ADRENOCORTICOTROPIC HORMONE IN HUMAN PLASMA *

By VERNON K. VANCE,† WILLIAM J. REDDY, DON H. NELSON‡ AND
GEORGE W. THORN

(From the Department of Medicine, Harvard Medical School, and the Peter Bent Brigham Hospital, Boston, Mass.)

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Utilizing the bioassay of Sayers, Sayers and Woodbury (1), based upon adrenal ascorbic acid depletion in the hypophysectomized rat, Paris (2), Taylor (3), and Sydnor (4, 5) and their co-workers, were unable to find detectable levels of ACTH in normal subjects. Gaarenstroom and his colleagues (6) observed detectable quantities in 7 of 33 normal subjects. Others (7-12) have reported very high concentrations. Sayers (13) discounted these latter reports as being inconsistent with the observation (14) that ACTH causes a maximal adrenal response in doses calculated to give much lower plasma concentrations. He concluded that the normal concentration is less than 0.5 mU per 100 ml of blood. Fujita (15) concluded from his studies that the normal concentration of ACTH in whole blood was 1.0 mU per 100 ml.

The bioassay, using the secretion of adrenal steroids in the hypophysectomized dog after administration of a test substance (16), provided a more direct method of measuring ACTH, but this method was not sensitive enough to allow measurement of ACTH in the plasma of normal humans. The development of a method for the measurement of corticosterone¹ in small quantities of rat plasma (17, 18) permitted the application of the principle of measurement of adrenal venous corticosteroids to the hypophysectomized rat (19). The rat bioassay for ACTH proved to be sensi-

tive to 0.01 mU per 100 g, or 0.02 mU per 200 g rat.

The present study applies the rat bioassay to the measurement of ACTH in the plasma of normal subjects, patients with pituitary or adrenal disorders or both, and subjects or patients given substances which presumably stimulate the pituitary-adrenal axis.

MATERIALS AND METHODS

Subjects and patients. Thirteen normal adult laboratory workers, 7 patients with Cushing's syndrome due to adrenal hyperplasia, 4 with ACTH-producing pituitary tumors following adrenalectomy for adrenal hyperplasia, 14 with hypoadrenalcorticism, and 3 with panhypopituitarism were studied. All patients and subjects were studied on the Metabolic Ward of the Peter Bent Brigham Hospital with the following exceptions: plasma from Patient M.R. was kindly supplied by Drs. Frederick Goetz and Walter Moran; plasma from Patients L.C. and R.A. was kindly supplied by Drs. Arnold Relman and James Hudson; plasma from Patient R.F. was kindly supplied by Dr. William Daughaday.

Preparation of plasma for assay. Blood for baseline studies was drawn between 8 and 9 a.m. into a syringe previously moistened with heparin, immediately placed in plastic tubes and centrifuged at high speed in a cold room (0 to 4° C) for 10 minutes. The plasma was separated into a test tube which was sealed and kept frozen until the assay for ACTH. All tubes and pipets were rinsed with 0.01 N HCl in 0.9 per cent saline solution prior to their use. Some of the normal specimens were prepared by lyophilizing 10-ml aliquots of plasma and diluting the dried portion to 5 ml with 0.01 N HCl.

Assay of ACTH. Assays were performed by a slight modification of the method of Lipscomb and Nelson (19). Female Sprague-Dawley rats weighing 200 to 225 g were anesthetized with ether. Anesthesia was maintained by cannulating the trachea with one end of a polyethylene catheter and placing the other end in a test tube containing a sponge saturated with ether. Hypophysectomy was performed via the transpharyngeal approach under direct observation with a dissecting microscope. Two hours later the animal was reanesthetized and a cut-down was performed on the right femoral vein. The inferior epigastric vein was dissected free, clamped, and divided dis-

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† This work was done as an Advanced Research Fellow of the American Heart Association.

‡ Investigator, Howard Hughes Medical Institute. Present address: Department of Medicine, University of Southern California, School of Medicine, Los Angeles, Calif.

