High Molecular Weight Forms of Adrenocorticotropic Hormone in the Mouse Pituitary and in a Mouse Pituitary Tumor Cell Line†

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ABSTRACT: Denaturing solvents have been used to determine the molecular weight of the adrenocorticotropic hormone (ACTH) activity in mouse pituitary, in an ACTH secreting mouse pituitary tumor cell line (AtT-20/D-16v), and in the tissue culture medium from the pituitary tumor cells. ACTH activity was quantitated by radioimmunoassay and by bioassay. It is possible to utilize guanidine hydrochloride or sodium dodecyl sulfate in characterizing the multiple forms of ACTH because treatment of porcine ACTH (the 39 amino acid polypeptide form of ACTH, $\alpha(1-39)$), pituitary extracts, tumor cell extracts, and tumor cell tissue culture medium with these denaturants does not diminish the immunological ACTH activity. Based on gel filtration in the presence of guanidine hydrochloride, extracts of the pituitary tumor cells and the mouse pituitary contain three distinct molecular weight classes of ACTH activity. The major form of ACTH has a molecular weight similar to $\alpha(1-39)$ (molecular weight 4000–5500), but there are significant amounts of two higher molecular weight forms of ACTH: molecular weight 6500–9000 and molecular weight 20,000–30,000. The 6500–9000 molecular weight form of ACTH is the major form of ACTH in the tissue culture medium; there is no peak of $\alpha(1-39)$ size ACTH in the medium. In the radioimmunoassay all three forms of ACTH generate competitive binding curves parallel to that of porcine $\alpha(1-39)$; in the bioassay (stimulation of steroidogenesis in a mouse adrenal tumor cell line) the dose response curve for each of the molecular forms of ACTH is parallel to that for porcine $\alpha(1-39)$.

Many polypeptide hormones have been shown to exist in multiple molecular forms, although the physiological significance of many of these different forms is not yet clear (Yalow, 1974; Tager and Steiner, 1974). Multiple forms of adrenocorticotropic hormone (ACTH)[1] have been observed in pituitary extracts of many different mammals (Orth et al., 1973; Lang et al., 1973; Scott and Lowry, 1974; Coslovsky and Yalow, 1974; Yalow and Berson, 1971, 1973; Gewirtz et al., 1974). The 39 amino acid polypeptide form of ACTH ($\alpha(1-39)$,[2] molecular weight 4600) has been purified from human, bovine, porcine, ovine, rat, and mouse tissue (Tager and Steiner, 1974; Scott et al., 1974b; Canfield et al., 1970). The higher molecular weight forms of ACTH (referred to as "big" and "intermediate" ACTH by Yalow and coworkers; Yalow and Berson, 1971, 1973; Coslovsky and Yalow, 1974) have not yet been well characterized. The distribution of immunological ACTH activity among the various molecular forms of ACTH in extracts of mouse and rat pituitaries varies greatly in studies by different authors (Orth et al., 1973; Lang et al., 1973; Scott et al., 1974b; Coslovsky and Yalow, 1974). For example, the purification of a 39 amino acid polypeptide form of ACTH from extracts of rat pituitary has been reported (Scott et al., 1974b); however, other studies suggest that rat pituitary extracts contain no ACTH of this size (Coslovsky and Yalow, 1974).

Most of the gel filtration procedures for separating the different molecular forms of ACTH do not eliminate aggregation or conformation as a cause of the heterogeneity observed. There are ACTH binding proteins in plasma (Fehm et al., 1973), and human and porcine $\alpha(1-39)$ take on apparent molecular weights greater than 4600 following infusion into human subjects (Upton et al., 1970). Based on studies using ultracentrifugation (Brown et al., 1955) and equilibrium dialysis (Stouffer and Hsu, 1966) porcine ACTH is tightly bound by serum albumin and other plasma proteins.

The need for the use of denaturing solvents in studying these high molecular weight forms of ACTH can be illustrated by gel filtration studies of purified parathyroid hormone and proparathyroid hormone under nondenaturing conditions; under some conditions parathyroid hormone (molecular weight 9600) appears to be larger than chymotrypsinogen A (molecular weight 25,000) and proparathyroid hormone (molecular weight 12,000) (Cohn et al., 1974).

Corticotrophs, ACTH secreting cells, normally comprise only a small percentage of the cells in the pituitary (Siperstein and Miller, 1970; Baker et al., 1970; Costoff, 1973). An ACTH secreting cell line (AtT-20/D-16v) derived from a mouse pituitary tumor provides a simplified system in which to examine some aspects of ACTH synthesis, storage, and secretion (Furth, 1955; Buonassisi et al., 1962; Yasamura, 1968). In the studies reported here, denaturing solvents

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[1] Abbreviations used are: ACTH, adrenocorticotropic hormone; RIA-ACTH, ACTH determined by radioimmunoassay; BIO-ACTH, ACTH determined by bioassay; MSH, melanocyte stimulating hormone; Gdn·HCl, guanidine hydrochloride.

[2] The nomenclature proposed by Li (1959) is used to refer to the various fragments of ACTH (denoted by $\alpha(1-39)$) and αMSH (denoted by *N*-acetyl-$\alpha(1-13)NH_2$); porcine $\alpha(1-39)$ is denoted by $\alpha_P(1-39)$.

have been used to determine the molecular weights of the different forms of ACTH in the tissue culture medium from the tumor cell line and in extracts of the tumor cells. Extracts of mouse pituitary have also been examined in order to demonstrate that the high molecular weight forms of ACTH observed in the pituitary tumor cells also occur in normal mouse tissue.

Materials and Methods

Growth of Cells. Pituitary Tumor Cells. Mouse pituitary tumor cells (AtT-20/D-16v) were obtained from Dr. U. I. Richardson (Harvard Medical School) and were adapted to growth in Dulbecco-Vogt Modified Eagle's Medium. Tissue culture medium was prepared from powdered medium (North American Biologicals, Inc., or Grand Island Biological Co.); the following substances were added to each liter of medium: 3.7 g of $NaHCO_3$, 3.5 g of glucose, 0.6 g of glutamine, 0.1 g of kanamycin sulfate (Sigma Chemical Co.), and 20–25 ml of horse serum (see below). Monolayer cultures were grown at 36° in a humidified 14% CO_2–86% air atmosphere. Horse serum was prepared from fresh clotted blood by centrifugation and sterile filtration. The cells were maintained by serial passage at weekly intervals; culture medium was changed every 3 days. For passage the cells were removed from the dish with either 2 m*M* EDTA or 0.125% trypsin (Nutritional Biochemicals Co., trypsin I-300) in phosphate buffer (19 m*M* KH_2PO_4–80 m*M* Na_2HPO_4 (pH 7.3)). The characteristics of the tumor cells have been examined further by E. Herbert (manuscript in preparation).

Adrenal Tumor Cells. Y-1 mouse adrenal tumor cells were obtained from Dr. G. Sato (University of California, San Diego) and were grown in Ham's F-10 medium. Tissue culture medium was prepared from powdered medium; the following were added to each liter of F-10: 1.2 g of $NaHCO_3$, 1.5 g of glucose, 0.6 g of glutamine, 0.1 g of kanamycin sulfate, 150 ml of horse serum, and 25 ml of fetal calf serum (Colorado Serum Co., Denver, Colo., or Reheis Chemical Co., Kankakee, Ill.). The cells were grown at 36° in a humidified 5% CO_2–95% air atmosphere. For weekly passage, the cells were removed from the dish with 0.125% trypsin in phosphate buffer and were plated into tissue culture dishes precoated with 0.5% gelatin in 0.9% NaCl. When plating cells for bioassays, the cells were suspended with 0.09% trypsin plus 0.5 m*M* EDTA in phosphate buffer. Related mouse adrenal tumor cell lines have been used for bioassays by Yasamura et al. (1966) and by Kowal and Fiedler (1968).

