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### Research Article

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# Stimulation of Gonadal Steroid Synthesis by Chronic Excess of Adrenocorticotropin in Patients with Adrenocortical Insufficiency

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## Abstract

Analysis of 24-h urinary steroid excretion was performed by capillary gas chromatography in six patients (five men, one woman) with adrenocortical insufficiency. Ten healthy subjects (five men, five women) served as controls. A complete absence of all 21-hydroxylated steroid metabolites was seen in patients with adrenocortical insufficiency, whereas the excretion of several steroids lacking hydroxylation in the 21-position (pregnenolone, pregnenetriol, and 11-ketoandrosterone) was markedly increased. In addition, the presence of 11 $\beta$ -hydroxyandrosterone was confirmed by mass-spectrometry in the urine of three patients. This pattern of steroid excretion was unchanged in patients with adrenocortical insufficiency, both after stimulation by 1-24 adrenocorticotropin (ACTH) and after short-term (3-d) suppression with dexamethasone. We conclude that patients with adrenocortical insufficiency present a pattern of steroid excretion characterized by the absence of 21-hydroxylated metabolites. In the absence of functional adrenocortical tissue, long-term pathologically elevated concentrations of ACTH apparently stimulate early steps of steroid synthesis, most likely in the gonads. In addition, the presence of 11-hydroxylated steroid metabolites (11-ketoandrosterone, 11 $\beta$ -hydroxyandrosterone) in the urine of patients with adrenocortical insufficiency demonstrates that chronic ACTH excess in this disorder may induce some activity of 11 $\beta$ -hydroxylase, an enzyme not found in the gonads under physiological conditions.

## Introduction

The simultaneous evaluation of major urinary steroid metabolites by capillary gas chromatography (1, 2) comprehensively characterizes steroid production in man. Since a differentiation of metabolites derived from adrenocortical or gonadal steroid synthesis is not possible from determinations performed under basal conditions, dynamic tests of adrenocortical function (i.e., stimulation by adrenocorticotropin [ACTH] or suppression by exogenous glucocorticoids) are used to characterize the contribution of the adrenal cortex to overall steroid secretion. The interpretation of the results thus obtained relies on the assumption that these maneuvers interfere with adrenal, but not with gonadal, steroid synthesis. Although it is undisputed that ACTH

predominantly influences the function of the adrenal cortex, an additional effect on gonadal steroid production has not yet been excluded. Patients with adrenocortical insufficiency provide an excellent model from which to study the effect of markedly increased endogenous concentrations of ACTH on gonadal steroid production in the absence of functioning adrenocortical tissue. In the present investigation we have compared steroid excretion rates in patients having adrenocortical insufficiency with those of healthy controls, both under basal conditions as well as after additional stimulation with exogenous ACTH 1-24 and after suppression with dexamethasone.

## Methods

**Healthy subjects.** The subjects were five healthy female volunteers, aged 19–27 yr, who had not taken oral contraceptives for at least 6 mo, and five healthy male volunteers, aged 21–28 yr. All subjects were within  $\pm 10\%$  of their ideal body weight (according to Metropolitan Life Insurance Tables). The aim and potential risks of the investigation were explained to each subject and written consent was obtained in each case. In healthy women, the study was begun between the fifth and the seventh day of the menstrual cycle. Following the collection of a 24-h urine for the determination of steroid excretion, an indwelling catheter was inserted into an antecubital vein and 0.25 mg synthetic ACTH (1-24 ACTH, Synacthen, 0.25 mg = 100 IU; CIBA-GEIGY Ltd. Basle, Switzerland) was administered at 8:00 a.m. as an intravenous bolus. This was followed by a continuous infusion (3 ml/h;  $t = 8$  h) containing 0.25 mg 1-24 ACTH in 24 ml of 0.9% NaCl solution. On days 3–6 of the experiment the subjects were treated with oral dexamethasone (0.5 mg four times a day; Schering A.G., Berlin, Federal Republic of Germany), but not with intravenous ACTH. A 24-h urine (8:00 a.m.–8:00 p.m.) was collected for the determination of urinary steroid excretion on day 1 (basal), day 2 (ACTH), and day 5 (dexamethasone) of the experiment. Blood samples for the determination of plasma concentrations of cortisol, 21-deoxycortisol and 11-ketoandrosterone, pregnenolone, and 17-hydroxy-pregnenolone were obtained before the infusion of ACTH. Plasma concentrations of cortisol were also determined after the administration of ACTH and of dexamethasone.

**Patients with adrenocortical insufficiency.** Six patients with adrenocortical insufficiency (five men, aged 26–48 yr, and one postmenopausal woman, aged 55 yr) consented to participate in the study.  $^{131}\text{I}$ -cholesterol scanning failed in all patients to reveal the presence of functional adrenocortical tissue. Adrenocortical insufficiency was of autoimmune origin in four patients and due to bilateral adrenalectomy for Cushing's disease in two cases. Pituitary surgery had not been performed on any of these patients. The patients had been on continuous glucocorticoid (37.5–50.0 mg cortisone acetate/d divided into two doses) and mineralocorticoid (0.05–0.1 mg 9 $\alpha$ -fluoro-hydrocortisone/d) replacement therapy for 2–17 yr before this investigation. Nevertheless, supranormal concentrations of ACTH ( $>100$ – $>1,000$  pg/ml; normal  $<60$  pg/ml) were found in each patient when estimated during routine controls on an outpatient basis. For the purpose of this study the patients were admitted to the hospital and taken off their individual substitution therapy. Consecutively, electrolyte equilibrium was monitored daily and maintained by intravenous administration of 0.9% saline. 84 h after the last dose of the respective substitution therapy the patients received an intravenous bolus and an

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infusion of 1-24 ACTH as described above for the healthy volunteers. Subsequently each patient was given 0.5 mg dexamethasone four times a day and 0.05 mg 9 $\alpha$ -fluoro-hydrocortisone/d by mouth for 3 d. A 24-h urine (08:00–08:00 h) for the determination of steroid excretion was collected under basal conditions (i.e., from 60 to 84 h after withdrawal of substitution therapy) during ACTH stimulation and on the third day of reinstated glucocorticoid (dexamethasone) therapy. Blood samples for the determination of plasma concentrations of cortisol were drawn at the beginning of each urine-collecting period and at the end of the ACTH infusion. Concentrations of ACTH, of 21-deoxycortisol, of 11-ketoandrostenedione, of pregnenolone, and of 17-hydroxypregnenolone were obtained before the infusion of ACTH. Concentrations of ACTH were also estimated after the administration of dexamethasone.

