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URINARY STEROID EXCRETION IN 17α-HYDROXYLASE DEFICIENCY

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SUMMARY

Urinary steroids from a patient with 17α-hydroxylase deficiency syndrome have been identified. The steroids were obtained by enzymatic hydrolysis of urine, Amberlite XAD-2 extraction and Sephadex LH-20 column chromatography. Following preparation of volatile derivatives, the steroids were separated and estimated by gas chromatography on open-tubular capillary columns (OV-1, OV-101 and SE-52) and were identified by combined gas chromatography–mass spectrometry. The major steroids excreted were metabolites of pregnenolone (e.g. 5-pregnene-3β,20α-diol and 5-pregnene-3β,16α,20α-triol), progesterone (pregnanediols and 5β-pregnane-3α,16α,20α-triol) and 11β-hydroxy progesterone or corticosterone (11-oxo-pregnanediol, 11β-hydroxypregnanediol, 1,3,20-trihydroxypregnan-11-one, tetrahydrocorticosterone, *allo*-tetrahydrocorticosterone, and their *20-dihydro* metabolites). It is probable that the 11-oxygenated 21-deoxysteroids are principally formed by microbial 21-dehydroxylation of the *tetrahydro* metabolites of corticosterone secreted into the bile. These metabolites are then reabsorbed in the intestine and excreted in the urine. Tetrahydrodeoxycorticosterone, 18-hydroxytetrahydro-deoxycorticosterone and 3α,18,21-trihydroxy-5β-pregnane-11,20-dione, were also identified.

The daily excretion of 17-oxosteroids and cortisol metabolites was extremely small (< 50 μg/24 h) but androsterone and tetrahydrocortisone were positively identified.

INTRODUCTION

Congenital adrenal hyperplasia caused by deficiency of steroid 17α-hydroxylase was the last form of the disorder to be characterised [1], and since the first report only just over 20 cases have been described in the literature and most of these have been reviewed by Biglieri [2]. Steroid 17α-hydroxylation is essential not only to the adrenal biosynthesis of cortisol but also to the formation of androgens and oestrogens by both gonads and adrenals. Primary amenorrhoea in females and male pseudohermaphroditism results from the inability to synthesize gonadal hormones. The fact that all the reported patients had both gonadal and adrenal hormone deficiencies strongly supported single gene control of 17α-hydroxylase. Patients with 17α-hydroxylase deficiency are invariably hypertensive due to ACTH stimulation of the intact mineralocorticoid pathway resulting in excessive secretion of deoxycorticosterone.

The chemical diagnosis of 17α-hydroxylase deficiency has previously been obtained only after the separate or group analysis of selected steroids. A recent publication by the authors has illustrated the value of urinary steroid multicomponent analysis in the investigation of disorders of steroid biosynthesis in infants [3], but to date this type of analysis has not been carried out on patients with the 17α-hydroxylase deficiency syndrome.

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EXPERIMENTAL

Case report

A full report on the case history of this patient has already been published [4]. The patient is now 51 years old and has been maintained for 3 years on 0.25 mg/day dexamethasone. Blood pressure has remained between 150/100 and 170/120 mm Hg. Treatment was withdrawn for three weeks prior to the collection of a 24 h urine specimen for the present study.

Steroid abbreviations

Tetrahydrodeoxycorticosterone (*tetrahydro DOC*), 3α,21-dihydroxy-5β-pregnan-20-one; *3β-allo-tetrahydroDOC*, 3β,21-dihydroxy-5α-pregnan-20-one. The nomenclature of other steroids has been abbreviated using similar prefixes.

Reference steroids

Almost all reference steroids were obtained from the M.R.C. Steroid Reference Collection, Westfield College, London. 5β-Pregnan-3α,11β,20β,21-tetrol was prepared by borohydride reduction of tetrahydrocorticosterone [5]. 3β-*allo*-Tetrahydro DOC and 3β-*allo*-tetrahydroaldosterone were purchased from Ikapharm, Ramat Gan, Israel.

Methodology

Urine extraction and hydrolysis. Steroids were extracted from a portion of the 24 h urine specimen by passage through a column of Amberlite XAD-2 resin. The column was washed with water prior to elution of the steroids with ethanol. Enzymic hydrolysis (*Helix pomatia* and Ketodase) in 0.1 mol acetate buffer, pH 4.6, released conjugates and the freed steroids were extracted with Amberlite XAD-2 and eluted as before. Sephadex LH-20 chromatography was used to purify the extracts prior to further analysis. The method to this point has been documented previously [3, 6]. However, it was necessary to change the volumes of fractions collected from those described previously due to the different polarity of the major steroids excreted in this disorder. Cyclohexane–ethanol (4:1, v/v) was used as the solvent system and eight fractions were collected: 12–25, 25–35, 35–45, 45–55, 55–80, 80–110, 110–200, and an ethanol eluate of the LH-20.

Steroid sulphates not hydrolysed by the sulphatase enzymes in the *Helix pomatia* preparation were hydrolyzed by solvolysis. This was carried out by addition of acidified ethyl acetate to the dried ethanol eluant of the Sephadex LH-20 column. After overnight incubation at 40°C the ethyl acetate extract was washed with 8% sodium bicarbonate and water and dried under vacuum. The extract was purified on a 1 g LH-20 column using cyclohexane–ethanol, (4:1, v/v) as eluting solvent. A single fraction 1–30 ml was collected.

Derivative formation. 200 μl of a 2% methoxyamine hydrochloride solution in pyridine was added to a portion of the dried extracts and the derivatization reaction was allowed to proceed for 72 h at ambient temperature. Trimethylsilylimidazole (100 μl) was then added and the reaction tube was heated at 100°C for 2.5 h. Excess reagents were removed by chromatography on Lipidex 5000 columns [3, 7]. Oximes were also prepared with other reagents (hydroxyamine hydrochloride and ethoxyamine hydrochloride) in order to facilitate resolution of gas chromatographic peaks unresolved as methoxime derivatives.

The extremely long derivatization period was considered necessary as it has been found that this period of time is required for complete oximation of 18-hydroxylated C_{21} steroids, the detection and measurement of which are important in 17α-hydroxylase deficiency where they have been found to be excreted in elevated amounts [1, 8]. Problems encountered with the derivatization of 18-oxygenated C_{21} steroids have been discussed in two recent publications by the authors [9, 10]. The reaction rate of oxime formation can be increased by heating the derivatization tube for 3 h at 60°C, but some degradation of steroid occurs under these conditions.

Gas chromatography. Gas chromatographic separations were carried out either on a 20 m × 0.3 mm i.d. OV–101 wall-coated open-tubular (WCOT) column housed in a Becker 409 gas chromatograph or 15 m × 0.4 mm SE-52 and 19 m × 0.4 mm i.d. OV-1 support-coated open-tubular (SCOT) columns in a Varian 3700. The former instrument was equipped with a solid injection device while a splitless injection system was used with the latter. After injection, the oven temperature was raised from 160 to 260°C by 2–3°C/min. Helium was used as carrier gas at a flow rate of approximately 2 ml/min.

