Rapid Radioimmunoassay for Corticotropin in Unextracted Human Plasma

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This RIA for corticotropin (ACTH) involves use of a commercially available antiserum and permits measurement of immunoreactive ACTH in unextracted plasma. The assay takes 2.5 days, detects as little as 5 pg/mL of plasma, and is specific: structurally and (or) biosynthetically related peptides do not cross react. Generally, data on dilutions of almost all human plasma specimens produce curves parallel to that for the ACTH reference standard. Values correlate well with those obtained by a well-established but more laborious RIA. We measured immunoreactive ACTH in normal subjects at various times of the day and after modulation of their pituitary-adrenal axis, and in patients with hypo- and hypersecretion of ACTH. We conclude that the full range of immunoreactive ACTH values, which accurately reflect the status of the subject's pituitary-adrenal axis, can be quickly and easily determined in samples of unextracted human plasma.

Additional Keyphrases: hormones · adrenal-pituitary status · direct vs extraction methods · reference interval · application to experimental and veterinary medicine

Several radioimmunoassays (RIAs) have been reported for measuring corticotropin (ACTH) in human plasma. The effectiveness (i.e., the sensitivity requisite to accurate measurement of concentrations less than 20 pg/mL frequently found in normal human plasma) of most of these RIAs depends upon partially purifying and concentrating the hormone before assay (1–5). One RIA for ACTH in unextracted human plasma has been reported, but the antibody was never made available to others (6). None of the established commercially available RIA kits for measuring ACTH in unextracted plasma has the sensitivity required to measure immunoreactive ACTH (IR-ACTH) in serial dilutions of plasma or to quantitate, accurately and with confidence, concentrations of IR-ACTH in the lower physiological range or less. From literature provided by the companies (7–9), the amount of reference standard ACTH required for 50% displacement of tracer (ED50) in RIA performed according to their protocols is 3.3–5.3– and 5.8-fold that required in the two-day, two-stage RIA described here. Although measurement of ACTH has wide clinical and research applicability, it is done by a relatively few investigators and commercial reference laboratories, who use time-consuming procedures requiring extraction of ACTH from plasma before the RIA. In this report we describe a relatively brief, in terms of time and complexity, RIA for ACTH, in which commercially available reagents are used to measure physiologically relevant ACTH concentrations in unextracted human plasma.

Materials and Methods

**Peptides.** Anti-ACTH serum "IgG-ACTH-1" was donated by IgG Corporation, Nashville, TN 37211. This antiserum was produced in a rabbit immunized with residues 1–24 of the ACTH molecule ("Synacthen"; Organon, Inc., West Orange, NJ 07005) conjugated to bovine serum albumin (BSA, cat. no. A9647; Sigma Chemical Co., St. Louis, MO 63178) by the carbodiimide method (5).

Synthetic intact human ACTH and the (1–16)-NH2, (1–18)-NH2, 11–24, and N5-1-acetyl-(1–13)-NH2 (α-melanotropin) fragments of it were gifts of Ciba-Geigy, Ltd., Basel, Switzerland. Synthetic fragments 5–18 and 5–24 were gifts of Dr. D. DeWeid, Rudolf Magnus Institute for Pharmacology, Utrecht, The Netherlands. Human β-lipotropin and its amino-terminal fragment, γ-lipotropin (β-lipotropin(1–56)), were purified from human pituitary glands in our laboratory (10). Synthetic hα-lipotropin(35–56) and (59–89), the former also called "hα-melanotropin" and the latter "hα-endorphin," were provided by Ciba-Geigy, Ltd., and Dr. J. Rivier, The Salk Institute, La Jolla, CA 92138, respectively.

**Phosphate-EDTA buffer.** A 63 mmol/L phosphate-EDTA buffer, pH 7.4, was prepared by dissolving 16.84 g of Na2HPO4·7H2O, 4.74 g of Na3EDTA·2H2O, and 0.2 g of NaN3 in de-ionized water, adjusting the pH to 7.4 with 1 mol/L NaOH if necessary, and diluting to 1 L. This buffer was stored at 4°C.

