Biologic and Immunologic Characterization and Physical Separation of ACTH and ACTH Fragments in the Ectopic ACTH Syndrome

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ABSTRACT Extracts of tumors from 32 patients with the ectopic ACTH syndrome were subjected to simultaneous bioassay and radioimmunoassays for ACTH. Radioimmunoassays were performed using three antisera, one of which reacts with the extreme N-terminal 1-13 amino acid sequence of ACTH, the second with the N-terminal 1-23 sequence of the ACTH molecule, and the third with the C-terminal 25-39 amino acid sequence of ACTH. There was, in general, good correlation between bioactivity and N-terminal ACTH immunoreactivity. However, there were large excesses of both extreme N-terminal and C-terminal immunoreactive materials in most tumor extracts, which were not found in extracts of three human pituitaries. Three tumor extracts were subjected to molecular sieve chromatography on Sephadex G-50 fine resin. The bioactive ACTH eluted in the same fractions as pituitary ACTH (mol wt $\simeq$ 4,500 daltons) and reacted equally in all three ACTH radioimmunoassay systems. The bioactive tumor ACTH was neutralized by incubation with the C-terminal antiserum, indicating it has an intact C-terminal sequence of amino acids. The next several fractions from the Sephadex column contained a material, mol wt $\simeq$ 3,100, which was biologically inactive and had C-terminal immunoreactivity but no N-terminal or extreme N-terminal immunoreactivity. Incubation with the N-terminal 1-23 ACTH antiserum did not adsorb these C-terminal fragments, indicating they lacked an intact sequence of amino acids in this region. A smaller ACTH fragment (mol wt $\simeq$ 1,800 daltons) eluted in still later fractions and reacted with the extreme N-terminal antiserum but not with the N-terminal or C-terminal antisera. It had no steroidogenic activity, but appeared to have significant melanocyte-stimulating activity. It is concluded that, in addition to an ACTH similar, if not identical, to pituitary ACTH, tumors of patients with the ectopic ACTH syndrome contain both N-terminal and C-terminal ACTH fragments.

INTRODUCTION

Partial characterization of the corticotropin extracted from tumors of patients with the ectopic adrenocorticotropic hormone (ACTH)[1] syndrome (1, 2) has shown it to be very similar to pituitary corticotropin in a variety of biologic, physicochemical, and immunologic test systems (3, 4). The precise structure of the tumor hormone has not been determined, however. In order to characterize tumor ACTH more fully, we have exploited a combination of ACTH bioassay, radioimmunoassays, immunologic neutralization, immunoadsorption, and gel filtration. Tumor extracts were subjected to bioassay and to radioimmunoassays utilizing ACTH antisera which react with three different portions of the ACTH molecule. The quantity of ACTH bioactivity was then correlated with the quantity of immunoreactive ACTH in each tumor extract. Finally, experiments were performed using gel filtration and immunologic neutralization and adsorption to determine if the major bioactive and immunoreactive components of a tumor extract were parts of the same molecule or were physically separable.

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[1] *Abbreviations used in this paper:* ACTH, adrenocorticotropic hormone. MSH, melanocyte-stimulating hormone.

METHODS

Preparation of tissue extracts. Freshly frozen human tumors or pituitaries were homogenized in glacial acetic acid and partially purified by cation-exchange chromatography on Amberlite CG-50 resin (Rohm & Haas Co., Philadelphia, Pa.) as described by Island et al. (5); heating was not employed. Extracts were dried in a flash evaporator and reconstituted in 0.001 N HCl. Portions were frozen at −70°C and thawed only once at 4°C immediately before assay.

Preparation of plasma extracts. Blood was withdrawn into lightly heparinized syringes, chilled, centrifuged immediately at 1,000–1,200 *g* at 4°C for 20 min, and the supernatant plasma aspirated and stored at −70°C. The portions were thawed once at 4°C and the ACTH extracted by modifications (6) of the method of Island et al. (5). Plasma extracts were then handled in a manner identical with that used for tissue extracts.

Preparation of plasma for direct radioimmunoassay. The availability of the polyethylene glycol method (7, and see below) for separation of antibody-bound ACTH from free ACTH allowed us to increase the volume of plasma added to the incubation mixtures, thereby permitting the assay of unextracted plasma. Plasma for this purpose was prepared in the same way as that for extraction, except that the 1,000 *g* plasma was carefully aspirated, transferred to a second tube, and centrifuged for 10 min at 6,000 *g* at 4°C. This yielded a pellet of additional formed blood elements. The plasma was carefully decanted and stored at −70°C. When prepared in this manner before freezing, thawed plasma was much less destructive to ACTH (8) and caused much less "incubation damage" in the radioimmunoassay system.

ACTH bioassay. Bioassay for ACTH was performed by modifications (9) of the method of Lipscomb and Nelson (10). Potency was related to that of highly purified human ACTH (Lerner-Upton-Lande preparation 8B) (11), which has a potency of 100 IU/mg (8), assuming that the Third International Standard for Corticotropin has 1.5 intravenous IU/ampoule (12). Each extract was assayed at two effective dilutions containing approximately 0.3 and 1.2 ng of ACTH, respectively, using four animals at each dilution. The mean potency for each extract was calculated and rounded off to the nearest nanogram.

ACTH radioimmunoassays. Immunoreactive ACTH concentration was estimated by modifications of the method previously reported from this laboratory (13). Highly purified human ACTH (Lerner-Upton-Lande preparation 8B) was used both for iodination and as reference standard. ACTH was labeled with ^{131}I or ^{125}I by minor modifications of the method of Hunter and Greenwood (14). Specific activities of 800–1,000 and 150–200 $\mu Ci/\mu g$ were achieved, respectively. Each extract or plasma sample was assayed in duplicate at three or more dilutions. Extracts were diluted in acidified human albumin solution (15); plasma samples were diluted in 6,000 *g* ACTH-free plasma from dexamethasone-treated subjects. The mixtures were incubated in 10 × 75-mm polypropylene tubes (Falcon Plastics, Div. of B-D Laboratories, Inc., Los Angeles, Calif.) for 3–6 days at 4°C. Separation of antibody-bound labeled ACTH from free labeled ACTH was achieved by the chromatoelectrophoretic system of Yalow and Berson (16) or, in more recent experiments, by the precipitation of antibody-bound ACTH with polyethylene glycol (7). When antibody-bound ACTH was separated from free ACTH in this manner, the volume of the incubation mixture was increased to 1.5 ml with a final plasma concentration of 10%. Trasylol (FBA Pharmaceuticals Inc., New York) was added at a concentration (200 KIU/ml) that did not interfere with binding. After incubation was complete, 1.5 ml freshly prepared 20% polyethylene glycol (Carbowax 6000, Union Carbide Corp., New York) in 0.05 M phosphate buffer pH 7.4 containing 0.45% NaCl at 4°C was added with a repipet (Labindustries, Berkeley, Calif). The tubes were mixed on a Vortex mixer (Scientific Industries, Inc., Queens Village, N. Y.) for 2 s, centrifuged at 6,000 *g* at 4°C for 15 min, and the supernate decanted and discarded. The precipitate was counted in a Packard Auto-Gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). This method of separation was also adapted to situations in which small incubation volumes were adequate and the only carrier protein in the incubation mixtures was the 0.25% human serum albumin in the diluent and buffer solutions (13) (e.g., assays of plasma and tissue extracts, Sephadex fractions of tissue extracts, and purified or synthetic ACTH and melanocyte-stimulating hormone (MSH) analogues). A volume of cold (4°C) phosphate buffer containing 150 μl of out-dated blood bank plasma sufficient to bring the total volume of the incubation mixture to 1.5 ml was added immediately before adding the polyethylene glycol.

