Human Corticotropin (ACTH) Radioimmunoassay with Synthetic 1–24 ACTH

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A corticotropin antiserum was obtained from rabbits immunized with synthetic 1–24 corticotropin conjugated with bovine serum albumin. The antiserum did not cross react with synthetic α-melanotropin or with synthetic β-endorphin and had a cross reactivity of 0.23% with human β-lipotropin. We developed a radioimmunoassay with the antiserum obtained, in which we used polyethylene glycol in conjunction with a second precipitating antibody for fast (15-min) separation of antibody-bound and free corticotropin. The assay had a sensitivity of 16 ng/L and was validated on patients with various pituitary and adrenal diseases. From 103 normal subjects, the median value for corticotropin in specimens collected during the morning was 34 ng/L of plasma; the upper 95% confidence limit of the normal range was 98 ng/L.

Additional Keyphrases: steroids • hormones • normal values • values in various adrenal and pituitary disorders and in other diseases • antiserum

Simultaneous measurement of plasma corticotropin (ACTH) and adrenal steroid amounts offers a more comprehensive assessment of pituitary–adrenal function than does measurement of steroid hormone alone. ACTH assay also could help differentiate primary and secondary hypercorticosteroidism. ACTH radioimmunoassay, practical for routine clinical use, was first published by Yalow et al. [1] and Berson and Yalow [2]. In their reports, porcine ACTH at a dose of 25 units (Duraston; Nordic Biochemicals, Montreal, Canada) was used to immunize guinea pigs at irregular intervals. Among the 50 guinea pigs immunized, some produced excellent antisera and gave an assay sensitivity as high as 1 ng/L. Orth [3] also reported that one guinea pig of 200 immunized with a similar procedure produced highly sensitive antiserum at one bleeding.

To increase immunogenicity, McGuire et al. [4] applied the carbodiimide method of Goodfriend et al. [5] to conjugate porcine ACTH with rabbit serum albumin. The initial injection of conjugate was 3 mg, and the booster injection was 2 mg [4]. Orth [3] modified the carbodiimide method by prolonging the reaction time from 30 min to 8 h and maintaining the pH at 6.0 throughout the reaction to conjugate synthetic 1–24 ACTH with albumin. A dose of 1250 μg was used for the initial injection to immunize either sheep or rabbits, and 50–100 μg was used for the booster injection. Excellent antibody with high titer was produced three months after the initial injection. Despite this information and nearly 14 years of experience after the first paper was published, few clinical laboratories use the ACTH radioimmunoassay.

We report here our experience in producing and characterizing a clinically useful ACTH antiserum from one of six rabbits immunized with 100-μg doses of conjugated synthetic 1–24 ACTH. With this antiserum, we developed an ACTH radioimmunoassay and studied normal subjects and patients.

Materials and Methods

Reagents and Solutions

Synthetic 1–24 corticotropin (gift from Dr. John R. Simoons, Organon, Inc., West Orange, NJ 07092).

Synthetic α-melanotropin and β-endorphin (from Bachem, Torrance, CA 90205).

Bovine serum albumin, essentially fatty acid free (from Sigma Chemical Co., St. Louis, MO 63178).

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (from Pierce Chemical Co., Rockford, IL 61105).

Complete Freund's adjuvant (from Calbiochem, La Jolla, CA 92037).

Human ACTH, human β-lipotropin (gift from Dr. C. H. Li, University of California, San Francisco, CA 94143). Dissolve 2.5 μg in 5 μL of 5 mmol/L HCl.

Bovine lactoperoxidase (EC 1.11.1.7; Sigma). Dissolve 1.08 mg in 2 mL of 50 mmol/L phosphate buffer, pH 7.4.

Na125I (New England Nuclear, North Billerica, MA 01862), specific activity 17 kCi/g.

Hydrogen peroxide, 300 g/L (Mallinckrodt, Inc., St. Louis, MO 63160). Dilute to 4 mg/L with water.