¹ Corticosterone: 11 β ,21-dihydroxy-4-pregnene-3,20-dione.

tal to the clamp. Applying light traction to the clamp anchored the femoral vein, facilitating venipuncture. The test solution was injected into the femoral vein within 30 seconds. Three minutes after injection the peritoneal cavity was entered through a wide transverse incision. The stomach, spleen and intestines were packed to the right and the liver suspended with a saline-moistened sponge, allowing good visualization of the left renal and adrenal veins and the left adrenal gland. The renal vein was grasped with forceps, and exactly 5 minutes after injection of the test solution the left adrenal vein was cannulated (20) by advancing a 21- to 23-gauge needle through the renal vein into the adrenal vein until it was lodged in the vein. Adrenal venous blood was then drawn for 4 minutes into a tuberculin syringe which previously had been moistened with heparin and attached to the needle. No attempt was made to ligate the non-adrenal tributaries to the adrenal vein, although some of them were bypassed by advancing the needle as far as possible into the adrenal vein. The blood drawn was thus a mixture of adrenal and systemic venous blood (21). The blood was placed in a hematocrit tube, centrifuged, and the plasma was separated. Total plasma removed in 4 minutes was computed from the readings on the hematocrit tube of the total and cellular components of the blood and from the known total volume of the previously calibrated hematocrit tube; 0.2-ml aliquots of plasma were analyzed for corticosterone concentration by estimation of H_2SO_4 -induced fluorescence (17, 18) and minute output of corticosterone was computed.

A typical dose-response curve, formed by plotting on semilogarithmic paper the ACTH dose against minute output of corticosterone in the adrenal vein, is shown in Figure 1. An increased response with increased ACTH

dosage is apparent; however, the plot is not that of a first-order reaction, and with increasing dosage the standard errors of the mean response increase (heteroscedasticity). When the square-root transformation of the response is plotted against the logarithm of the ACTH dosage, a straight-line relationship is achieved between the doses of 0.04 and 0.64 mU, and the standard deviation of the mean response remains constant over the same dosage range (Figure 2). The response to 0.02 mU of ACTH is significantly greater than the response to saline but does not fit the straight-line plot.

Analysis of 58 separate standard assays over a 1-year period revealed homogeneity of the entire series—there was no statistical difference in response to the various ACTH doses nor did the slopes of the dose-response lines differ. When all points were combined in a single plot, the standard deviation of the mean was 3.9, the slope 19.7, and the index of precision (λ) 0.188.

In Figure 3 the responses to ACTH standards and to human plasma are compared. The responses to plasma from 9 different patients with adrenal insufficiency or pituitary tumors, or both, are plotted. In each instance at least three doses of plasma were injected (0.25 to 4 ml), each successive dose being double the preceding one. Plasma from two of these patients was examined on two occasions, and the plasma from one of these was assayed in quadruplicate (three different doses) on one occasion. The points, therefore, represent 13 different 3-point and one 4-point assay of human plasma. It is obvious that the dose-response curve to plasma parallels that to ACTH standards. Figure 4 demonstrates the responses to 0.5, 1.0, and 2.0 ml of plasma from one patient with adrenal insufficiency, performed in quadruplicate. Lines are drawn between successive doses. Again, parallelism is

FIG. 1. TYPICAL RESPONSE CURVE. ACTH plotted logarithmically against minute output of corticosterone (millimicrograms per minute) in adrenal venous blood. Note increasing slope and standard error of the mean with increasing ACTH dosage.

FIG. 2. DOSE-RESPONSE CURVE FORMED BY PLOTTING THE ACTH DOSE LOGARITHMICALLY AGAINST THE SQUARE ROOT OF THE MINUTE OUTPUT OF CORTICOSTERONE. Note the straight-line relationship and constancy of the standard deviations of the mean.

obvious. Therefore it seems reasonable that the response produced by plasma is due to its ACTH content and that the response can be expressed as concentration of ACTH.

Assays of ACTH were designed as follows. Initially a "screening" dose of plasma, "guessed" at from a knowledge of the patients or subject's clinical status, was tested; from the response obtained, the amount of plasma con-

taining approximately 0.08 mU was estimated and this amount was assayed—at least in duplicate. Two standard doses (0.04 and 0.16 mU), each in duplicate or triplicate, were administered for comparison, and potency of the unknown plasma specimens was estimated from the standard response.

Experiments in this laboratory indicated a recovery of

FIG. 3. DOSE-RESPONSE RELATIONSHIPS OF PLASMA FROM NINE PATIENTS WITH ADRENAL INSUFFICIENCY OR PITUITARY TUMOR, OR BOTH, AS COMPARED WITH ACTH STANDARDS.