ACTH antisera. The antisera used for radioimmunoassay and for neutralization and immunoadsorption experiments were chosen empirically on the basis of their binding affinities and their specificities for different portions of the ACTH molecule. Specificity was defined by determining the ability of a variety of synthetic analogues of ACTH and MSH to compete with labeled human ACTH (Lerner-Upton-Lande 8B) for binding sites on the antibody. The *extreme N-terminal antiserum* was the gift of Doctors L. H. Rees, D. Cook, and J. W. Kendall. It was developed by injecting a rabbit subcutaneously with synthetic α^{1-24}ACTH[2] (Cortrosyn, Organon, Inc., West Orange, N. J.) conjugated by the carbodiimide reaction to rabbit serum albumin and emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). The *N-terminal ACTH antiserum* was raised in a guinea pig by subcutaneous injections of zinc-porcine ACTH (Organon, Inc.) emulsified in complete Freund's adjuvant, as previously described (13). The two *C-terminal antisera* were both raised in rabbits in response to subcutaneous injections of partially purified human ACTH (Raben) emulsified in complete Freund's adjuvant. The available quantity of the first C-terminal antiserum was so limited that a second antiserum was required for further studies. The second antiserum was "mixed," with antibody populations that reacted both with the N-terminal and with the C-terminal portions of the ACTH molecule (13). Therefore, the antiserum was adsorbed with α^{1-24}ACTH

[2] In this study we have adhered to the conventions for nomenclature proposed by Dr. C. H. Li (35) for various analogues of ACTH and MSH. The first ACTH isolated from the pituitary of a given species is designated "α-ACTH" and the second, if one were to be found, "β-ACTH," and so forth. Any polypeptide with an amino acid sequence similar to that of the α-ACTH of a given species is also designated "α-ACTH." A superscript is used to indicate the portion of the ACTH molecule represented by the analogue (for example, α^{11-24}ACTH) and a C-terminal amide, if present, is also indicated (for example, α^{7-18NH_2}ACTH). If the analogue contains species-specific portions of the molecule, the appropriate species is indicated by a subscript (for example, $\alpha_p{}^{25-39}$ACTH contains the C-terminal 15 amino acids of porcine ACTH).

covalently coupled to diazotized cellulose (17); 1.8 ml of the antiserum was diluted 1:100 in 0.05 M phosphate buffer pH 7.4 and mixed with 1 mg α^{1-24}ACTH coupled to diazocellulose. It was incubated at 4°C for 7 days while being stirred slowly with a Teflon-coated magnetic stirring bar. The mixture was then centrifuged, and the supernatant antiserum was aspirated from the immunoadsorbent pellet. The antiserum was similarly adsorbed four more times.

Immunoadsorption procedure. Immunoadsorption of C-terminal immunoreactive ACTH by the N-terminal ACTH antiserum was assessed by mixing standard quantities (1 ng) of various preparations with 0.2 ml of the N-terminal ACTH antiserum diluted 1:100 in 0.05 M phosphate buffer pH 7.4. These mixtures were incubated for 5 days at 4°C. As a control in each experiment, an identical quantity of the same preparation was similarly incubated in a 1:100 dilution of nonimmune guinea pig serum. The materials incubated were partially purified extracts of two tumors, highly purified α_hACTH, synthetic α^{1-24}ACTH, and synthetic α_p^{25-39}ACTH (Ciba-Geigy, Ltd., Basel, Switzerland). After incubation, the peptides not bound to antibody were extracted from the incubation mixture by stirring with 5 mg silicic acid (Mallinckrodt Chemical Works, St. Louis, Mo.) (18) for 30 min at 4°C, centrifuging, and aspirating the supernatant medium, which was then reextracted with another 5 mg silicic acid. The pooled 10 mg silicic acid was washed three times with 2 ml distilled water at 4°C. The peptides were eluted with 1 ml 40% acetic acid at 4°C. The eluates were lyophilized and reconstituted in 0.001 N HCl containing 0.25% human serum albumin. The reconstituted eluates were then assayed in the C-terminal ACTH radioimmunoassay. The degree of immunoadsorption of C-terminal immunoreactive material was determined by comparing the quantity remaining after incubation with the N-terminal ACTH antiserum with that remaining after incubation with nonimmune guinea pig serum.

Gel filtration procedure. Partially purified extracts of tumors obtained at surgery from three patients with ectopic ACTH syndrome and an extract of a normal human pituitary were applied in 1% acetic acid to a Sephadex G-50 fine resin column 85 × 2.5 cm at 25°C, saturated with 100 mg bovine serum albumin applied in 1 ml 1% acetic acid at least 4 h previously. They were eluted with 1% acetic acid by descending flow at a rate of 48 ml/h. The void volume of the column was 142 ml; 9.5-ml fractions were collected. Fractions were subjected both to bioassay and to radioimmunoassays. Reproducibility to within 9.5 ml was observed with repeated calibration of the column with reference standard ACTH, synthetic human β-MSH and synthetic α-MSH. Recoveries of biologic ACTH activities for the standard and unknowns were 30–100%, averaging 61%; recoveries of immunologic ACTH reactivities were 55–112%, averaging 92%.

Neutralization procedure. Neutralization of biologic ACTH activity by the C-terminal ACTH antiserum was assessed by mixing standard quantities of various preparations (usually 0.25 mU/rat) with the first C-terminal antiserum diluted 1:5 with 0.05 M phosphate buffer pH 6.8 containing 0.5% bovine globulin. The mixtures were incubated at 37°C for 30 min before injection into the hypophysectomized rat. As a control in each experiment, an identical quantity of the same preparation was similarly incubated in nonimmune rabbit serum diluted 1:5 in the same buffer solution. The materials tested were three tumor extracts, highly purified human ACTH, and synthetic α^{1-24}ACTH. The degree of neutralization of ACTH bioactivity was determined by comparing the adrenal-stimulating potency of the preparation that had been incubated with the C-terminal antiserum with that incubated with nonimmune rabbit serum (19).

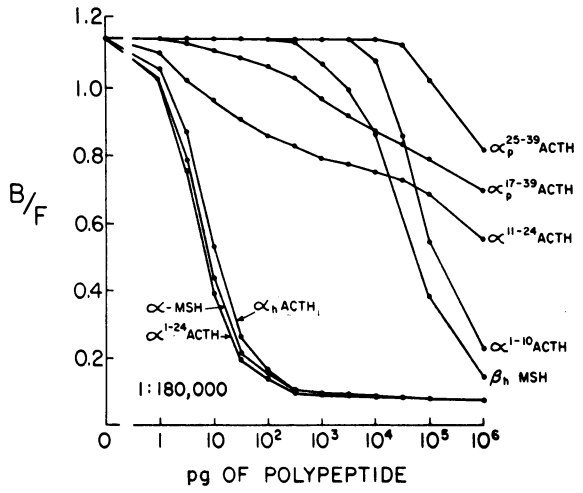

FIGURE 1 Specificity of the extreme N-terminal ACTH antiserum. Competition with labeled human ACTH (Lerner-Upton-Lande 8B) for antibody-binding sites by various synthetic analogues of ACTH and MSH is shown. The ratio of antibody-bound to free-labeled ACTH (B/F) is plotted on the ordinate. The picograms of unlabeled polypeptide added to the incubation mixture are plotted on the abscissa on a logarithmic scale. Final antiserum dilution was 1:180,000.

RESULTS

Characterization of ACTH antibody specificity [3]

Extreme N-terminal ACTH antiserum. The results of studies of its specificity are shown in Fig. 1. The antibodies reacted equally with all ACTH analogues (α-MSH, α^{1-24}ACTH, and α_hACTH; also α^{1-16NH_2}ACTH and α^{1-23}ACTH, not shown) that share the N-terminal 13 amino acid sequence of ACTH, β_hMSH, which contains amino acid sequence 4-10 of ACTH, and α^{1-10}ACTH were far less reactive with the antibodies. Thus, this antiserum appeared to be directed toward the extreme N-terminal 1-13 amino acid sequence of the ACTH molecule.