Bioassays for ACTH. Standard porcine ACTH (Schwarz/Mann, 120 IU/mg; purified by gel filtration on Sephadex G-75 in 1% acetic acid) and unknown samples were dissolved in test medium. Test medium consists of Ham's F-10 containing 10% charcoal-treated horse serum in place of the standard mixture of horse serum and fetal calf serum. Charcoal-treated horse serum was prepared by mixing 40 ml of horse serum with 1.0 g of charcoal (Norit A; Matheson Coleman and Bell) for 15 min at room temperature; the charcoal was removed by centrifugation and sterile filtration. Plastic tubes and plastic or siliconized glass pipets were used throughout. Sets of identical dishes (35 mm) of adrenal cells were incubated in 1.0 ml of test medium for 1 hr at 36°. Following incubation of duplicate samples, the medium was extracted and reacted as described by Peterson (1957) and simplified by Sayers et al. (1971). Fluorescence was determined on a Hitachi MPF-2A fluorimeter. A standard curve (five concentrations of $\alpha_p(1-39)$) was carried out for each assay.

The following substances were all less than 0.003 times as active as $\alpha_p(1-39)$ on a weight basis: αMSH, $\alpha(1-13)NH_2$, $\alpha(1-16)NH_2$, $\alpha(11-24)$, $\alpha_p(25-39)$, bovine βMSH, rat prolactin (NIAMD-PRL-RP-1), rat follicle stimulating hormone (NIAMD-FSH-RP-1), and rat luteinizing hormone (NIAMD-LH-RP-1). All ACTH and MSH fragments were kindly provided by Dr. W. Rittel (CIBA-GEIGY Ltd., Basel).

Radioimmunoassay of ACTH. The radioimmunoassay for ACTH was performed by the method of Rees et al. (1971) with minor modifications. Antiserum to ACTH was obtained from the National Pituitary Agency (Baltimore, Md; Fred 699). Porcine ACTH was used both as the standard for the radioimmunossay and for iodination. Concentrations of ACTH stock solutions were determined by amino acid analysis (Spackman et al., 1958). ^{125}I-labeled $\alpha_p(1-39)$ was prepared with chloramine T as described by Rees et al. (1971), or with lactoperoxidase (Sigma Chemical Co.) as described by McIlhinney and Schulster (1974). Iodinated ACTH was purified on a 100-μl column of Amberlite CG50 (Island et al., 1965) or on a 2-ml column of Sephadex G-10 in 1% (v/v) acetic acid. Incubations were done in a final volume of 200 μl; 4000–8000 cpm of ^{125}I-labeled $\alpha_p(1-39)$ (about 20 pg) was added per tube. Antibody concentration was adjusted to give between 40 and 70% binding; the midpoint of the assay was between 50 and 200 pg of $\alpha_p(1-39)$. A standard (ten serial twofold dilutions of $\alpha_p(1-39)$) was assayed along with each group of unknowns; six serial twofold dilutions were assayed for each unknown.

The antiserum used gives parallel competition curves for $\alpha_p(1-39)$ and synthetic $\alpha(1-24)$. αMSH and the synthetic fragments $\alpha(1-16)NH_2$ and $\alpha(1-13)NH_2$ cross react with this antiserum, but the competition curve generated is not parallel to that for $\alpha_p(1-39)$ (Figure 1). There is less than 1% cross-reactivity with $\alpha_p(25-39)$ and $\alpha(11-24)$ and less than 0.1% cross-reactivity with bovine βMSH, $\alpha(1-10)$, rat prolactin, rat follicle stimulating hormone, and rat luteinizing hormone.

Radioimmunoassay of αMSH. αMSH antiserum was obtained from Dr. J. W. Kendall (University of Oregon Medical School) and showed less than 0.1% cross-reactivity with $\alpha_p(1-39)$, $\alpha(1-10)$, and $\alpha(1-16)NH_2$; it shows significant cross-reactivity with $\alpha(1-13)NH_2$. αMSH was iodinated by the hypochlorite procedure of Redshaw and Lynch (1974); ^{125}I-labeled αMSH was purified by gel filtration on Sephadex G-10 in 1% acetic acid. The αMSH radioimmunoassay was performed in the same way as the ACTH radioimmunoassay described above.

Gel Filtration. Gel Filtration in Gdn·HCl. Bio-Gel A 0.5m was equilibrated and eluted with 4.0 *M* Gdn·HCl (Sigma, Grade I)–0.02% bovine serum albumin; the flow rate of the column (0.9 × 30 cm) was 3–5 ml/hr. Sephadex G-75 was equilibrated and eluted with 6.0 *M* Gdn·HCl–0.02% bovine serum albumin; the flow rate of the column (0.9 × 53 cm) was 3–4 ml/hr. The following proteins were used to calibrate the columns: bovine serum albumin (68,000), aldolase (40,000), soybean trypsin inhibitor (22,000), ribonuclease (13,700), cytochrome *c* (11,700), lima bean trypsin inhibitor (9195), Trasylol (6520; FBA Pharmaceuticals), $\alpha_p(1-39)$ (4570), glucagon (3480), $\alpha(1-24)$ (2390), and αMSH (1660). K_d values were reproducible to within ±0.05. Gdn·HCl in amounts up to 20 m*M*

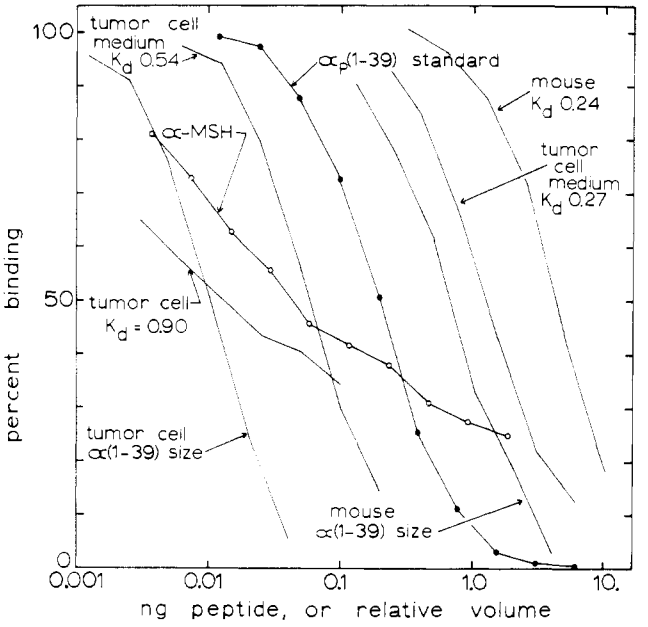

FIGURE 1: Radioimmunoassay of the different molecular forms of ACTH activity. The results from several separate ACTH radioimmunoassays were normalized for presentation in one curve. The competition curves for α_p(1-39) (●) and αMSH (○) were determined simultaneously. For each assay, the difference between damage and the total binding was adjusted to 100%; the α_p(1-39) standards from the separate radioimmunoassays agreed well when normalized in this fashion. Representative competition curves for each of the different molecular weight forms of ACTH activity are shown. For α_p(1-39) and αMSH the abscissa is ng of peptide. The competition curves for the other samples have been arbitrarily shifted along the abscissa for clarity of presentation.

does not interfere with the bioassay of ACTH. The radioimmunoassay for ACTH can be done with up to 50 m*M* Gdn·HCl; a blank containing 50 m*M* Gdn·HCl alone must be assayed to determine background. Unless specified otherwise, samples for the Gdn·HCl columns were dissolved in 6 *M* Gdn·HCl and 0.5–2.5% 2-mercaptoethanol, heated to 70° for approximately 10 min, and allowed to incubate at room temperature for several hours.