**Reference steroids.** Reference steroids were purchased from Steraloids Inc., Wilton, NH; and from Makor Chemicals, Jerusalem, Israel. Purity was checked by controlling melting point in each case.

**Radioimmunoassays.** For the simultaneous radioimmunologic determination of cortisol, 21-desoxycortisol, and 11-ketoandrostenedione, 2.0 ml of plasma were diluted with 8.0 ml H<sub>2</sub>O. To determine recoveries 20,000 dpm of [<sup>3</sup>H]cortisol (NET 396, specific activity 115 Ci/mmol; New England Nuclear, Boston, MA), 1,000 dpm of 21-[<sup>3</sup>H]desoxycortisol (specific activity 43 Ci/mmol; Amersham International, Amersham, Buckinghamshire, England), and 10,000 dpm of [<sup>3</sup>H]DOC (specific activity 42 Ci/mmol; Amersham International) were added to each sample. [<sup>3</sup>H]DOC was used to estimate the recovery of 11-ketoandrostenedione, since the latter is not available in a labeled form and shows a chromatographic behavior in the chromatographic system we employed which is identical to that of DOC. Subsequently the samples were extracted with 150 ml of cold CH<sub>2</sub>Cl<sub>2</sub> and the organic extract was washed once with 10 ml of cold 0.1 N NaOH and twice with 10 ml of cold H<sub>2</sub>O. Separation of the steroids was achieved by thin-layer chromatography (benzene/acetone = 75:25). For the simultaneous determination of pregnenolone and 17-hydroxypregnenolone, 2 ml of plasma were diluted with 8 ml of H<sub>2</sub>O. To determine recoveries 10,000 dpm of [<sup>3</sup>H]pregnenolone (specific activity 12 Ci/mmol; Amersham Corp.) and 17-[<sup>3</sup>H]hydroxypregnenolone (specific activity 19 Ci/mmol; Amersham Corp.) were added to each sample. Subsequently the samples were extracted with 150 ml of cold CH<sub>2</sub>Cl<sub>2</sub> and the organic extract was washed with 10 ml of cold H<sub>2</sub>O. Separation of steroids was achieved by two-dimensional thin-layer chromatography (I: benzene/acetone, 75:25; II: ethylacetate/cyclohexane, 55:45). Radioimmunoassays were performed in triplicate using antisera against cortisol and 21-desoxycortisol obtained from Dr. Vecsei, Division of Pharmacology, University of Heidelberg, Heidelberg Federal Republic of Germany (3). Antisera for the determination of pregnenolone and 17-hydroxypregnenolone were obtained from Steranti Research Ltd., St. Albans, England; and from Radioassay Systems Laboratories, Inc., Carson, CA, respectively. Since no specific antiserum was available for the determination of 11-ketoandrostenedione, this steroid was determined using the cross-reactivity (12.5%) of an antibody raised against androstenedione (Nr. XXA001; Steranti Research Ltd.). Cross-reactivity of this antibody against DOC is <0.1%. Furthermore, the behavior of androstenedione and 11-ketoandrostenedione permits complete separation in the used chromatographic system. Urinary concentrations of cortisol and plasma concentrations of ACTH were determined as previously reported (4).

**Urine processing.** Urine samples (5 ml) of 24-h collections were acidified to pH 5.2 by addition of 0.2 N HCl.  $\beta$ -glucuronidase/arylsulfatase (20,000 Fisher units, Calbiochem-Behring Corp., La Jolla, CA) was added, the hydrolysis taking place at 37°C. Hydrolysis did not affect pH values. After a total hydrolysis period of 24 h, 50,000 dpm [<sup>3</sup>H]cortisol were added to estimate recovery. The unconjugated steroids were extracted with three times 20 ml of ethylacetate and the combined extract was washed once with 20 ml of 0.1 N NaOH and at least twice with 20 ml of double distilled water until neutrality was reached. Anhydrous sodium sulfate (5–10 g) was then added to each sample. After 12 h the solvent was distilled off at 35°C at reduced pressure. The residue was transferred with 3  $\times$  1 ml ethylacetate into a centrifuging tube and the solvent was evaporated in a stream of dry nitrogen at 35°C.

**Derivatization** (5, 6). Samples were dissolved in 100  $\mu$ l of a solution

of methoxyamine hydrochloride (2% in pyridine; Pierce Chemical Co., Rockford, IL) and heated at 80°C for 60 min. After evaporation with dry N<sub>2</sub> at 30°C, 100  $\mu$ l of a mixture of trimethylsilylimidazole, *N,O*-bis(trimethylsilyl)acetamide, and trimethylchlorosilane (3:3:2, TRI-SIL TBT; Pierce Chemical Co.) was added and the mixture was heated to 60°C for 20 h. After evaporation to dryness by dry N<sub>2</sub> (25°C, overnight), 2 ml of dichloromethane and 1.5 ml of 0.1 N H<sub>2</sub>SO<sub>4</sub> were added to each sample, the mixture was shaken, and the aqueous (lighter) phase was pipetted off. The organic layer was washed once with 1 ml of double distilled water. If the pH of the washing was >7, the organic layer was diluted with 1 ml 0.1 N H<sub>2</sub>SO<sub>4</sub>. The organic layer was then diluted with 1 ml CH<sub>2</sub>Cl<sub>2</sub> and washed with H<sub>2</sub>O until the washings were neutral. Sodium sulfate was added to the organic solutions and the samples were shaken for 20 min and centrifuged. The clear solutions were transferred into reactivials; the solvent was evaporated with dry N<sub>2</sub> at room temperature and the residue was dissolved in 100  $\mu$ l of a mixture of ethylacetate/*n*-hexane (9:1) containing 100 ng/ $\mu$ l of alkanes *n*-C<sub>24</sub>H<sub>50</sub> and *n*-C<sub>32</sub>H<sub>66</sub>.