N-terminal ACTH antiserum. The specificity of this antiserum is shown in Fig. 2. Its affinity was limited to analogues of ACTH containing at least the 1-23 sequence

[3] The designations of the specificities of these antisera as "extreme N-terminal," "N-terminal," and "C-terminal" are intended as convenient abbreviations. Although these antisera are more completely characterized than any used in radioimmunoassay, it is still not possible to define the exact primary sequence requisite for maximum binding to any one of them. The sequences 1–13, 1–23, and 25–39, respectively, are the best approximations that can be made with the available data.

of amino acids. Thus, α^{1-23}ACTH, α^{1-24}ACTH, α_p^{1-39}-ACTH, and α_hACTH all reacted with the antibodies to generate parallel competition curves. In contrast, α^{1-10}-ACTH, α-MSH (N-α-acetyl-α^{1-13NH_2}ACTH), α^{1-16NH_2}-ACTH, β_hMSH, and α^{11-24}ACTH had little affinity for the antibodies, and C-terminal α_p^{25-39}ACTH failed to react. Thus, the antibodies appeared to be directed at the N-terminal 1-23 portion of the molecule. The antibodies also reacted fully with N-α-acetyl-α^{1-24}ACTH and with analogues having 1-D-serine and 4-norleucine substitutions, suggesting that the first four residues may not be critical for binding. It is interesting that α_p^{17-39}ACTH reacted with the antibodies, although with reduced binding affinity.

C-terminal ACTH antisera. Specificity analysis of the first antiserum revealed that it reacted with the C-terminal portion of the ACTH molecule. Four analogues that share an identical 8 amino acid C-terminal sequence, α_hACTH, α_p^{1-39}ACTH, α_p^{17-39}ACTH, and α_p^{25-39}ACTH, all generated parallel competition curves (Fig. 3). In contrast, α^{7-13NH_2}ACTH, α^{11-24}ACTH, α-MSH, α^{1-10}-ACTH, α^{1-24}ACTH, β_hMSH failed to displace labeled α_hACTH from the antibodies. The fragments α_p^{17-39}ACTH and α_p^{25-39}ACTH were slightly more effective than equimolar quantities of 1-39 ACTH, yet they failed to displace all of the labeled α_hACTH from the antibodies. This appeared to be due to the presence of a very minor fraction of antibody that reacted with the N-terminal portion of the ACTH molecule, as demonstrated by the fact that complete displacement was achieved by the further addition of α^{1-24}ACTH.

The specificity of the second antiserum before adsorption is shown in Fig. 4*a*. The antiserum was "mixed,"

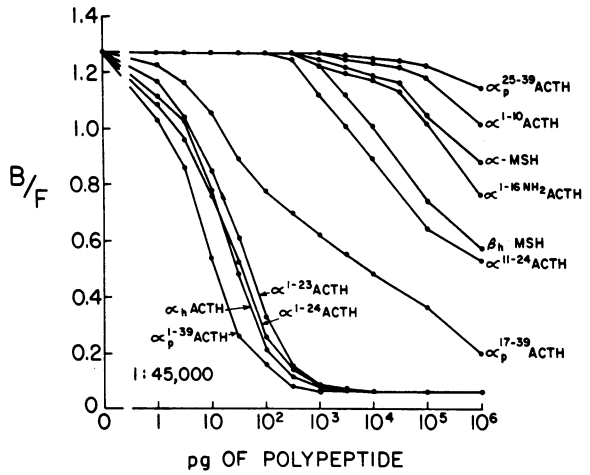

FIGURE 2 Characterization of the specificity of the N-terminal ACTH antiserum. Final antiserum dilution was 1:45,000. Competitive binding curves for a variety of synthetic ACTH and MSH analogues are plotted in the same manner as in Fig. 1.

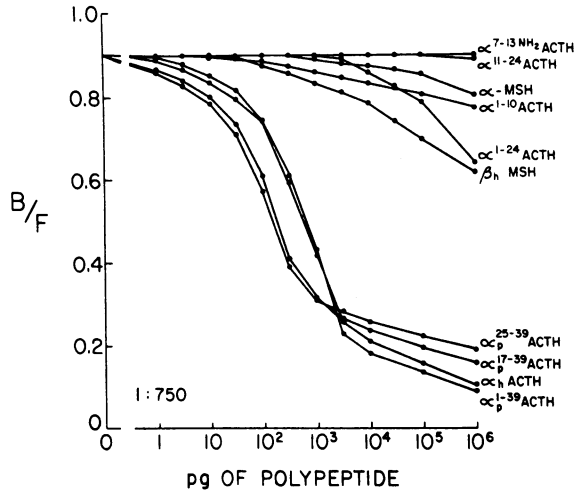

FIGURE 3 Specificity of the first C-terminal ACTH antiserum. Competitive binding curves for various synthetic ACTH and MSH analogues are plotted in the same manner as in Fig. 1. Final antiserum dilution was 1:750.

with antibody populations that reacted both with the N-terminal α^{1-24}ACTH fragment and the C-terminal α_p^{17-39}ACTH and α_p^{25-39}ACTH sequences. The specificity of this antiserum after immunoadsorption is shown in Fig. 4*b*. Although not all of the N-terminal antibodies were removed, the specificity of this antiserum was now similar to that of the first C-terminal antiserum, and its sensitivity was considerably greater than that of the first antiserum.

Precision of ACTH radioimmunoassays

Duplication. Six duplicate estimates of endogenous ACTH concentrations in four plasma pools in one assay yielded the results summarized in Table I. They are typical of the assays, in that they represent a single experiment, performed in a routine manner, and not the best of several experiments.

Replication. Replication of 32 plasma samples with levels ranging from 50 to 5,000 pg/ml in assays performed on different days yielded the following correlation coefficients: + 0.98 (extreme N-terminal), + 0.97 (N-terminal), and + 0.80 (C-terminal).

Assay of ACTH in tissue

Tumor tissue. Extracts of tumors from 32 patients with ectopic ACTH syndrome obtained at autopsy or at surgery were subjected to bioassay and to radioimmunoassays employing the extreme N-terminal, N-terminal, and C-terminal ACTH antibodies. The competition curves generated by the tumor extracts were parallel to those of standard α_hACTH in all three immunoassay systems (Figs. 5–7). Thus, there were components in the tumor extracts which were immunologically indistin-

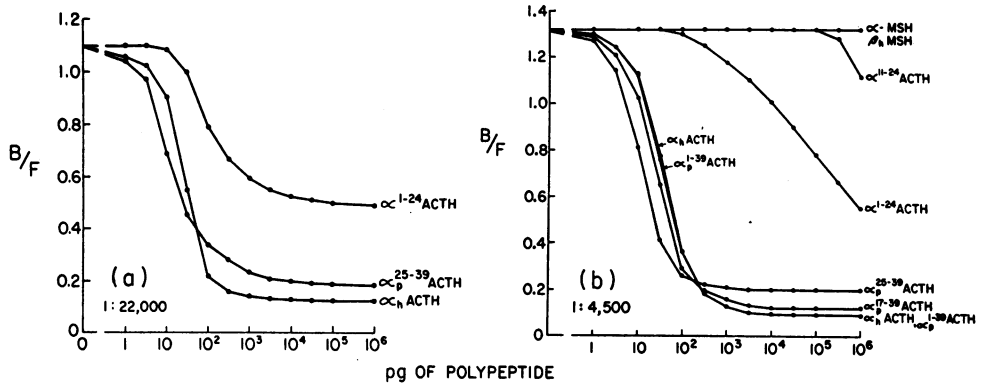

FIGURE 4 (*a*) Mixed specificity of the second C-terminal antiserum before immunoadsorption. Competitive binding curves for unlabeled human ACTH, N-terminal α^{1-24}ACTH, and C-terminal α_p^{25-39}ACTH are shown. Although the major antigenic determinant resides in the 25–39 C-terminal sequence of the ACTH molecule, there is significant contamination with antibodies that react, albeit with lower affinity, with the N-terminal 1–24 amino acid sequence. (*b*) Specificity of the second C-terminal antiserum after immunoadsorption with α^{1-24}ACTH covalently linked to diazocellulose (17). The majority of the N-terminal antibody has been removed. There was a fivefold loss in antibody titer during immunoadsorption.

guishable from authentic ACTH in each of these three radioimmunoassay systems. The quantitative results of the assays are summarized in Table II. Although there was a several 1,000-fold range in tumor ACTH concentrations, there was reasonably close agreement between the amounts of bioactive and N-terminal immunoreactive ACTH found in individual tumor extracts. There was, however, an unexpected superabundance of both extreme N-terminal and C-terminal immunoreactive ACTH in most of the tumor extracts. The mean ratio of N-terminal immunoreactive to bioactive ACTH in the 32 tumor extracts was 2.9, that of extreme N-terminal immunoreactive to bioactive ACTH was 24.7, and that of C-terminal immunoreactive ACTH to bioactive ACTH was 25.6.