**RIA buffer.** To the phosphate-EDTA buffer was added 1 mL of Triton X-100 (cat. no. 111020; Research Products International Corp., Mount Prospect, IL 60056) and 250 000 kalikrein inhibitory units of aprotinin (Trasylol; FBA Pharmaceuticals, New York, NY 10022) per liter (10). This buffer was also stored at 4°C.

**Bovine serum albumin-RIA buffer.** A 35 g/L solution of BSA (cat. no. A9647, Sigma) in phosphate-EDTA buffer was prepared, and 100 g of polysilic acid (cat. no. SIL-A-200, Sigma) that had previously been sieved to exclude particles smaller than 100 mesh was added per liter. This suspension was rotated to mix end-over-end for 30 min at room temperature. The purpose of this procedure is to extract corticotropin and any other contaminating peptides that may interfere in the RIA. The suspension was centrifuged (6000 × g, 10 min), and the supernatant BSA-RIA buffer was stored at −20°C.

**RIA diluent.** Diluent, used to dilute the standard and plasma samples for RIA, was prepared by adding, per liter, 1 mL of Triton X-100 and 250 000 kalikrein inhibitory units of aprotinin to the BSA-RIA buffer.

**Standards.** About 0.5 mg of human ACTH was dissolved in 10 mmol/L HCl, and the concentration was adjusted to 1 g/L, as determined spectrophotometrically (11). Two-microliter aliquots containing 2 μg of ACTH were pipetted into 1.5-mL conical polypropylene tubes (cat. no. 5-407-5; Fisher Scientific Co., Philadelphia, PA 19406) and stored at −70°C.

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until used for iodination. The remaining ACTH was diluted to 1 mg/L with RIA buffer and stored in 100-μL (100-ng) aliquots at −70 °C until used as reference standard. The ACTH analogs and other peptides were similarly diluted, aliquoted, and stored frozen.

Procedures

Samples. Blood was collected in a chilled syringe and enough was transferred to fill either a 5- or 10-mL Vacutainer Tube (Becton, Dickinson and Co., Rutherford, NJ 07070) containing Na2EDTA. Plasma was prepared and stored as previously described (5). ACTH was extracted from some plasma samples with silicic acid before RIA (5).

Radioiodination. ACTH was labeled with 125I and purified as previously described (5) with two exceptions: (a) the 0.9 × 57 cm column of Sephadex G-50 fine resin (cat. no. 17-0042-02; Pharmacia Fine Chemicals, Piscataway, NJ 08854) that was used to separate 125I-labeled ACTH from 125I after iodination was equilibrated and developed in RIA buffer; this column was extensively washed with RIA buffer and was used to repurify the labeled ACTH immediately before each assay; and (b) BSA was omitted from the purification procedure.

Assay procedure. The entire RIA procedure was carried out at 4 °C by performing all procedures in ice-water baths, refrigerated centrifuges, and cooled centrifuge carriers, and incubating solutions in refrigerators.

Day 1. Standard human ACTH and plasma samples were diluted in RIA diluent so that the desired concentrations were contained in 100 μL. We assayed at least two threefold dilutions of each plasma sample. The standard curve was prepared in triplicate; plasma sample dilutions were prepared in duplicate. "IgG-ACTH-1" antiserum was diluted 10 000-fold in RIA buffer containing 10 mL of normal rabbit plasma (cat. no. 31141-1BY; Pel-Freeze, Rogers, AR 72756) per liter, which serves as a carrier and coprecipitant for the anti-ACTH immunoglobulin G. We added 100 μL of human ACTH standard or plasma sample to 100 μL of diluted antiserum in 12 × 75 mm polypropylene tubes (cat. no. 2002, Falcon Plastics, Oxnard, CA 93030; or cat. no. 55 526, Sarstedt, Princeton, NJ 08554), mixed the solutions by brief horizontal shaking of the racks, and incubated the mixtures for 24 h in a humidified atmosphere by enclosing in sealed plastic bags the racks containing the tubes.