Quantification was achieved by the method described previously [3]. Where possible, values were corrected by employing peak height response factors relative to the internal standards determined by gas chromatographic analysis of reference compounds. Retention times of individual peaks were determined relative to a series of *n*-alkanes (methylene units, MU).

Mass spectrometry. The samples were analysed by repetitive scanning either on a Hitachi M-52 instrument equipped with 50 m × 0·5 mm i.d. OV-101 SCOT column or on a Varian Mat 731 instrument with 3 m × 2 mm i.d. OV-1 column. Complete mass spectra over the mass range 0–800 mass units were acquired, data were stored in mass converted format and processed to obtain either complete mass spectra or to plot the intensities of selected ions in a series of spectra. In spite of the poorer resolution of the packed or SCOT columns, compared to the WCOT columns used for preliminary analysis, the chromatograms were very similar and there was little difficulty in assigning identity to the peaks in the capillary column separation.

Description of mass spectra. For clarity the *base peak* in the spectra of compounds reported or illustrated has been taken as the major ion above mass 100. The major peaks were almost invariably at *m/e* 73 or 75, fragments formed by loss of silyl groups ($(CH_3)_3Si)^+$, ($HO = Si(CH_3)_2^+$).

Selected-ion-monitoring. For increased sensitivity and specificity it was necessary to determine certain steroids by selected-ion-monitoring (SIM). Where this has been necessary a brief description of the method will be given in the Results section. The analyses were carried out on Varian Mat 731 and Varian Mat 112 instruments, both with 3 m × 2 mm i.d. OV-1 packed columns operated isothermally (250°C for tetrahydrocortisone, tetrahydroaldosterone and 3α,18,21-trihydroxy-5β-pregnane-11,20-dione determination; 220°C for 17-oxo-steroids). It was possible to analyse six ions simultaneously on the Varian Mat 112 and four ions on the Varian Mat 731.

RESULTS

Urinary steroid gas chromatographic profile

Many gas chromatographic analyses were carried out during the study as each of the fractions from LH-20 chromatography was analysed as three derivatives (methyloxime, ethyloxime and trimethylsilyloxime). However, for illustrative purposes Fig. 1 shows the total profile (methyloxime-trimethylsilylether de-

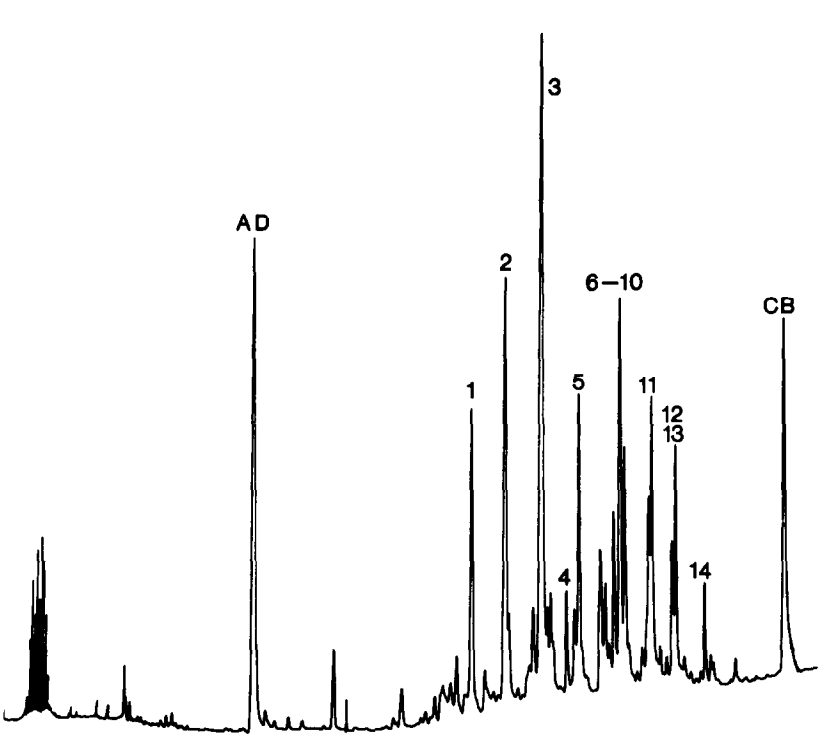

Fig. 1. Gas chromatogram of the major steroids excreted by the patient with 17α-hydroxylase deficiency. For this analysis, portions of individual Sephadex LH-20 fractions were combined and the methoxime-trimethylsilyl derivatives were prepared. A 20 m OV-101 WCOT column was used for this analysis. Because of its complexity this chromatogram was not used for quantitation. Some of the major steroids are indicated. **1.** 5β-pregnane-3α,20α-diol; **2,** 5-pregnene-3α,20α-diol; **3.** 3α,20α-dihydroxy-5α(and β)-pregnan-11-one (including 5-pregnene-3α,16α,20α-triol and 5β-pregnane-3α,16α,20α-triol); **4.** 5β-pregnane-3α,11β,20α-triol; **5.** 5α-pregnane-3α,11β,20α-triol and 5-pregnene-3β,16α,20α-triol; **6.** 3α,21-dihydroxy-5β-pregnane-11,20-dione; **7.** 3α,21-dihydroxy-5α-pregnane-11,20-dione; **8.** tetrahydrocorticosterone; **9.** *allo*-tetrahydrocorticosterone; **10.** 1ξ,3ξ,20ξ-trihydroxy-5ξ-pregnan-11-one; **11.** 3α,20α,21-trihydroxy-5α(and β)-pregnan-11-one and second peaks of **6–9**; **12,13.** 5α(and β)-pregnane-3α,11β,20α,21-tetrol; **14.** 5-pregnene-3β,16α,20α,21-tetrol and *hydroxy*tetrahydrocorticosterone. **AD** (5α-androstane-3α,17α-diol) and **CB** (cholesteryl butyrate) were internal standards.

rivatives) prepared by combining portions of each of the chromatographic fractions.

Table 1 reports the retention data (methylene units, MU) for each of the peaks identified, and results of the quantitative analysis.

Identification of metabolites of pregnenolone

5-Pregnene-3α,20α-diol and 5-pregnene-3β,20α-diol. The major urinary pregnenediol had the MU value and mass spectrum of 5-pregnene-3β,20α-diol. The parent ion was at *m/e* 462 and base peak at *m/e* 117. An important fragment was at *m/e* 129, a common ion in trimethylsilyl derivatives of 3-hydroxy-5-ene steroids. A mass spectrum of 5-pregnene-3β,20α-diol has been published [11]. The pregnenediol with short retention time could only have a 3α-hydroxy-5-ene structure and is probably 5-pregnene-3α,20α-diol.

5-Pregnene-3α,16α,20α-triol and 5-pregnene-3β,16α,20α-triol. The major pregnenetriol had mass spectrum and retention time identical to reference 5-pregnene-3β,16α,20α-triol [12]. The minor pregnenetriol had short retention time and could only be 5-pregnene-3α,16α,20α-triol. The mass spectra of both compounds are dominated by base peaks at *m/e* 117 and major fragments at *m/e* 460 (M − 90) and *m/e* 445 (M − 90 − 15). Important fragments are also seen at *m/e* 156 and 157, ions indicative of a 16,20-dihydroxy structure.

