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# THE PLASMA CORTISOL RESPONSE TO ACTH IN "IDIOPATHIC HIRSUTISM" *

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The biochemical abnormalities in patients with "idiopathic hirsutism" are imperfectly understood. The fractionation and measurement of urinary 17-ketosteroids in these patients have been studied in several laboratories. It appears that this disorder is associated with increased production of an adrenal androgen which is reflected by increased excretion of urinary $C_{19}O_2$ 17-ketosteroids, in particular, androsterone and etiocholanolone[1] (1–7). Gallagher and co-workers (1), Kappas, Pearson, West and Gallagher (2) and Brooks and Prunty (3) reported that the urinary 11-oxy-17-ketosteroids may also be increased in these patients, and Bush and Mahesh (4) noted, in particular, increased excretion of 11-hydroxyandrosterone. These 11-oxygenated steroids are presumed to be derived principally from cortisol and 11-hydroxyandrostenedione metabolism (8). By inference cortisol production in patients with idiopathic hirsuitism has been thought to be either normal or high. To define further the nature of this adrenal abnormality, plasma cortisol levels have been studied in hirsute patients during 8 hours of intravenous ACTH stimulation.

The determination of plasma 17-hydroxycorticoids has received wide acceptance and application in studies of abnormal adrenal function. These methods generally depend upon the specificity of the Porter-Silber (9) reaction after partial purification of a chloroform or dichloromethane extract of plasma. While it appears that these methods satisfactorily reflect cortisol content of plasma, work by Bondy and co-workers (10) and Morris and Williams (11), in which the plasma extract was carried through more rigorous purification procedures before final quantitation, have given blood cortisol levels which were significantly lower than those obtained by the less specific procedures. Since "idiopathic hirsutism" has been postulated to represent a partial enzymatic block in cortisol synthesis (2, 5), similar to the enzymatic blocks now recognized in the congenital adrenal hyperplasias (12–14), it was felt desirable to measure blood cortisol in these patients. A paper chromatographic step to separate plasma cortisol from other adrenal products and their derivatives was used. A reliable, technically feasible method has been devised.

## MATERIALS AND METHODS

*Control subjects.* Control subjects used in this experiment were healthy hospital personnel; they were supplemented with appropriate hospitalized patients and patients seen in our outpatient department for routine physical examination, minor psychoneurotic complaints or minor surgical or orthopedic problems. These subjects ranged in age from 20 to 40 years.

*Hirsute patients.* These were 10 consecutive women, referred to us with primary complaints of hypertrichosis; their mean age was 28 years. The onset of hirsutism was noted in each during her first postpubertal decade, and the duration of the complaint had ranged from 3 to 20 years at the time of study. None had received estrogen treatment and one had previously received a 1-year course of prednisone.

It is realized that adult hirsutism remains a poorly understood condition and may well represent a spectrum of different biochemical abnormalities. The patients studied met the following criteria. All had apparent hirsutism of the face and extremities without signs of virilization. Body habitus and proportions were normal

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[1] In this paper the trivial names of the steroids have been used. Cortisol: 11β,17α,21-trihydroxy-4-pregnene-3,20-dione. Prednisone: 17α,21-dihydroxy-1,4-pregnene-3,11,20-trione. Cortisone: 17α,21-dihydroxy-4-pregnene-3,11,20-trione. Etiocholanolone: 3α-hydroxy-etiocholane-17-one. Androsterone: 3α-hydroxy-androstane-17-one. 11-Hydroxyandrosterone: 3α,11β-dihydroxy-androstane-17-one. 11-Hydroxyandrostenedione: 11β-hydroxy-4-androstene-3,17-dione. Pregnanetriol: 5β-pregnane-3α,17α,20α-triol. Pregnanediol: 5β-pregnane-3α,20α-diol.

in each case, none had clitoral enlargement, angular baldness or any of the clinical stigmata of Cushing's syndrome —i.e., ecchymosis, plethora, hypertension, manifest diabetes, characteristic fat distribution, striae. Some of the patients indicated minor menstrual abnormalities, but the five married members of this group conceived without difficulty. While patients with idiopathic hirsutism may have normal or elevated total urinary 17-ketosteroids (1, 3), these patients were chosen only if their total 17-ketosteroid and corticoid excretion in a 24-hour urine specimen was within normal range.