**Recovery.** 20  $\mu$ l of each sample were measured in a  $\beta$ -counter (Packard Instruments Co., Delft, Netherlands). Mean recovery ( $n = 75$ ) was 55.7  $\pm$  5.5%.

**Gas chromatography.** Gas chromatography was carried out using a Packard Instrument Co. 428 gaschromatograph equipped with a 50 m OV-101 wall-coated open tubular column (0.25 mm i.d., film thickness 0.25  $\mu$ m, pretested at 180,000 effective plates), a solid injection device, and a flame ionization detector. N<sub>2</sub> was used as a carrier gas with a flow rate of  $\sim$ 0.8 ml/min. After sample injection (300°C), the temperature of the oven was immediately programmed between 180°C and 265°C at 0.4°C/min followed by an isothermal run for 15 min at end temperature.

**Steroid identification.** Steroids were identified by comparison of retention times expressed as methylene units as determined by interpolation between those of the alkanes *n*-C<sub>24</sub>H<sub>50</sub> and *n*-C<sub>32</sub>H<sub>66</sub>; (Table I). Trivial names of steroid metabolites determined by gas chromatography and of the other steroids mentioned in this report are given in Table I.

**Mass spectrometry.** Positive identification of 11-hydroxyandrostosterone was attempted by gas chromatography-mass spectrometry using a 5995 gas chromatography-mass spectrometry system equipped with a 25-m fused silica capillary column coated with OV 101 stationary phase (Hewlett-Packard Co., Palo Alto, CA). The GC injector was maintained at 300°C and the oven was programmed from 180°C to 260°C at a rate of 1°C/min. Temperatures of the analyzer, transfer line, and ion source were kept at 180°C, 280°C, and 150°C, respectively, and the electron energy was 70 eV. To increase sensitivity it was necessary to determine 11-hydroxyandrostosterone by selected ion monitoring, the instrument having been set to monitor the ions at *m/e* 479 (M<sup>+</sup>), *m/e* 449 (M<sup>+</sup> - 30), *m/e* 448 (M<sup>+</sup> - 31), and *m/e* 358 (M<sup>+</sup> - 121).

**Calculation of steroid excretion.** An automatic integrator (3358 A; Hewlett-Packard Co.) was used for the calculation of the area under the respective peaks. Response factors (*f*) for the two *n*-alkanes (*f* C24 and *f* C32) and for each reference steroid standard (*f* St) were determined by their injection in varying amounts. These response factors were checked and adjusted regularly (i.e., after every 15th injection of biological samples) by derivatizing and injecting defined mixtures of the reference compounds. Response factors for the two *n*-alkanes were practically identical (*f* C24, 1.05  $\pm$  0.18; *f* C32, 1.07  $\pm$  0.20) over the complete series of injections. It was unnecessary to introduce additional correction factors for the increasing elution temperatures. The excreted amounts of individual steroids were calculated by the following formula:

$$\text{mg/24 h} = \text{area} \times f \text{ C24} \times f \text{ St} \times 100/\text{recovery}(\%) \times \text{dilution},$$

where area equals measured peak area; *f* C24 equals response factor for hydrocarbon C24 (provides for the actually injected amount); *f* St equals response factor for individual steroid; recovery equals the yield of the complete treatment of the samples before gas chromatography by measuring recovery of [<sup>3</sup>H]cortisol; and dilution equals the aliquotization of original urinary samples.

**Sensitivity and precision.** The sensitivity of the procedure varied according to the gas chromatography conditions used. Applying standard

Table I. Trivial Names and Gas-chromatographic Characteristics of Evaluated Standard Steroid Compounds\*