Normal human pituitary tissue. Extracts of three normal human pituitaries obtained at autopsy were subjected to bioassay and to the three radioimmunoassays. These normal ACTH-producing tissues were used as controls for the study of the ectopic ACTH-producing tumor tissues. The competition curves were parallel to those of highly purified α_hACTH in all three radioimmunoassay systems. There was reasonably close agreement between the amounts of bioactive and extreme N-terminal, N-terminal and C-terminal immunoreactive ACTH found in the pituitary extracts (Table III).

TABLE I

Duplication in the Three ACTH Radioimmunoassays

Immunoreactive ACTH		
Extreme N-terminal	N-terminal	C-terminal
pg/ml	*pg/ml*	*pg/ml*
3,400±260	2,082±151	3,763±638
925±35	799±55	993±111
488±13	360±10	482±82
133±9	115±14	190±39

Immunoreactive ACTH concentrations in six duplicate samples from each of four plasma pools were determined on a single run in each assay. The results are expressed as the mean ±SEM.

Assays of plasma ACTH

Since there was a disproportionately high concentration of extreme N-terminal and C-terminal immunoreactive ACTH material in the tumor extracts, it seemed possible that a similar discrepancy might be found in the circulating plasma of patients with the ectopic ACTH syndrome.

Plasma from patients with ectopic ACTH syndrome. Plasma or plasma extracts from 18 patients with ectopic ACTH syndrome were subjected to extreme N-terminal, N-terminal, and C-terminal radioimmunoassay. The competition curves generated by these materials were parallel to those of the α_hACTH standard in each assay system. The results of the assays are summarized in Table IV. There was a consistent excess of both extreme N-terminal and C-terminal ACTH over N-terminal immunoreactive ACTH, with mean ratios of 3.0 and 3.3, respectively. In one patient (Gr) from whom sufficient plasma was obtained for bioassay, the value (2,900 pg/ml) agreed well with N-terminal immunoreactive ACTH.

Plasma from patients with Cushing's disease. As a control group, plasma or plasma extracts from 19 patients with Cushing's disease (pituitary ACTH-dependent Cushing's syndrome) were similarly assayed. The competition curves were parallel to those of standard α_hACTH in all three radioimmunoassays. The results are

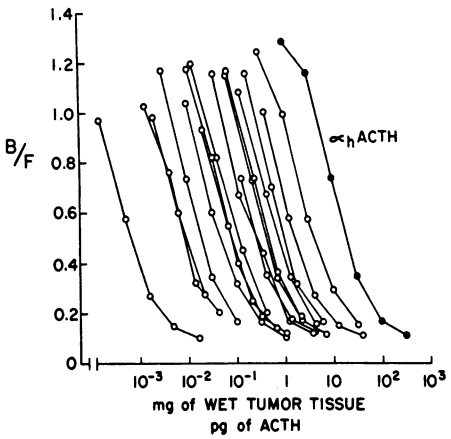

FIGURE 5 Competitive binding curves for extracts of 16 tumors causing ectopic ACTH syndrome (open circles) and the curve for reference standard human ACTH (closed circles) in the extreme N-terminal ACTH radioimmunoassay are shown. The milligram equivalents of wet tumor tissue and the picograms of unlabeled standard ACTH are plotted on the abscissa on a logarithmic scale.

summarized in Table IV. There were consistent excesses of both extreme N-terminal and C-terminal ACTH over N-terminal immunoreactive ACTH, similar to those observed in the plasma of patients with ectopic ACTH syndrome. In one patient (O'Br) in whom there was sufficient plasma for bioassay, the value obtained (2,120 pg/ml) agreed well with N-terminal immunoreactive ACTH. The ratio of extreme N-terminal and C-terminal immunoreactive ACTH to N-terminal immunoreactive ACTH (3.1 and 2.8, respectively) did not appear to differ from those found in the plasma of patients with ectopic ACTH syndrome.

Immunoadsorption of tumor extracts

In order to determine whether the excess C-terminal ACTH immunoreactivity found in the tumor extracts was due to a single ACTH molecule with enhanced C-terminal immunoreactivity or indicated the additional presence of C-terminal ACTH fragments, extracts of tumors from two patients with the ectopic ACTH syndrome were incubated with the N-terminal ACTH antiserum. Purified α_hACTH, which possesses an immunoreactive 1-23 amino acid sequence, and α_p^{25-39}ACTH, which does not, were similarly incubated. Control mixtures were incubated with equal concentrations of nonimmune guinea pig serum. The amounts of C-terminal immunoreactive ACTH adsorbed by the N-terminal ACTH antiserum were expressed as the percent of the controls and are summarized in Table V. Most of the C-terminal immunoreactivity was adsorbed when α_h-ACTH was incubated with the N-terminal ACTH antiserum. In contrast, most of the C-terminal immunoreactivity was not adsorbed when the C-terminal fragment, α_p^{25-39}ACTH, was incubated with the antiserum. Since most of the C-terminal immunoreactive material in the tumor extracts also was not adsorbed, it appeared that the tumors contained C-terminal ACTH fragments that lacked an intact 1-23 sequence of amino acids.

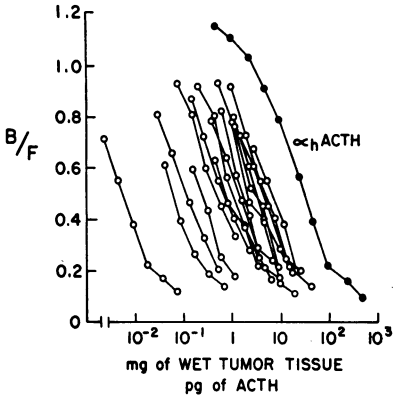

FIGURE 6 Parallelism of the competitive binding curves of 16 ectopic ACTH-producing tumor extracts (open circles) with that of highly purified α_hACTH (closed circles) in the N-terminal ACTH radioimmunoassay. The data are plotted in the same manner as in Fig. 5.

Gel filtration

Three tumor extracts were subjected to Sephadex G-50 fine gel filtration to determine if the extreme N-terminal and C-terminal ACTH immunoreactive components of the tumor extracts could be physically separated from the bioactive, N-terminal immunoreactive ACTH and from each other. An extract of a normal human pituitary was used as a control.

Tumor extracts. Extracts of three tumors that had been obtained at surgery and frozen immediately were

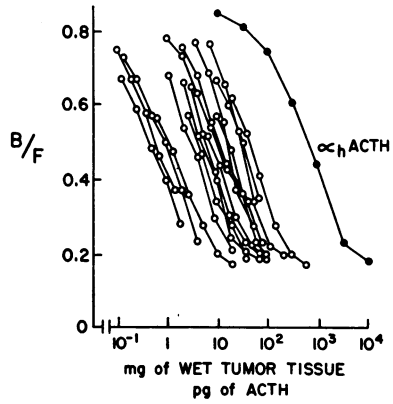

FIGURE 7 The competitive binding curves of extracts of 16 tumors (open circles) and that of reference standard ACTH (solid circles) in the C-terminal ACTH radioimmunoassay, plotted in the same way as in Fig. 5.