Adult hirsutism may be a manifestation of ovarian neoplasms and Cushing's syndrome and is associated with the postpubertal analog of congenital adrenal hyperplasia, and the Stein-Leventhal syndrome (3, 15). It is most unlikely that the patients studied here represented hirsutism secondary to ovarian neoplasia or Cushing's syndrome, since they were selected from a clinical population in which either of these two disease entities is extremely rare. The duration of the hirsutism and the criteria used in choosing the patients for study make these possibilities even less likely.

Patients with the postpubertal form of congenital adrenal hyperplasia excrete increased quantities of urinary 17-ketosteroids and manifest virilism (3). While neither of these criteria was fulfilled in any of our patients, their urinary pregnanediol and pregnanetriol excretion was not studied. This form of adult hirsutism may therefore have been included in this series. Since none of these patients was surgically explored, the Stein-Leventhal syndrome is not rigorously excluded and may also be represented in these 10 patients. However, pelvic examination was normal in all the patients studied, fertility problems were not noted in this group, and menstrual disturbance was unusual.

One of the patients in this series had previously received a course of corticosteroid suppressive therapy. This patient, B.C., at age 19 when originally seen 2 years prior to this study, had control urinary 17-ketosteroid excretion of 29 and 27 mg per 24 hours on two separate collections, and urinary corticoids in mid-normal range. She had been subsequently treated with 7.5 mg prednisone daily for 1 year without change in extent or rate of growth of hair, but had been off therapy for 1 year prior to being studied. At this time her urinary 17-ketosteroids were 10.0 and 11.5 mg per 24 hours.

None of the control or hirsute subjects had evidence of hepatic, cardiac or renal disease, and all were normotensive.

*Urinary corticoids and 17-ketosteroids.* These determinations were done on aliquots of 24-hour urine collections. Urinary corticoids were measured on an alkali- and acid-washed ethyl acetate extract of urine after glucuronidase hydrolysis, by the blue tetrazolium reduction method of Izzo, Keutmann and Burton (16). Normal adult values in this laboratory are 4 to 15 mg per 24 hours.

17-Ketosteroids were determined by the method of Peterson and Pierce (17). The normal range for adult females with this method is 7 to 15 mg per 24 hours. Urinary total 17-ketosteroids were used only as a criterion for our initial selection of patients. No attempt was made to fractionate the 17-ketosteroids into individual components.

*ACTH tests.* These were started at approximately 9 a.m. Forty U of ACTH [2] was added to 800 ml of 5 per cent glucose in water and infused intravenously at an even rate over an 8 hour period; 25-ml blood samples were drawn in heparinized syringes before the start of the infusion and again at 2, 4 and 8 hours. The plasma was separated from its red cells within 30 minutes of collection and frozen until analyzed.

*Plasma cortisol determination.* A 10 to 15 ml aliquot of plasma was brought to room temperature and transferred to a 90 ml heavy-walled centrifuge tube. The plasma was adjusted to pH 9 with a 1 N solution of lithium hydroxide and immediately extracted with three 15-ml aliquots of chloroform freshly distilled from anhydrous potassium carbonate. Mixing was achieved with glass loop stirrers. After centrifugation the chloroform layer was drawn off with a 20 ml syringe equipped with a 4 inch no. 18 blunt needle. A drop of 5 N acetic acid was added to the combined extract which was then taken to dryness under reduced pressure at 43°C.

Sheets of Whatman no. 1 filter paper were washed for 3 days with distilled water followed by 2 days with absolute methanol in a large chromatography cabinet. The plasma extracts were transferred to 2-cm wide strips of this paper, and concentrated at the origin by dipping the upper edge of the chromatogram into a mixture of ethyl acetate : methanol (1 : 1); the solvent ascended by capillary action (18). The paper was allowed to equilibrate overnight at 28°C in the Bush benzene : methanol : water (100 : 55 : 45) system (18). Reference cortisol was chromatographed alongside the extracts. The plasma was carried through the extraction procedure to the paper chromatogram in one working day and was not allowed to stand overnight at any intermediate stage.

The chromatograms were run for 3 hours after which the standard cortisol was located with ultraviolet light, and corresponding areas 4 to 5 cm long were cut from the paper strips containing the plasma extract. Occasionally, paper blanks of 4 to 5 cm length were cut above and below the areas corresponding to the reference cortisol. The cortisol area of the paper was placed on a coarse, sintered glass filter and eluted with three 0.5-hour soaks in 2 ml of methanol. Each methanol eluate was pulled through the filter by application of mild suction. The methanol was taken off *in vacuo*. The remaining material was transferred to 10 × 57 mm test tubes with methanol and the solvent removed under a stream of nitrogen.