5-Pregnene-3β,16α,20α,21-tetrol. The spectrum of this compound had a parent ion at *m/e* 638 and prominent ions at *m/e* 535, 445, 355 and 265. The spectrum was identical to that given by reference 5-pregnene-3β,16α,20α,21-tetrol [12].

Identification of metabolites of progesterone and/or deoxycorticosterone (DOC)

Pregnanediol. Two peaks gave spectra indicative of pregnanediols [11]. Parent ions were at *m/e* 464 and base peak at *m/e* 117. The retention indices were identical to those of reference 5β-pregnane-3α,20α-diol and 5α-pregnane-3α,20α-diol and could be clearly distinguished from all other epimers.

5β-Pregnane-3α,16α,20α-triol. This compound co-eluted with 5-pregnene-3α,16α,20α-triol on both SE-52 and OV-101 columns (Peak 3). The major identifiable fragments were those at *m/e* 462 (M − 90) and *m/e* 447 (M − 90 − 15). The compound had the retention time of 5β-pregnane-3α,16α,20α-triol.

TABLE 1. Steroids Identified in Urine of Patient with 17α-Hydroxylase Deficiency Syndrome; Sephadex LH-20 Fractionation, Gas Chromatographic Retention Indices and Daily Excretion Rates.

Steroids Identified	Sephadex LH-20 Fraction-6g Column (ml)	M.U. Values (15 m WCOT SE-52)[a]			Excretion Rate μg/24
		MO-TMS	EO-TMS	TMS-O-TMS	
5-Pregnene-3α,20α-diol	12-25	27.41	27.37	27.40	230
5α-Pregnane-3α,20α-diol	12-25	27.55	27.50	27.52	300
5β-Pregnane-3α,20α-diol	12-25	27.72	27.71	27.72	2450
5-Pregnene-3β,20α-diol	12-25	28.31	28.34	28.31	3520
5β-Pregnane-3α,16α,20α-triol	25-35	28.72	28.71	28.72	2600
5-Pregnene-3α,16α,20α-triol	25-35	28.72	28.71	28.72	
3α-20α-Dihydroxy-5α(and β)pregnan-11-one	25-35	28.93	28.87	28.90	4520
3α,21-Dihydroxy-5β-pregnan-20-one[b]	25-35	28.93 (29.72)	29.44 (30.00)	29.70 (29.85)	160
5α-Pregnane-3α-11β,20α-triol	44-55	29.18	29.13	29.11	895
5β-Pregnane-3α,11β,20α-triol	44-55	29.37	29.30	29.36	2980
5-Pregnene-3β,16α,20α-triol	25-35	29.81	29.77	29.78	1860
3α,21-Dihydroxy-5β-pregnane-11,20-dione [b]	45-55	30.15 (30.90)	30.58 (31.18)	30.73 (30.92)	1650
3α,21-Dihydroxy-5α-pregnane-11,20-dione [b]	45-55	30.40 (31.10)	30.84 (31.40)	31.10 (31.23)	1200
Tetrahydrocorticosterone [b]	55-80	30.15 (30.77)	30.46 (31.00)	30.60 (30.88)	2000
allo Tetrahydrocorticosterone [b]	55-80	30.31 (30.77)	30.58 (31.11)	30.76 (31.00)	5400
1ξ,3ξ,20ξ-Trihydroxy-5ξ-pregnan-11-one	45-55	30.42	30.39	30.36	2700
3α,20α,21-Trihydroxy-5α(and β)-pregnan-11-one	45-55	30.92	30.85	30.89	3680
5β-Pregnane-3α,11β,20α,21-tetrol	55-80	31.15	31.06	31.15	2240
5α-Pregnane-3α,11β,20α,21-tetrol	55-80	31.27	31.25	31.28	2880
5-Pregnene-3β,16α,20α,21-tetrol	55-80	31.88	31.80	31.82	380
Hydroxy tetrahydrocorticosterone[b]	80-110	32.04(33.10)	33.00(33.80)	33.20(22.25)	580

[a] Appropriate reference compounds (where available) gave MU values almost identical to those of the urinary steroids.

[b] Derivatives of steroids with α-ketol side chain invariably give two peaks equivalent to syn and anti forms. The retention indices (MU values) for both peaks are given. Under the derivatisation conditions used, the ratio of first to second peak was approximately 2:1.

TetrahydroDOC. A high secretion of DOC is one of the hallmarks of 17α-hydroxylase deficiency and is a causative agent of the hypertension associated with the disease. Urinary tetrahydroDOC, the major metabolite of DOC, is frequently elevated in the disease (100–700 μg/24 h) [2], but even high levels can be difficult to determine by gas chromatography as tetrahydroDOC as a methyloxime-trimethylsilyl ether is not easily resolved from 3α,20α-dihydroxy-5β-pregnan-11-one trimethylsilyl ether (11-oxo-pregnanediol). Since approximately 4 mg of 11-oxo-pregnanediol is excreted, a great problem arises when an assay is required of a compound with similar retention time excreted in the 100–200 μg range. The initial fractionation by Sephadex LH-20 chromatography does not help materially, since both steroids are obtained by elution with approximately the same volume (25–35 ml). Ethyloxime and trimethylsilyloxime derivatives of the urinary steroid fractions were also prepared in anticipation of improved resolution of tetrahydroDOC from interfering steroids but mass spectrometry confirmed that the desired derivative was still contaminated with 11β-hydroxypregnanediol and 5-pregnene-3β,16α,20α-triol, respectively.

It was therefore necessary to resort to selective-ion-monitoring for tetrahydroDOC measurement. Following addition of 1 μg 3β-*allo*-tetrahydroDOC (internal standard) to 5 ml of urine, the urine was hydrolysed and the extract separated on a 1 g LH-20 column, the fraction eluted between 1 and 6 ml being collected. After derivatization the sample was analysed by gas chromatography-mass spectrometry, the instrument having been set to monitor the ions at *m/e* 507 (M^+) and *m/e* 476 (M − 31). The peaks given by tetrahydroDOC (*syn*- and *anti*- forms of the oxime) were well separated from those given by 3β-*allo*-tetrahydroDOC and quantitation was achieved by relating the peak height of the natural metabolite to the inter-

nal standard. Using this technique the excretion of tetrahydroDOC was estimated to be 160 μg/24 h.