Cellulose dialysis tubing, 1 cm flat width, retains material with a relative molecular mass of 12000 or more (Fisher Scientific Co., Chicago, IL 60143).

Potassium iodide solution. Dissolve 16 g of sucrose, 1 g of potassium iodide, and 20 mg of sodium azide in 100 mL of water.

Sodium metabisulfite solution for lactoperoxidase method. Dissolve 5 mg of sodium metabisulfite (Mallinckrodt) in 100 mL of phosphate buffer (10 mmol/L, pH 7.5) immediately before use.


Acid-acetone solution. To 40 mL of acetone, add a mixture of acetic acid and water (1:99 by vol) to give 100 mL and mix well.

Talcum-treated human plasma. Mix 15 mL of aged human plasma (with sodium citrate as anticoagulant) from the blood bank with four 25-mg tablets of talcum (Ormont Drug and Chemical Co., Englewood, NJ 07631) for 1 h at room temperature and centrifuge (27 000 × g, 20 min). Decant the supernatant and save for future use.

Sodium barbital buffer, 20 mmol/L. Dissolve 3.68 g of sodium barbital and 7.85 mg of benzamidine-HCl in 900 mL of water; adjust the pH to 8.6 with 1 mol/L NaOH, add water to make volume 1.0 L, and store at 4°C.

Assay buffer. Dissolve mg of soybean trypsin inhibitor (Sigma) in 100 mL of barbital buffer.

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Normal rabbit serum solution. Dilute 0.8 mL of normal rabbit serum to 20.0 mL with barbital buffer.

Chloramine T solution: Dissolve 2.5 mg of Chloramine T (Eastman Kodak, Rochester, NY 14650) in 10 mL of phosphate buffer (0.5 mol/L, pH 7.5) immediately before use.

**Sodium metabisulfite solution for Chloramine T method.** Dissolve 5 mg of sodium metabisulfite (Mallinckrodt) in 10 mL of phosphate buffer (0.5 mol/L, pH 7.5) immediately before use.

**Procedure**

**Iodination of ACTH.** We iodinated human ACTH either by the lactoperoxidase method (6) or the Chloramine T method (2) with some modification.

**Lactoperoxidase method:** To 2.5 μL of human ACTH in 5 μL of HCl, 5 mmol/L, add in the following sequence: 20 μL of phosphate buffer (50 mmol/L, pH 7.4), 2.7 μg of bovine lactoperoxidase, 500 μCi of Na125I, and 5 mg of hydrogen peroxide, each in 5-μL volumes. Let the mixture react for 20 s at room temperature, then add 100 μL of sodium metabisulfite and potassium iodide solution to stop the reaction. To the reaction mixture add 1 mL of tallow-treated plasma and 5 mg of Quso G-32; mix. Separate the Quso from the reaction mixture by centrifugation, wash with 3 mL of water, and elute with 0.5 mL of acid-acetone solution. To the acetone eluate, add 1.5 mL of water. Divide the acid-acetone-water mixture into 0.2-mL aliquots and store in the freezer.

**Chloramine T method:** To 2.5 μg of human ACTH in 5 mL of hydrochloric acid (5 mmol/L), add 25 μL of phosphate buffer (50 mmol/L, pH 7.5), 500 μCi of Na125I, and 2.5 μg of Chloramine T in 10 μL. Let the mixture react for 20 s at room temperature, then add 100 μL of sodium metabisulfite solution to stop the reaction. To the mixture add 1 mL of tallow-treated plasma and 5 mg of Quso; mix. Separate the Quso from the reaction mixture by centrifugation, and elute the labeled ACTH from Quso, as described in the lactoperoxidase method.