FIG. 4. DOSE-RESPONSE RELATIONSHIP OF PLASMA FROM A PATIENT WITH ADDISON'S DISEASE, AS COMPARED WITH ACTH STANDARDS. Three-dose assay performed in quadruplicate.

TABLE I

Comparison of response of adrenal venous corticosteroid to saline, lyophilized outdated plasma, and lyophilized normal human plasma

Test solution	Corticosterone <i>μg/min</i>
Saline controls Mean $\pm$ SE	8, 14, 13, 9, 20, 14 13 $\pm$ 1.92
Lyophilized outdated plasma Mean $\pm$ SE <i>t</i> , 0.35; <i>p</i> > 0.05	17, 14, 16, 20, 8, 9 14 $\pm$ 2.09
Lyophilized normal human plasma Mean $\pm$ SE <i>t</i> , 4.43; <i>p</i> > 0.005	24, 66, 49, 64, 38, 30 45 $\pm$ 6.96

only about 30 per cent, by either oxycellulose absorption or acid-acetone extraction, of ACTH added to outdated plasma. Furthermore, it was found that rats can tolerate (for the 9 minutes necessary to complete the assay) 5 ml of plasma or 10 ml of plasma lyophilized to dryness and diluted to 5 ml with 0.01 N HCl. Plasma or lyophilized reconstituted plasma was used throughout all studies reported here.

Table I shows the response to 10-ml aliquots of fresh, normal human plasma as compared with the response to 10-ml aliquots of outdated blood-bank plasma and 5 ml of 0.9 per cent sodium chloride solution. The differences in response are highly significant (*p* < 0.005).

RESULTS

Concentration of ACTH plasma of normal subjects. ACTH was detected in 5 ml of plasma from 8 of 12 normal subjects and in 10 ml of plasma (lyophilized and diluted to 5 ml with 0.01 N HCl) from 8 subjects (Table II). Although lyophilization resulted in an apparent loss of ACTH (the

response to 10 ml being equal to that of 5 ml of fresh plasma in G.C., E.B. and B.G.), concentration by this means permitted detection of ACTH in plasma of subjects in whose unconcentrated plasma no ACTH was demonstrated. The concentration in normal subjects ranged from 0.4 to 1.0 mU per 100 ml plasma.

Concentration of ACTH in plasma of patients with Cushing's syndrome due to adrenal hyperplasia. With one exception ACTH levels in patients with Cushing's syndrome were within the range found in normal subjects (Table III).

The one patient with elevated levels, a 35 year old white female, had developed signs of Cushing's syndrome 8 years earlier and had undergone bi-

TABLE II
ACTH concentration in normal human plasma *

Subject	ACTH	
	5 ml	10 ml
	<i>mU/100 ml</i>	
G.C. ♂	1.0	0.4
V.V. ♂	0.8	0.6
R.W. ♂	1.0	
E.B. ♂	0.8	0.6
B.G. ♀	0.8	0.4
A.K. ♀	0.8	
F.P. ♂	ND	0.4
J.L. ♂	ND	0.8
D.S. ♂		0.6
W.R. ♂	ND	0.4
L.O. ♂	1.0	
E.P. ♀	0.8	
J.S. ♂	0.8	

* Range, not detectable (ND) to 1.0; 8 a.m. to 9 a.m.

TABLE III
ACTH levels in Cushing's syndrome

Patient	ACTH
	<i>mU/100 ml plasma</i>
F.C. ♂	0.6
G.T. ♀	0.8
M.R. ♂	0.8
G.A.* ♀	2.2-4.0
F.G. ♀	0.8
F.S. ♀	0.8
M.F. ♀	0.8

* Recurrent Cushing's syndrome following subtotal adrenalectomy.

lateral adrenalectomy three years previously. The left adrenal gland was ruptured during removal. Postoperative steroid excretion was low and she was given cortisone ² maintenance therapy. However, there was no remission of the signs of Cushing's syndrome, but rather a progression; on admission she presented the classical clinical and laboratory signs of the disease. Skull X-rays were suggestive of sellar enlargement, and laminograms of the left suprarenal area suggested a suprarenal mass. This area was explored and 6 g of histologically hyperplastic adrenal tissue was removed. Postoperative steroid excretion was consistent with total adrenalectomy, and substitution therapy was prescribed. There was marked amelioration of the signs of Cushing's syndrome. Unfortunately the patient died of a coronary occlusion at home 3 months postoperatively. No postmortem examination was performed. Because of the elevated plasma ACTH and suggestive skull X-rays, the presence of a pituitary tumor was suspected.