TABLE II

Concentrations of Bioactive and Immunoreactive ACTH in Tumors of Patients with Ectopic ACTH Syndrome

		Immunoreactive ACTH					
	Bioactive ACTH	Extreme N-terminal		N-terminal		C-terminal	
Patient	μg ACTH/g tumor	μg ACTH/g tumor	(Imm/Bio)	μg ACTH/g tumor	(Imm/Bio)	μg ACTH/g tumor	(Imm/Bio)
He	0.001	0.021	(21.0)	0.001	(1.0)	0.017	(17.0)
Ol	0.001	0.037	(37.0)	0.017	(17.0)	0.029	(29.0)
Py	0.001	0.005	(5.0)	0.005	(5.0)	0.015	(15.0)
McM	0.002	0.004	(2.0)	0.006	(3.0)	0.044	(22.0)
Th	0.002	0.011	(5.5)	0.005	(2.5)	0.021	(10.5)
So	0.002	0.003	(1.5)	0.009	(4.5)	0.061	(30.5)
Ph	0.002	0.019	(9.5)	0.002	(1.0)	0.036	(18.0)
Ne	0.002	0.043	(21.5)	0.012	(6.0)	0.197	(98.5)
Br	0.003	0.172	(57.3)	0.010	(3.3)	0.354	(118.0)
Al	0.004	0.225	(63.7)	0.020	(5.0)	0.123	(30.8)
As	0.006	0.058	(9.7)	0.024	(4.0)	0.057	(9.5)
Gr	0.006	0.019	(3.3)	0.007	(1.1)	0.024	(4.0)
Hg	0.009	0.096	(10.7)	0.030	(3.3)	0.180	(20.0)
Sw	0.010	0.038	(3.8)	0.008	(0.8)	0.035	(3.5)
Wi	0.012	0.018	(1.5)	0.012	(1.0)	0.100	(8.3)
McG	0.014	0.019	(1.4)	0.021	(1.5)	0.117	(8.4)
Bro	0.019	2.550	(134.0)	0.020	(1.0)	0.140	(7.4)
Mg	0.020	0.170	(8.5)	0.155	(7.8)	0.054	(2.7)
Og	0.020	0.017	(0.8)	0.025	(1.2)	0.630	(31.5)
Pl	0.028	0.123	(4.4)	0.039	(1.4)	0.062	(2.2)
Hu	0.043	0.456	(10.6)	0.037	(0.9)	1.047	(24.4)
Ny	0.053	0.300	(5.7)	0.056	(1.1)	0.260	(4.9)
Ta	0.053	0.087	(1.6)	0.044	(0.8)	0.571	(10.8)
Bri	0.073	0.185	(2.5)	0.067	(0.9)	0.728	(10.0)
Ev	0.103	2.260	(21.9)	0.444	(4.3)	1.090	(10.6)
Gre	0.217	1.220	(5.6)	0.220	(1.0)	0.800	(3.7)
Brd	0.480	1.050	(2.2)	1.045	(2.2)	1.800	(3.7)
Pa	0.664	32.800	(49.4)	1.001	(1.5)	19.200	(29.0)
Ca	0.957	24.200	(25.3)	1.520	(1.6)	18.800	(19.6)
Sa	0.981	11.300	(11.5)	1.080	(1.1)	17.500	(17.8)
Swa	3.700	1,090.000	(294.0)	12.200	(3.3)	590.000	(159.0)
Cap	28.500	844.000	(29.6)	64.600	(2.3)	1,100.000	(38.6)

Each tumor extract was assayed simultaneously in the Lipscomb-Nelson bioassay and the extreme N-terminal, N-terminal, and C-terminal radioimmunoassay systems.

TABLE III

Concentrations of Bioactive and Immunoreactive ACTH in Extracts of Three Normal Human Pituitary Glands

	Immunoreactive ACTH					
Bioactive ACTH	Extreme N-terminal		N-terminal		C-terminal	
μg ACTH/g tissue	μg ACTH/g tissue	(Imm/Bio)	μg ACTH/g tissue	(Imm/Bio)	μg ACTH/g tissue	(Imm/Bio)
176	192	(1.09)	230	(1.31)	148	(0.84)
260	272	(1.05)	252	(0.97)	252	(0.97)
108	146	(1.35)	110	(1.02)	120	(1.11)

Each pituitary extract was assayed simultaneously in the Lipscomb-Nelson bioassay and in the extreme N-terminal, N-terminal, and C-terminal radioimmunoassays.

TABLE IV

Concentrations of Immunoreactive ACTH in Plasma of Several Patients with Ectopic ACTH Syndrome or Cushing's Disease

Patient	Diagnosis	Immunoreactive ACTH		
		Extreme N-terminal	N-terminal	C-terminal
		pg ACTH/ml plasma	pg ACTH/ml plasma	pg ACTH/ml plasma
Gr*	Ectopic ACTH	6,740	3,295	12,800
Je	"	3,160	1,080	2,590
Bu	"	5,960	1,060	6,670
Zi	"	512	536	410
Sh	"	470	510	660
Al	"	1,590	410	995
Ph	"	430	390	505
Ca	"	747	373	1,390
So	"	3,500	338	1,170
Ta	"	433	325	2,710
Le	"	746	318	342
Wi	"	344	291	810
Ma	"	220	224	1,400
Be	"	590	174	200
Sw	"	240	156	396
Ka	"	300	130	810
La	"	555	125	415
McC	"	760	119	230
O'Br‡	Cushing's disease	—	1,840	7,080
Phi	"	1,910	1,140	7,840
Ar	"	3,120	1,140	2,350
Lee	"	5,680	782	1,800
Mu	"	4,780	773	2,760
But	"	620	619	410
Jo	"	970	580	900
Wy	"	—	422	1,720
Ri	"	—	377	1,500
He	"	505	258	525
Ja	"	920	179	250
Di	"	285	166	508
Ed	"	1,200	132	537
Mi	"	394	120	154
Lo	"	—	112	455
Br	"	58	73	59
Sc	"	165	69	49
Ne	"	81	69	274
Fo	"	48	61	49

Each plasma or plasma extract was assayed simultaneously in the extreme N-terminal, N-terminal, and C-terminal ACTH radioimmunoassay systems.

* Biologic ACTH activity (Lipscomb-Nelson) = 2,900 pg/ml.

‡ Biologic ACTH activity (Lipscomb-Nelson) = 2,120 pg/ml.

applied to the Sephadex G-50 fine column. The original extract and each fraction, from the void volume (142 ml) to beyond the elution volume for standard α-MSH, were assayed for ACTH bioactivity and for extreme N-terminal, N-terminal, and C-terminal ACTH immunoreactivity. Each fraction was assayed to exclude as little as 1% of the bioactive or immunoreactive ACTH added to the column. The results are shown in Figs. 8–10. Each of the tumor extracts contained a material that eluted in the early standard ACTH fractions (Fig. 11) and had equivalent ACTH biologic activity and extreme N-terminal, N-terminal, and C-termi-

TABLE V

Immunoadsorption of C-terminal ACTH Immunoreactive Material by Incubation with the N-Terminal ACTH Antiserum

Material	N-terminal 1–23 ACTH sequence present	% Adsorbed
α_h ACTH	Yes	80
α_p^{25-39} ACTH	No	5
Tumor extract 1	?	15
Tumor extract 2	?	5

Results are expressed as percent of C-terminal immunoreactive ACTH adsorbed as compared with that remaining in control mixtures incubated with nonimmune rabbit serum.

nal ACTH immunoreactivity. No higher molecular weight bioactive or immunoreactive ACTH materials were present. However, each tumor contained two other materials that eluted later than bioactive ACTH. The first eluted in the fractions between standard ACTH and β_hMSH, had no ACTH bioactivity, and reacted only with the C-terminal antiserum. Its molecular weight appeared to be approximately 3,100 daltons. The second material eluted in the fractions between β_hMSH and α-MSH standards, had no ACTH bioactivity, and reacted with the extreme N-terminal ACTH antiserum but not with the N-terminal or C-terminal antisera. Its molecular weight appeared to be approximately 1,800 daltons. The fractions containing the immunoreactive N-terminal ACTH fragment had significant biologic MSH activity, but little α-MSH or β_hMSH immunoreactivity, as previously reported (20). Thus, in addition to bioactive ACTH, the three tumor extracts contained two components that appeared to be N-terminal and C-terminal fragments of ACTH.

