For use in immunoprecipitation of ACTH, crude antiserum Bertha was purified by affinity chromatography. Synthetic α(1-24) was covalently linked to cyanogen bromide-activated Sepharose 4B (11); the affinity resin contained 0.8 mg of protein/ml of resin. In order to purify up to 4 ml of ACTH antiserum, a 0.5-ml column of α(1-24)-Sepharose 4B was prewashed immediately before use with 4 ml of 6 m guanidine HCl, 6 ml of Buffer A (see Table I for definitions of buffers), 4 ml of Buffer B, 10 to 15 ml of Buffer A, and 0.5 ml of nonimmune rabbit serum. Antiserum diluted 3-fold with Buffer A was applied to the column at room temperature at a flow rate of 10 ml/hour. The column was washed with 5 ml of Buffer A, 10 ml of 1 m guanidine HCl, 10 ml of Buffer A, and eluted with 5 ml of Buffer B. The eluate was dialyzed briefly against 900 ml of Solution A (2 x 2 hours) and then dialyzed repeatedly into Buffer C for 2 days. This purified α(1-24) antiserum was treated for 30 min at room temperature with 1% diution of Solution D and then dialyzed for 2 days against Buffer E containing 0.5% 2-mercaptoethanol, clarified by centrifugation, and stored at 4°C with 0.02% sodium azide added. More than 80% of the binding capacity for 35I-α(1-39) in crude antiserum Bertha was followed at monthly intervals; additional booster injections were given at 2- to 3-month intervals by multiple subcutaneous injections.

The abbreviations used are: ACTH, adrenocorticotropic hormone; RIA-ACTH, ACTH activity determined by radioimmunoassay and expressed as equivalent weight of purified porcine ACTH; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; SDS, sodium dodecyl sulfate.

* This work was supported by National Institutes of Health Grant AM 16879 and National Science Foundation Grant GB 38163 to B.A.E., University of Colorado Medical Center.
† Supported by the Helen Hay Whitney Foundation.
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The nomenclature proposed by Li (10) is used to refer to the various fragments of ACTH (denoted by α(1-39)).
Acids-AtT-BO/DlGv cells were cultured in Dulbecco-Vogt medium containing 2.5% horse serum (1). Equal aliquots of AtT-20 cells were incubated medium containing L-\[3,5-'H\]tyrosine or L-\[4,5(n)-'H\]lysine were 0.4 and 0.8 (average molecular weight 15,500; Schwarz/Mann); dissolved in Buffer F.

Recovery of Radioactivity-A double antibody immunoprecipitation scheme was used. Each sample to be analyzed was dissolved in Solution E and then split into two equal aliquots; one aliquot received a large excess of α(1-24) (Solution G) and served as a measure of any nonimmune binding. Purified α(1-24) antiserum (20 µl), 20 µl of either Buffer F or G, and 10 µl of sample (containing approximately 5 ng of RIA-ACTH) were incubated at 4°C for 12 to 16 hours in 0.5 ml plastic centrifuge tubes (Brinkmann). Samples were diluted with 175 µl of Buffer E and any precipitate present was removed by centrifugation. Nonimmune rabbit γ-globulin, goat anti-rabbit γ-globulin, and 75 µl of Buffer H were added to 200 µl of this supernatant; the final volume was brought to 575 µl by addition of Buffer E. Samples were further incubated for 4 hours at 4°C; immunoprecipitates of cell extracts were washed once in 500 µl of Buffer H and once in 500 µl of Buffer I. Immunoprecipitates of tissue culture medium were washed twice in Buffer H and twice in Buffer I. The washed immunoprecipitates were dissolved in Buffer J and incubated in boiling H₂O for 5 min; aliquots were counted and the remainder was analyzed by SDS-polyacrylamide gel electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis—SDS-gel electrophoresis (10% acrylamide/0.5% N,N' methylenebisacrylamide) was performed as described by Davies and Stark (18). Molecular weight markers used were: bovine serum albumin, heavy and light chains of rabbit γ-globulin, aldolase, yeast alcohol dehydrogenase, carbonic anhydrase, myoglobin, ribonuclease, cytochrome c, and α(1-29). All samples contained denatured aldolase and denatured myoglobin (19) as internal standards; mobilities were calculated with respect to the myoglobin marker. After electrophoresis, gels were cut into 2-mm discs. For radioimmunoassays, gel slices were smashed and eluted into 0.06 m sodium phosphate/0.06% SDS/0.2 mg/ml of bovine serum albumin/0.05 M sodium phosphate/0.06% SDS/O.5 M urea/0.5 m NaHCO₃ at 37°C for 10 hours (20). 10 µl of scintillation fluid (25% Triton X-114/75% xylene; Ref. 21) were added. The recovery of radioactivity after gel electrophoresis was always greater than 90%; tritium was counted at 38% efficiency.