**Purification of labeled ACTH.** Labeled ACTH was further purified by use of the modified McIlhinney and Schulster method (6); CM-Sephadex was used instead of CM-cellulose, and the ammonium acetate linear gradient was pH 6.0 instead of 4.8. The details are as follows: hydrate CM-Sephadex 25-120 (Pharmacia, Piscataway, NJ 86584) in ammonium acetate solution (10 mmol/L, pH 6.0) for 24 h and pour into a 1.2 x 20 cm column. Charge the column with 200 mL of ammonium acetate solution, 10 mmol/L, pH 6.0, at 4 °C. On the day of assay, mix 0.2 mL of acid-acetone-water extract of labeled ACTH, obtained either from the lactoperoxidase method or from the Chloramine T method, with 1.8 mL of tallow-treated plasma and apply to the CM-Sephadex column. Elute the column with a linear gradient of ammonium acetate solutions of 500 mL of 0.1 mol/L and 500 mL of 0.6 mol/L, each having a pH of 6.0 at 4 °C. Collect 5-mL fractions.

**Conjugation of synthetic ACTH.** We conjugated synthetic 1–24 corticotropin (5 mg) with 5 mg of fatty acid-free bovine serum albumin by using 100 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl. To monitor the percentage of corticotropin conjugated, we modified the method of McGuire et al. (4) by adding trace amounts of 125I labeled ACTH (32 000 cpm). The mixture was reacted for 30 min at room temperature, transferred into 1-cm cellulose dialysis tubing, and dialysed for 48 h against 2 L of water with three changes; 32% of the radioactivity remained inside the dialysis bag. On the assumption that 125I-labeled ACTH reacts with bovine serum albumin in a manner identical to that of 1–24 corticotropin, this indicates that 1.6 g of the 5 mg of 1–24 corticotropin was conjugated with the albumin or that 8 mol of 1–24 corticotropin was conjugated with 1 mol of albumin.

**Immunization of rabbits.** We dissolved 100 μg of conjugated 1–24 corticotropin in 1 mL of saline, homogenized it with 1 mL of complete Freund's adjuvant to form a thick emulsion, and injected this intradermally at 30 sites on the backs of each of six 0.9-kg white, female New Zealand rabbits. Each rabbit received a monthly booster injection of 100 μg of conjugated ACTH and 1 mL of complete Freund's adjuvant in the same manner. Test bleeding was drawn 10 days after each booster injection. Three months after the initial injection, the rabbits were fed dexamethasone, 0.25 mg in 2.5 mL of elixir added to each 400 mL of drinking water. After six booster injections, one of the six rabbits produced an antiserum having a titer of 1:100 000 final dilution.

**Sample preparation.** Ten milliliters of blood was drawn in an ice-cooled tube containing 1.0 mL of sodium citrate solution (38 g/L) and centrifuged immediately at 4 °C to separate the plasma. The plasma was frozen at −20 °C or packed in solid CO2 for shipping. After assay, the plasma was thawed and recentrifuged at 35 000 × g for 10 min to remove any precipitate that formed during storage.

**Extraction of ACTH from tumor tissue.** A piece of frozen tumor tissue was weighed and further cut with a surgical knife into small portions of 3 mm² each. A homogenate in the proportion of 100 g/L was made by homogenizing the diced tissue in 0.1 mol/L acetic acid at 80 °C in a homogenizer (Polytron; Kinematische, Lucerne, Switzerland) for 40 s at a setting of 4. We heated the homogenate in a boiling water bath for 10 min to elimate protease activity (7) and centrifuged it at 12 000 × g for 10 min. The supernate was diluted with tallow-treated human plasma for assay.