Day 2. Freshly purified 125I-labeled human ACTH was diluted in RIA buffer so that each 100 μL contained ~ 2000 cpm (~ 6 pg of peptide), 100 μL of this was added to each assay tube, the solutions were mixed by shaking the racks, and the incubation was continued for another 24 h under the same conditions.

Day 3. Goat antiserum to rabbit γ-globulin (cat. no. 539846; Calbiochem-Behring Corp., La Jolla, CA 92037) was diluted 30-fold with RIA buffer, 100 μL was added to each tube, the racks were shaken, and the incubation was continued for 3 h under the same conditions. To separate bound from free ACTH, we added 1.6 mL of ice-cold phosphate–EDTA buffer containing 20 mg of BSA per milliliter, then immediately centrifuged (6000 × g, 20 min) the tubes. Immediately after centrifugation, each centrifuge carrier, which contained 35 assay tubes, was covered with stainless steel mesh wires mesh inverted to drain the supernates. Keeping the wire mesh inverted, we pressed it against eight layers of paper towel to blot each tube simultaneously. The tubes, which contained the antibody-bound 125I-labeled human ACTH pellets, were counted for 5 min in a gamma-scintillation spectrometer (Model no. 4/600; Micromedic Systems, Horsham, PA 19044).

Results

Radioimmunoassay Characteristics

Time-course of antibody binding. To examine the binding of labeled ACTH tracer in the absence of added unlabeled ACTH, we used a concentration of antiserum (30 000-fold final dilution) that had been demonstrated to bind approximately 32% of the 125I-labeled ACTH at equilibrium after three days of incubation. Binding progressed rapidly and reached near-equilibrium levels (~ 28%) by 24 h (Figure 1).

Sensitivity. Assay sensitivity can often be improved by using a two-stage RIA protocol, delaying addition of labeled tracer antigen to the system (12). The sensitivity of our two-stage, two-day RIA procedure was fourfold that of a single-stage, one-day assay, the only changes being incubation of unlabeled standards and samples with the antiserum for one day prior to adding the labeled tracer, followed by incubation for another day prior to separation. With the two-stage RIA, 10% of the labeled tracer was displaced (ED10) by only 0.5 pg (0.11 fmol) of unlabeled ACTH per tube, the ED50 being 3 pg (0.66 fmol) per tube and the ED90 9 pg (2 fmol) per tube (Figure 2). The routine level of the detection of plasma IR-ACTH with the two-stage, two-day RIA was 5–10 pg/mL (1.1–2.2 pmol/L).

The one-day, single-stage assay protocol can be used to obtain results more rapidly if less sensitivity is acceptable. Antibody binding of labeled antigen is almost at equilibrium by 24 h (Figure 1). Thus, a single-stage assay, in which 125I-labeled ACTH in 100 μL of RIA diluent is added at the beginning of the incubation, can be performed with the same dilution of first antibody. We add the second antibody after 22–24 h, incubate for another 3 h, and separate bound from free labeled antigen as in the two-stage assay. There was only a slight decrease in B10, but there was a significant loss in sensitivity: the ED10 increased to about 2 pg/tube, and the ED50 and ED90 were proportionately increased to 15 and 40 pg/tube, respectively. Almost the same sensitivity (ED10, ED50, and ED90 of 2.5, 15, and 50 pg/tube, respectively) can be achieved by an even more rapid protocol in which the second antiserum is added at the beginning of the
incubation and a total volume of 300 µL is incubated at 4 °C overnight (≈ 16 h), with phase separation carried out as usual. This protocol requires about 40% more IgG-ACTH-1 antibody to achieve trace binding (B0) of 30–35%. However, it yields rapid results and can measure ACTH in the midphysiological range. It would, therefore, be especially useful in quickly excluding above-normal values for plasma ACTH in patients suspected of having ACTH hypersecretion.