Concentration of ACTH in plasma of patients with ACTH-producing tumors of the pituitary gland. Plasma levels ranged from 12 to 330 mU

TABLE IV
Plasma ACTH in patients with pituitary tumors

Patient	ACTH			
	Initial	After suppression with cortisol	X-ray, R _x	Hypophysectomy
	<i>mU/100 ml</i>		<i>mU/100 ml</i>	
N.C.	12	1	1	
L.C.	330	6	12	
E.C.	32	32		30
R.F.	26			

² Cortisone: 17 α ,21-dihydroxy-4-pregnene-3,11,20-trione.

per 100 ml among the four patients studied (Table IV). Patient L.C., with the highest initial concentration, had marked suppression of plasma ACTH after the intravenous infusion of 20 mg cortisol ³ over a 4-hour period and a marked reduction also followed pituitary irradiation. Patient N.C., with recurrent Cushing's syndrome following subtotal adrenalectomy, was deeply pigmented and plasma ACTH was found to be elevated. Routine skull films were normal, but subsequent laminograms revealed asymmetrical enlargement of the sella turcica. Intravenous cortisol caused a reduction in plasma ACTH. After pituitary irradiation there was amelioration of the

TABLE V
Plasma ACTH in patients with hypoadrenalcorticism

Patient	ACTH 24 hrs after steroid
	<i>mU/100 ml</i>
Addison's disease	
M.McG. ♀	19
M.L. ♀	2
	55 (48 hrs)
H.H.W. ♂	3
L.W. ♂	2
E.D. ♂	4
M.C. ♀	14
	20 (48 hrs)
M.M. ♀	36
F.L. ♀	2
Adrenalectomy	
D.L. ♀	2
L.D. ♀	3
B.A. ♀	4
A.K. ♀	12
A.C. ♀	4
R.A. ♂	3

clinical and laboratory signs of Cushing's syndrome, a loss of pigmentation, and a marked reduction of plasma ACTH. Patient E.C. had no decrease in plasma ACTH after intravenous cortisol; hypophysectomy was attempted but only a portion of the tumor could be removed. There was no decrease in plasma ACTH postoperatively. Tissue culture of the tumor was done ⁴ and ACTH was found in aliquots from three consecutive changes of culture medium. Histologically, the tumor was a chromophobe adenoma.

³ Cortisol: 11 β ,17 α ,21-trihydroxy-4-pregnene-3,20-dione.

⁴ By Dr. Daniel Pugh, Laboratory for Surgical Research, Peter Bent Brigham Hospital and Harvard Medical School, Boston, Mass.

Concentration of ACTH in plasma of patients with hypoadrenalcorticism. Concentration ranged from 2 to 36 mU per 100 ml (Table V) 24 hours after the last dose of steroid and had increased in the two patients who were given no medication for 48 hours. All patients studied had detectable levels and all had levels higher than those found in normal subjects. Five mg cortisol (Figure 5) administered intravenously over a 2-hour period to one patient with Addison's disease caused a marked reduction in plasma ACTH. The level rose to the baseline 2 hours after completion of the infusion. In contrast, 50 mg intravenous deoxycortisol,⁵ caused no decrease in ACTH concentration (Figure 6).

FIG. 5. EFFECT OF CORTISOL (HYDROCORTISONE) ON PLASMA ACTH IN ADDISON'S DISEASE.

Concentrations of ACTH in plasma of patients with panhypopituitarism. No ACTH was detected in plasma from three patients with hypopituitarism (Table VI).

Effect of methopyrapone⁶ (SU-4885 ditartrate) on the concentration of ACTH in plasma. After collection of a control specimen, four normal subjects were given methopyrapone⁷ intravenously in doses ranging from 10 to 40 mg per kg body weight over periods of 2 to 4 hours. One subject was given methopyrapone on three separate occasions. Blood specimens were drawn at dif-

⁵ Deoxycortisol: 17 α ,21-dihydroxy-4-pregnene-3,20-dione (Compound S).

⁶ 2-Methyl-1,2-bis-(3-pyridil)-1-propanone.

⁷ Kindly supplied by Dr. C. H. Sullivan of CIBA Pharmaceutical Products, Inc.