Gel Filtration in 1% Acetic Acid. Sephadex G-75 was equilibrated with 1% acetic acid. The column (1.1 × 55 cm) was calibrated with the same protein standards used above; the flow rate was 5–10 ml/hr. The elution volume observed for a given protein was reproducible between analyses, but it was not possible to fit all of the protein standards to a single calibration curve.

Other Columns. Columns of Bio-Gel P-6 and Sephadex G-10 were used as described (Mains and Eipper, 1975). The presence of bovine serum albumin in the elution buffers for both columns is necessary to obtain adequate recovery of ACTH activity.

Preparation of Tissue Extracts. Male mice were purchased from Jackson Labs (LAF_1/J) or obtained through the Bio-Social Research Facility, University of Oregon (DBA/2J and C57BL/6J). ACTH activity was extracted from pituitary tissue and from packed pituitary tumor cells by the method of Jacobowitz et al. (1963) using either 95% acetic acid or 5.0 *N* acetic acid. ACTH activity was also extracted by homogenization of tissue into 6.0 *M* Gdn·HCl and 0.5% 2-mercaptoethanol. The Gdn·HCl extracts were heated to 70° for 10 min and allowed to incubate at 20–25° for 10–24 hr, and insoluble material was removed by centrifugation.

Table I: Effect of Denaturants on ACTH Immunoactivity.

A. Recovery of ACTH Activity after Denaturation

Sample	Treatment	μg of RIA-ACTH/ml[c]
α_p(1–39)	10 m*M* acetic acid	10.0 ± 0.5
	6 *M* Gdn·HCl[a]	9.9 ± 1.2
	1% sodium dodecyl sulfate[b]	8.9 ± 1.1
AtT-20/D-16v culture medium (without serum)	Control	0.78 ± 0.07
	6 *M* Gdn·HCl[a]	0.84 ± 0.06
	1% sodium dodecyl sulfate[b]	0.78 ± 0.08

B. Extraction of ACTH Immunoactivity

Sample	Method	μg of RIA-ACTH/mg[f]
AtT-20/D-16v cells	5 *N* acetic acid[d]	1.86 ± 0.28
	6 *M* guanidine-HCl[e]	2.11 ± 0.28
Lyophilized bovine anterior pituitary powder	5 *N* acetic acid[d]	1.55 ± 0.18
	6 *M* Gdn·HCl[e]	1.58 ± 0.16

[a] One volume of sample was added to 3 volumes of 8 *M* Gdn·HCl and 2.7% 2-mercaptoethanol; the sample was incubated in boiling water for 2 min. [b] Nine volumes of sample were added to 1 volume of 10% sodium dodecyl sulfate, 0.1 *M* Tris-HCl (pH 7.4), and 2.2% 2-mercaptoethanol; the sample was incubated in boiling water for 2 min. [c] The values given have been corrected for dilution during the treatment with denaturant; mean ± standard deviation. [d] The sample was heated to 70° for 1 hr in 5 *N* acetic acid, incubated at 4° for 12 hr, centrifuged, and assayed after dilution into assay buffer or after lyophilization. [e] The cell pellet was dissolved in 6 *M* Gdn·HCl, 0.2% bovine serum albumin, and 0.5% 2-mercaptoethanol; the bovine anterior pituitary powder (Miles Laboratories) was dissolved in 6 *M* Gdn·HCl and 0.1% 2-mercaptoethanol; samples were heated to 70° for 10 min, incubated at 20° for 12 hr, centrifuged, and assayed. [f] For the tumor cells, μg of RIA-ACTH/mg of protein; for the bovine pituitary powder, μg of RIA-ACTH/mg of powder; mean ± standard deviation.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate urea polyacrylamide gel electrophoresis (6.0% polyacrylamide, 0.24% *N,N'*-methylenebisacrylamide) was performed as described by Swank and Munkres (1971). The gels were calibrated with the same proteins used to calibrate the Gdn·HCl columns. The ACTH radioimmunoassay is unaffected by concentrations of sodium dodecyl sulfate as high as 0.06 mg/ml, so it is possible to carry out ACTH radioimmunoassays after gel electrophoresis in sodium dodecyl sulfate. After electrophoresis gels were cut into discs of 2 mm length and eluted into 0.4 ml of 0.05% sodium dodecyl sulfate and 5 m*M* $NaHCO_3$ for 8 hr at 37°; the liquid was removed from the pieces of polyacrylamide and radioimmunoassayed as described above.

Protein Determination. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Results

Use of Denaturants to Study ACTH. Purified porcine ACTH can be incubated in either 6 *M* Gdn·HCl or 1% sodium dodecyl sulfate without decreasing its immunological activity (Table IA). Since the ACTH immunoactivity in tissue culture medium from the AtT-20/D-16v cells can also be recovered following denaturation with Gdn·HCl or sodium dodecyl sulfate (Table IA), these denaturants can be used to fractionate different molecular weight forms of ACTH in the culture medium. ACTH activity has often been solubilized from pituitary tissue by extraction with

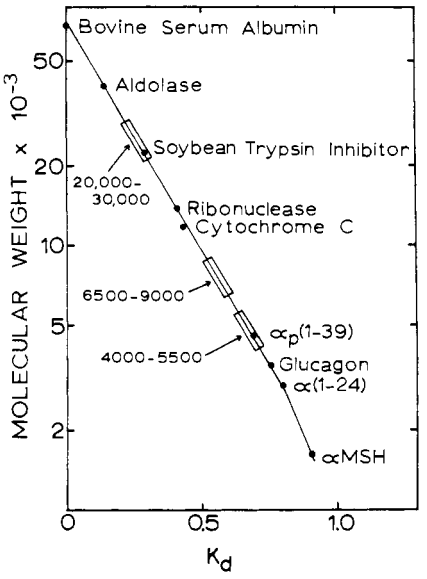

FIGURE 2: Calibration curve for Bio-Gel A 0.5m in 4 *M* Gdn·HCl. Protein standards were incubated in Gdn·HCl as described under Materials and Methods. When possible a void volume and a total volume marker were included in each sample. K_d values of replicate samples agreed to within ±0.05. Iodinated $\alpha_p(1-39)$ and [^{14}C]formaldehyde labeled $\alpha_p(1-39)$ (prepared by the method of Rice and Means (1971)) elute at the same position as $\alpha_p(1-39)$. The elution positions and molecular weights of the three different classes of ACTH activity are also indicated.