RESULTS

Resolution of Forms of ACTH on SDS Gels—Earlier studies using gel filtration in guanidine HCl resolved the ACTH activity in normal mouse pituitaries and in the AtT-20/D-16 cells into three size classes: Mₓ = 4,000 to 5,500 ACTH, Mₓ = 6,500 to 9,000 ACTH, and Mₓ = 20,000 to 30,000 ACTH (1, 2). The use of SDS-polyacrylamide gel electrophoresis (18) has made it possible to achieve better resolution of the different forms of ACTH (Fig. 1). Reference to peaks of immunoreactivity will be based on their apparent molecular weight in this gel system. ACTH activity in the Mₓ = 20,000 to 30,000 size class was resolved into two distinct forms: Mₓ = 23,000 ACTH and 31,000 ACTH. Cell extracts and tissue culture medium both contained Mₓ = 23,000 ACTH and 31,000 ACTH; there was relatively more Mₓ = 93,000 ACTH in the medium than in the cell extracts. The peak of Mₓ = 13,000 ACTH seen in both cell extracts and culture medium corresponds to the Mₓ = 6,500 to 9,000 ACTH previously described; this substantial difference in apparent molecular weight is considered further in the accompanying paper (23). As was seen using gel filtration in guanidine HCl, the predominant form of ACTH in cell extracts comigrated with 131I-labeled α(1-39) (Mₓ = 4,500 ACTH), but there was little if any of this form of ACTH in the culture medium. It has not been determined whether the differences between the ACTH pattern in cell extracts and in culture medium are due to selective secretion of various forms, selective proteolysis, or interconversion after secretion.

Immunoprecipitation of ACTH Activity—To study the kinetics of labeling of the different forms of ACTH, it was necessary to extract all four forms quantitatively and separate them from other labeled cell products. Extraction of cultures into cold acetic acid and subsequent solubilization in low ionic strength medium has been seen in many systems (13-16) and was verified directly with the AtT-20 cells. Cultures were incubated with α-H-amino-acid for 10 min, rinsed twice with isotonic saline (0.9% NaCl solution), and extracted with ice-cold 1 N HCl. The specific activity of the acid-soluble pools was determined using amino acid analysis to measure the amount of each amino acid, and paper electrophoresis at pH 1.9 and pH 6.5 (17) to determine the radioactivity associated with each amino acid. The pools of free lysine and tyrosine inside the cells were found to be 95 ± 8% (mean ± S.D.) equilibrated with lysine and tyrosine in the culture medium in 10 min.
strength sodium phosphate buffer containing 0.1% Triton X-100 (Solution E) was found to solubilize 95 ± 8% (mean ± S.D.) of the RIA ACTH solubilized by heating cultures in 6 M guanidine HCl plus 5% 2-mercaptoethanol (1). The technique of double antibody immunoprecipitation using purified α(1-24) antiserum was used to separate the four forms of ACTH from other radioactively labeled proteins in a single step. Nonspecific binding was monitored by performing control immunoprecipitations in the presence of sufficient α(1-24) to exceed the binding capacity of the purified α(1-24) antiserum by 100-fold. The adequacy of the immunoprecipitation procedure was determined by including trace amounts of 125I-α(1-39) during analysis of AtT-20 cell extracts. Immunoprecipitation and charcoal adsorption (24) both indicated that over 90% of the 125I-α(1-39) was bound to antibody. At least 95% of the immunoprecipitated 125I-α(1-39) was retained in the pellet through the washing procedure described under “Methods.” The presence of cell extract containing 5 to 10 ng of RIA-ACTH activity depressed the binding of 125I-α(1-39) by less than 5%; the amount of specific 3H-labeled immunoprecipitable material was not altered by doubling or halving the standard amount of purified α(1-24) antiserum used. Duplicate immunoprecipitations of a sample performed on different days agreed within ±10%.

The method of immunoprecipitation and separation of the forms of ACTH was tested using [3H]tryptophan as the labeled precursor (Fig. 2). The same four forms of ACTH detected with the ACTH radioimmunoassay (Fig. 1) were found in the immunoprecipitate (Fig. 2, open circles); all four forms of ACTH were displaced from the immunoprecipitates by excess α(1-24) (Fig. 2, closed circles). Again, immunoprecipitates of tissue culture medium did not show the same pattern as immunoprecipitates of cell extracts; there was very little [3H]tryptophan labeled M, = 4,500 ACTH in the medium and the amount of M, = 23,000 ACTH was relatively enhanced.