**Assay procedure.** To duplicate sets of polystyrene tubes, 12 × 75 mm, add 0.5 mL of unextracted plasma or human ACTH standard (range 7.8 to 1000 pg) dissolved in 0.5 mL of tallow-treated plasma, 100 μL of labeled ACTH (containing 2000 cpm), and 1.9 mL of antiserum diluted (1:76 000) with assay buffer. For zero standard tubes, use 0.5 mL of tallow-treated plasma; for blank tubes, use 1.9 mL of assay buffer to replace the sample and diluted antiserum, respectively. Incubate all tubes at 4 °C for four days. For separation of free and bound ACTH, add 375 μL of normal rabbit serum solution and 80 μL of goat antirabbit antiserum to each tube. Incubate the reaction for another 15 min at room temperature; after adding 300 μL of polyethylene glycol (mol. wt. 6000), 200 g/L of water, centrifuge the tubes at 3000 × g for 20 min at 4 °C. Decant the supernate, and count the precipitate for 5 min in an automatic gamma counter. Construct a standard curve of B/B₀ against the concentration of ACTH, and determine the concentration of ACTH in unknowns from this standard curve.

**Results**

**Iodination and Purification of Labeled ACTH**

After iodination, either by the lactoperoxidase method or by the Chloramine T method, between 30 and 35% of the radioactivity added to the reaction mixture was recovered in the fraction of acid-acetone eluate. This fraction was diluted with water, divided into 0.2-mL aliquots, and stored in the freezer. To ensure the quality of labeled ACTH for each assay, we mixed 0.2 mL of the aliquot with 1.8 mL of tallow-treated plasma; we further purified this mixture immediately before use on a CM-Sephadex column and eluted it with linear gradient ammonium acetate solutions (see Materials and Methods and Figure 1). After elution we determined the trichloroacetic acid-precipitable radioisotope counts of the first and second peak. We found that 50% of the radioactive counts of the first peak and 100% of those of the second peak were precipitable, which indicates that at least 50% of the radioactive material in the
Table 1. Recovery of ACTH Added to a Pooled Plasma

<table>
<thead>
<tr>
<th>Plasma alone</th>
<th>Plasma plus 25 pg</th>
<th>Plasma plus 50 pg</th>
<th>Plasma plus 100 pg</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Mean, pg/tube</td>
<td>16</td>
<td>41</td>
<td>61</td>
</tr>
<tr>
<td>SD, pg/tube</td>
<td>1.8</td>
<td>4.9</td>
<td>2.9</td>
</tr>
<tr>
<td>% recovery</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Recovery Study

Human ACTH in amounts of 25, 50, and 100 pg was added to 0.5 mL of pooled patient’s sample, and ACTH concentrations were determined by the assay. The recoveries were 100, 92, and 107% for 25, 50, and 100 pg of added ACTH, respectively (Table 1).

Assay Variation

A clinical sample was divided into aliquots and determined in three consecutive assays within a period of two weeks, with eight to 10 tubes in each assay. The ACTH values of individual tube determinations, the standard deviations, and the means within each assay determination are shown in Table 2. Pooling the values of all 27 tubes in the three assays gave a mean of 83.4 ng/L and a standard deviation of 8.2 ng/L.

ACTH is not very stable in a single sample or pooled clinical samples, if the sample is stored for a long time even in frozen condition. For this reason, ACTH samples or pooled samples are not suitable to use as long-term quality-control samples. Thus, we routinely have stored aliquots of 100 pg of human ACTH per tube and added 500 μL of talmuc-treated plasma

Table 2. Assay Variation of a Single Clinical Sample

<table>
<thead>
<tr>
<th>Assay, ng/L</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of tubes assayed</td>
<td>9</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Mean, ng/L</td>
<td>73.6</td>
<td>88.3</td>
<td>88.4</td>
</tr>
<tr>
<td>SD, ng/L</td>
<td>3.2</td>
<td>5.8</td>
<td>4.1</td>
</tr>
<tr>
<td>CV, %</td>
<td>4.3</td>
<td>6.5</td>
<td>4.6</td>
</tr>
</tbody>
</table>
per tube before each assay as quality-control samples. Every assay, in addition to clinical quality-control samples, also includes two tubes of 100 pg of human ACTH plus 500 µL of talcum-treated human plasma. The results of 12 assays in a period of three months, as calculated by Rodbard's method (8), are shown in Table 3. The mean of duplicate tubes obtained from 12 assays was 103 pg/tube, the standard deviation between 12 assays was 10.97 pg/tube, and the coefficient of variation was 10.55%. The combined within-assay variance was 7.79 pg/tube, and the within-assay coefficient of variation was 7.56%.