No.	Steroid	Trivial name	Methylation units (as MO-TMS derivatives)
1		<i>n</i> -C <sub>24</sub> -Alkane	24.00
2	5 $\alpha$ -Androstan-3 $\alpha$ -ol-17-one	Androsterone	25.09
3	5 $\beta$ -Androstan-3 $\alpha$ -ol-17-one	Etiocholanolone	25.28
4	5-Androsten-3 $\beta$ -ol-17-one	Dehydroepiandrosterone	25.68
5	5 $\alpha$ -Androstan-3 $\beta$ -ol-17-one	Epiandrosterone	25.83
6	5-Androsten-3 $\beta$ ,17 $\beta$ -diol	Androstenediol	25.92
7	5 $\alpha$ -Androstan-3 $\alpha$ -ol-11,17-dione	11-Ketoandrosterone	26.01
8	1,3,5(10)-Estratrien-3-ol-17-one	Estrone	26.01
9	5 $\beta$ -Androstan-3 $\alpha$ -ol-11,17-dione	11-Ketoetiocholanolone	26.14
10	4-Androsten-3,17-dione	Androstenedione	26.19/26.24
11	1,3,5(10)-Estratrien-3,17 $\beta$ -diol	Estradiol	26.38
12	4-Androsten-17 $\beta$ -ol-3-one	Testosterone	26.47
13	5 $\beta$ -Pregnan-3 $\alpha$ -ol-20-one	Epipregnanolone	26.87
14	5 $\alpha$ -Androstan-3 $\alpha$ -11 $\beta$ -diol-17-one	11 $\beta$ -Hydroxyandrosterone	26.96
15	5 $\beta$ -Androstan-3 $\alpha$ -11 $\beta$ -diol-17-one	11 $\beta$ -Hydroxyetiocholanolone	27.13
16	5 $\beta$ -Pregnan-3 $\beta$ -ol-20-one	Pregnanolone	27.15
17	5 $\alpha$ -Pregnan-3 $\alpha$ ,20 $\alpha$ -diol	Allo-Pregnanediol	27.42
18	5 $\beta$ -Pregnan-3 $\alpha$ ,20 $\alpha$ -diol	Pregnanediol	27.62
19	5-Pregnen-3 $\beta$ -ol-20-one	Pregnenolone	27.67
20	5 $\beta$ -Pregnan-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol	Pregnanetriol	27.95
21	5-Androsten-3 $\beta$ -16 $\alpha$ ,17 $\alpha$ -triol	17 $\alpha$ -Androstenetriol	28.16
22	4-Pregnen-3,20-dione	Progesterone	28.20/28.25
23	5-Androsten-3 $\beta$ ,16 $\alpha$ ,17 $\beta$ -triol	17 $\beta$ -Androstenetriol	28.41
24	5 $\beta$ -Pregnan-3 $\alpha$ ,17 $\alpha$ ,21-triol-20-one	TH-"S"	28.67
25	5 $\beta$ -Pregnan-3 $\alpha$ ,21-diol-20-one	TH-"DOC"	28.76
26	1,3,5(10)-Estratrien-3,16 $\alpha$ ,17 $\beta$ -triol	Estriol	28.76
27	5 $\alpha$ -Pregnan-3 $\alpha$ ,21-diol-20-one	Allo-TH-"DOC"	28.91
28	5 $\alpha$ -Pregnan-3 $\alpha$ ,17 $\alpha$ ,21-triol-20-one	Allo-TH-"S"	29.02
29	5 $\beta$ -Pregnan-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol-11-one	Pregnanetriolone	29.02
30	5-Pregnen-3 $\beta$ ,17 $\alpha$ ,20 $\alpha$ -triol	Pregnenetriol	29.47
31	5 $\beta$ -Pregnan-3 $\alpha$ ,17 $\alpha$ ,21-triol-11,20-dione	TH-"E"	29.66
32	5 $\beta$ -Pregnan-3 $\alpha$ ,21-diol-11,20-dione	TH-"A"	29.75
33	5 $\beta$ -Pregnan-3 $\alpha$ ,11 $\beta$ ,21-triol-20-one	TH-"B"	30.00
34	5 $\alpha$ -Pregnan-3 $\alpha$ ,11 $\beta$ ,21-triol-20-one	Allo-TH-"B"	30.12
35	5 $\alpha$ -Pregnan-3 $\alpha$ ,17 $\alpha$ ,21-triol-11,20-dione	Allo-TH-"E"	30.25
36	5 $\beta$ -Pregnan-3 $\alpha$ ,11 $\beta$ ,17 $\alpha$ ,21-tetrol-20-one	TH-"F"	30.27
37	5 $\alpha$ -Pregnan-3 $\alpha$ ,11 $\beta$ ,17 $\alpha$ ,21-tetrol-20-one	Allo-TH-"F"	30.37
38	5 $\beta$ -Pregnan-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ ,21-tetrol-11-one	$\alpha$ -Cortolone	30.49
39	5 $\beta$ -Pregnan-3 $\alpha$ ,17 $\alpha$ ,20 $\beta$ ,21-tetrol-11-one	$\beta$ -Cortolone	30.77
40	5 $\beta$ -Pregnan-3 $\alpha$ ,11 $\beta$ ,17 $\alpha$ ,20 $\beta$ ,21-pentol	$\beta$ -Cortol	30.77
41	5-Cholesten-3 $\beta$ -ol	Cholesterol	30.89
42	5 $\beta$ -Pregnan-3 $\alpha$ ,11 $\beta$ ,17 $\alpha$ ,20 $\alpha$ ,21-pentol	$\alpha$ -Cortol	31.22
43		<i>n</i> -C <sub>32</sub> -Alkane	32.00

\* Trivial names of other steroids mentioned in this paper:

4-Androsten-11 $\beta$ -ol-3,17-dione	11 $\beta$ -Hydroxyandrostenedione
4-Androsten-3,11,17-trione	11-Ketoandrostenedione
5-Pregnen-3 $\beta$ ,17 $\alpha$ -diol,20-one	17 $\alpha$ -Hydroxypregnenolone
4-Pregnen-11 $\beta$ ,17 $\alpha$ -diol-3,20-dione	21-Desoxycortisol
4-Pregnen-17 $\alpha$ -ol-3,20-dione	17 $\alpha$ -Hydroxyprogesterone ("17OH-P")
4-Pregnen-17 $\alpha$ ,21-diol-3,11,20-trione	Cortisone ("E")
4-Pregnen-11 $\beta$ ,17 $\alpha$ ,21-triol-3,20-dione	Cortisol ("F")
4-Pregnen-11 $\beta$ ,21-diol-3,20-dione	Corticosterone ("B")
4-Pregnen,21-ol-3,20-dione	Desoxycorticosterone ("DOC")
4-Pregnen-17 $\alpha$ ,21-diol-3,20-dione	11-Desoxycortisol ("S")

conditions sensitivity was  $\sim 1$  ng per injected sample corresponding to about 0.02 mg of steroid per 24 h. The coefficient of variation for multiple determinations ( $n = 8$ ) of the whole spectrum of standard compounds was  $12 \pm 3\%$  (range, 10–20%).

**Statistics.** Data are presented as mean  $\pm$  SD. Student's *t* test (two-tailed) for matched and for unmatched pairs was used for statistical evaluation.