Steady Labeling Experiments—In order to observe the kinetics of labeling of the different forms of ACTH in the tumor cells, several identical cultures were incubated with [3H]lysine and harvested after increasing periods of time from 10 min to 6 hours (Fig. 3). Incorporation of [3H]lysine into trichloroacetic acid-precipitable material proceeded linearly for the entire 6-hour incubation. Cell extracts were analyzed by immunoprecipitation and gel electrophoresis. After a 10-min incubation the only form of ACTH labeled with [3H]lysine was M, = 31,000 ACTH. After longer periods of incubation more [3H]lysine was incorporated into M, = 31,000 ACTH, and radioactivity was also incorporated into M, = 23,000, 13,000, and 4,500 ACTH. This pattern of labeling is consistent with, but does not demonstrate, a precursor-product relationship between M, = 31,000 ACTH and the smaller forms of ACTH; the smaller...
forms of ACTH might be synthesized by slower pathways not involving $M_r = 31,000$ ACTH.

Similar results were found in steady labeling experiments using [3H]tyrosine instead of [3H]lysine.

In studying the kinetics of labeling of the four forms of ACTH, it is necessary to take into account ACTH secreted into the culture medium. For example, after the 6 hour incubation in [3H]lysine (Fig. 3C), the medium contained approximately the same amount of [3H]-labeled immunoprecipitable material as the cell extract (data not shown). However, at the short times used for pulse-labeling experiments (see below), the amount of labeled ACTH secreted is not significant; after 30 min there was less than 1% as much [3H]-labeled immunoprecipitable material in the medium as in the cells, and after 2 hours that ratio was about 10%.

**Pulse-labeling Experiments**—In order to expose the cells to a short pulse of [3H]-labeled amino acid with minimal disturbance to the system, the concentration of amino acids present in normal growth medium was used throughout the pulse and chase periods. Fig. 4 shows the results of exposing the AtT-20 cells to a 20-min pulse of [3H]tyrosine and then analyzing the cultures after varying periods in nonradioactive medium. Immunoprecipitation of a sample exposed to [3H]lysine for 20 min showed that label had been incorporated only into $M_r = 31,000$ ACTH (Fig. 4A). After a 20-min chase incubation in unlabeled tyrosine (Fig. 4B) the amount of label in $M_r = 31,000$ ACTH had decreased and label had begun to appear in the smaller forms of ACTH. After a 100-min chase incubation (Fig. 4C) there was very little label in $M_r = 31,000$ ACTH or in $M_r = 23,000$ ACTH, but the amount of label in $M_r = 13,000$ ACTH and $4,500$ ACTH continued to increase. Results of a similar pulse chase experiment with [3H]lysine are summarized in Fig. 5. A progression of label from $M_r = 31,000$ ACTH to $23,000$ ACTH to $13,000$ ACTH to $4,500$ ACTH was observed. The label in $M_r = 31,000$ ACTH disappeared with a half-life of 15 to 20 min. Label appeared in $M_r = 23,000$ ACTH after a lag period and then disappeared with a half-life of 15 to 20 min. Finally, label appeared in $M_r = 13,000$ ACTH and $4,500$ ACTH more slowly than in the two higher molecular weight forms of ACTH; $M_r = 13,000$ ACTH did not label and chase more rapidly than $M_r = 4,500$ ACTH. The results obtained with [3H]lysine and [3H]tyrosine were identical.

**DISCUSSION**

In order to examine the kinetics of synthesis of ACTH, it is necessary to purify all four forms of ACTH away from all other cell products in high yield. The immunoprecipitation procedure described here was shown to be reproducible, specific ([3H]-labeled material was displaced by $a(1-24)$) and quantitative. In addition, pituitaries are known to contain enzymes that can destroy ACTH activity (9, 25); therefore the protease inhibitors phenylmethylsulfonyl fluoride and iodoacetamide were used in these studies to prevent degradation or interconversion during the extraction and immunoprecipitation procedures. Pulse-chase experiments were performed without protein synthesis inhibitors; the rapid equilibration of cellular amino acid pools with the tissue culture medium indicated that a pulse of label could be introduced into the biosynthetic pathway simply by replacing the culture medium. The fact that the amount of label in $M_r = 31,000$ ACTH decreased steadily throughout the incubation in unlabeled amino acid indicates that the chase procedure was effective; in the continued presence of radioactive amino acids, $M_r = 31,000$ ACTH would have continued to label linearly for at least 40 min (Fig. 3).