Linearity of Results on Sample Dilution

A sample was diluted with talcum-treated plasma by twofold, fourfold, and eightfold. The undiluted sample and diluted samples were determined. The results were 800, 405, 210, and 98 pg, respectively. The weighted regression line of volume of sample vs. ACTH content passed through the origin (r = 0.9996) (Figure 3). This suggested that samples at higher concentrations can be diluted to obtain an accurate value for their concentration.

Determination of ACTH Content in Tumor Tissue

We homogenized an ACTH-producing tumor and extracted it with hot acetic acid, as described in Materials and Methods. After diluting the supernate in 100-fold with talcum-treated human plasma, we determined that 5, 10, and 20 µL of diluted supernate contained 165, 380, and 700 pg of ACTH, respectively. A weighted regression line of the volume of tumor extract vs. ACTH content intercepted the y-axis at 5 pg (r = 0.9970) (Figure 3), which suggests that the tumor extract may have contained some substance that slightly interfered with the assay. The assay, however, still can be used to estimate the ACTH content in a tumor.

Determination of ACTH in Normal and Disease States

Normal values. Normal values were determined on two occasions five months apart. Plasma of apparently healthy persons was collected between 7:30 and 8:30 a.m. On the first determination, we studied 11 males, eight females, and eight females taking contraceptive pills. The results (mean ± SD) were 54 ± 27 ng/L for males, 51 ± 37 ng/L for females, and 51 ± 35 ng/L for females taking contraceptive pills. The difference among the groups was not statistically significant. The amount of corticosteroid in females taking contraceptive pills (380 ± 140 mg/L) was twice as high as that in normal males and females (160 ± 40 mg/L), but the ACTH values were not different. (In our laboratory, the normal range for morning-determined corticosteroids is 70–280 mg/L.)

On the second determination, we studied 42 males and 34 females. The results were 40 ± 38 ng/L for males and 54 ± 32 ng/L for females. The differences between males and females and between the first and second determinations were not significant. Thus, we pooled all the data to establish normal values for males and females. In all, 105 apparently healthy persons were studied, and the result was 41 ± 34 ng/L (Table 4). The median of the 105 subjects was 34 ng/L and the upper 95% confidence limit of the normal range was 98 ng/L (Figure 4).

Fig. 3. Linear regression lines of ACTH content vs. dilution of plasma or tumor extract from patients Plasma (■) or tumor extract (○) were diluted with talcum-treated plasma, and their ACTH contents were determined.

Fig. 4. ACTH concentration in apparently normal persons and in patients with Cushing's syndrome, Addison's disease, 17- or 21-hydroxylase deficiency, or ectopic ACTH syndrome. ○-○, ACTH of same patient determined on different dates; •-•, same patient after therapy or operation; Rx = steroid replacement therapy; op. = operation; Open horizontal arrows indicate the upper 95% confidence limit of normal subjects. Horizontal bar indicates median.
**Table 4. Determination of Normal Values**

<table>
<thead>
<tr>
<th>No. of subjects</th>
<th>ACTH, ng/L</th>
<th>Corticosteroids, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td><strong>First group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>11</td>
<td>0–86</td>
</tr>
<tr>
<td>Females</td>
<td>8</td>
<td>0–100</td>
</tr>
<tr>
<td>Females taking contraceptive pill</td>
<td>8</td>
<td>0–90</td>
</tr>
<tr>
<td><strong>Second group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>42</td>
<td>0–136</td>
</tr>
<tr>
<td>Females</td>
<td>34</td>
<td>0–98</td>
</tr>
<tr>
<td>Total</td>
<td>103</td>
<td>0–136</td>
</tr>
</tbody>
</table>

* Upper 95% confidence limit.