## Results

In unsubstituted patients with adrenocortical insufficiency, plasma concentrations of cortisol were  $1.1 \pm 0.5$   $\mu\text{g}/\text{dl}$  (controls,  $6.8 \pm 2.2$   $\mu\text{g}/\text{dl}$ ). Concentrations of 11-ketoandrostenedione and of 21-deoxycortisol were  $64 \pm 18$  ng/dl (controls,  $279 \pm 139$  ng/dl) and  $2.0 \pm 0.3$  ng/dl (controls,  $5.1 \pm 1.0$  ng/dl) respectively, and the concentrations of pregnenolone and 17-hydroxypregnenolone were  $38.2 \pm 14.2$  ng/dl (controls,  $53.3 \pm 6.2$  ng/dl) and  $12.9 \pm 6.4$  ng/dl (controls,  $45.0 \pm 18.9$  ng/dl), respectively. Thus, when compared with healthy controls, mean estimated concentrations of cortisol, 11-ketoandrostenedione, 21-deoxycortisol, pregnenolone, and 17-hydroxypregnenolone in patients with adrenocortical insufficiency were 13, 23, 39, 72, and 29%, respectively. In patients with adrenocortical insufficiency, plasma concentrations of cortisol were unchanged by the administration of ACTH ( $1.5 \pm 0.7$   $\mu\text{g}/\text{dl}$ ; controls,  $24.0 \pm 10.6$   $\mu\text{g}/\text{dl}$ ) and of dexamethasone ( $0.6 \pm 0.2$   $\mu\text{g}/\text{dl}$ ; controls,  $0.8 \pm 0.2$   $\mu\text{g}/\text{dl}$ ). Plasma concentrations of ACTH in patients with adrenocortical insufficiency were  $968 \pm 410$  pg/ml (range, 462–1670 pg/ml) after withdrawal of substitution therapy and  $151 \pm 144$  pg/ml (range, 54–429 pg/ml) after 3 d of dexamethasone by mouth.

Steroid excretion rates of healthy men, healthy women, and of patients with adrenocortical insufficiency are given in Table II. Comparable excretion rates of cholesterol were found in patients with adrenocortical insufficiency and in healthy controls. Urine samples of patients were characterized by a lack of all 21-hydroxylated metabolites (TH-"A", TH-"B", allo-TH-"B", TH-"DOC", allo-TH-"DOC", TH-"S", allo-TH-"S", TH-"E", allo-TH-"E", TH-"F", allo-TH-"F",  $\alpha$ -cortol,  $\beta$ -cortol,  $\alpha$ -cortolone, and  $\beta$ -cortolone, Fig. 1). In addition, patients with adrenocortical insufficiency presented with a reduced excretion of androsterone, etiocholanolone, dehydroepiandrosterone, androstenediol, 11 $\beta$ -hydroxyetiocholanolone, 11 $\beta$ -hydroxyandrosterone, 11-ketoetiocholanolone, pregnanediol, and allopregnanediol, whereas the mean excretion of pregnanetriol ( $3.92 \pm 3.07$  mg/24 h), pregnetriol ( $2.22 \pm 2.47$  mg/24 h), pregnenolone ( $1.85 \pm 2.47$  mg/24 h), and 11-ketoandrosterone ( $1.34 \pm 1.64$  mg/24 h) was increased as compared with healthy controls. In regard to the low excretion rates, both in patients with adrenocortical insufficiency and in healthy controls, a comparison of the excreted amounts was not possible for the remaining compounds listed in Tables I and II. Finally, the excretion rates of cortisol in patients with adrenocortical insufficiency were  $<0.5$   $\mu\text{g}/24$  h after withdrawal of substitution therapy, during stimulation by ACTH, and after 3 d of dexamethasone by mouth. Whereas the administration of ACTH and of dexamethasone induced the expected changes in the excretion of steroid metabolites in healthy men and women, the respective excretion rates remained unchanged in patients with adrenocortical insufficiency (Table II).

Gas-chromatographic analysis indicated the presence of 11-hydroxyandrosterone in the urine of three patients with adrenocortical insufficiency. This was confirmed by additional mass

spectrometry using selected ion monitoring. As shown in Fig. 2 for one single case the chosen characteristic ions were readily identified, both in a mixture of derivatized reference compounds and in the patient's urine.

## Discussion

The prerequisite for the simultaneous estimation of a large number of urinary steroid metabolites has been provided by the development of glass capillary gas chromatography (1, 2). The variety of techniques and hydrolytic procedures used by different laboratories implies that the only valid reference material is a material obtained using that very method. In order to study the steroid excretion rates in patients with adrenocortical insufficiency, it was therefore pivotal to establish reference data in a group of healthy subjects. These data are included in this report.

Although the relative share of the gonads and the adrenal cortex in overall steroid production can easily be studied in patients with adrenocortical insufficiency, few such data have been reported as yet in this disorder (7–9).

Under physiological conditions the enzymes 21-hydroxylase, 11 $\beta$ -hydroxylase, and 18-hydroxylase are unique to the adrenal cortex, whereas other enzymes, such as 3 $\beta$ -steroid dehydrogenase, 17 $\alpha$ -hydroxylase, and the desmolases are shared by the adrenals and by the gonads and probably are controlled by a single gene (10). The lack of 21-hydroxylated steroid metabolites observed in the urine of our patients can therefore be easily deduced from the differences in the enzymatic equipment of the adrenal cortex as compared with that of the gonads, whereas the presence of two 11-hydroxylated metabolites, 11 $\beta$ -hydroxyandrosterone and 11-ketoandrosterone, and the elevated excretion of pregnenolone, pregnanediol, and pregnanetriol requires further discussion.

Although adrenal scanning is believed to be superior to other noninvasive techniques in the detection of adrenal rest tissue (11), the negative outcome of  $^{131}\text{I}$ -cholesterol scanning per se may not completely rule out this possibility in our patients. Additional evidence against a residual adrenal function is, however, provided by the lack of urinary cortisol and by plasma cortisol concentrations close to the lower limit of the used radioimmunological method. Finally, the increased excretion of some metabolites not requiring 21-hydroxylation (e.g., pregnanetriol and pregnetriol) in the absence of all urinary 21-hydroxylated metabolites and a similarly distorted relationship among the estimated plasma steroids implies that the site of steroid hormone production in our patients is characterized by a pattern of steroidogenesis different from that of normal adrenal tissue.

In the absence of functional adrenal tissue the presence of 11-hydroxylated steroid metabolites must be due to an extraadrenal 11-hydroxylase. Such a speculation may be based on the previous demonstration of an extraadrenal 21-hydroxylase (12) and of a hepatic 3 $\beta$ -steroid dehydrogenase (10). However, in regard to the increased excretion of early steroid metabolites mentioned above, a gonadal origin of 11 $\beta$ -hydroxylated steroids in our patients appears more likely. The minor alterations in gonadotropin secretion seen in patients with adrenocortical insufficiency (13) are not likely to account for these pronounced changes in steroid excretion.