Identification of metabolites of 11β-hydroxyprogesterone and corticosterone

11-oxo-Pregnanediol. The major peak illustrated in Fig. 1 (Peak 3) gave the spectrum illustrated in Fig. 2a. The base peak was at *m/e* 117 and parent ion at *m/e* 478. Prominent ions were seen at *m/e* 388 (M − 90) and *m/e* 334 (M − 144) the latter fragmentation being a common feature among 11-oxo-3α-hydroxy steroids (e.g. 11-oxo-androsterone). The spectrum was virtually identical to that of reference 3α,20β-dihydroxy-5β-pregnan-11-one which unfortunately was the only epimer readily available, but the peak given by the urinary steroid had a longer retention time. The width of the peak suggested that there may be more than one component, furthermore separation by thin layer chromatography revealed two spots containing approximately equal amounts. Previous investigations have shown that 3α,20α-dihydroxy-5β-pregnan-11-one was an important metabolite of corticosterone [13, 14], so one of the major components must be this steroid. It may be assumed with confidence that the other component was 3α,20α-dihydroxy-5α-pregnan-11-one, since both steroids are almost certainly formed principally by 21-dehydroxylation of the tetrahydro- and *allo*-tetrahydro- derivatives of corticosterone and 21-hydroxy-4-pregnene-3,11,20-trione. A third isomer was prominent in the solvolysis extract but has a slightly different mass-spectrum characterised by a strong peak at *m/e* 434 (M − 44) and had a considerably longer retention time. The spectrum was identical to that published by Eriksson [15] of 3β,20β-dihydroxy-5α-pregnan-11-one present in the faeces of female rats.

11β-Hydroxy pregnanediols. Two peaks gave spectra of pregnanetriols and a representative spectrum is illustrated in Fig. 2b. The parent ions were at *m/e* 552 and prominent fragments were seen at *m/e* 462,

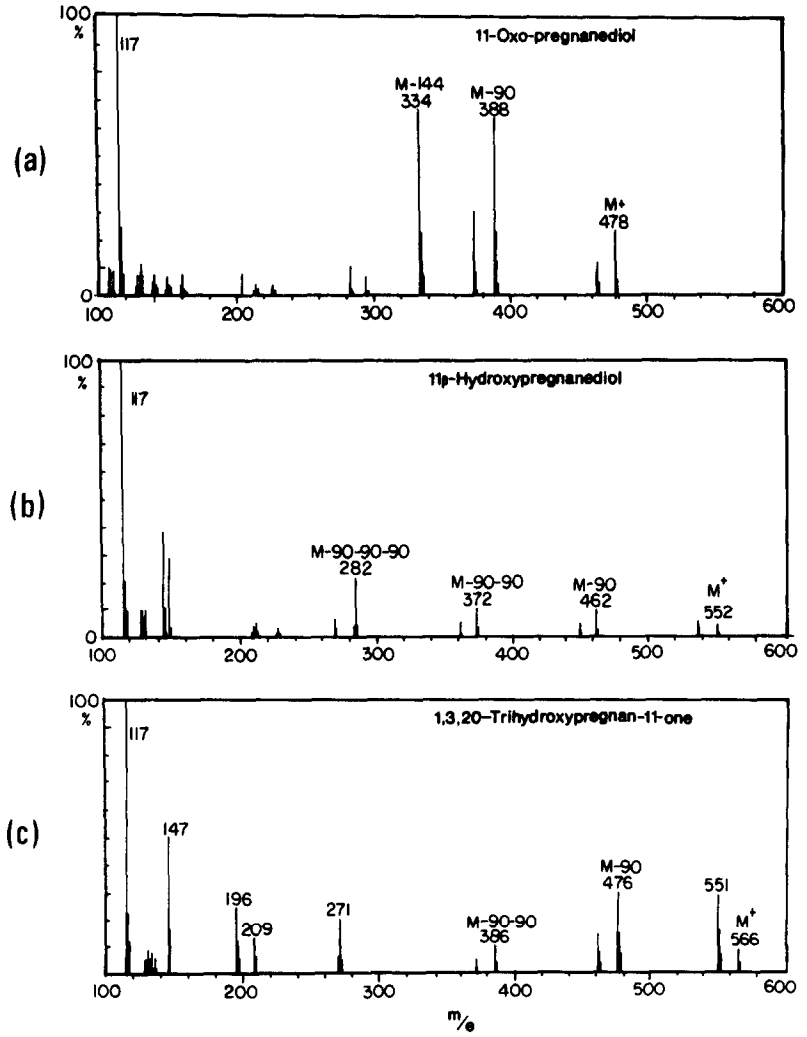

Fig. 2(a). Mass spectrum of 3α,20α-dihydroxy-5α(and β)-pregnan-11-one (11-oxo-pregnanediol) trimethylsilyl ether.

Fig. 2(b). Mass spectrum of 5β-pregnane-3α,11β,20α-triol (11β-hydroxypregnanediol) trimethylsilyl ether.

Fig. 2(c). Mass spectrum of 1ξ,3ξ,20ξ-trihydroxy-5ξ-pregnan-11-one trimethylsilyl ether.

372 and 282. The base peak was at *m/e* 117. The spectrum was identical to that of reference 5β-pregnane-3α,11β,20β-triol but both urinary compounds had shorter retention time. Since these compounds are almost certainly formed by 21-dehydroxylation of tetrahydrocorticosterone and *allo*-tetrahydrocorticosterone and urinary 20α-hydroxy-17-deoxy steroids predominate over 20β-hydroxy-17-deoxy steroids, these unknowns have been tentatively identified as 5α-pregnane-3α,11β,20α-triol and 5β-pregnane-α,11β,20α-triol.

1ξ,3ξ,20ξ-Trihydroxy-5ξ-pregnan-11-one. A peak eluted just after *allo*-tetrahydrocorticosterone has the mass spectrum illustrated in Fig. 2c. The base peak was at *m/e* 117 indicating the presence of a 20-hydroxy-21-deoxy side-chain and the parent ion was at *m/e* 566 indicating the presence of an underivatised 11-carbonyl group. However, the most characteristic ions were those at *m/e* 196, 209 and 271 which have been found to be extremely characteristic of steroids with a hydroxyl at 1β associated with hydroxyl at position 3 and carbonyl at position 11 [16]. These ions are prominent in the spectra of trimethylsilyl derivatives of 1β-hydroxytetrahydrocortisone, 1β-hydroxycortolone, 1β,3α-dihydroxy-5β-pregnane-11,17-dione and 1β,3α,17β-trihydroxy-5β-pregnan-11-one. The structure of the compound is almost certainly a 1ξ,3ξ,20ξ-trihydroxy-5-pregnan-11-one.

3α,21-Dihydroxy-5α(and β)-pregnane-11,20-dione. Both these compounds were identified in the urine fractions. The mass spectra have been published previously [16]. The parent ions were at *m/e* 521 and prominent fragments were seen at *m/e* 490 (M − 31), 400 (M − 90 − 31) and *m/e* 188 and 175. The latter two fragments are diagnostic of steroids having a derivatised α-ketol side-chain without 17α-hydroxyl.

Tetrahydrocorticosterone and allo-tetrahydrocorticosterone. Tetrahydrocorticosterone and *allo*-tetrahydrocorticosterone were identified in the 55–80 ml fraction from Sephadex LH-20. The spectra have been published previously [16] and have parent ions at *m/e* 595, with prominent fragments at *m/e* 564, 474, 446, 281, 188 and 175.