**Primary hypercorticosteroidism (Cushing's syndrome).** There were only three patients with primary hypercortico-
steroiodynamics treated in our clinic during the development of this assay. The first patient had ACTH-independent nodular hyperplasia of the adrenals. ACTH content, determined on separate days two weeks apart, was 26 and 0 ng/L. The second patient had one ACTH determinations, 0 ng/L, later, a 21-g tumor was surgically removed from the left adrenal gland of this patient. The third patient had three ACTH determinations—0, 29, and 39 ng/L. A 2- to 3-g tumor was surgically removed from each side of the adrenal. The data indicated that low amounts of ACTH sometimes may be found in some patients with Cushing's syndrome (Figure 4).

**Primary hypocorticosteroidism (Addison's disease).** Eight patients with defined Addison's disease had their ACTH amounts determined. Among them, four untreated patients had a higher median ACTH value than the four patients who were receiving replacement steroid. In one of the patients receiving replacement, ACTH values determined on different days were 26 and 32 ng/L (Figure 4).

**Enzymatic block or adrenogenital syndromes.** Eleven pediatric patients, all less than 13 years old, had a deficiency of either 17- or 21-hydroxylase. Four patients were without treatment, including one who had had no treatment for six months and whose ACTH values increased from 122 to 351 ng/L. Of seven patients with steroid treatment, one had an ACTH value of 85 ng/L before treatment and 10 ng/L after treatment (Figure 4).

**Ectopic ACTH syndrome.** We studied six preoperative patients with ectopic ACTH syndrome caused by oat-cell carcinoma of the lung, medullary carcinoma of the thyroid gland, or pancreatic tumor. After resection of a 1-cm cystadenoma of the pancreas, one patient's ACTH decreased from 1280 to 20 ng/L (Figure 4). Ten months later, when the patient was rechecked, ACTH was determined to be 112 ng/L. The median value for patients with an ectopic ACTH syndrome who received surgical treatment was 70 ng/L and the range was 20–160 ng/L.

**Cushing's disease (ACTH-dependent adrenal hyperplasia).** Of four patients with pituitary Cushing's disease but not operated on, two patients had determinations of ACTH repeated on different days. The results were 108, 108, 110, and 130 ng/L for one patient and 85, 88, and 96 ng/L for the other. The mean and range for all four patients are given in Figure 5. Their ACTH values were not too much higher than the average for normal subjects, but all had increased amounts of plasma corticosteroid.

Three other patients with Cushing's disease had been successfully treated with radiation therapy to their pituitary glands; the median ACTH value was 14 ng/L, and the range was 10–17 ng/L (Figure 5). Four patients with Cushing's disease had a remission induced by removal of pituitary tumor through a transsphenoidal approach; after the operation, their median ACTH value was 40 ng/L and the range was 10–74 ng/L.

Patients with pituitary-dependent Cushing's syndrome who were treated by adrenalectomy had a high tendency to develop Nelson's syndrome. Several of these patients, even without increasing pigmentation, had increases in ACTH (Figure 5).

**Nelson's syndrome or increasing pigmentation after adrenalectomy.** Some patients, in whom pigmentation increased after adrenalectomy, or who had defined Nelson's syndrome, had much higher ACTH values (Figure 5). Usually, these patients had evidence of pituitary tumors. Some of them were further treated by transsphenoidal removal of pituitary tumor, but in general, after surgery, their ACTH values did not seem to be substantially decreased. Two of these patients, however, who showed some improvement before the transsphenoidal removal of the pituitary tumor had ACTH values of 240 and 1920 ng/L; after surgery, the ACTH values were 156 and 195 ng/L, respectively. In one patient, before the transsphenoidal operation ACTH was more than 2000 ng/L; two days after the operation, the patient's ACTH was 13 ng/L.