The samples were quantitated by the method of Silber and Porter (19). They stood overnight at room temperature with 0.5 ml of the phenylhydrazine : ethanol : sulfuric acid reagent and were centrifuged for 3 min-

[2] Supplied as corticotropin ACTH by Armour Pharmaceutical Co.

utes before being transferred to microcuvets for spectrophotometry. Routinely, known quantities of cortisol were similarly reacted.

## RESULTS

*Cortisol recovery from plasma.* Table I summarizes the recoveries obtained with six different plasma samples. Known quantities of cortisol were added to aliquots of plasma and the plasma then carried through the entire procedure. The cortisol recovery was 73.5 ± 4.2 per cent (mean ± SD). It appears that plasma volumes of 10 to 20 ml can be satisfactorily and reproducibly processed. The recovery was sufficiently reproducible so that a correction using $C^{14}$-cortisol carrier was not necessary.

During preliminary investigations, marked variability of recovery was noted. The probable explanation was that traces of sodium hydroxide used to alkalinize the plasma were carried over into the chloroform extract, resulting in alkaline degradation. Two lines of investigation supported this explanation: *1*) losses were detected upon adding cortisol directly to a chloroform extract of alkalinized plasma; *2*) losses were accentuated by allowing the dried chloroform extract of alkalinized plasma to stand overnight before further processing. Lithium hydroxide served as well as sodium hydroxide in diminishing the lipid content of the extract, and in combination with the drop of acetic acid appeared to yield a more stable dry extract.

*Paper blanks.* Two paper blanks were taken from each of 55 cortisol determinations on morning plasma samples. The mean value for paper background was equivalent to 0.04 μg of cortisol, with a SD of ± 0.04 μg. If it is assumed that the paper background in the cortisol area is roughly equivalent to the average paper background obtained above and below this area, mean cortisol

TABLE I
*Cortisol recovery from plasma*

| Plasma no. | Volume extracted | Cortisol added | Cortisol recovered | Recovery, added cort. | Recovery |
|---|---|---|---|---|---|
| | *ml* | *μg* | *μg* | *μg* | % |
| 1 | 15 | 0 | 0.80 | | |
| 1 | 15 | 0.44 | 1.12 | 0.32 | 73 |
| 1 | 15 | 0.88 | 1.42 | 0.62 | 70 |
| 2 | 15 | 0 | 0.90 | | |
| 2 | 15 | 0.44 | 1.24 | 0.34 | 77 |
| 2 | 15 | 0.88 | 1.58 | 0.68 | 77 |
| 3 | 15 | 0 | 0.84 | | |
| 3 | 15 | 0.44 | 1.18 | 0.34 | 77 |
| 3 | 15 | 0.88 | 1.46 | 0.62 | 71 |
| 3 | 15 | 1.18 | 1.80 | 0.96 | 81 |
| 3 | 15 | 1.18 | 1.75 | 0.91 | 77 |
| 4 | 15 | 0 | 0.58 | | |
| 4 | 15 | 0.42 | 0.88 | 0.30 | 71 |
| 4 | 15 | 0.84 | 1.16 | 0.58 | 69 |
| 4 | 15 | 0.84 | 1.17 | 0.59 | 70 |
| 4 | 15 | 1.12 | 1.47 | 0.89 | 79 |
| 4 | 15 | 1.40 | 1.59 | 1.01 | 72 |
| 4 | 15 | 1.40 | 1.72 | 1.14 | 81 |
| 5 | 10 | 0 | 0.99 | | |
| 5 | 15 | 0 | 1.38 | | |
| 5 | 20 | 0 | 1.97 | | |
| 5 | 15 | 0.42 | 1.78 | 0.29 | 69 |
| 5 | 20 | 0.84 | 2.60 | 0.63 | 75 |
| 5 | 10 | 0.84 | 1.56 | 0.57 | 68 |
| 5 | 15 | 1.12 | 2.23 | 0.74 | 66 |
| 5 | 15 | 1.40 | 2.45 | 0.96 | 69 |
| 6 | 10 | 0 | 0.78 | | |
| 6 | 15 | 0 | 1.12 | | |
| 6 | 20 | 0 | 1.65 | | |
| 6 | 10 | 0.42 | 1.08 | 0.30 | 72 |
| 6 | 10 | 0.84 | 1.44 | 0.66 | 78 |
| 6 | 10 | 1.12 | 1.65 | 0.87 | 78 |
| 6 | 10 | 1.40 | 1.78 | 1.00 | 71 |
| 6 | 20 | 1.40 | 2.55 | 0.99 | 71 |

values obtained with this method would be about 0.4 μg per 100 ml too high. In routine use this correction adds little to interpreting the values obtained in the resting state or during ACTH stimulation, but for purposes of comparing our values with those published by other workers, the blood cortisol levels were corrected by the above value. All figures were also corrected for 74 per cent recovery.