Difficulties arise with the analysis of corticosterone metabolites since the four so far described are poorly separated, even by capillary column gas chromatography. In addition each forms two well separated peaks caused by *syn*- and *anti*- forms of the oxime derivatives. However, separation on Sephadex LH-20 can be used to advantage since 3α,21-dihydroxy-5α-(and β)-pregnane-11,20-dione are eluted between 45 and 55 ml. This fractionation greatly increases the accuracy of determination of these compounds.

3α,20α,21-Trihydroxy-5α(and β)-pregnan-11-one. The mass spectrum of this steroid has been published [16]. The parent ion was at *m/e* 566 and prominent fragments are seen at *m/e* 463 (M − 103), 373 and 283. Although only one gas chromatographic peak is seen, it probably contains both the 5α and 5β epimers since these are not separated by the columns used.

5α(and β)-Pregnane-3α,11β,20α,21-tetrol. The mass spectrum of a pregnane-3,11β,20,21-tetrol has been published by Eriksson [17]. The spectrum has a small parent ion at *m/e* 640 and prominent ions at *m/e* 537 (M − 103), 447, 357 and 267. The urinary steroids had shorter retention times than reference 5α(and β)-pregnane-3α,11β,20β,21-tetrols and their structures must be 5α(and β)-pregnane-3α,11β,20α,21-tetrols.

Other corticosterone metabolites. One polar compound had a mass spectrum indicative of a *hydroxy* tetrahydrocorticosterone. The base peak was at *m/e* 280 and major peaks were seen at *m/e* 683 (M^+), 652 (M − 31), 562 (M − 31 − 90), 549 (M − 103 − 31), 534 (M − 103 − 31 − 15), 459, 444, 432, 369 and 342. Sodium borohydride reduction of this compound gave a hexol of M^+ 728 and major fragmentation at *m/e* 625 (M − 103), 535, 445 and 355. Unfortunately, it has not been possible to assign a structure to this compound. However, likely positions for the addition of a hydroxyl group would be C-1 and C-6.

Many other unidentified steroids were seen in polar fractions from the LH-20 chromatography. Some of these were hexols and pentols and some were compounds with the 1β-hydroxy-11-carbonyl structure (giving rise to the prominent fragments at *m/e* 271 and 196). These were considered to be too minor to warrant extensive identification.

Identification of 18-hydroxylated steroids

18-Hydroxylated C_{21} steroids as methoxime-trimethylsilyl derivatives can now be recognised by a characteristic fragmentation on electron impact during mass spectrometry [9]. An ion at *m/e* M − 152 is given by the methyloxime-trimethylsilyl ethers of 3α,18,21-trihydroxy-5β-pregnane-11,20-dione, 18-hydroxytetrahydrocorticosterone and 18-hydroxytetrahydroDOC [18].

Unless metabolites of 18-hydroxylated steroids are present in elevated amounts, they are difficult to distinguish by gas chromatography alone since quantitatively they are of minor importance and frequently have contaminated spectra. Selected-ion-monitoring methods are obviously required and a method has already been established for the measurement of 3α,18,21-trihydroxy-5β-pregnane-11,20-dione [10]. Using this method, the excretion of this major metabolite of 18-hydroxytetrahydrocorticosterone was found to be 78 μg/24 h which is the normal range for adults.

The data obtained following repetitive scanning GC–MS were searched for 18-hydroxytetrahydroDOC by requesting output of the intensity of the ions at *m/e* 595 (M^+), *m/e* 564 (M − 31) and *m/e* 443 (M − 152). This gave the output illustrated in Fig. 3. One peak responded to all three ions and, moreover, the relative intensity of the *m/e* 564 and 443 ions was similar to that reported by Bournot *et al.* [18]. This peak was therefore identified as 18-hydroxytetrahydroDOC.

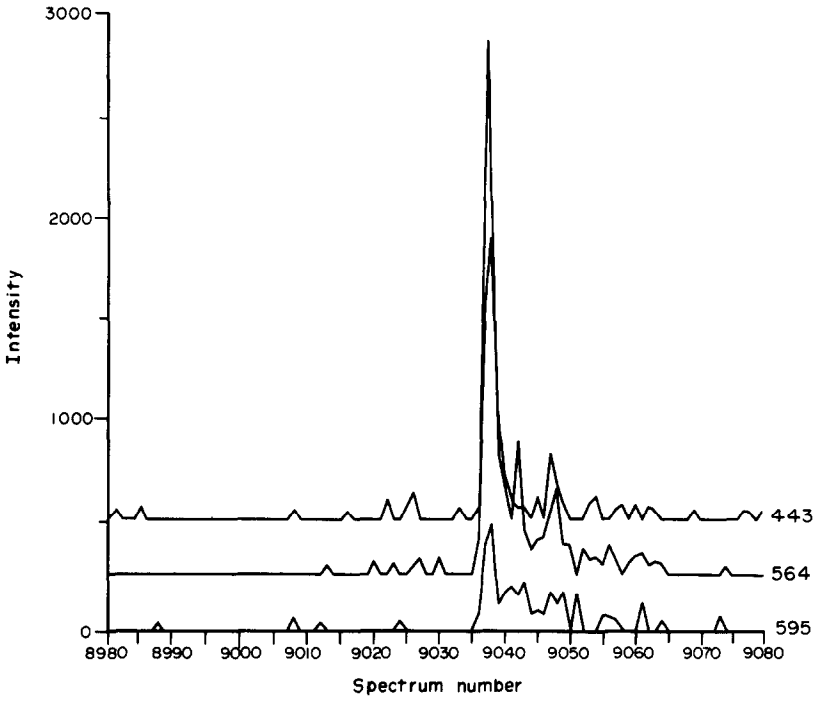

Fig. 3. Identification of 18-hydroxytetrahydroDOC. Plots of three major ions in the spectrum of the methyloxime-trimethylsilyl ether or urinary 18-hydroxytetrahydroDOC: The ion at *m/e* 595 (parent ion), the ion at *m/e* 564 (M − 31) and the ion at *m/e* 443 (M − 152). Loss of 152 mass units is highly specific for 18-hydroxylated C_{21} steroids. Spectrum 9037 was similar to that of published 18-hydroxytetrahydroDOC but it was considerably contaminated by other compounds.

Identification of 17-oxo-steroids and cortisol metabolites

Group estimations and individual assay of oxosteroids and cortisol metabolites present in urine from patients with this disorder have indicated that the 17α-hydroxylase deficiency may not be complete since these steroids have frequently been detected [2, 19]. We felt therefore that a thorough search should be made for these compounds in the urine of our patient.