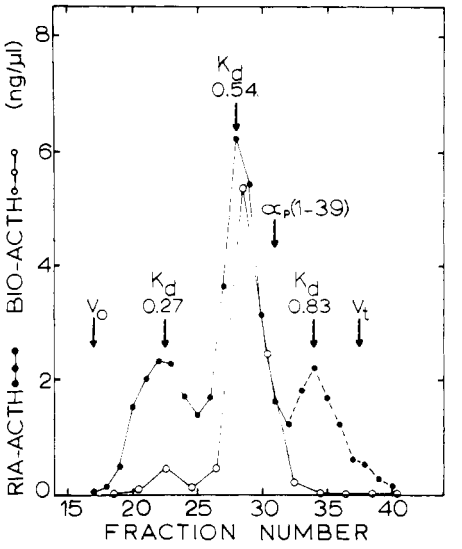

FIGURE 3: Gel filtration of mouse pituitary tumor cell culture medium in Gdn·HCl. AtT-20/D-16v cells were incubated in medium with 2.5% horse serum for 24 hr; medium was removed from the plates and cell debris was removed by centrifugation. The ACTH activity from 21 ml of medium was concentrated by ion exchange chromatography on Amberlite CG-50 (Island et al., 1965). One volume of medium was mixed with two volumes of 0.025 *M* acetic acid and immediately applied to a 0.5-ml column; the flow rate was kept below 1 ml/min. The column was washed with 0.025 *M* acetic acid until the pH was 3.5. The column was washed with 10 ml of 5% acetic acid and the ACTH activity was eluted with 3 ml of 60% acetic acid; Gdn·HCl was added and the sample was lyophilized. The recovery of RIA-ACTH activity by this method is greater than 95%. The sample was incubated with Gdn·HCl as described in Methods and applied to the column of Bio-Gel A 0.5m in 4 *M* Gdn·HCl and 0.02% bovine serum albumin; 0.46-ml fractions were collected. Fractions were pooled for bioassays. The overall recovery of RIA-ACTH activity was 83% and the overall recovery of BIO-ACTH activity was 72%. The material in fractions 32-40 generates competitive binding curves in the ACTH radioimmunoassay that are not parallel to the standard. Medium analyzed directly on the Gdn·HCl column without the CG-50 step shows an identical distribution of RIA-ACTH activity. RIA-ACTH, ng/μl (●); BIO-ACTH, ng/μl (○).

acetic acid (Jacobowitz et al., 1963). Extraction of mouse pituitary tumor cells or bovine anterior pituitary powder with Gdn·HCl solubilizes the same amount of ACTH activity as extraction with acetic acid (Table IB), indicating that Gdn·HCl can also be used to study high molecular weight forms of ACTH in tissue extracts. With the antiserum used in these studies, radioimmunoassays of ACTH activity in samples denatured with Gdn·HCl or sodium dodecyl sulfate can be performed when the denaturant is diluted to less than 50 m*M* or 0.006%, respectively; a denaturant blank must be performed for each assay. Bioassays for ACTH with the Y-1 adrenal tumor cell line can be performed when Gdn·HCl is diluted to less than 20 m*M*; bioassays were not performed following denaturation with sodium dodecyl sulfate.

The effectiveness of 6 *M* Gdn·HCl as a denaturant has been studied extensively by Tanford and coworkers (Fish et al., 1969). In many of the studies reported here the concentration of Gdn·HCl was lowered to 4 *M* in order to reduce interference in subsequent radioimmunoassays and bioassays. Gel filtration in 4 *M* Gdn·HCl gives a linear relationship of K_d to the logarithm of the polypeptide molecular weight for all the standard proteins examined (Figure 2). For all samples analyzed in both 4 and 6 *M* Gdn·HCl molecular weight determinations have been identical.

ACTH Activity in Mouse Pituitary Tumor Cell Tissue Culture Medium. As a first step in characterizing the ACTH activity secreted by the AtT-20/D-16v pituitary tumor cell line, the size distribution of the ACTH activity in the culture medium was analyzed by gel filtration in Gdn·HCl (Figure 3). After gel filtration in Gdn·HCl, ACTH activity was localized by diluting the fractions and carrying out radioimmunoassays and bioassays. The culture medium contains peaks of BIO-ACTH activity and RIA-ACTH activity at K_d = 0.27 (molecular weight 24,000 ± 5000) and K_d = 0.54 (molecular weight 8200 ± 1800). There is no peak of ACTH activity at the position of $\alpha_p(1-39)$. There is a peak of low molecular weight material (K_d = 0.83; molecular weight 2400 ± 700) that generates competition curves similar to those of $\alpha(1-16)NH_2$ and αMSH in the ACTH radioimmunoassay (Figure 1); this material is not biologically active. Radioimmunoassays of the culture medium fractionated on the Gdn·HCl column indicate there is less than 1% as much immunological αMSH activity as there is immunological ACTH activity.

The molecular weight distribution of the ACTH activity found in the tumor cell tissue culture medium is not altered when the concentration of serum in the tissue culture medium is raised from 2% horse serum to 12.5% horse serum plus 2% fetal calf serum during a 24-hr collection period. The pituitary tumor cells can be maintained in tissue culture medium without added horse serum. Although the growth rate drops to near zero under these conditions, the rate of secretion of RIA-ACTH activity is maintained for at least five consecutive 24-hr periods (data not shown). The size distribution of the ACTH activity found in zero serum medium is similar to that observed in normal growth medium (Figure 4). ^{125}I-labeled $\alpha_p(1-39)$ was included in the sample analyzed in Figure 4 in order to verify the absence of a peak of RIA-ACTH activity in the tissue culture medium at the position of $\alpha_p(1-39)$. Identical results were obtained when zero serum tissue culture medium was frac-

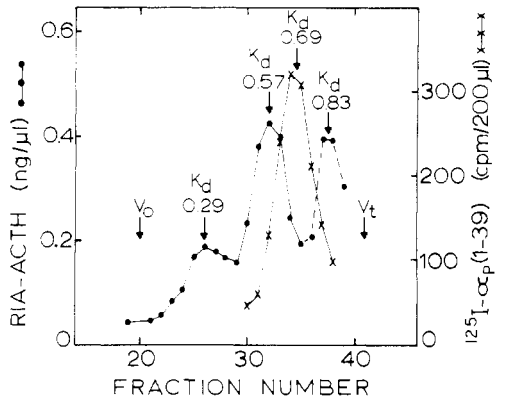

FIGURE 4: Gel filtration of zero serum mouse pituitary tumor cell culture medium in Gdn·HCl. AtT-20/D-16v cells were grown for 4 days in medium without serum; the medium was changed daily. A 0.6-ml aliquot of medium from the fourth day was made 6 *M* in Gdn·HCl and 1.5% in 2-mercaptoethanol; ^{125}I-labeled α_p(1–39) (3700 cpm) and 0.5 mg of bovine serum albumin were added. The sample was incubated as described in Methods and fractionated on the column of Bio-Gel A 0.5m in 4 *M* Gdn·HCl and 0.02% bovine serum albumin; 0.40-ml fractions were collected. The recovery of radioactivity was 95%; the recovery of RIA-ACTH activity was 77%. Fractions 36–39 did not generate competitive binding curves parallel to α_p(1–39) in the ACTH radioimmunoassay; it is not possible to quantitate the amount of low molecular weight cross-reacting material in these fractions. Iodination of α_p(1–39) does not alter its elution position from the Gdn·HCl column. RIA-ACTH, ng/μl (●); ^{125}I-labeled α_p(1–39), cpm/200 μl (x).