Under the appropriate experimental conditions, ACTH, the tropic hormone of the adrenal, may stimulate both rat and human testicular cells at the molecular level in terms of RNA syn-

Table II. Excretion of Urinary Steroids in Healthy Men (n = 5) and Women (n = 5) and in Patients with Adrenocortical Insufficiency (n = 6)

Steroid (mg/24 h)	Basal				ACTH				Dexamethasone				
	Male		Female		Male		Female		Male		Female		ACI
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	ACI
Androstero	3.35±0.49‡	2.11±1.07	<0.10-1.28	—	6.11±1.01	3.04±1.29	<0.08-1.18	—	2.21±0.78§	0.52±0.22	0.23-1.59	—	
Etiocholanolone	2.67±0.75	2.40±0.75	<0.10-0.41	—	4.87±1.46	3.46±1.67	<0.08-0.87	—	1.41±0.43‡	0.63±0.43	<0.07-0.58	—	
Dehydroepiandrosterone	0.26±0.17	-0.26	—	—	1.18±0.72	0.45±0.43	—	—	-0.03	-0.07	—	—	
Epiandrosterone	-0.83	-0.41	—	—	-0.13‡	-0.06	—	—	-0.09§	-0.15	—	—	
Androstendiol	0.26±0.13‡	0.11±0.06	0.00-0.05	—	1.06±0.67	1.31±1.40	0.00-0.04	—	0.18±0.07	0.06±0.03	0.00-0.03	—	
11-O-Androsterone	-0.06	-0.30	0.36-4.63	—	-0.24	-0.26	0.38-1.56	—	-0.08	-0.14	0.46-1.92	—	
Estrone	0.09±0.05‡	0.16±0.05	—	—	0.28±0.13	0.27±0.07	—	—	-0.04	-0.03	—	—	
11-O-Etiocholanolone	-0.11	-0.23	—	—	-0.18	-0.22	—	—	-0.07	-0.12	—	—	
Androstendione	—	-0.13	—	—	—	-0.12	—	—	—	-0.09	—	—	
Estradiol	—	—	—	—	—	—	—	—	—	—	—	—	
Testosterone	—	—	—	—	—	—	—	—	—	—	—	—	
Epipregnanolone	-0.10	-0.07	—	—	-0.10	-0.12	0.00-0.18	—	-0.07‡	-0.02	0.00-0.12	—	
11β-OH-Androsterone	1.20±0.40	0.76±0.26	0.00-0.37	—	3.62±0.63	3.15±1.56	0.00-0.53	—	0.51±0.21	0.19±0.07	0.00-0.73	—	
11β-OH-Etiocholanolone	0.27±0.15	0.20±0.11	0.00-0.05	—	1.07±0.44	0.80±0.21	—	—	0.08±0.02	0.08±0.02	—	—	
Pregnanolone	-0.05	-0.54	—	—	-0.13	-0.10	—	—	-0.04	-0.02	—	—	
allo-Pregnanediol	0.15±0.07	-0.13	—	—	-0.47	0.19±0.13	—	—	-0.11	-0.10	—	—	
Pregnanediol	0.33±0.10	0.47±0.35	—	—	1.16±0.42	1.18±0.67	—	—	0.15±0.09	0.21±0.17	—	—	
Pregnenolone	-0.07	-0.06	—	—	-0.14‡	-0.08	0.69-2.33	—	-0.06	-0.07	0.44-2.60	—	
Pregnanetriol	1.53±0.34	1.31±0.47	1.80-10.00	—	3.43±1.08	1.67±0.14	1.53-4.44	—	1.19±0.21‡	0.75±0.21	2.16-4.91	—	
17α-Androstenetriol	-0.05	-0.05	—	—	0.49±0.31‡	-0.14	—	—	-0.03	-0.02	—	—	
Progesterone	-0.14	-0.05	—	—	-0.19‡	-0.08	—	—	0.13±0.10	-0.02	—	—	
17β-Androstenetriol	-0.09	-0.13	—	—	-0.30	-0.21	—	—	-0.05	-0.05	—	—	
TH-“S”	0.15±0.04	0.14±0.10	—	—	0.47±0.13	0.43±0.20	—	—	-0.09	-0.07	—	—	
TH-DOC	-0.21	-0.08	—	—	-0.15	-0.11	—	—	-0.05	-0.07	—	—	
Estril	-0.13	-0.24	—	—	-0.13	-0.14	—	—	-0.11	-0.07	—	—	
allo-TH-DOC	-0.10	-0.10	—	—	0.58±0.52	-1.80	—	—	-0.04	-0.34	—	—	
Pregnanetriolone	0.30±0.06	0.22±0.09	0.54-7.06	—	1.64±0.86	0.91±0.53	0.73-2.56	—	-0.19	-0.17	0.71-3.14	—	
TH-“E”	1.67±0.60	1.81±0.36	0.00-0.07	—	10.13±3.73	6.66±1.83	—	—	-0.08	0.05±0.04	—	—	
TH-“A”	-0.43‡	0.52±0.14	—	—	2.27±0.62	1.60±0.38	—	—	-0.04	-0.06	—	—	
TH-“B”	0.29±0.14	0.32±0.11	—	—	2.10±0.52	2.06±0.89	—	—	-0.16	0.07±0.03	—	—	
allo-TH-“B”	0.33±0.18	0.43±0.21	0.00-0.06	—	6.21±0.91§	3.95±1.03	—	—	0.15±0.05	0.16±0.11	—	—	
allo-TH-“E”	1.14±0.10	1.40±0.26	—	—	13.76±3.94	9.65±1.66	—	—	-0.04	-0.07	—	—	
TH-“F”	0.60±0.10	0.58±0.30	—	—	7.57±2.22‡	4.46±1.96	—	—	-0.03	-0.04	—	—	
allo-TH-“F”	0.47±0.21	0.64±0.20	—	—	2.59±0.91	1.71±0.36	—	—	-0.04	-0.04	—	—	
a-Cortolone	-0.18	0.21±0.13	—	—	1.92±0.80‡	0.85±0.36	—	—	-0.04	-0.04	—	—	
β-Cortolone	—	—	—	—	—	—	—	—	—	—	—	—	
β-Cortol	—	—	—	—	—	—	—	—	—	—	—	—	
Cholesterol	0.35±0.13	0.52±0.12	0.28-2.45	—	1.06±0.50	0.84±0.17	0.07-1.28	—	0.38±0.13	0.48±0.34	0.19-1.58	—	
a-Cortol	-0.08	0.05±0.06	—	—	0.87±0.35	0.60±0.33	—	—	-0.04	-0.03	—	—	