**Precision.** Intra- and interassay variation was determined at three concentrations of plasma IR-ACTH. The intra-assay coefficient of variation, determined by measuring the IR-ACTH in five replicates of three separate plasma samples in a single assay, was 7.3, 4.6, and 13.2% when the respective plasma concentrations of IR-ACTH were 15, 60, and 125 pg/mL. The interassay coefficient of variation, determined by measuring the IR-ACTH in the same three samples of plasma in 10 successive assays, was 9.2, 8.6, and 12.4%, respectively.

**Specificity.** Hormones structurally and (or) biosynthetically related to ACTH (i.e., the lipotropins and melanotropins) all reacted less than 0.5% as much as the ACTH standard with the IgG-ACTH-1 antiserum. The antiserum is directed toward the steroidogenic amino-terminal (1-18) sequence of the ACTH molecule (13; the minimum sequence that we have defined as being required for complete immunoreactivity is ACTH(5-18) (Figure 2). This sequence is identical in all species thus far investigated. Thus, this RIA should be capable of measuring plasma or tissue ACTH concentrations not only in humans, but in many other species.

**Incubation damage.** As specifically measured by adding fourfold excess of first antibody (14), incubation damage was less than 2%. Nonspecific precipitated radioactivity (i.e., that in the pellet of tubes containing no first antibody, sometimes incorrectly referred to as "damage" in RIAs in which second antibody is used for phase separation (14]) was regularly less than 3% of the total added 125I cpm; we determined nonspecific radioactivity in each assay and subtracted it from antibody-bound cpm before calculating the percent bound 125I labeled ACTH. None of the plasma samples affected the nonspecific precipitated cpm.

**Comparison Studies**

**Correlation with an established ACTH RIA.** Initially, unextracted plasma, appropriately diluted in RIA buffer, was added to the ACTH RIA system. Data for plasma IR-ACTH obtained with this method (Figure 3) correlated well with values obtained when aliquots of the same plasma samples were extracted and assayed (5) with R1543 antibody to ACTH (15).

**Lack of physiologic correlation due to plasma interference in the ACTH RIA.** As more individual plasma samples were studied, it became apparent that samples from individuals expected to have low concentrations of plasma IR-ACTH, such as normal subjects whose ACTH secretion had been suppressed by administration of dexamethasone, frequently appeared to contain significant amounts of IR-ACTH (Figure 4). Because we had not encountered this problem when ACTH was extracted from similar plasma samples before measurement by RIA (5), we assumed that nonspecific inhibition of antigen–antibody binding by plasma components contributed to the apparent IR-ACTH content of the samples. Three lines of evidence supported this hypothesis: (a) "hormone-free plasma," prepared as the 6000 × g supernate of plasma from which the hormone had been removed by adsorption to silicic acid one to four times (5), consistently contained significant amounts of "IR-ACTH" (Figure 5); (b) the slopes of the displacement curves generated by serial dilutions of "hormone-free plasma" were consistently not parallel to that of the standard (Figure 5), indicating that the component(s) causing the displacement of 125I-labeled human ACTH from antibody was not immunologically identical to ACTH; and (c) each undiluted sample of "hormone-free plasma" inhibited antigen–antibody binding by a relatively constant amount, 24 ± 1.4% (mean ± SEM) (Figure 5), irrespective of the original IR-ACTH content of the plasma.

**Source of plasma interference in the ACTH RIA.** The "IR-ACTH" content of "hormone-free plasma" appeared to be the result of interference with antigen–antibody binding by plasma proteins, because most of the "IR-ACTH" in such samples eluted with the higher-Mr plasma proteins during gel filtration, rather than with authentic human ACTH (Figure 6). This "IR-ACTH" was not decreased by repeated
extractions with silicic acid (5), and labeled ACTH incubated for 1 h with "hormone-free plasma" co-eluted with authentic ACTH (data not shown). Thus, this "IR-ACTH" did not appear to represent hormone bound to plasma proteins.