*Normal values.* Plasma cortisol was determined between 10:30 a.m. and 12 noon on 19 men and 19 women. The concentration in males was $8.0 \pm 2.4$ μg per 100 ml, and the concentration in females was $6.7 \pm 2.9$ (mean ± SD). The levels were not significantly different ($p > 0.2$). The value for the combined groups was $7.3 \pm 2.7$ μg per 100 ml with a maximal range of 3.2 to 15. Plasma samples taken from a corresponding group of subjects between 8:30 and 9 a.m. showed a level of $10.6 \pm 3.1$ μg per 100 ml. The early values were significantly higher ($p < 0.001$) than those taken in the late morning. This observation has been previously reported in plasma 17-hydroxycorticoid determinations (20, 21).

*ACTH tests.* Seven male and five female control subjects were studied with intravenous ACTH for 8 hours. The individual cortisol values, means, and standard deviations are plotted in Figure 1.

Figure 2 shows a similar plot of the patients with idiopathic hirsutism. Patient M.S. responded differently; a separate plot of her values is made. With this one exception, the hirsute group showed increased blood cortisol levels starting 2 hours after the beginning of ACTH stimulation (p values shown relate to the significance of the difference between the two groups at each time-interval). We could not show different pre-ACTH values in the two groups. Several of the hirsute patients

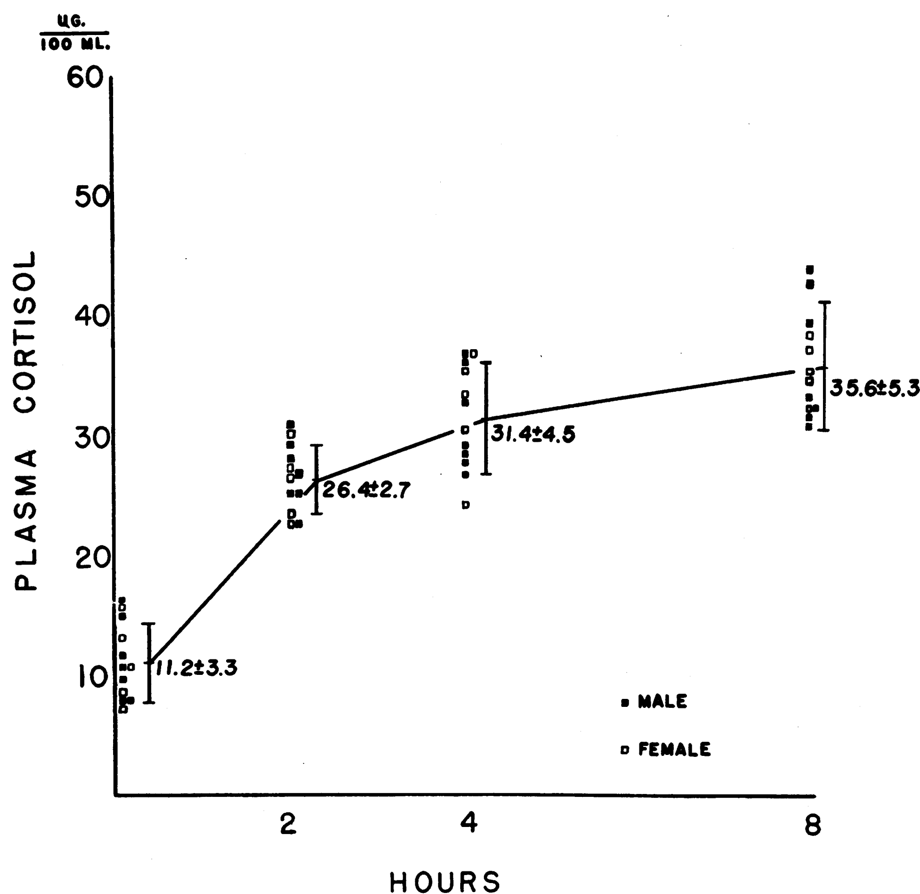


Fig. 1. The plasma cortisol response to intravenous ACTH in normal subjects.