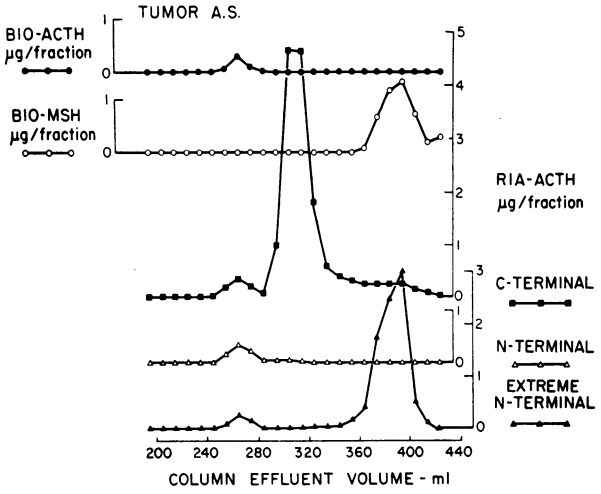

FIGURE 8 Sephadex G-50 fine gel filtration elution pattern of an extract of an ectopic ACTH-producing tumor (Patient A. S.). The column was 85 × 2.5 cm; void volume was 142 ml. The total biologic ACTH activity in each 9.5 ml fraction is plotted at the top, the total biologic MSH activity (20) next, and then the C-terminal, N-terminal, and extreme N-terminal immunoreactive ACTH. The bioactive and immunoreactive ACTH values are plotted as micrograms of a α_hACTH (100 mU/µg); the bioactive MSH is plotted as micrograms of α-MSH.

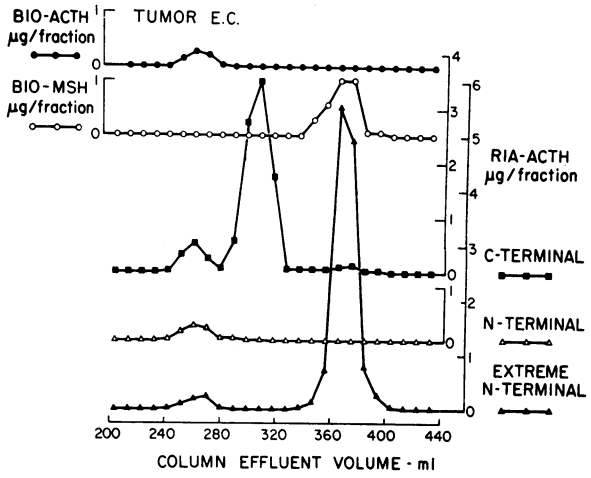

FIGURE 9 The bioactive ACTH and MSH, and the C-terminal, N-terminal, and extreme N-terminal immunoreactive ACTH in each 9.5 ml fraction eluted from a Sephadex G-50 fine resin column. The data are for an extract of the tumor removed at surgery from patient E. C. and are plotted in the same manner as in Fig. 8.

Pituitary extract. An extract of a normal human pituitary gland, obtained at autopsy and frozen immediately, was applied to the same Sephadex G-50 fine column. The bioactive and immunoreactive ACTH eluted in the same fractions as reference standard ACTH (Fig. 11). No higher molecular weight bioactive or immunoreactive ACTH materials were present, nor were there immunoreactive ACTH materials present that eluted later than bioactive ACTH from the column. Thus, the N-terminal and C-terminal fragments observed in the three tumor extracts were not present in the normal pituitary extract.

Neutralization of ACTH biologic activity

The C-terminal 15 or 16 amino acid sequence of ACTH is not essential for steroidogenic activity. Thus, it was at least theoretically possible that the bioactive and C-terminal immunoreactive tumor ACTH components which eluted from Sephadex G-50 fine in the same volume as reference standard ACTH were separate entities, although similar in size. In order to exclude this possibility, the biologically active ACTH extracted from tumors of three patients with ectopic ACTH syndrome was incubated with C-terminal antiserum. Purified α_h-

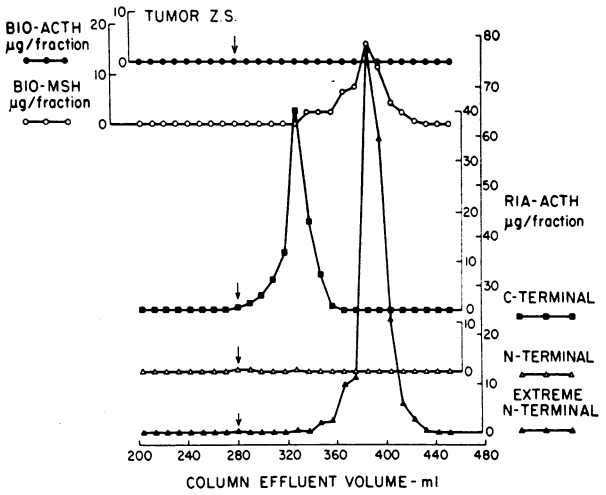

FIGURE 10 The elution pattern for the bioactive and immunoreactive components in an extract of the tumor of patient Z. S. Although this tumor had the second highest bioactive ACTH concentration in this series of 32 tumors, the scale required to plot the N-terminal and C-terminal immunoreactive ACTH fragments is such that the bioactive ACTH is not visible. The arrows indicate the peak of bioactive (and corresponding immunoreactive) ACTH. Otherwise, the data are plotted in the same manner as in Fig. 8.

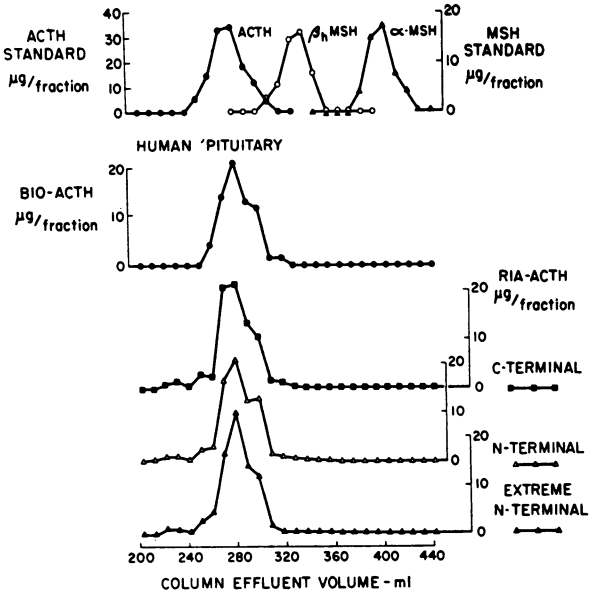

FIGURE 11 The elution pattern of an extract of a normal human pituitary gland from the same 85 × 2.5 cm Sephadex G-50 fine column that was used for the three tumor extracts. The column was calibrated with purified human ACTH, synthetic human β-MSH and synthetic α-MSH, as shown at the top. The bioactive ACTH and the immunoreactive ACTH measured in the three ACTH radioimmunoassays are plotted below in the same way as in Fig. 8.