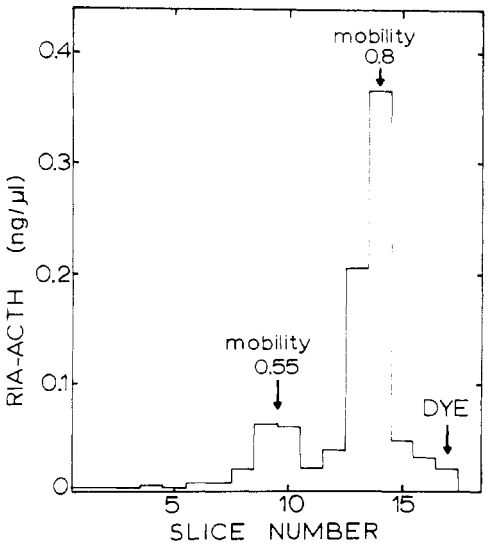

FIGURE 5: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of pituitary tumor cell culture medium. AtT-20/D-16v cells were incubated in medium without added horse serum; the medium was harvested and dialyzed against 10% acetic acid (at 4°), lyophilized, dissolved in 2% sodium dodecyl sulfate, 6 *M* urea, half-strength gel buffer, and 2.5% 2-mercaptoethanol, and incubated in boiling H_2O for 1 min. The sample was analyzed by gel electrophoresis as described under Materials and Methods. The direction of electrophoresis was from left to right. The recovery of RIA-ACTH activity was 70%. Yeast alcohol dehydrogenase (polypeptide molecular weight 37,000) had a mobility of 0.47; cytochrome *c* (molecular weight 11,700) had a mobility of 0.80; α_p(1–39) migrates close to the position of the tracker dye in this system.

tionated in 6 *M* Gdn·HCl (data not shown). Tissue culture medium containing 2.5% horse serum has a serum protein concentration of about 1.5 mg/ml; the ACTH concentration in the medium seldom exceeds 1–3 μg of RIA-ACTH/ml. By incubating the cells in medium without serum the products secreted by the cells can be obtained free from serum proteins, making purification of the molecular species with ACTH activity easier.

When tissue culture medium from the AtT-20/D-16v cells is analyzed by gel filtration on columns of Sephadex G-75 in acetic acid, there are two peaks of ACTH activity: one peak is between the positions of bovine serum albumin and carbonic anhydrase and accounts for about one-third of the ACTH immunoactivity; the other peak is slightly before the position of ribonuclease and accounts for about two-thirds of the ACTH immunoactivity in the tissue culture medium (data not shown).

Analysis of ACTH Activity in Tissue Culture Medium by Gel Electrophoresis. The existence of high molecular weight forms of ACTH was confirmed by analysis of the tissue culture medium on polyacrylamide gels in sodium dodecyl sulfate and urea. AtT-20/D-16v cells were incubated in medium without added horse serum (see above) and the medium was prepared for polyacrylamide gel electrophoresis as described in the legend to Figure 5. Two peaks of RIA-ACTH are present: about 15% of the RIA-ACTH activity has a molecular weight of between 20,000 and 30,000; 80% of the RIA-ACTH activity has a molecular weight of 8000–12,000. More accurate molecular weights were not assigned based on gel electrophoresis because calibration of the 10,000–20,000 molecular weight range of these gels with cytochrome *c*, ribonuclease, lysozyme, myoglobin, and hemoglobin did not yield a straight line; as noted in the original description of this gel electrophoresis system, ACTH and some other small peptides do not migrate according to molecular weight alone (Swank and Munkres, 1971). The size distribution of RIA-ACTH activity observed on sodium dodecyl sulfate polyacrylamide gels is consistent with the pattern observed using gel filtration in Gdn·HCl (Figures 3 and 4).

ACTH Activity in Mouse Pituitary Tumor Cell Extracts. Fractionation of 6 *M* Gdn·HCl extracts of tumor cells by gel filtration on Bio-Gel A 0.5m in 4 *M* Gdn·HCl indicates that there are two regions with both RIA-ACTH and BIO-ACTH activity: K_d = 0.29 (molecular weight 23,000 ± 5000) and K_d = 0.66 (molecular weight 5000 ± 800) (data not shown). The crude cell extracts also contain a peak of low molecular weight material (K_d = 0.90; molecular weight 1600 ± 500) that generates competitive binding curves similar to those of α(1–13)NH_2, α(1–16)NH_2, and αMSH in the ACTH radioimmunoassay; this material is not biologically active. In comparison to the amount of ACTH activity present, there is much more of this low molecular weight material present in cell extracts than in the tissue culture medium.

When the cell extract is first fractionated by gel filtration on Bio-Gel P-6 to remove material with a molecular weight below about 3000, it is possible to resolve three peaks of ACTH activity in the tumor cell extracts (Figure 6): K_d = 0.22 (molecular weight 30,000 ± 5000); K_d = 0.55 (molecular weight 7900 ± 1500); and K_d = 0.66 (molecular weight 5000 ± 800). These three forms of mouse tumor cell ACTH all generate competition curves parallel to α_p(1–39) in the ACTH radioimmunoassay (Figure 1). Although the peak of ACTH at K_d = 0.55 is resolved by only a single fraction in the radioimmunoassay (Figure 6), rechromatography experiments confirm the fact that the tumor cells contain three molecular weight classes of ACTH activity.

ACTH Activity in Mouse Pituitary Extracts. Since studies on the ACTH activity in the mouse pituitary tumor cells and in their tissue culture medium indicated that there were

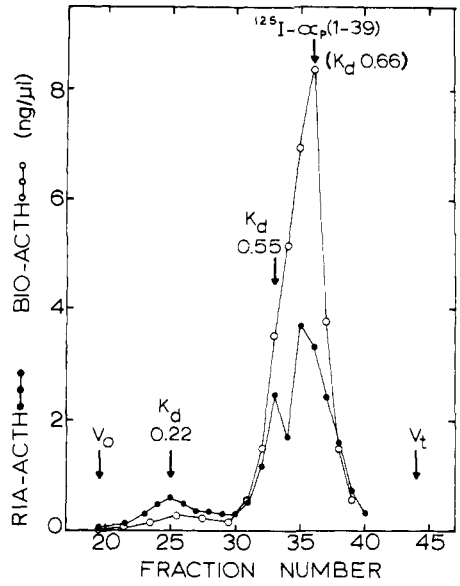

FIGURE 6: Gel filtration of mouse pituitary tumor cell extracts in Gdn·HCl. AtT-20/D-16v cells grown in medium with 2.5% horse serum were scraped from the dishes with a rubber policeman and collected by centrifugation. The medium had been changed 3 hr before harvesting. The cell pellet was extracted into 0.5 ml of 95% acetic acid as described under Materials and Methods. The sample was centrifuged and the supernatant was diluted and lyophilized. The residue was dissolved in 0.6 ml of 5% acetic acid plus 4% 2-mercaptoethanol; 16,000 cpm of ^{125}I-labeled α_p(1-39) was added and low molecular weight material was removed by gel filtration on Bio-Gel P-6. The fractions from the P-6 column with K_d values of 0-0.25 were pooled, lyophilized, dissolved in 6 *M* Gdn·HCl plus 6% 2-mercaptoethanol, and incubated as described under Materials and Methods. The sample was applied to the Bio-Gel A 0.5m column in 4 *M* Gdn·HCl and 0.02% bovine serum albumin; 0.44-ml fractions were collected. Fractions 19-30 were pooled pairwise for the bioassay. Recovery of radioactivity was 82%; ^{125}I-labeled α_p(1-39) and α_p(1-39) elute from the Gdn·HCl column at the same position. RIA-ACTH, ng/μl (●); BIO-ACTH, ng/μl (○).