Minimizing plasma protein interference in the ACTH RIA.

Because the addition of unextracted plasma to the assay resulted in relatively constant nonspecific displacement of $^{125}$I-labeled human ACTH from antibody, we anticipated that this artifact could be minimized by including in the assay appropriate concentrations of "hormone-free plasma," so that the binding in all assay tubes would be similarly reduced. However, use of "hormone-free plasma" is not practical in a high-volume RIA laboratory, because of the difficulty and expense in procuring sufficient quantities of human plasma. Therefore, we evaluated preparations of several readily available animal serum proteins, among them solutions of BSA roughly equivalent in protein concentration to that of normal human plasma. At this concentration (i.e., 70 g/L), each BSA preparation caused more interference with antigen–antibody binding than did "hormone-free plasma" (Figure 7), and extracting them with silicic acid to remove contaminating endogenous ACTH had little effect on the binding interference of most of them (Figure 7). For one preparation of BSA (Sigma cat. no. A9647), however, treatment with silicic acid extraction and...
dilution to 35 g/L in RIA buffer resulted in a level of binding inhibition similar to that seen with "hormone-free plasma" over the whole range of the RIA standard curve (Figure 8). We therefore considered this protein solution to be a suitable diluent for samples and standard in the unextracted plasma ACTH RIA.

Comparison of results of RIA for ACTH in unextracted and extracted plasma. Using the IgG-ACTH-1 antibody, we determined the IR-ACTH content of extracts (5) of 65 samples of plasma and compared the results with those obtained with the present method for unextracted aliquots of the same plasma specimens (Figure 9). The values covered the normal range of plasma IR-ACTH concentrations, and there was good correlation between the results obtained by both methods, including those results in the low normal range (Figure 9). Very occasionally, some patients' unextracted plasma still generated competitive binding curves that were less steep than that of standard ACTH. This property was characteristic of certain individuals, such that curves generated by plasma specimens obtained from them on several occasions many days to several weeks apart were always parallel to each other, but not parallel to the standard. Subjects whose plasma exhibited this property included both normal individuals and patients. Extracted samples of the same plasma specimens almost always generated parallel curves in the RIA for ACTH, indicating that it was not a difference in the structure of the hormone that caused this phenomenon. The artifact can be detected by assaying more than one dilution of plasma and can be corrected, when necessary, by extracting ACTH from the plasma of such subjects before assay (5).

Physiologic Correlations

The data shown in Figure 10 represent only a very small fraction of our overall experience with this RIA. None of the results from those very occasional subjects or patients whose plasma generated nonparallel displacement curves is included.

Normal subjects. Plasma concentrations of IR-ACTH ranged from 8 to 30 pg/mL in 17 normal volunteers between 0500 and 0700 h while they remained supine; they had been hospitalized as part of a study reported elsewhere (16). A wider range of plasma IR-ACTH, 8–79 pg/mL, was found between 0700 and 0800 h in a separate group of nine volunteers whose activities were unrestricted before blood collection. Six hours after these nine subjects were given 0.5 mg of dexamethasone intravenously at 0800 h, plasma IR-ACTH in each had fallen to undetectable concentrations.

Fig. 9. Correlation between concentrations of IR-ACTH measured in unextracted plasma with the present RIA and those measured in extracts of the same plasma specimens with RIA (5) also by using IgG-ACTH-1 antiserum

Solid and broken lines as in Fig. 3.

Fig. 10. Plasma IR-ACTH and physiologic correlations as determined in samples diluted with RIA diluent

Normal subjects were studied as described in the text. The patients with pituitary ACTH-dependent Cushing's syndrome were studied 1 h after falling asleep; the other patients were sampled between 0800 and 0900 h.