ACTH, which possesses an immunoreactive 25-39 C-terminal sequence of amino acids, and synthetic α^{1-24}ACTH, which does not, were similarly incubated. The neutralization of ACTH was determined by comparing the biologic potency of the mixtures which had been incubated with the C-terminal ACTH antiserum with the potency of control mixtures incubated with equal concentrations of nonimmune rabbit serum (19). The results are summarized in Table VI. Human 1-39 ACTH was largely neutralized by incubation with the antiserum, whereas 1-24 ACTH was not. Incubation with the C-terminal antiserum largely neutralized the biologic ACTH potency of the tumor extracts. Neutralization could be reversed by acidifying the incubation mixtures before injection into the rat, demonstrating that the ACTH was not destroyed, but was inactivated by reversibly binding to the antibodies (19). Since most of the biologic ACTH activity of the tumor extracts was neutralized, the tumor ACTH appeared to have a C-terminal amino acid sequence similar to that of normal pituitary ACTH.

DISCUSSION

The quantity of ACTH contained in the tumors of patients with the ectopic ACTH syndrome is so small that it has not yet been possible to isolate enough of the hormone to determine its amino acid sequence. Previous studies, however, have indicated that tumor ACTH behaves like pituitary ACTH in several biologic assay systems and in several physicochemical systems designed to achieve partial purification (3, 4). Thus, both hormones cause adrenal hypertrophy, both stimulate the release of corticosteroids from the adrenal of man and of the rat, both cause darkening of frog skin in vitro and in vivo, and both demonstrate in vitro lipolytic activity. The dose-response curves of the two hormones are parallel in the rat bioassay system, and the steroidogenic activity of both appears to be mediated by the intraadrenal formation of cyclic AMP. Ectopic ACTH, like pituitary ACTH, can be extracted from

TABLE VI

Neutralization of ACTH Biologic Activity, which Resides in the 1–24 N-terminal Sequence of Amino Acids, by Incubation with the C-terminal ACTH Antiserum

Material	C-terminal 25–39 ACTH sequence present	% Neutralized
α_h ACTH	Yes	79
α^{1-24}ACTH	No	0
Tumor extract 1	?	75
Tumor extract 2	?	74
Tumor extract 3	?	50

Results are expressed as percent of control mixtures incubated with nonimmune rabbit serum.

tissues with glacial acetic acid and can then be precipitated with ether. Both are adsorbed from dilute acetic acid onto Amberlite CG-50 cationic-exchange resin, from which they can be eluted with 50% acetic acid, or onto oxycellulose, from which they can be eluted with 0.1 N HCl. Both can be separated from MSH on a column of SE-Sephadex. Ectopic ACTH, like authentic pituitary ACTH, is stable at a pH of less than 2 but is relatively labile in alkaline solution. Both are susceptible to inactivation by trypsin and chymotrypsin and are labile in plasma. Both can be dialyzed from plasma only at acid pH. Like pituitary ACTH, ectopic ACTH is readily inactivated by exposure to H_2O_2 but can be reactivated by cysteine, an observation that suggests methionine is an important constituent of the molecule (21). Both ectopic ACTH and pituitary ACTH are inactivated by periodate, an observation suggesting that the N-terminal amino acid residue of both is serine (22).

Most previous reports of radioimmunoassay of ACTH in the plasma or tumors of patients with the ectopic ACTH syndrome provide little qualitative information about the tumor hormone; either the specificities of the antisera were not well-documented, parallelism of the competition curves of the tumor ACTH with that of standard ACTH was not demonstrated, or simultaneous assays of biologic ACTH potency were not performed (23–28). The present study provides evidence that the tumors of patients with the ectopic ACTH syndrome produce a material that not only has biologic ACTH activity but also has at least three areas of immunochemical similarity to the authentic human pituitary ACTH molecule. The first of these is located in the extreme N-terminal sequence of amino acids 1–13, and the second in the extended N-terminal sequence 1–23; these two sequences encompass the steroidogenic portion of the ACTH molecule. The third area of immunochemical similarity is in the biologically inactive C-terminal sequence of amino acids 25–39. Since the C-terminal antiserum reacts equally well with porcine and human ACTH, the extreme C-terminal 8 amino acid sequences of which are identical, it may be presumed that the antibodies are directed toward this portion of the molecule.

There was generally good correlation between the quantities of bioactive ACTH and the quantities of N-terminal immunoreactive ACTH found in the tumor extracts and in normal human pituitaries. Modest disparities between the amounts of immunoreactive ACTH and the amounts of bioactive ACTH would not constitute an unexpected finding, since the receptor on the adrenal cell membrane presumably has different structural requirements for binding ACTH than does an antibody (8). In contrast to the relatively close agreement between the amounts of bioactive ACTH and the amounts of extreme N-terminal and C-terminal immunoreactive ACTH found in the pituitary extracts, however, there were unexpected excesses of both immunoreactive ACTH components in several of the tumor extracts. Since we first reported the presence of excess C-terminal immunoreactive ACTH fragments in tumors of patients with the ectopic ACTH syndrome (4, 29), a study has appeared which corroborates our findings (28). These authors described simultaneous bioassay and radioimmunoassays of ACTH in tumors of nine patients with ectopic ACTH syndrome. Two ACTH antisera were used, the specificities of which were similar to the N-terminal and C-terminal ACTH antisera employed in the present study. The average ratios of the amounts of N-terminal immunoreactive ACTH to the amounts of bioactive ACTH (1.6) and of the amounts of C-terminal immunoreactive ACTH to the amounts of bioactive ACTH (14.0) in their tumors were similar to those we have found.

There are a number of possible explanations for the presence of ACTH fragments in extracts of tumors causing ectopic ACTH syndrome. The excess of immunoreactive materials in the tumor extracts might have been an artifact of the standard extraction procedure. However, the discrepancy was not observed in normal human pituitaries extracted by the same technique, and similar results have been observed using another extraction method (28). A systematic study of the efficiency of various extraction procedures for these ACTH fragments has not been performed, however. The excess might have been the result of autolytic processes which cleaved the ACTH molecule somewhere in the 1–23 sequence, destroying both biologic activity and N-terminal immunoreactivity. However, several of the tumors in the present series and in that of Ratcliffe, Knight, Besser, Landon, and Stansfeld (28) were obtained at the time of surgery and were frozen immediately. Furthermore, a similar discrepancy was not observed in the extracts of pituitary glands, some of which were obtained as long as 5 h after death. It has been reported that proteolytic activity is higher in tumor tissue than in normal tissue (30, 31), although neutral proteases, which might be expected to cause the greatest problem in the radioimmunoassay system, were not increased (31). Thus, although it appeared that the presence of these immunoreactive ACTH fragments was associated only with nonpituitary ACTH-producing tumors, it has not been possible to establish definitively that they were present *in situ*. The magnitude of the excess did not appear to be related either to the tumor cell type or to the ACTH concentration of the tumor tissue.

In order to determine if all the ACTH activities

measured resided in a single molecule, rather than representing properties of several different molecules, three tumor extracts were subjected to gel filtration. Each of these tumors (tumor A. S. = "small cell" carcinoma of lung, tumors E. C. and Z. S. = bronchial carcinoid adenomas) were obtained at the time of surgery, frozen immediately, and stored at −70°C until extracted. A single gel filtration procedure using Sephadex G-50 fine resin resolved the components into three distinct peaks.

The first material cochromatographed with pituitary ACTH. There was comparable bioactivity and extreme N-terminal, N-terminal, and C-terminal immunoreactivity in each fraction containing this material. Furthermore, its biologic potency was reversibly neutralized by incubation with the C-terminal antiserum, demonstrating that the N-terminal steroidogenic sequence was part of the same molecule that reacted with the C-terminal antiserum. The tumor ACTH eluted in the earliest fractions containing standard pituitary ACTH. Thus, the possibility that it is very slightly larger than pituitary ACTH cannot be excluded. Otherwise, it is biologically, immunologically, and physicochemically indistinguishable from pituitary ACTH. We have previously described two bioactive and immunoreactive ACTH species synthesized by normal mouse pituitary and by a mouse pituitary tumor cell line in vivo and in tissue culture (32). One of these was somewhat larger (mol wt $\simeq$ 7,800 daltons) than the 39 amino acid molecule (mol wt $\simeq$ 4,500 daltons) extracted from the pituitaries of man and other species. Others have more recently described a much larger immunoreactive "ACTH" in human tissues and plasma (33). We found no immunoreactive or bioactive ACTH of this size in the glacial acetic acid extracts of normal human pituitary or of the three tumors examined in the present study.