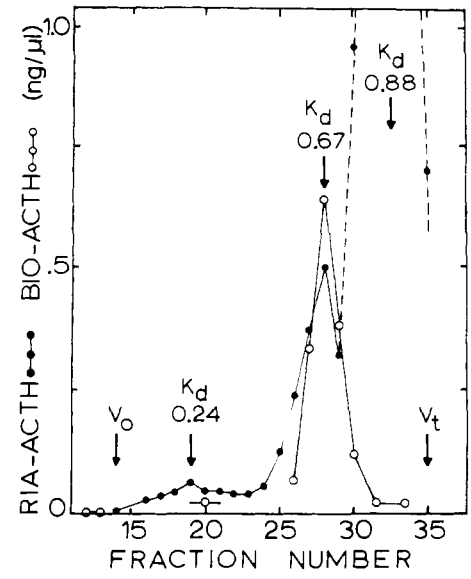

FIGURE 7: Gel filtration of mouse pituitary extract in Gdn·HCl. Pituitaries from six mice were extracted into Gdn·HCl as described under Materials and Methods. An aliquot of the sample (0.9 of the total) was analyzed on the Bio-Gel A 0.5m column in 4 *M* Gdn·HCl and 0.02% bovine serum albumin; fractions of 0.50 ml were collected. Fractions 26-34 were bioassayed directly. BIO-ACTH activity in fractions 12-13, 19-21, and 28 was determined after desalting the samples. The BIO-ACTH values plotted for fractions 12-13 and 19-21 were calculated from the BIO-ACTH/RIA-ACTH ratio of the desalted samples and the RIA-ACTH activity determined with the direct radioimmunoassay of ACTH. The binding curves for fractions 30-35 in the ACTH radioimmunoassay were not parallel to the curve for the α_p(1-39) standard; the location of the peak fraction can be determined, but the amount of cross-reacting material present cannot be accurately quantitated. Although a single sample in the radioimmunoassay defines the peak of RIA-ACTH at K_d = 0.67, this peak was found consistently, and the peak of RIA-ACTH activity coincided with the peak of BIO-ACTH activity. RIA-ACTH, ng/μl (●); BIO-ACTH, ng/μl (○).

three distinct classes of molecules with ACTH activity, it was of interest to determine whether these high molecular weight forms of ACTH occurred in the mouse pituitary. Whole mouse pituitaries were extracted with Gdn·HCl and fractionated by gel filtration in 4 *M* Gdn·HCl. RIA-ACTH activity was again observed at K_d values of approximately 0.24 (molecular weight 27,000 ± 5000) and 0.67 (molecular weight 4800 ± 800) (Figure 7). The amount of αMSH present in whole mouse pituitary extracts made it impossible to obtain ACTH radioimmunoassay data in the low molecular weight regions of the Gdn·HCl column with the antiserum used in these experiments. When extracts of mouse pituitary were fractionated on Bio-Gel P-6 to remove low molecular weight material, subsequent gel filtration in Gdn·HCl resolved three peaks of ACTH activity corresponding in size to those in tumor cell extracts (Mains and Eipper, 1975).

Relative Amounts of Different Forms of ACTH. Each of the forms of ACTH observed in the mouse pituitary extracts, mouse tumor cell extracts, and mouse tumor cell tissue culture medium generated parallel competitive binding curves to α_p(1-39) in the ACTH radioimmunossay (Figure 1) and parallel dose response curves in the ACTH bioassay (Figure 8). A summary of the distribution of ACTH activity among the different molecular forms is presented in Table II. The distribution of ACTH activity among the three different molecular weight classes is quite similar in extracts of mouse pituitary and mouse pituitary tumor cells. In contrast, tissue culture medium from the mouse pituitary tumor cells has a markedly different distribution of ACTH activity. The major form of ACTH in the tumor cell extracts (molecular weight 4000-5500) is not the major form of ACTH in the tissue culture medium (molecular weight 6500-9000). Further studies are required before it will be possible to determine whether these differences are caused by proteolytic modification of ACTH after secretion into the tissue culture medium or by selective secretion of high molecular weight forms of ACTH.

Each of the different molecular weight forms of ACTH has a characteristic BIO-ACTH/RIA-ACTH ratio regardless of its source (mouse pituitary, mouse pituitary tumor cells, or tumor cell medium) (Table III); these three values differ significantly from each other ($p < 0.001$). The biological and immunological activity per mole were not determined for any of these forms of ACTH (this will require highly purified preparations of the active peptides), and the varied BIO-ACTH/RIA-ACTH ratios observed could reflect a change in both BIO-ACTH activity/mole and RIA-ACTH activity/mole.

Discussion

The occurrence of forms of ACTH activity with apparent molecular weights greater than that of the 39 amino acid polypeptide form of ACTH has been reported in pituitary extracts from many species (Yalow and Berson, 1971, 1973;

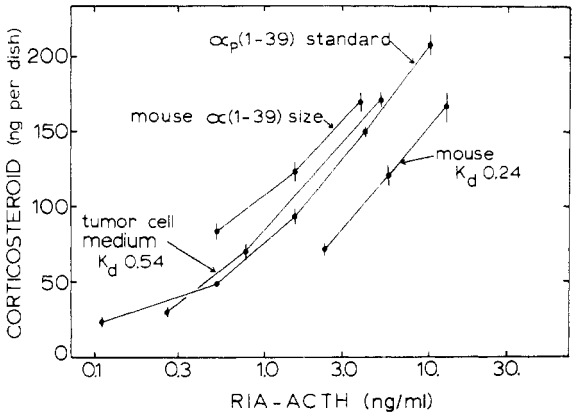

FIGURE 8: Bioassays of different molecular forms of ACTH activity. Mouse adrenal tumor cells were incubated in the presence of α_p(1-39) or various test samples, and the steroid production per dish was determined as described under Materials and Methods. Representative curves for the different molecular forms of ACTH activity are shown. The samples tested were separated by gel filtration in Gdn·HCl. The tumor cell 6500-9000 molecular weight ACTH (K_d = 0.54) and the mouse α(1-39) size ACTH (4000-5500 molecular weight) samples were bioassayed without desalting (similar results were obtained with desalted mouse 4000-5500 molecular weight ACTH). The high molecular weight mouse sample (K_d = 0.24) was desalted; both the RIA-ACTH activity and the BIO-ACTH activity were determined on the desalted sample. Similar curves were obtained with samples isolated by gel filtration in 1% acetic acid. The vertical bars indicate the spread in the duplicate samples used for each point.