Excretion rates in healthy subjects are given as mean±SD. If one or more of the individual excretion rates were below the sensitivity of the method only mean values±1SD are given as the upper range. If the respective steroid was not detectable in any subject, no value is given. For patients with adrenocortical insufficiency the range in individual excretion rates is indicated. P values indicate differences between healthy males and healthy females.

\* ACI, adrenocortical insufficiency. ‡ P < 0.05. § P < 0.01.

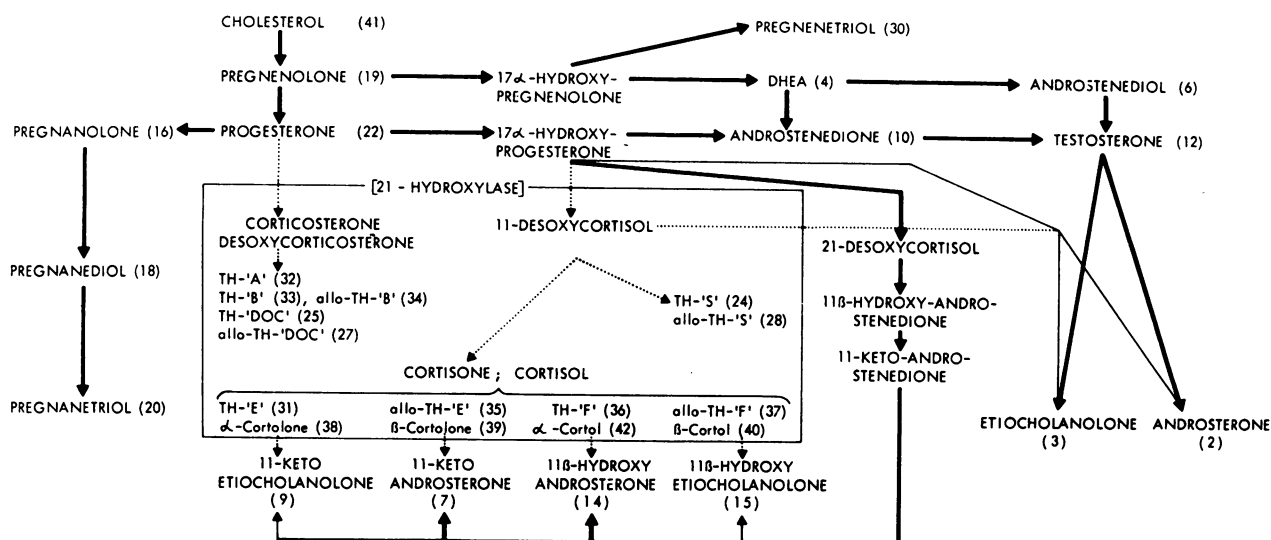


Figure 1. Simplified scheme of steroidogenesis in patients with adrenocortical insufficiency. The numbers in brackets refer to the numbering system of steroid standards given in Table I.

thesis and of steroid hormone synthesis (14). It appears possible that concentrations of ACTH elevated, as in our patients, for periods of several years may influence gonadal steroidogenesis in adrenocortical insufficiency.

In the adrenal cortex the presence of elevated concentrations of ACTH for prolonged periods of time has been shown to alter the relative activities of the various steroid hydroxylases (15). Chronic overexposure to ACTH apparently modifies gonadal