The second material that eluted from the Sephadex G-50 fine resin was smaller (mol wt $\simeq$ 3,100) than human pituitary ACTH (mol wt $\equiv$ 4,539), had no biologic ACTH activity, and reacted only with the C-terminal ACTH antiserum. Although it eluted in fractions that contained standard β_hMSH, previous studies of these fractions revealed negligible amounts of bioactive MSH or immunoreactive β_hMSH or α-MSH (20). This material is presumed to represent the 25 or 26 C-terminal amino acid sequence of ACTH ($\alpha_h{}^{14-39}$ACTH or $\alpha_h{}^{15-39}$ACTH), although determination of its exact composition must await more detailed chemical analyses.

The third material eluted from the resin column still later and appeared to have a molecular weight of about 1,800, corresponding to a polypeptide of 15 or 16 residues. This material had no detectable steroidogenic activity and reacted with the extreme N-terminal ACTH antiserum, but not with the N-terminal or C-terminal ACTH antisera. Thus, it appeared to represent the N-terminal portion of the ACTH molecule. It eluted in fractions between those containing reference standard β_hMSH and α-MSH, but did not cross-react significantly in either the β_hMSH or α-MSH radioimmunoassays (20). However, it did elute in those fractions having biologic MSH activity. ACTH itself has an intrinsic melanocyte-stimulating potency of about 0.7% that of α-MSH, the most potent MSH known (34). As the length of the amino acid chain of ACTH is decreased from 24 to 13 residues by progressive deletion of the C-terminal amino acid residues (Table VII), ACTH bioactivity is rapidly lost and MSH bioactivity is progressively increased. On the basis of the approximate molar concentration of the fragment as measured in the extreme N-terminal ACTH radioimmunoassay, its MSH potency is about 8% that of α-MSH. This is consistent with the predicted MSH activity of a fragment 14–15 residues in length. Thus, the unknown MSH previously reported from this laboratory (20) appears to be the N-terminal fragment of the ACTH molecule.

TABLE VII

*Effect of Decreasing Chain Length and N-terminal Acetylation upon Relative ACTH and MSH Bioactivities**

Analogue	ACTH bioactivity	MSH bioactivity
	%	%
$\alpha_p{}^{1-39}$ ACTH	100	0.7
N-α-acetyl-$\alpha_p{}^{1-39}$ ACTH	3.5	1.2
α^{1-24} ACTH	122	0.8
N-α-acetyl-α^{1-24} ACTH	8.1	8.2
α^{1-23} ACTH	83	1.4
α^{1-19} ACTH	52	1.3
α^{1-17} ACTH	6.0	3.5
α^{1-16} ACTH	<0.1	2.5
$\alpha^{1-13}NH_2$ ACTH	<0.1	13
N-α-acetyl-$\alpha^{1-13}NH_2$ ACTH $\equiv \alpha$-MSH	0.2	100

* Reference 34.

It was anticipated that the excessive quantities of extreme N-terminal and C-terminal ACTH fragments in the tumors might be reflected in plasma and thereby serve as a useful diagnostic test for ectopic ACTH production. Although there were threefold higher levels of immunoreactive ACTH as measured by these two assays than by the N-terminal radioimmunoassay or by bioassay, similar ratios were found in the plasma of patients with Cushing's disease (Cushing's syndrome caused by excessive secretion of ACTH by the pituitary). It is not surprising to find more immunoreactive ACTH than bioactive ACTH in plasma, since we have found that the disappearance rate of biologic ACTH activity from plasma is more rapid than that of immunoreactive ACTH measured in two radioimmunoassay systems (8). It was remarkable, however, that

the discrepancies found in the tumors were not reflected in the plasma. In patient Z. S., for example, the tumor extreme N/N and C/N ratios were 89 and 20, respectively, whereas the corresponding plasma ratios were only 1.5 and 2.5. This suggests that, if these fragments are produced in vivo, either the fragments are much more rapidly metabolized once they reach the circulation, or very small percentages of the fragments reach the circulation. Two lines of evidence suggest that the latter may be the correct hypotheis. First, our previous data indicate that ACTH fragments have a prolonged half-life in circulating plasma, not a shortened one (8). Second, there has appeared to be no close correlation between the concentration of ACTH in the tumor and in the plasma of the same patient, either in our own experience or that of others (28). ACTH has been found in postmortem tumor tissue of patients who were not suspected of having had ectopic ACTH syndrome in concentrations similar to those of patients in whom the syndrome was readily apparent (4, 28). Thus, tumor concentrations of ACTH or ACTH fragments may not accurately reflect the rate of secretion of these polypeptides by the tumor.

The explanation for the presence of the ACTH fragments is not yet known. There are at least three possible mechanisms: faulty transcription or translation of the ACTH genome, resulting in the synthesis of both ACTH and ACTH fragments; proteolytic cleavage of intact ACTH after normal synthesis, either in vivo or after the tissue was removed; or inability of the tumor cell to condense efficiently two normal ACTH precursor molecules to form whole ACTH. The first seems unlikely, since each of the three tumors appeared to contain identical ACTH fragments. The second is a strong possibility, since the 14–18 sequence of ACTH is Gly-Lys-Lys-Arg-Arg, which contains bonds that can be cleaved by several known proteolytic enzymes. The tumor cell may contain an enzyme not active in the pituitary ACTH-secreting cell, or the ACTH in the pituitary cell may be unavailable to an enzyme present in both it and the tumor cell, by being contained within a secretory granule, for example. However, preliminary studies involving incubation of intact ACTH with a 105,000 *g* tumor cell cytosol preparation at 37°C rapidly destroyed biologic ACTH activity and produced immunoreactive ACTH fragments of many sizes, with no evidence for preferential production of these two specific fragments. The third possibility, while intriguing, suffers from the fact that there is as yet no evidence for such precursor subunits of ACTH in the normal pituitary. If their synthesis, rather than their subsequent condensation, were the rate-limiting step in pituitary ACTH synthesis, however, their turnover could be so rapid that they would never be present in more than trace quantities. These possibilities are currently being investigated.

It is apparent that tumors causing the ectopic ACTH syndrome may contain, in addition to ACTH that is indistinguishable from authentic pituitary ACTH, at least two ACTH fragments. One of these appears to be the C-terminal portion of the ACTH molecule and has no detectable biologic activity. The other appears to be the complementary N-terminal amino acid sequence. This fragment has negligible ACTH biologic activity, but has significant melanocyte-stimulating activity. Thus, tumors causing ectopic humoral syndromes may contain, not only intact hormones, but hormone fragments, some of which may have a spectrum of biologic activities and immunologic reactivities different from that of the parent hormones.

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Purified human ACTH (α_hACTH; Lerner-Upton-Lande preparation 8B) was obtained from The National Pituitary Agency, University of Maryland and National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. Synthetic α^{1-24}ACTH (Cortrosyn) was generously provided by Dr. H. Strade of Organon, Inc., West Orange, N. J. The following synthetic analogues of ACTH and MSH were made available to us for study through the generosity of Drs. H. Bein, P. A. Desaulles, and W. Rittel of Ciba-Geigy, Ltd., Basel, Switzerland: α-MSH, β_hMSH, $\alpha_p{}^{1-39}$ACTH, α^{1-10}ACTH, α^{7-13NH_2}ACTH, α^{1-16NH_2}-ACTH, α^{1-23}ACTH, α^{1-24}ACTH, α^{11-24}ACTH, $\alpha_p{}^{17-39}$ACTH, and $\alpha_p{}^{25-39}$ACTH.

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