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**Fig. 8.** Effect of various proteins added to the RIA buffer on the RIA standard curves for ACTH

ACTH standard was diluted either with RIA buffer alone (Θ). RIA buffer containing 35 g/L BSA from which endogenous ACTH had been removed (RIA diluent) (○), or "hormone-free plasma" (△). Each point represents the mean of three determinations.

**Fig. 9.** Correlation between concentrations of IR-ACTH measured in unextracted plasma with the present RIA and those measured in extracts of the same plasma specimens with RIA (5) also by using IgG-ACTH-1 antiserum

Solid and broken lines as in Fig. 3.

**Fig. 10.** Plasma IR-ACTH and physiologic correlations as determined in samples diluted with RIA diluent

Normal subjects were studied as described in the text. The patients with pituitary ACTH-dependent Cushing's syndrome were studied 1 h after falling asleep; the other patients were sampled between 0800 and 0900 h.
(i.e., <5 pg/mL) at 1400 h. Plasma IR-ACTH determined between 1800 and 1700 h in a third group of 50 normal volunteers who had been supine for at least 45 min before sampling (17) ranged from 7 to 30 pg/mL. Thirty to 45 min after eight of these subjects received intravenous insulin (0.15 units/kg body wt) to induce hypoglycemia (plasma glucose =400 mg/L), their plasma IR-ACTH had increased to 135–370 pg/mL. A fourth group of eight normal volunteers, who were hospitalized as part of previous study (12) and had been recumbent for at least 1 h before sampling, all had plasma IR-ACTH concentrations of 10 pg/mL or less at midnight.

Patients with pituitary or adrenal disorders. Eight patients with untreated pituitary ACTH-dependent Cushing's syndrome, or Cushing's disease, had plasma IR-ACTH concentrations of 23–95 pg/mL when sampled 1 h after falling asleep. Only one of six patients with Cushing's syndrome caused by a cortisol-producing adrenal tumor had detectable plasma IR-ACTH, whereas the concentrations in four patients whose syndrome was caused by ectopic production of ACTH ranged from 125 to 800 pg/mL. Adrenal insufficiency and Nelson's syndrome were both associated with high concentrations of plasma IR-ACTH, up to 7250 pg/mL. Plasma IR-ACTH was relatively low (10-20 pg/mL) and was unresponsive to insulin-induced hypoglycemia in two patients with hypopituitarism.

Discussion

RIAs for ACTH have not been more widely applied primarily because, along with a handful of other RIAs, this assay has remained very difficult to perform. The normal concentrations of ACTH that circulate in plasma are very low, about 2–15 pmol/L, lower than the sensitivity of the available assays for ACTH. Consequently, virtually all RIAs for ACTH (1–5) have required prior extraction and concentration of the hormone from plasma and (or) incubation times of four to seven days to achieve sensitivity similar to that of the present assay. One possible exception (6) involved an antiseraum that was never made available to others.

The present RIA method overcomes these technical difficulties. A commercially available antiseraum of unique sensitivity and requisite specificity is used and low-normal to subnormal concentrations of IR-ACTH can be measured in unextracted plasma with an incubation time of two days. Binding is rapid because the concentration of reagents is maximized by minimizing the volume of the incubation mixture. The sensitivity achieved is related to the binding affinity of the antibody and the use of the two-stage RIA protocol. Incubation damage is negligible, due to the use of properly prepared (5) plasma samples, relatively short incubation at 4°C, and the presence of EDTA and apronitin to inhibit proteases (which might degrade both labeled ACTH tracer and unlabeled endogenous ACTH in the plasma samples). Bound and free labeled ACTH are separated simply, inexpensively, and nearly quantitatively by addition of second antibody. The BSA in the phosphate-EDTA buffer added just before centrifugation is not precipitated by the second antibody, but helps produce a pellet that is stable during decanting. The extra volume of the buffer reduces nonspecific trapping of free labeled ACTH in the pellet and thereby eliminates the need to wash the pellet to obtain optimal reproducibility and minimal nonspecific precipitated label.