Large amounts of appropriate chromatographic fractions were subjected to repetitive scanning GC–MS analysis. Following data acquisition, selected-ion-chromatograms were prepared of the ions *m/e* 391 (M^+), 360 (M − 31) and 270 (M − 31 − 90), ions indicative of the methyloxime-trimethylsilyl ether of androsterone and aetiocholanolone. However, no peak gave response for all three ions. Since repetitive scanning GC–MS analysis was not sufficiently sensitive for detection of 17-oxosteroids in this patient, an attempt was made to identify these compounds by selected-ion-monitoring. The chromatographic fraction which should contain the 17-oxosteroids was analysed on the Varian Mat 112 instrument using a 3 m OV-1 packed column relative to 5α-androstane-3α,17α-diol as internal standard. The following ions were determined simultaneously: for androstanediol (internal standard) *m/e* 346 (M − 90), 331 (M − 90 − 15), 256 (M − 90 − 90), 241 (M − 90 − 90 − 15); for androsterone and aetiocholanolone-360 (M − 31) and 270 (M − 31 − 90). The methyloximetrimethylsilyl ethers of androsterone and aetiocholanolone were not resolved by the column used. Following determination of a sample containing equal amounts of reference androsterone and androstanediol (for response factor determination), an appropriate fraction of the urinary steroids was analysed with the internal standard. Small peaks were given by both ions (360 and 270) at the correct retention time for androsterone and from this analysis it was estimated that the combined daily excretion rate of androsterone and aetiocholanolone was 13 μg.

The selected-ion-monitoring method for 3α,18,21-trihydroxy-5β-pregnane-11,20-dione was used for determination of tetrahydrocortisone since the same internal standard 3β-*allo*-tetrahydrocortisone was appropriate for both steroids. The ions at *m/e* 609 (M^+) and 578 (M − 31) were determined. It was possible to positively identify tetrahydrocortisone although the excretion rate was extremely low (about 30 μg/24 h).

We attempted to identify 3β-hydroxy-5-ene C_{19} steroids since it is possible that the 17α-hydroxylase that acts on pregnenolone may differ from the progesterone enzyme, enabling C_{19} steroids to be synthesized. We searched the data for ions indicative of dehydroepiandrosterone (DHA), 16α-hydroxyDHA and 5-androstene-3β,16α,17β-triol but with negative results. However, an excretion of DHA by a patient with this disorder has been described [19].

Analysis of tetrahydroaldesterone was determined using the selected-ion-monitoring method described by Honour and Shackleton [10] using 3β-*allo*-tetra-

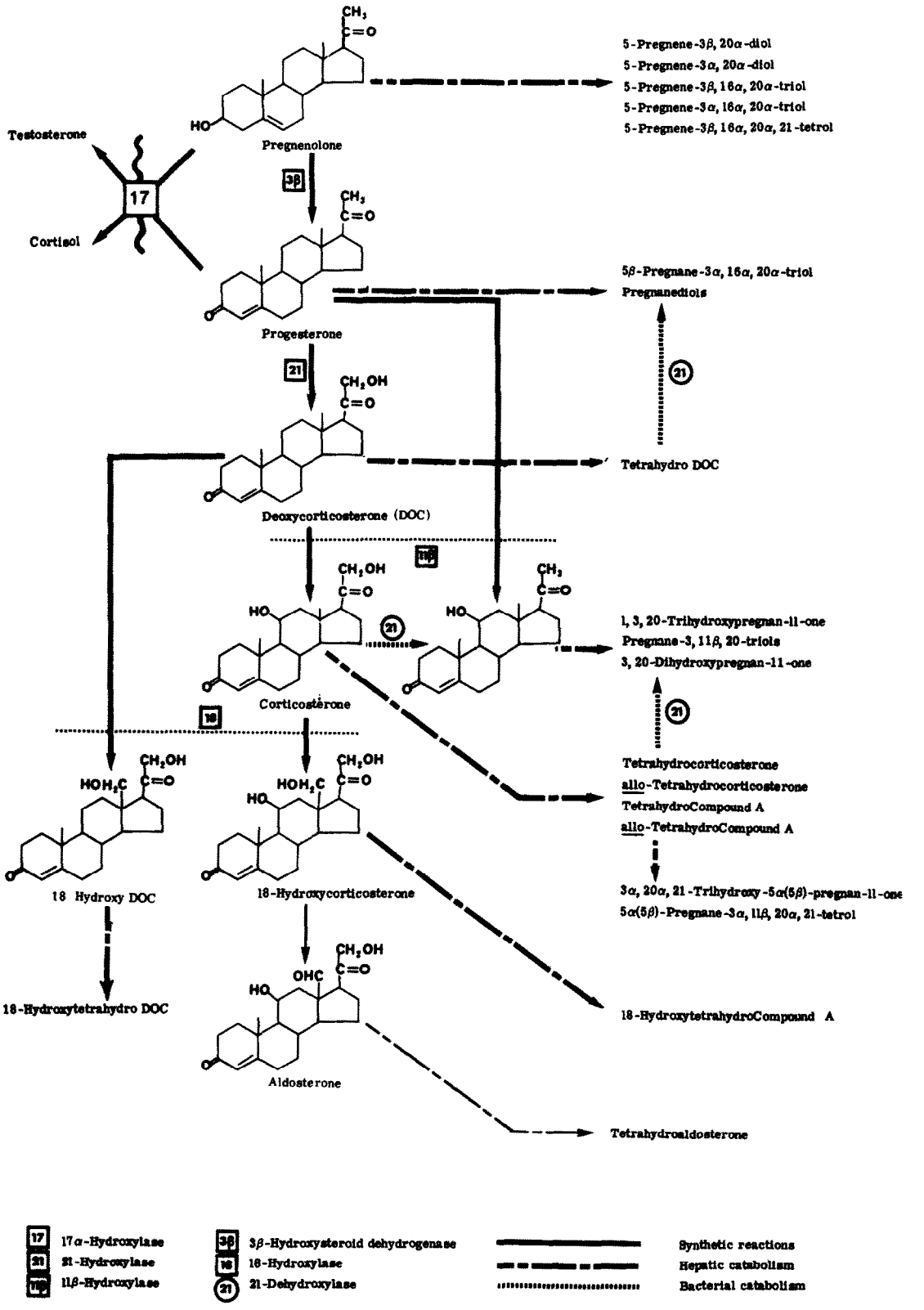

Fig. 4. Biosynthetic and metabolic pathway in 17α-hydroxylase deficiency.

hydroaldosterone as internal standard. The result obtained was extremely low and indicated an excretion rate of approximately 2 μg/24 h.

Steroids released by solvolysis

Very little steroid was recovered by solvolysis after enzymic hydrolysis. However, the following steroids

were identified: pregnenediol, pregnanediol, two 11-oxo-pregnanediols, 16α-hydroxypregnenolone, 5-pregnene-3β,16α,20α-triol, *allo*-tetrahydrocorticosterone and a corticosterone metabolite with long retention time tentatively identified as 3β-*allo*-tetrahydrocorticosterone. These compounds were not quantified.