**Fig. 5. ACTH concentration of patients with Cushing's disease, pituitary adenoma, or disease unrelated to pituitary–adrenal disease.**

Patients with Cushing's disease were classified as having no notable increase in pigmentation or no increase in pigmentation. No RoRx = radiation therapy to pituitary gland; TS = transsphenoidal removal of pituitary; ADX = adrenalectomy; ADX+TS = adrenalectomy followed by transsphenoidal removal of pituitary after pigmentation increased; A = patient with prolactin value greater than 5000 ng/L; *, patient with subternal goiter; other symbols same as for Fig. 4.
operation, ACTH decreased to 1560 ng/L; a few days later, the ACTH value increased again to more than 2000 ng/L, indicating that a residual secretory mass probably remained.

*Non-ACTH-producing pituitary adenoma and acromegaly.* The ACTH values of patients with non-ACTH-producing pituitary adenoma and acromegaly had a median of 42 ng/L and a range of 10–84 ng/L. One patient had a prolactin-producing pituitary tumor. The prolactin was greater than 5000 ng/L, but the ACTH value was only 70 ng/L (Figure 5).

*Diseases unrelated to pituitary–adrenal disease.* Patients with other diseases had a median ACTH value of 17 ng/L and a range of 10–210 ng/L. The highest ACTH value (210 ng/L) was found in a hospitalized patient who had a large substernal goiter (Figure 5).

**ACTH Change During Insulin Tolerance Test**

This assay also can be used to monitor the change of ACTH secretion caused by insulin-induced hypoglycemia. Two patients with pituitary tumors had their blood sampled at 30-min intervals after injection of insulin (0.15 U/kg of body wt). Both ACTH and corticosteroid values increased and reached their peak 60 min after injection (Figure 6). At the peak, the corticosteroids and ACTH increased from basal values about 200% and 42%, respectively, for patient 1 and 160% and 54%, respectively, for patient 2. Recently, Lindholm et al. (9) showed that there was no significant correlation between ACTH and cortisol increase during insulin-induced hypoglycemia.

**Discussion**

The primary difficulty of the radioimmunoassay for ACTH is that normal subjects have very low amounts of ACTH in plasma, and hence a highly sensitive antiserum is required. Production of highly sensitive antiserum against ACTH is difficult, primarily for two reasons: (a) ACTH is a relatively small molecule (mol. mass 4500), and the immunogenicity of molecules with a molecular mass of less than 10,000 is generally low; and (b) because no species difference is found in the biologically active sequence of 1–24 amino acid residues of ACTH (among its whole 39 residues), different species of ACTH used to immunize animals also may cause the adrenal gland to produce a large amount of cortisol, which inhibits the immunologic response.

To overcome these difficulties, Yalow et al. (1) and Berson and Yalow (2) immunized 50 guinea pigs by administering metyrapone (75 mg) simultaneously with ACTH. Metyrapone, an 11-hydroxylase inhibitor, blocked the synthesis of cortisol. McGuire et al. (4) and Orth (3) immunized rabbits or sheep with conjugated ACTH, which not only increased the immunogenicity but also decreased the biologic activity of ACTH after it was conjugated with albumin.

The dose of ACTH used for immunization in these successful reports was 1.25–3 mg per animal (2–4). It is now believed that the antigen used for immunization should be highly pure and the dose should be small (10). Vaitukaitis et al. (11), in their work with human chorionic gonadotropin, reported that 100 μg was the smallest effective immunization dose that could produce antiserum in rabbits. For these reasons, we decided to use synthetic 1–24 corticotropin, which is pure, and injected a small amount of synthetic 1–24 corticotropin–bovine serum albumin conjugate containing 100 μg of 1–24 corticotropin and 308 μg of bovine serum albumin (38 nmol and 4.5 nmol, respectively). We used the same dose of conjugate for initial and booster injections. The labeled ACTH, which we added during the conjugation procedure, served as a conjugation indicator for calculating the amount of conjugate containing 100 μg of 1–24 corticotropin. With this immunization procedure, two of six rabbits after six booster injections produced antiserum. One of the antisera, with a titer of 1:100,000 of the final dilution, was characterized and used for this clinical study. It did not cross react with α-melanotropin or with β-endorphin and showed a cross reactivity of 0.23% with β-lipotropin.