ACTH is synthesized in humans and other species as a large precursor of several structurally related peptides that appear to be secreted concomitantly by the pituitary gland (18). Even though these peptides share a similar (19) or identical (20) heptapeptide steroidogenic core sequence with ACTH (i.e., ACTH(4–10)), none cross reacts significantly with the IgG-ACTH-1 antibody. This antibody reacts fully with ACTH(5–18), but not at all with ACTH(11–24). Thus, full reactivity requires both a part of the common ACTH(5–10) sequence and a carboxyl-terminal extension of eight residues or less. α-Melanotropin has an identical tripeptide extension, but the final residue is amidated; none of the other peptides has any sequence in common with ACTH(11–18). Presumably, the affinity of the antibody for this region of the ACTH molecule confers its specificity for ACTH alone. Furthermore, because the antibody is directed at the ACTH(5–18) sequence, it will react neither with corticotropin-like intermediate-lobe peptide [ACTH(18–39)] nor with nonsteroidogenic carboxy-terminal ACTH fragments that circulate in the blood of some patients (21). It should, however, react with ACTH in the plasma of most, if not all mammals, the ACTH(1–24) sequence being identical in all species thus far examined. Using this antiseraum, we have measured plasma ACTH in horses, sheep, dogs, cats, rats, and mice.

One problem did arise during the development of this RIA: unextracted plasma expected to contain low concentrations of ACTH (e.g., from patients with Cushing's syndrome due to adrenal tumor, patients with panhypopituitarism, and normal subjects administered dexamethasone) frequently had decreased bound label and were, therefore, interpreted as containing significant concentrations of IR-ACTH. We found that this was an artifact caused by nonspecific inhibition of antigen–antibody interaction by high-Mr components in plasma, presumably proteins; to correct this, we diluted standard hormone and plasma specimens with RIA diluent containing 35 g of BSA per liter, from which endogenous ACTH (and or) other unidentified contaminants had been removed by adsorption to silicic acid. When we used this diluent, most unextracted plasma specimens generated parallel competitive-binding curves, and their plasma IR-ACTH concentrations correlated well with those determined by more laborious RIA of silicic acid extracts of the same plasma specimens.

As determined with this method, plasma concentrations of IR-ACTH in a variety of conditions have been similar to those previously reported (1–5), and thus far our data have accurately reflected the status of the subjects' pituitary–adrenal axis. Normal subjects receiving dexamethasone had undetectable plasma IR-ACTH (<5 pg/mL); plasma IR-ACTH in normal subjects tended to be less at midnight than earlier in the day; midnight concentrations of plasma IR-ACTH in eight patients with Cushing's disease exceeded those of eight normal subjects studied at the same hour; in patients with Cushing's syndrome due to autonomous cortisol production by an adrenal tumor, plasma IR-ACTH was invariably low and was undetectable in five of six cases; four patients with Cushing's syndrome due to ectopic production of ACTH had markedly increased concentrations of plasma IR-ACTH, as did four patients with untreated Addison's disease and two patients with Nelson's syndrome; and although low concentrations of plasma IR-ACTH were detected in two patients with hypopituitarism, the values did not increase during insulin-induced hypoglycemia, as they did in normal subjects.

Very occasional subjects' unextracted plasma still reproducibly generated nonparallel binding curves. This did not appear to be caused by structural alterations in their ACTH, because extracts of plasma did not cause this phenomenon and could be used to quantify more correctly their concentrations of IR-ACTH.

With this RIA method, plasma ACTH concentrations can
now be rapidly and specifically measured throughout the range of values for normal and abnormal pituitary-adrenal function in humans.

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CLINICAL CHEMISTRY, Vol. 30, No. 2, 1984 265