DISCUSSION

Multicomponent steroid profile analysis by gas chromatography demonstrated an abnormal, but predictable, steroid excretion pattern. The overall results of this disorder on adrenal steroid synthesis, hepatic metabolism and metabolism during enterohepatic circulation is illustrated in Fig. 4. Neither pregnenolone nor progesterone are hydroxylated at position 17 and therefore cannot act as precursors to cortisol, C_{19} or C_{18} steroids. Those steroids not dependent on 17α-hydroxylation (e.g. progesterone, DOC and corticosterone) are secreted in elevated amounts and abnormal adrenal steroids may be secreted and metabolised. 11-Oxygentated 21-deoxycompounds (e.g. pregnane-3α,11β,20α-triols, 3α,20α-dihydroxy-5β(and α)-pregnan-11-one and 1ξ,3ξ,20ξ-trihydroxypregnan-11-one) have not previously been identified in urine from patients with this disorder. These may be formed by hepatic metabolism of adrenal 11β-hydroxyprogesterone or 21-dehydroxylation of corticosterone or metabolites of corticosterone. Several studies have demonstrated that 3α,20α-dihydroxy-5β-pregnan-11-one is a metabolite of 21-hydroxy-4-pregnene-3,11,20-trione or corticosterone [13, 14, 20], and since there is no evidence available which suggests that 21-dehydroxylation can be carried out by mammalian tissue, they must be formed by bacterial metabolism following biliary excretion. The 21-deoxy corticosterone metabolites may be excreted in the faeces or returned to the liver for further metabolism or conjugation. They may then be passed on to the blood for renal excretion or they may be returned to the bile and undergo several more enterohepatic circulations. Similarly, the pregnanediols identified could partially be formed from DOC since 5β-pregnane-3α,20α-diol glucuronide has been shown to be a DOC metabolite [21, 22]. A recent publication by Bokkenheuser *et al.* [23] describes the 21-dehydroxylation of DOC by human faecal flora preparations and these workers have recently identified the active organism as *Eubacterium lentum* (W. G. Kelly, personal communication). Eriksson in a thesis containing a series of publications, reports at length on 21-dehydroxylation of corticosterone metabolites by coecal and faecal flora of rats [24]. The results described relating to the biliary excretion and metabolism of *17-deoxy* corticosteroids are at variance with results obtained following administration of labelled cortisol which is only minimally excreted in the bile and is not subjected to 21-dehydroxylation to a significant extent [25]. Laatikainen (1970) confirmed this observation by failing to identify cortisol metabolites in human bile even though the tetrahydro metabolites of corticosterone were identified [26].

At this stage it is not possible to determine what proportion of the 21-deoxy steroids found in the urine are formed from adrenal steroid precursors (e.g. progesterone or 11β-hydroxyprogesterone) or are formed by 21-dehydroxylation. Answers to these questions must await determination of the specific activity of each of the urinary metabolites isolated following administration of labelled DOC or corticosterone. It is also probable that there will be considerable variation between individuals and a significant effect of diet on these excretions.

The severe or complete inability to make cortisol results in the increased release of and/or production of ACTH, thereby increasing the secretion of the mineralocorticoid hormones which have no feedback control. Aldosterone production, however, is reduced because the high level of DOC secretion causes suppression of renin release. During the period of the urine collection (dexamethasone withdrawn for 15 days), both the plasma renin activity and plasma aldosterone concentration were low at 3.3 ng/L/min and <20 pg/ml respectively, both measurements being taken under supine conditions. This agrees with similar data on the slow recovery of aldosterone secretion reported by Biglieri and Mantero [19] and Bricaire [8]. A major discrepancy exists between the result of the corticosterone secretion rate previously reported (250 mg/24 h) [4] and the results of urinary steroid measurements reported here. Even if the results for all the known and suspected corticosterone metabolites are summed, the figure is only about 30 mg, or 12% of the daily secretion rate. This is not atypical since similar discrepancies have been found in almost all 17α-hydroxylase deficiency syndromes so far described. Admittedly, the multicomponent urinary assay was carried out on a different urine sample than was used for the secretion rate determination and may have been obtained before the results of dexamethasone suppression had completely worn off, thereby reducing the steroid output. However, it is unlikely that this would be completely responsible for the large difference. A high faecal excretion of corticosterone metabolites is also unlikely since we have found that the recovery of radioactivity in urine during the excretion rate measurement was relatively high ($>65\%$ of injected labelled corticosterone dose). Probable explanations are that new corticosterone metabolites are present in fractions discarded during the work-up procedures or that known corticosterone metabolites are excreted as conjugate types not hydrolysed by the enzyme preparations.

Although recent work has demonstrated that steroid acids are quantitatively important metabolites of cortisol in man [27–30], it is believed that relatively little corticosterone is metabolised in this fashion [27]. In addition determination of secretion rates of corticosterone is subject to greater errors than those obtaining for cortisol since the basic assump-

tions underlying the analysis do not apply; for example enterophepatic and bacterial metabolism probably ensure that the fraction of corticosterone converted to a given metabolite is far from constant during the course of the experiment. Even in cortisol secretion rate determinations where single compartment metabolism applies, different specific activities are obtained for different urinary cortisol metabolites following administration of labelled precursor [31].

The available data on steroids in patients with 17α-hydroxylase deficiency have been reviewed by Biglieri [2, 19]. The data, collected from publications by several authors, indicates that in most cases some urinary analyses had been carried out. The results reported are interesting in that they suggest that the enzyme deficiency is often far from complete since 17-oxosteroids and metabolites of cortisol were frequently found; for example, Goldsmith [32] reports 17-oxosteroid and cortisol metabolite excretions of 8.0 mg/24 h and 1.6 mg/24 h, respectively, and the corresponding figures obtained for a patient studied by New [33] were 5.9 and 2.7 mg/24 h. However, the accuracy of the figures reported for 17-oxosteroid and cortisol metabolite excretions may be questioned since the specificity of the tests has not been proven in the presence of the large amounts of progesterone and corticosterone metabolites excreted. As far as could be determined our patient had a virtually complete deficiency of 17α-hydroxylase since androsterone, aetiocholanolone and tetrahydrocortisone were present in extremely low concentration, results which were in agreement with the extremely low levels of plasma C_{19} steroids and cortisol previously found [4]. Whether the block is invariably almost complete must await analyses of further urine specimens collected from patients with the disorder. If the block is generally total, then the disorder differs considerably from the 21-hydroxylase defect which is frequently incomplete, and appear often to affect only the 21-hydroxylase which acts on 17α-hydroxyprogesterone. Our study has demonstrated that in this patient, at least, little 17-hydroxylation of pregnenolone or progesterone occurs.

Although aldosterone secretion in patients with this disorder remains low for a long period after discontinuation of therapy, secretion of 18-hydroxycorticosterone is generally increased under the conditions of the treatment [8, 19]. In our patient during the period of withdrawal from dexamethasone treatment, the urinary excretion of 3α,18,21-trihydroxy-5β-pregnane-11,20-dione was in the normal range for adults. However, 18-hydroxytetrahydroDOC was excreted in elevated amounts and may reflect the increase in ACTH activity during the withdrawal period.

Further studies are in progress to determine the urinary excretion of steroids by other patients with this disorder in order to verify results previously obtained by more standard laboratory procedures. These data will be the subject of a future communication.