FIG. 6. EFFECT OF DEOXYCORTISOL (COMPOUND S) ON PLASMA ACTH IN ADDISON'S DISEASE.

ferent time intervals after completion of the infusion (Table VII). There was no demonstrable effect on plasma ACTH concentration in any of the normal subjects.

One patient with Cushing's syndrome was given methopyrapone, 20 mg per kg body weight over a 4-hour period, and blood specimens were drawn 0, 2, 3, and 4 hours after completion of the infusion. Again, no change in ACTH concentration in the plasma was demonstrated. Two other patients with Cushing's syndrome were given methopyrapone orally, 750 mg every 6 hours for eight doses, and specimens were obtained after the last dose. No change in plasma levels of ACTH was demonstrated.

Two patients with hypoadrenalcorticism were given 10 mg per kg body weight of methopyrapone, and blood specimens were drawn before and at the end of the infusion. There was a decrease of ACTH concentration in the plasma of one patient but no change in the other; 400 μ g methopyrapone was added to control plasma from these subjects. The bioassay was carried out and no difference

TABLE VI
ACTH levels in patients with hypopituitarism

Patient	Etiology	ACTH mU/100 ml
T.C. ♂	Unknown	ND
J.H. ♀	Hand-Schüller-Christian disease	ND
G.E. ♂	Chromophobe adenoma of pituitary	ND

TABLE VII
Effect of methopyrapone on plasma ACTH

Subject	Diagnosis	Methopyrapone		Control	Plasma ACTH				
		Dose	Time		Hours after end of infusion				
					0	$\frac{1}{2}$	1	2	4
		mg/kg	hrs		mU/100 ml				
B.G.	Normal	10	2	1.0	ND	ND	ND	ND	
A.K.	Normal	10	2	1.0	ND	ND			
B.G.	Normal	20	2	1.0	ND	ND	ND	ND	
B.G.	Normal	20	4	1.0	0.8		ND	ND	1.0
J.S.	Normal	30	4	1.0	0.8			1.0	1.0
E.P.	Normal	40	4	0.8	0.8			0.8	1.6
M.L.	Addison's	10	2	40.0		24	26	28	
M.C.	Addison's	10	2	14.0	13	13	13	14	
F.S.	Cushing's	20	4	0.8	1.0			1.0	ND

was found between the samples with and without added methopyrapone.

*Effect of purified lipopolysaccharide pyrogen*⁸ on concentration of ACTH in plasma. Lipexal, in doses of 0.1 μ g (0.2 ml) and 0.25 μ g (0.5 ml) was administered intravenously to one normal subject on two different occasions. Elevations of temperature to 101.8° and 102.4° F, respectively, occurred 90 and 120 minutes after injection on both occasions and persisted for 5 to 6 hours. There was no demonstrable increase in plasma ACTH concentration on either occasion (Table VIII).

DISCUSSION

ACTH was found in the plasma of 13 normal subjects in concentrations ranging from 0.4 to 1.0 mU per 100 ml plasma (Table II). These values are of the same magnitude as those predicted by Sayers (13) and found by Fujita (15).

In patients with Cushing's syndrome, concentrations were within the normal range with one

exception. As mentioned, a pituitary tumor was suspected but not proven in this patient. The finding of elevated levels in a patient with Cushing's syndrome should suggest the presence of a pituitary tumor and, if the tumor cannot be demonstrated radiologically, the possibility of an occult tumor must be considered and follow-up X-rays taken periodically.

The patients with proven pituitary tumors following adrenalectomy for Cushing's syndrome presented here have not been reported previously. As was expected, all were found to have elevated levels of ACTH in the plasma. In contrast to the group of patients previously reported by Nelson and co-workers, (22-24), only one of the present group had a level of ACTH higher than those found in some patients with Addison's disease. There appeared to be a correlation between the ability to cause a decrease in plasma ACTH with cortisol and the response to irradiation or hypophysectomy, or both, in the three patients in whom the suppression test was performed. Although one cannot generalize from this small series, the suppression test may have prognostic value.

The concentrations found in patients with hypoadrenalism are of the same order as those found by Sydnor, Sayers, Brown and Tyler (4) and by Bethune, Nelson and Thorn (25). However, in the current study, all of the patients had detectable levels and all of the levels were higher than those found in normal subjects. The difference can be explained by the much greater sensitivity of the present bioassay. The finding of elevated levels in patients suspected of having Addison's disease appears to be diagnostic of that condition.