The studies reported here indicate that synthesis of $M_r = 4,500$ ACTH in AtT-20 mouse pituitary tumor cells proceeds through a complex biosynthetic pathway beginning with the

![Fig. 3. Steady labeling with [3H]lysine: analysis of immunoprecipitates. Six identical cultures were each incubated in 100 ml of medium containing [3H]lysine (1.1 Ci/mmol). Individual cultures were harvested after 10, 20, 40, 60, 120, and 360 min of incubation; the results of the 10-, 60-, and 360-min incubations are shown. Approximately 5 ng of RIA-ACTH activity were applied to each gel. For short incubation times, immunoprecipitable counts represented 0.6% of the trichloroacetic acid-insoluble radioactivity. $\bullet$, specific immunoprecipitate; performed using Solution F; $\bigcirc$, $a(1-24)$ competed immunoprecipitate; performed using Solution G.](http://www.jbc.org/)

![Fig. 4. Pulse-chase with [3H]lysine: analysis of immunoprecipitates. Six identical cultures were each incubated in 60 ml of growth medium containing [3H]lysine (7.0 Ci/mmol) for 20 min; radioactive medium was removed, cultures were rinsed with 100 ml of unlabeled growth medium and then incubated in 200 ml of unlabeled growth medium. Individual cultures were analyzed after 0, 20, 40, 60, 100, or 150 min in unlabeled medium; the results of the 0-, 20-, and 100-min chase periods are shown. Approximately 7 ng of RIA-ACTH activity were applied to each gel. After the 20-min incubation in [3H]lysine, 1.4% of the trichloroacetic acid-insoluble radioactivity in the cell extracts was immunoprecipitable; trichloroacetic acid-insoluble counts (incorporated during the brief incubation in [3H]lysine) disappeared during incubation in unlabeled growth medium with a half-life of 3 to 4 hours. $\bullet$, specific immunoprecipitate; performed using Solution F; $\bigcirc$, $a(1-24)$ competed immunoprecipitate; performed using Solution G.](http://www.jbc.org/)
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Fig. 5. Pulse-chase with [3H]lysine: summary of analysis of immunoprecipitates. Twelve identical cultures were each incubated in 50 µl of growth medium containing [3H]lysine (2.0 Ci/mmol) for 20 min; radioactive medium was removed and cultures were incubated in 200 µl of unlabeled growth medium and duplicate cultures were analyzed at each of the times shown. About 18 ng of RIA-ACTION activity was applied to each gel; in order to precipitate this amount of RIA-ACTION activity, the amounts of purified α(1-24) antiserum and either Solution F or G were increased 4-fold over the standard amounts described under "Methods." After the 20-min incubation in [3H]lysine, 0.5% of the trichloroacetic acid-insoluble radioactivity in the cell extracts was immunoprecipitable; trichloroacetic acid-insoluble counts disappeared during incubation in unlabeled growth medium with a half-life of 3 to 4 hours. The amount of specific immunoprecipitable radioactivity assigned to each size class of ACTH was determined by subtracting the radioactivity remaining in the immunoprecipitate from the total radioactivity assigned to each size class of ACTH. The data presented indicate that the process of labeling of Mₖ = 31,000 ACTH to 13,000 ACTH and 4,500 ACTH is not simply aggregates of Mₖ = 4,500 ACTH but is a biosynthetic intermediate in this pathway. The quantitative question of whether all Mₖ = 31,000 ACTH molecules are processed through the Mₖ = 23,000 ACTH intermediate or whether some Mₖ = 31,000 ACTH can bypass the Mₖ = 23,000 ACTH intermediate cannot be answered yet. The data presented indicate that Mₖ = 13,000 ACTH and 4,500 ACTH are derived from a biosynthetic precursor (Mₖ = 31,000 ACTH and possibly 23,000 ACTH), but it is not yet clear whether Mₖ = 13,000 ACTH can or must serve as a precursor to Mₖ = 4,500 ACTH. The present data indicate that the process of labeling of Mₖ = 31,000 ACTH to 13,000 ACTH and 4,500 ACTH may be similar to the processing of proinsulin to insulin (26) and of preproparathyroid hormone to parathyroid hormone (27). The products of the in vitro translation of ACTH mRNA will have to be studied in order to determine whether ACTH biosynthesis involves a short-lived precursor analogous to preimmunoglobulin (28), preproparathyroid hormone (29, 30), and pregrowth hormone (31).

It was not clear whether any of the high molecular weight forms of ACTH are normally secreted by the pituitary and thus might have a functional role in the periphery in addition to their presumptive role as intermediates in the biosynthesis of Mₖ = 7,000 ACTH.

Acknowledgments—We thank Professor Edward Herbert for his advice and encouragement; Doris Guenzi for valuable technical assistance; and F. C. Dancioft, M. C. Neville, J. L. Roberts, and M. Budarf for critical reading of the manuscript.

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Biosynthesis of adrenocorticotropic hormone in mouse pituitary tumor cells.
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