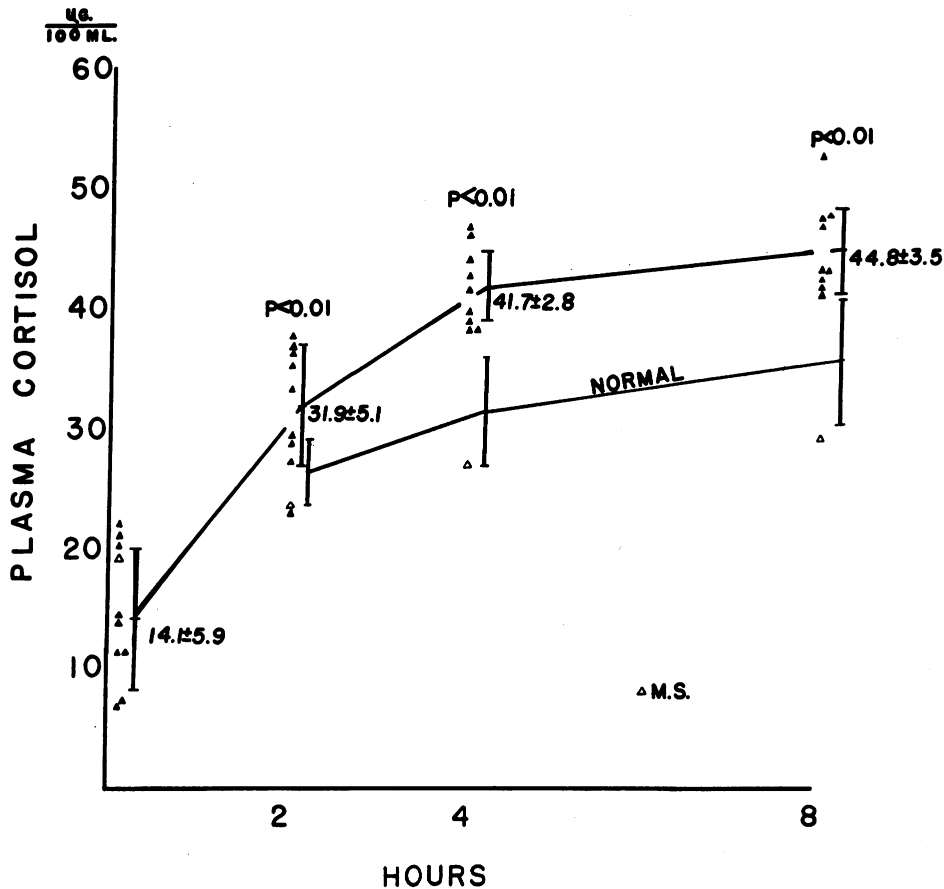


FIG. 2. THE PLASMA CORTISOL RESPONSE TO INTRAVENOUS ACTH IN PATIENTS WITH "IDIOPATHIC HIRSUTISM." The mean and standard deviation of the normal response are plotted for comparison.

started with unusually high resting cortisol levels. These were outpatients and had not been seen by us prior to the test day. The high pre-ACTH cortisol levels may be a reflection of the apprehension manifest in these patients. Cortisol levels attained with ACTH stimulation showed no correlation with their respective pre-ACTH values.

*Urinary corticoids.* Urinary corticoid values in the control group were 9.2 ± 1.1 mg per 24 hours before ACTH stimulation and 35 ± 4.4 mg per 24 hours during the day in which the ACTH test was performed. Corresponding values for the hirsute group were 9.5 ± 3.0 and 47 ± 9.4 mg per 24 hours. The difference between the urinary corticoid levels in the two groups during ACTH stimulation is significant ($p < 0.01$). Patient M.S. had a control value of 7.8 mg per 24 hours and 26.0 mg per 24 hours on the day she received ACTH.

DISCUSSION

The plasma cortisol method described here utilizes procedures well known in steroid methodology. It demands technical excellence, familiarity with paper chromatography and the routine capacity to carry the procedure through in slightly more than 2 days' time, but it offers the rewards of specificity and reliability.