Table II: Distribution of ACTH Activity among the Different Molecular Weight Forms.[a]

	Source					
	Mouse Tumor Cell Medium		Mouse Tumor Cell Extract		Mouse Pituitary Extract[b]	
Molecular Weight Class	RIA-ACTH	BIO-ACTH	RIA-ACTH	BIO-ACTH	RIA-ACTH	BIO-ACTH
20,000–30,000	34	4	16	4	9	5
6,500–9,000	54	78	21	25	34	24
4,000–5,500	12[c]	17[c]	62	72	57	72

[a] Extracts from the sources indicated were applied to a column of Bio-Gel A 0.5m in 4 *M* Gdn·HCl (Materials and Methods) and analyzed for RIA-ACTH and BIO-ACTH activity (Figures 3–7). The activity in each peak is expressed as a percentage of the ACTH activity recovered from the column (usually greater than 75% of the applied activity). The low molecular weight, cross-reacting material was not included in these calculations, since it does not have the characteristics of ACTH. [b] Sum of results for anterior and intermediate-posterior lobes analyzed separately (Mains and Eipper, 1975). [c] No peak of α(1–39) size ACTH was observed (Figures 3, 4); ACTH activity eluting in fractions with a K_d value of 0.65–0.74 was assigned to the 4000–5500 molecular weight pool.

Table III: BIO-ACTH/RIA-ACTH Ratio of Different Molecular Weight Forms of ACTH.[a]

	Source		
Molecular Weight Class	Mouse Tumor Cell Medium	Mouse Tumor Cell Extract	Mouse Pituitary Extract
20,000–30,000	0.36 ± 0.23	0.35 ± 0.12	0.43 ± 0.12
6,500–9,000	0.96 ± 0.37	1.07 ± 0.33	0.95 ± 0.13
4,000–5,500		1.58 ± 0.41	1.72 ± 0.36

[a] The ratio of BIO-ACTH activity to RIA-ACTH activity was determined for samples isolated by gel filtration in either 4 *M* Gdn·HCl or 1% acetic acid. Since no significant differences were observed among acetic acid, Gdn·HCl, and desalted Gdn·HCl samples, the results obtained with all three methods were pooled. Data shown are the mean ± standard deviation.

Coslovsky and Yalow, 1974; Orth et al., 1973; Lang et al., 1973; Gewirtz et al., 1974). The use of well-characterized denaturants such as Gdn·HCl (Fish et al., 1969) and sodium dodecyl sulfate (Weber et al., 1972; Fish et al., 1970) in studying multiple forms of polypeptide hormones reduces the risk of studying high molecular weight forms created by aggregation, the presence of hormone binding proteins, or conformational effects. Since the ACTH activity in extracts of pituitary and pituitary tumor cells is not irreversibly inactivated by treatment with these denaturants (Table I), different molecular weight forms of ACTH can be fractionated in the presence of Gdn·HCl or sodium dodecyl sulfate. Using these methods, the ACTH activity in mouse pituitaries, mouse pituitary tumor cells, and tumor cell tissue culture medium can be grouped into three distinct molecular weight classes (Figure 2): 4000-5500; 6500-9000; and 20,000-30,000. The three different molecular weight forms of ACTH generate curves parallel to those observed with α_p(1-39) in the ACTH radioimmunoassay and bioassay (Figures 1 and 8); since these curves are all parallel, it is possible to compare the distribution of immunological or biological ACTH activity among the three molecular weight classes in different samples.

Extracts of mouse pituitary and mouse pituitary tumor cells have a very similar molecular weight distribution of ACTH activity (Table II). A high molecular weight form of ACTH thought to be similar to the 6500-9000 molecular weight class of ACTH described here has been observed in mouse pituitary extracts by Orth et al. (1973) and by Coslovsky and Yalow (1974). The distribution of mouse pituitary ACTH activity between the 6500-9000 molecular weight form and α(1-39) size ACTH observed in studies with denaturing solvents (Mains and Eipper, 1975) is similar to that observed using gel filtration in acetic acid (Orth et al., 1973). However, studies on mouse pituitary ACTH using gel filtration in buffered 10% plasma (Coslovsky and Yalow, 1974) indicate that there is no α(1-39) size ACTH activity present. An α(1-39) size form of ACTH has been purified from mouse pituitary tumor tissue (Canfield et al., 1970). Variation in the amount of the different high molecular weight forms of ACTH observed could be due to the specificity of the particular assay systems used, proteolytic degradation of high molecular weight forms, or the use of different solvents for extraction and gel filtration. In particular, α(1-39) size ACTH has been shown to assume high apparent molecular weights in the presence of plasma proteins (Brown et al., 1955; Stouffer and Hsu, 1966; Upton et al., 1970; Fehm et al., 1973).

The pituitary tumor cell line studied here (AtT-20/D-16v) grows in monolayer culture and was derived from the AtT-20/D-1 line, which grows in suspension culture (Yasamura, 1968). Orth and coworkers (Orth et al., 1973) observed a high molecular weight form of ACTH (molecular weight approximately 6000) in addition to α(1-39) size ACTH in the AtT-20/D-1 cells. Analysis of extracts of AtT-20/D-1 cells (kindly provided by D. Orth, Vanderbilt University) by gel filtration in 6 *M* Gdn·HCl indicates the presence of a 20,000-30,000 molecular weight form of ACTH as well as smaller forms of ACTH (data not shown). For both cell lines, ACTH activity in the tissue cul-

ture medium does not have the same molecular weight distribution as ACTH activity inside the cells, and α(1-39) size ACTH is not the major form of ACTH in the medium (Orth et al., 1973; text Figures 3-6). The distinctly different molecular weight distribution of ACTH activity in the tissue culture medium compared to ACTH in cell extracts raises interesting questions about cellular control of the storage and secretion of ACTH. In order to interpret these differences, information is needed on the presence of proteolytic activity in the tissue culture medium and the structural relationship of the different molecular weight forms of ACTH to each other. The nature of the low molecular weight cross-reacting material observed in the cell extracts and tumor cell medium is not yet known; it lacks steroidogenic activity and could arise from the breakdown of ACTH or the postulated conversion of ACTH to αMSH (Scott et al., 1973).

The 20,000-30,000 molecular weight ACTH has a lower BIO-ACTH/RIA-ACTH ratio than the α(1-39) size ACTH (Table III). This low BIO-ACTH/RIA-ACTH ratio may involve a change in both the BIO-ACTH/mole and the RIA-ACTH/mole, or in only one of these parameters. Gewirtz et al. (1974) observed an extremely low BIO-ACTH/RIA-ACTH ratio for a form of human ACTH with a high apparent molecular weight ("big" ACTH) when it was bioassayed in vitro with rat adrenal cells. The bioassay used in the studies reported here utilized mouse adrenal tumor cells to assay mouse ACTH. Species specificity may be important in determining the BIO-ACTH/RIA-ACTH ratio observed for high molecular weight forms of ACTH. Species specificity has been reported with α(1-39) size human, porcine, and mouse ACTH (Shapiro et al., 1972; Pearlmutter et al., 1974; Schenkel-Hulliger et al., 1974). The occurrence of degradation of the high molecular weight forms of ACTH during incubation with mouse adrenal tumor cells in the bioassay has not yet been investigated. Isolated rat adrenal cells are known to cleave α(1-39) to smaller fragments during in vitro incubations (Voigt et al., 1974); it is not known whether the high molecular weight forms of ACTH are intrinsically biologically active or whether the steroidogenic activity observed involves proteolytic cleavage of these forms during the incubation.