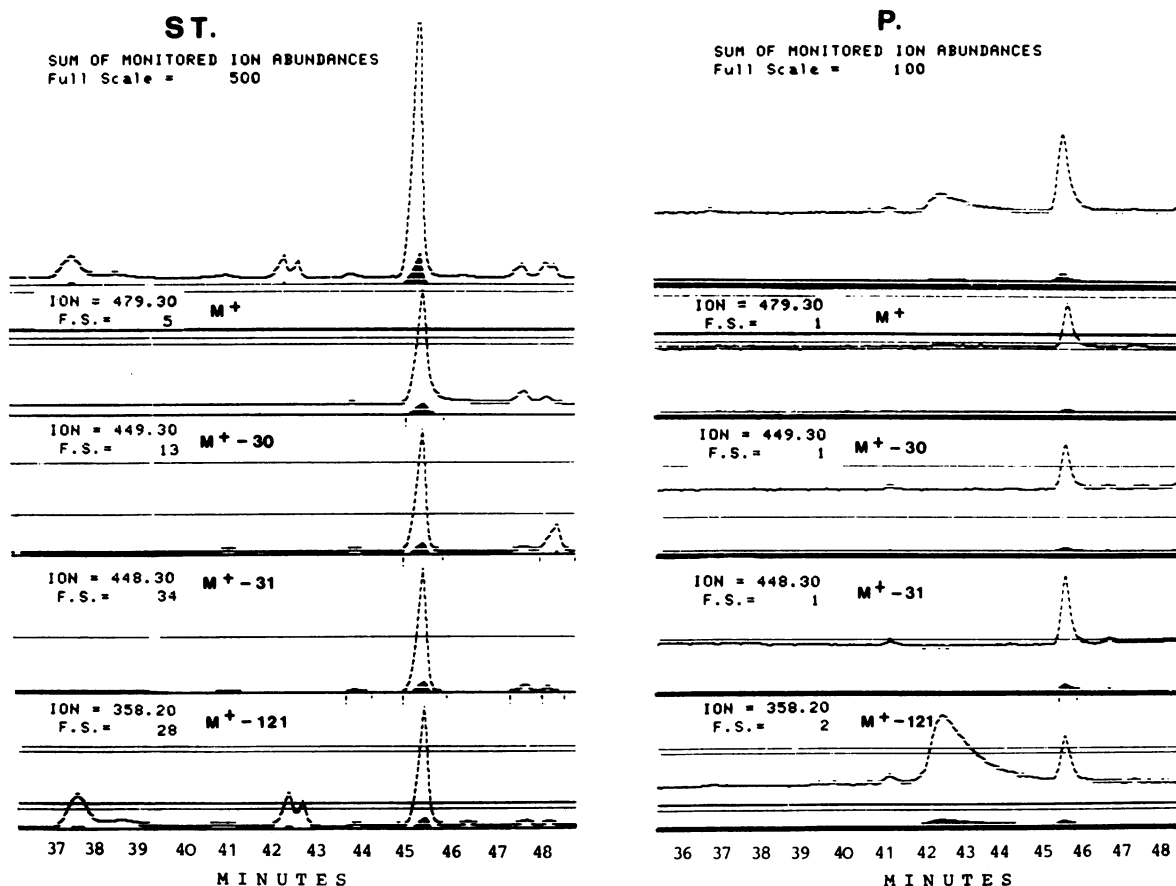


Figure 2. Identification of 11-hydroxyandrosterone by gas chromatography-mass spectrometry (selected ion monitoring) in a mixture of derivatized reference compounds (St.) and in the urine of a patient with adrenocortical insufficiency (P.). Plots of the following four ions are shown:  $m/e$  479 ( $M^+$ ),  $m/e$  449 ( $M^+ - 30$ ),  $m/e$  448 ( $M^+ - 31$ ), and  $m/e$  358 ( $M^+ - 129$ ). The abscissa represents the retention time (minutes).

steroidogenesis in an analogous manner, thus explaining the increased excretion of early steroid metabolites not requiring 21-hydroxylation. This would also imply that the attempt to qualify the origin of any given steroid as gonadal or adrenal by its absence or presence in patients with adrenocortical insufficiency (9) is an oversimplification. Steroid excretion rates remained unchanged in our unsubstituted patients during the administration of 1-24 ACTH and of dexamethasone, indicating that the additional administration of ACTH or the short-term partial suppression of endogenous ACTH by 2 mg dexamethasone for 3 d is apparently of little relevance in this pathological situation. Concerning the presence of 11-hydroxylated metabolites, i.e., of 11 $\beta$ -hydroxyandrosterone and 11-ketoandrosterone, in the urine of patients with adrenocortical insufficiency, it should be remembered that some testicular tumors in patients with congenital adrenal hyperplasia and in adrenalectomized patients with Cushing's syndrome possess both 11 $\beta$ -hydroxylase and 21-hydroxylase activity in vitro (16-19). The question of whether these tumors arise from regular testicular tissue (20) or from adrenal remnants within the testes (21) is as yet unsettled (19), but prolonged exposure to markedly elevated concentrations of ACTH has generally been implied in their pathogenesis (17). Thus, during prolonged overexposure to ACTH, the human gonad is capable of producing 11-hydroxylated steroids.

Since the side chain at C17 found in C<sub>21</sub> steroids orients the reduction of the double bond at C4/C5 in the 5 $\beta$  direction (22), C<sub>19</sub>O<sub>3</sub> steroids with the 5 $\beta$ -configuration (i.e., 11-ketoetiocholanolone, and 11 $\beta$ -hydroxyetiocholanolone) are metabolites of C<sub>21</sub> precursors such as cortisol. Compounds with the 5 $\alpha$  configuration (i.e., 11-ketoandrosterone, 11 $\beta$ -hydroxyandrosterone) are derived from 11 $\beta$ -hydroxyandrostenedione and from 11-ketoandrostenedione (22-24) via side-chain cleavage of 21-deoxycortisol. The synthesis of these steroids, therefore, requires the activity of 11 $\beta$ -hydroxylase, but not of 21-hydroxylase. The assumed presence of 11 $\beta$ -hydroxylase in the gonads of our patients thus not only explains the observed excretion of C11-oxygenated steroids, but also the preponderance of the 5 $\alpha$ - vs. the 5 $\beta$ -configured metabolites.

The estimated plasma concentrations of 21-deoxycortisol and 11-ketoandrosterone are also in keeping with the presence of 11-hydroxylase activity in our patients, and it is of note that the reduction in the plasma concentrations of both steroids was less pronounced than that of cortisol. In regard to the elevated excretion rates of 11-ketoandrosterone, pregnanetriol, pregnetriol, and pregnenolone, elevated plasma concentrations of the respective precursor steroids (i.e., 11-ketoandrostenedione, 21-deoxycortisol, pregnenolone, and 17-hydroxypregnenolone) might have been expected. This was clearly not the case and there is no ready explanation for this observation, although one might point out that plasma concentrations of steroids do not necessarily reflect the excretion rates of their metabolites, since both values also depend on the metabolic clearance rate of the respective precursor (25).

Under physiological conditions both the adrenal cortex and the gonads contribute to the urinary excretion of 11-desoxy-17-ketonic steroid metabolites (etiocholanolone, androsterone), although direct glandular secretion of both compounds is small (26). In our patients with adrenocortical insufficiency the lack of adrenal precursors apparently accounts for the diminished excretion of etiocholanolone and androsterone. The latter is in keeping with the 65% reduction in plasma androsterone as reported by others (26).

In conclusion, patients with adrenocortical insufficiency present a pattern of steroid excretion characterized by the complete absence of 21-hydroxylated, but not of 11-hydroxylated, metabolites. The obtained data suggest that chronic ACTH exposure may result in the activation of 11 $\beta$ -hydroxylase in the human gonad.

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