The resting cortisol values obtained with this method are 10.6 ± 3.1 and 7.3 ± 2.7 μg per 100 ml for early and late morning plasma samples. These values agree quite well with those cited in the literature. Sweat (22) reported 10.8 ± 2.6 μg per 100 ml; Bondy and co-workers (10) gave

values of 7.7 ± 3.5 and 8.6 ± 3.7 μg per 100 ml for men and women, respectively; and Morris and Williams (11) gave a range of 6.5 to 10.5 μg per 100 ml in a small series. In contrast, plasma 17-hydroxycorticoid determinations generally give mean values 3 to 6 μg per 100 ml higher, with a relatively wide normal range and a larger standard deviation from the mean (20, 23, 24). It is of interest that the differences between the types of methodology appear to be maintained and perhaps emphasized during ACTH stimulation. Eik-Nes and colleagues (25) reported that, after 2, 4 and 6 hours of intravenous ACTH stimulation, plasma 17-hydroxycorticoid levels were 28 ± 7, 37 ± 12 and 42 ± 13 μg per 100 ml, respectively, and Christy, Wallace and Jailer (26) gave a range of 35 to 54 μg per 100 ml after 4 hours of ACTH stimulation. The values reported here for 2, 4 and 8 hours of ACTH are 26 ± 2.7, 31 ± 4.5 and 36 ± 5.3 μg per 100 ml, respectively. It appears likely that part of the difference may be due to the presence of variable quantities of cortisone, and the "A" ring reduction products of cortisone and cortisol, in addition to nonspecific material, being measured in the 17-hydroxycorticoid determinations (11, 27). Other workers (10, 28) who have done both determinations on corresponding plasma samples have shown similar differences.

Of the ten patients with idiopathic hirsutism, nine clearly showed increased plasma cortisol response to ACTH. It is realized that the blood cortisol level is a balance between adrenal production and peripheral removal of this hormone. It is unlikely that the rate of cortisol removal was appreciably altered in these patients for two reasons. As a group they were young, healthy individuals without evidence of hepatic or renal disease. In addition, their urinary corticoids, which should reflect impairment of peripheral removal, were significantly higher in the hirsute group than in the controls when stimulated with ACTH. It appears clear, therefore, that there was no limitation in the ability of their adrenals to produce cortisol. On the other hand, these individuals reacted sensitively to ACTH to produce supranormal plasma cortisol levels. Any defect in peripheral metabolism of the adrenal steroids in idiopathic hirsutism has yet to be demonstrated; an adrenal abnormality remains probable.

Patient M.S. showed marked hirsutism and could not be distinguished from the rest of our group by clinical or the more usual laboratory criteria. Her plasma cortisol response to ACTH, however, was in the lower range of our normal patients, and this response was reflected by her low urinary corticoid excretion. Bush and Mahesh (4) have suggested that postpubertal virilism is not an entity that can be defined by a single functional classification but may represent a wide spectrum of biochemical abnormalities. How this one patient differs from the other nine remains unclear. Patients with congenital adrenal hyperplasia characteristically have low levels of plasma 17-hydroxycorticoids and these levels fail to respond to ACTH stimulation (29, 30). This patient conceivably may represent a mild form of this group, but detailed study of urinary steroid fractions is necessary for further elucidation.

Several workers have suggested that a normal pattern of 17-ketosteroid excretion may be regained in idiopathic hirsutism after a course of suppressive therapy with a corticosteroid, and this therapy may apparently result in prolonged correction of the biosynthetic abnormality (2, 31, 32). Patient B.C., whose elevated urinary 17-ketosteroid excretion fell to within normal range subsequent to prednisone therapy, still showed plasma cortisol values at about the mean of our hirsute group when tested with ACTH. While all our patients had normal total urinary 17-ketosteroid values when studied, it is evident that they may still have had marked abnormalities in excretion of individual 17-ketosteroid components. The question remains, whether correction of the pattern of 17-ketosteroid excretion by corticosteroid therapy in these patients is accompanied by any alteration in the abnormal cortisol response to ACTH stimulation.

## SUMMARY

A method has been described for the determination of cortisol in human plasma by which normal plasma cortisol levels have been measured. These values are 10.6 ± 3.1 μg per 100 ml between 8:30 and 9 a.m. and 7.3 ± 2.7 μg per 100 ml between 10:30 a.m. and noon.

Ten women with "idiopathic hirsutism" were studied with intravenous ACTH tests. Plasma cortisol response and urinary corticoids were

measured. Nine of the ten patients responded to ACTH with increased levels of plasma cortisol and increased urinary corticoids, in comparison with a control group of subjects.

The data support the view that in most patients with "idiopathic hirsutism" there is no adrenal enzymatic limitation to cortisol synthesis. If there is an increased production of adrenal androgen by these nine patients, it appears to be accompanied by an increase in cortisol synthesis.

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