In studying the distribution of ACTH activity among the different molecular weight forms, care must be taken to eliminate proteolytic activities that might alter the original distribution in the tissue (the possible precursor product relationship of the three size classes of ACTH has not been examined in these studies). An important role for proteases in the pituitary has been suggested by the occurrence of C-terminal fragments of ACTH in the pituitary (Scott et al., 1973; 1974a,b) and the suggestion that the observation of βMSH in human pituitary extracts depends on proteolytic degradation of β-lipotropin during extraction (Scott and Lowry, 1974). The extracts studied here were heated to inactivate proteolytic activity rapidly, but further studies with protease inhibitors must be done to determine whether or not proteolytic activities are altering the molecular weight distribution observed. Proteolytic activity may also create a family of closely related molecular species. Studies of proparathyroid hormone have indicated the existence of heterogeneity in the N-terminal sequence of the molecule (Hamilton et al., 1974) and partially cleaved proinsulin intermediates have been observed in pancreas (Kitabchi et al., 1972).

In order to determine in detail the molecular nature of the high molecular weight forms of ACTH, and their possible relationships to α(1-39) size ACTH, the forms must be purified. The AtT-20/D-16v cell line provides a particularly convenient source of material for such studies.

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References

Baker, B. L., Pek, S., Midgley, A. R., Jr., and Gersten, B. E. (1970), *Anat. Rec. 166,* 557-568.

Brown, R. A., Moyer, A. W., Davies, M. C., and Cox, H. R. (1955), *Arch. Biochem. Biophys. 58,* 68-72.

Buonassisi, V., Sato, G., and Cohen, A. I. (1962), *Proc. Natl. Acad. Sci. U.S.A. 48,* 1184-1190.

Canfield, R. F., Fried, J. E., and Cohen, H. (1970), *Endocrinology 86,* 700-702.

Cohn, D. V., MacGregor, R. R., Sinha, D., Huang, D. W. Y., Edelhoch, H., and Hamilton, J. W. (1974), *Arch. Biochem. Biophys. 164,* 669-673.

Coslovsky, R., and Yalow, R. S. (1974), *Biochem. Biophys. Res. Commun. 60,* 1351-1356.

Costoff, A. (1973), Ultrastructure of the Rat Adenohypophysis, New York, N.Y., Academic Press.

Fehm, H. L., Voigt, K. H., Lang, R., Özyol, M. B., and Pfeiffer, E. F. (1973), *FEBS Lett. 36,* 109-112.

Fish, W. W., Mann, K. G., and Tanford, C. (1969), *J. Biol. Chem. 244,* 4989-4994.

Fish, W. W., Reynolds, J. A., and Tanford, C. (1970), *J. Biol. Chem. 245,* 5166-5168.

Furth, J. (1955), *Recent Prog. Horm. Res. 11,* 221-255.

Gewirtz, G., Schneider, B., Krieger, D. T., and Yalow, R. S. (1974), *J. Clin. Endocrinol. Metab. 38,* 227-230.

Hamilton, J. W., Niall, H. D., Jacobs, J. W., Keutmann, H. T., Potts, J. T., Jr., and Cohn, D. V. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 653-656.

Island, D. P., Shimizu, N., Nicholson, W. E., Abe, K., Ogata, E., and Liddle, G. W. (1965), *J. Clin. Endocrinol. Metab. 25,* 975-983.

Jacobowitz, D., Marks, B. H., and Vernikos-Danellis, J. (1963), *Endocrinology 72,* 592-597.

Kitabchi, A. E., Duckworth, W. C., Stentz, F. B., and Yu, S. (1972), *Crit. Rev. Biochem. 1,* 59-94.

Kowal, J., and Fiedler, R. P. (1968), *Arch. Biochem. Biophys. 128,* 406-421.

Lang, R. E., Fehm, H. L., Voigt, K. H., and Pfeiffer, E. F. (1973), *FEBS Lett. 37,* 197-199.

Li, C. H. (1959), *Science 129,* 969-970.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265-275.

Mains, R. E., and Eipper, B. A. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72* (in press).

McIlhinney, J., and Schulster, D. (1974), *Endocrinology 94,* 1259-1264.

Orth, D. N., Nicholson, W. E., Mitchell, W. M., Island, D. P., Shapiro, M., and Byyny, R. L. (1973), *Endocrinology 92,* 385-393.

Pearlmutter, A. F., Franco-Saenz, R., Rapino, E., and Saffran, M. (1974), *J. Clin. Endocrinol. Metab. 39,* 150-153.

Peterson, R. E. (1957), *J. Biol. Chem. 225,* 25–37.
Redshaw, M. R., and Lynch, S. S. (1974), *J. Endocrinol. 60,* 527–528.
Rees, L. H., Cook, D. M., Kendall, J. W., Allen, C. F., Kramer, R. M., Ratcliffe, J. G., and Knight, R. A. (1971), *Endocrinology 89,* 254–261.
Rice, R. H., and Means, G. E. (1971), *J. Biol. Chem. 246,* 831–832.
Sayers, G., Swallow, R. L., and Giordano, N. D. (1971), *Endocrinology 88,* 1063–1068.
Schenkel-Hulliger, L., Maier, R., Barthe, P. L., Desaulles, P. A., Jarret, A., Riniker, B., Rittel, W., and Sieber, P. (1974), *Acta Endocrinol. (Copenhagen) 75,* 24–32.
Scott, A. P., and Lowry, P. J. (1974), *Biochem. J. 139,* 593–602.
Scott, A. P., Lowry, P. J., Bennett, H. P. J., McMartin, C., and Ratcliffe, J. G. (1974a), *J. Endocrinol. 61,* 369–380.
Scott, A. P., Lowry, P. J., Ratcliffe, J. G., Rees, L. H., and Landon, J. (1974b), *J. Endocrinol. 61,* 355–367.
Scott, A. P., Ratcliffe, J. G., Rees, L. H., Landon, J., Bennett, H. P. J., Lowry, P. J., and McMartin, C. (1973), *Nature (London), New Biol. 244,* 65–67.
Shapiro, M., Nicholson, W. E., Orth, D. N., Mitchell, W. M., Island, D. P., and Liddle, G. W. (1972), *Endocrinology 90,* 249–256.
Siperstein, E. R., and Miller, K. J. (1970), *Endocrinology 86,* 451–486.
Spackman, D. H., Stein, W, H., and Moore, S. (1958), *Anal. Chem. 30,* 1190–1206.
Stouffer, J. E., and Hsu, J.-S. (1966), *Biochemistry 5,* 1195–1201.
Swank, R. T., and Munkres, K. D. (1971), *Anal. Biochem. 39,* 462–477.
Tager, H. S., and Steiner, D. F. (1974), *Annu. Rev. Biochem. 43,* 509–538.
Upton, G. V., Hollingsworth, D. R., Lande, S., Lerner, A. B., and Amatruda, T. T., Jr. (1970), *J. Clin. Endocrinol. Metab. 30,* 190–195.
Voigt, K. H., Fehm, H. L., Lang, R., and Pfeiffer, E. F. (1974), *Acta Endocrinol. (Copenhagen), Suppl. 184,* 165.
Weber, K., Pringle, J. R., and Osborn, M. (1972), *Methods Enzymol. 26C,* 3–27.
Yalow, R. S. (1974), *Recent Prog. Horm. Res. 30,* 597–626.
Yalow, R. S., and Berson, S. A. (1971), *Biochem. Biophys. Res. Commun. 44,* 439–445.
Yalow, R. S., and Berson, S. A. (1973), *J. Clin. Endocrinol. Metab. 36,* 415–423.
Yasamura, Y. (1968), *Am. Zool. 8,* 285–305.
Yasamura, Y., Buonassisi, V., and Sato, G. (1966), *Cancer Res. 26,* 529–535.