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REFERENCES

1. Biglieri E. G., Herron M. A. and Brust M.: 17-Hydroxylation deficiency in man. *J. Clin. Invest.* **45** (1966) 1946–1954.
2. Biglieri E. G.: Adrenal enzymatic defects resulting in hypertension. *In*: *Hypertension*: *Physiopathology and Treatment* (Edited by J. Genest, E. Koin and O. Kuchel). McGraw-Hill, New York (in press).
3. Shackleton C. H. L. and Honour J. W.: Simultaneous estimation of urinary steroids by semi-automated gas chromatography. Investigation of neonatal infants and children with abnormal steroid synthesis. *Clin. chim. Acta* **69** (1976) 267–283.
4. Tourniaire J., Audi-Parera L., Loras B., Blum J., Castelnovo P. and Forest M. G.: Male pseudohermaphroditism with hypertension due to a 17α-hydroxylation deficiency. *Clin. Endocr.* **5** (1976) 53–61.
5. Bush I. E.: *Chromatography of Steroids*. Pergamon, Oxford (1961).
6. Setchell K. D. R. and Shackleton C. H. L.: The group separation of plasma and urinary steroids by column chromatography on Sephadex LH-20. *Clin. chim. Acta* **47** (1973) 381–388.
7. Axelson M. and Sjövall J.: Separation and computerised gas chromatography-mass spectrometry of unconjugated neutral steroids in plasma. *J. steroid Biochem.* **5** (1974) 733–738.
8. Bricaire H., Luton J. P., Laudat P., Legrand J. C., Turpin G., Corvol P. and Lemmer M.: A new male pseudohermaphroditism associated with hypertension due to a block of 17α-hydroxylation. *J. clin. Endocr. Metab.* **35** (1972) 67–72.
9. Shacketon C. H. L. and Honour J. W.: Identification and measurement of 18-hydroxycorticosterone metabolites by gas chromatography–mass spectrometry. *J. steroid Biochem.* **8** (1977) 199–203.
10. Honour J. W. and Shackleton C. H. L. Mass spectrometric analysis for tetrahydroaldosterone. *J. steroid Biochem.* **8** (1977) 299–305.
11. Vihko R.: Gas chromatographic–mass spectrometric studies on solvolyzable steroids in human peripheral plasma. *Acta endocr., Copenh.* **52** Suppl. 109 (1966).
12. Gustafsson J.-Å., Shackleton C. H. L. and Sjovall J.: Steroids in newborn and infants. C_{19} and C_{21} steroids in faeces from infants. *Eur. J. Biochem.* **10** (1969) 302–311.
13. Migeon C. J., Sandberg A. A., Carl Paul A., Samuels L. T.: Metabolism of 4-[C^{14}]-corticosterone in man. *J. clin. Endocr. Metab.* **16** (1956) 1291–1298.
14. Engel L. L., Carter P. and Fielding L. L. Urinary metabolites of administered corticosterone. I. Steroids liberated by glucuronidase hydrolysis. *J. biol. Chem.* **213** (1955) 96–106.
15. Eriksson H.: Steroids in germfree and conventional rats. Unconjugated metabolites of 4-[^{14}C]-pregnenolone and 4-[^{14}C]-corticosterone in faeces from female rats. *Eur. J. Biochem.* **16** (1970) 261–267.
16. Shackleton C. H. L. and Snodgrass G. H. A. I. Steroid excretion by an infant with an unusual salt-losing syndrome: A gas chromatographic–mass spectrometric study. *Ann. clin. Biochem.* **11** (1974) 91–99.
17. Eriksson H.: Steroids in germfree and conventional rats. Metabolism of 4-[^{14}C]-pregnenolone and 4-[^{14}C]-corticosterone in urine and faeces from male rats. *Eur. J. Biochem.* **18** (1971) 86–93.

18. Bournot P., Prost M. and Maume B. G. Separation and characterisation of the reduced metabolites of the 18-hydroxy deoxycorticosterone hormone by gas liquid chromatography–mass spectrometry. Occurrence of stereoisomeric forms in rat adrenals and liver. *J. Chromatogr.* **112** (1975) 615–630.
19. Biglieri E. G. and Mantero F.: The characteristics, course and implication of the 17-hydroxylation deficiency in man. *In*: *Research in Steroids*, V (Edited by M. Finkelstein). Societa Editrice Universo, Roma (1977) pp. 385–399.
20. Harris J. J., Hoegel C. and Crane M. G.: Deoxycorticosterone secretion rates before and during metyrapone administration in normal subjects. *J. clin. Endocr. Metab.* **27** (1967) 106–113.
21. Campagnoli C., De Martinis C. and Doglio R.: Dimostrazione della conversione *in vivo* del desossicorticosterone in pregnandiol. *Folio Endocr.* **18** (1965) 550–555.
22. Horvitt B. N., Dorfman R. I., Shipley R. A. and Fish W. R.: Metabolism of the steroid hormones. IV. Conversion of deoxycorticosterone to pregnanediol 3α,20α in man and in the chimpanzee. *J. biol. Chem.* **155** (1944) 213–218.
23. Bokkenheuser V. D., Winter J., Dehazya P., De Leon O. and Kelly E. G.: Formation and metabolism of tetrahydrodeoxycorticosterone by human faecal flora. *J. steroid Biochem.* **7** (1976) 837–843.
24. Eriksson H.: Thesis entitled: Studies on the Metabolism of Steroid Hormones in the Rat. Opuscula Medica, Stockholm (1971).
25. Migeon C. J., Sandberg A. A., Decker H. A., Smith D. K., Paul A. C. and Samuels L. T.: Metabolism of 4-[^{14}C]-cortisol in man: body distribution and rates of conjugation. *J. clin. Endocr. Metab.* **16** (1956) 1137–1150.
26. Laatikainen T.: Identification of $C_{21}O_3$ and $C_{21}O_4$ steroids in human bile. *Eur. J. Biochem.* **14** (1970) 372–378.
27. Bradlow H. L., Zumoff B., Monder C., Lee H. J. and Hellman L.: Isolation and identification of four new carboxylic acid metabolites of cortisol in man. *J. clin. Endocr. Metab.* **37** (1973) 811–818.
28. Monder C., Zumoff B., Bradlow H. L. and Hellman L.: Studies on the biotransformation of cortisol to the cortoic acids in man. I. Metabolism of 21-dehydrocortisol. *J. clin. Endocr. Metab.* **40** (1975) 86–92.
29. Weiss C., Monder C. and Bradlow H. L.: 17-Deoxygenation: a new pathway of cortisol metabolism. Isolation of 17-deoxycortolonic acids. *J. clin. Endocr.* **43** (1976) 696–699.
30. Zumoff B., Monder C. and Bradlow H. L.: Studies on the biotransformation of cortisol to the cortoic acids in man. II. The central role of tetrahydrocortisol and tetrahydrocortisone as intermediates. *J. clin. Endocr.* **44** (1970) 647–650.
31. Kelly W. G.: Questions concerning the validity of one of the assumptions underlying the determination of the secretory rate of cortisol. *Steroids* **16** (1970) 579–602.
32. Goldsmith O., Solomon D. H. and Horton R.: Hypogonadism and mineralocorticoid excess. The 17-hydroxylase deficiency syndrome. *New Engl. J. Med.* **277** (1967) 673–677.
33. New M.: Male pseudohermaphroditism due to 17α-hydroxylase deficiency. *J. clin. Invest.* **49** (1970) 1930–1941.