In our radioimmunoassay system, we did not find any significant difference in labeled ACTH produced by the Chlomramine T or by the lactoperoxidase iodination method. Rae and Schimmer (12) also found that the biologic activity of labeled ACTH produced by the lactoperoxidase method was not necessarily better than that produced by the Chloramine T method. However, the labeled ACTH produced by either method was further purified by ion-exchange column chromatography. The method used to purify labeled ACTH for biologic studies (6) was modified; we used the CM-Sephadex column, which technically is easier to operate than the CM-cellulose column, and a linear gradient of acetate solutions of 0.1–0.6 mol/L was at pH 6.0 instead of at pH 4.8.

The average specific activity of labeled ACTH prepared by either iodination method was 68 Ci/g of ACTH. At present, we cannot increase the specific activity without increasing the amount of damaged ACTH or decreasing the binding ability with antiserum. Based on this specific activity (68 Ci/g) and 60% counting efficiency for an average counter, 22 pg of labeled ACTH had a radioactivity of only 2000 cpm. To achieve a sensitivity of 16 ng/L in our assay, we used 2000 cpm of labeled ACTH per tube and counted every tube for 5 min to accumulate a significant count. By doing so, we obtained a between-assay coefficient of variation of 10.55% (Table 3), which is acceptable for general radioimmunoassay methods. This indicates that, with care, 2000 cpm per tube can be used without unduly sacrificing the quality of the assay.

For economic reasons, we used benzamidine (13) and soybean trypsin inhibitor in our assay buffer instead of Transylol.
(2). We did not find any significant protective function of mercaptoethanol in assay buffer, just as Orth (3) mentioned, and also deleted it from our assay buffer.

Some clinical samples have high lipid contents that will interfere with the ACTH assay and cause falsely high results. For this reason, we centrifuged all the clinical samples indiscriminately at 35000 × g for 10 min to remove the lipids.

The values for plasma ACTH determined in normal persons had a range comparable to that reported for radioimmunoassay of human ACTH (2, 14, 15). In general, there was good clinical correlation between the plasma ACTH values determined and the clinical state of our patients with pituitary-adrenal disease. For example, the high-normal or above-normal plasma ACTH values noted in patients with Cushing’s disease (ACTH-dependent adrenal hyperplasia) are inappropriate when considered in light of the increased plasma corticosteroid values seen in these patients. Pronounced increases of plasma ACTH have uniformly been seen in the patients with hyperpigmentation (Nelson’s syndrome), and in the few instances available, a decrease in ACTH values has generally correlated with diminished pigmentation after therapy of the pituitary tumor even though their ACTH values may not decrease to normal. Increased values were noted in the patients with untreated primary adrenal insufficiency, and these values tended to normalize after replacement therapy was started. Similar values were noted in the patients with untreated congenital adrenal hyperplasia because of the enzymatic adrenal synthetic blocking of cortisol synthesis. Very high ACTH values generally are associated with ectopic ACTH-producing tumors. In a few selected cases of documented pituitary-adrenal problems, the individual ACTH values did not correlate well with the clinical situation. In some instances, multiple measurements tended to give a better correlation with the clinical situation.

These are too few cases to present as a comprehensive clinical analysis. Yet, when other laboratories realize from using our technique that ACTH is not an impossible assay, more data will become available for evaluating the relationship between ACTH values and pituitary-adrenal problems.

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