TABLE VIII
Effect of Lipexal on plasma ACTH in a normal subject

Time after injection	ACTH	
	Lipexal, 1 μ g	Lipexal, 2.5 μ g
	mU/100 ml	
0	1.0	0.8
5 min	0.8	0.8
15 min	0.8	0.8
60 min	ND	0.8
90 min	0.8	ND
2 hrs	1.4	1.0
3 hrs	1.0	1.0
5 hrs		1.0

⁸ Kindly supplied as Lipexal by Dr. Fred H. Schultz, Jr., of the Wander Co.

The three highest plasma ACTH values in patients with hypoadrenalcorticism were in those with Addison's disease. However, there was no apparent difference between the levels in patients with Addison's disease and those who had been adrenalectomized. Williams, Island, Oldfield and Liddle (26) have reported that patients who have previously been adrenalectomized for Cushing's syndrome have definitely higher plasma ACTH concentrations after being given sufficient oral cortisol to maintain normal cortisol levels than have patients with Addison's disease. These findings are surprising in view of the present observation that patients with Cushing's syndrome do not have elevated levels, and that in certain cases the secretion of ACTH by pituitary tumors is easily suppressed by the intravenous infusion of cortisol.

Increases in plasma 17-hydroxycorticosteroids or urinary 17-hydroxycorticosteroids or both, or of 17-ketosteroids or 17-ketogenic steroids after administration of methopyrapone have been reported by Liddle (27) and Jenkins (28) and their colleagues, and by Gold, Di Raimondo and Forsham (29). These authors have postulated an increased ACTH secretion secondary to a decreased cortisol secretion as the mechanism of this response. Increases in plasma or urinary steroids after the infusion of bacterial pyrogens, presumably due to increased secretion of ACTH by the pituitary, also have been demonstrated (30-32).

In this study neither methopyrapone nor Lipexal caused a clear-cut increase in plasma ACTH concentration. By the present technique a rise of plasma ACTH can be demonstrated readily after discontinuing intravenously administered cortisol (ascending line of curve, Figure 5), or discontinuing maintenance therapy in patients with Addison's disease. The failure to demonstrate an increase of plasma ACTH after methopyrapone suggests that this compound does not act simply by suppression of cortisol production. The lack of ACTH response cannot be attributed to the presence of methopyrapone itself in the plasma samples, since the bioassay of ACTH was not affected by the addition of methopyrapone to plasma *in vitro*. Since deoxycortisol did not lower the ACTH level when 50 mg was infused over a 4-hour period in a patient with Addison's disease, it does not seem likely that the increase in deoxy-

cortisol secretion associated with methopyrapone administration would inhibit a measurable rise in ACTH. In contrast to the reported observation that plasma ACTH increases in the adrenalectomized dog after infusion of methopyrapone (33), no increase was noted in two patients with hypoadrenalcorticism.

In the above situations it is possible that a slight increase in ACTH did occur which the bioassay was not capable of detecting. It is also possible that there was an increase in ACTH production that was not reflected in an increase in the plasma level because of a corresponding increase in the rate of removal. In view of the data presented it appears most likely that the increase in steroid secretion after methopyrapone or bacterial pyrogens is due to a direct effect of these substances on the adrenal cortex. However, the presence of ACTH appears to be necessary in order to demonstrate an effect of methopyrapone—as indicated by a negative response in patients with hypopituitarism and in patients or subjects receiving supplementary fluorocortisol.⁹

SUMMARY

Adrenocorticotrophic hormone was assayed by the measurement of corticosterone in the adrenal venous plasma of the hypophysectomized rat after the intravenous administration of plasma. The assay is more sensitive than are previous techniques, enabling the detection of 0.04 mU of ACTH. Plasma from normal subjects was found to contain ACTH in levels ranging from 0.4 to 1.0 mU per 100 ml. Patients with Cushing's syndrome due to adrenal cortical hyperplasia did not have elevated levels. All the patients with hypoadrenalcorticism have elevated levels, and no ACTH was detected in patients with panhypopituitarism. No elevations of plasma ACTH were noted after administration of methopyrapone (SU-4885) or a bacterial pyrogen.

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⁹ Fluorocortisol: 9 α ,fluoro-11 β ,17 α -21-trihydroxy-4-pregnene-3,20-dione.

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