Isolation of 16 OH Pregnenolone from Urine of Newborn Infants.* (28550)

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Hydroxylation at carbon 16 has been shown to be a prominent route of progesterone metabolism in *in vitro* studies of human fetal adrenals(1) and hyperplastic adrenal tissues from patients with Cushing's disease (2). A number of 16 hydroxylated C-21 steroids have been isolated from human urine. Most have been found in urine from pregnant women(3) and in urine from patients with adrenal tumors(4).

The fetal adrenal cortex has been shown by *in vitro* studies to be relatively deficient in the 3β hydroxy steroid dehydrogenase system(5). Not until after mid-gestation is an appreciable amount of steroid with the Δ4-3 keto configuration produced. Large excretions of neutral steroids with the Δ5-3β ol configuration have been reported for young infants with congenital adrenal hyperplasia (CAH). This finding has been made in patients with 3β hydroxy steroid dehydrogenase deficiency(6) and in patients with the more common C-21 hydroxylase deficiency(7).

The present paper is concerned with the isolation and identification of 3β,16α-Dihydroxypregn-5-ene-20-one (16 OH pregnenolone). This C-21 steroid, containing both a hydroxyl group at carbon 16 and the Δ5-3β ol configuration, was found to be excreted in large amounts by an infant with the C-21 hydroxylase deficiency type of congenital adrenal hyperplasia. It was also identified as a prominent excretory product of an endocrinologically normal newborn male. To our knowledge, the only prior isolation of this steroid from human biological material was by Bongiovanni(6), who reported its presence in the urines of 2 infants with the 3β hydroxy steroid dehydrogenase deficiency type of congenital adrenal hyperplasia.

Materials and methods. A series of complete 24-hour urine collections from 2 patients in University of Minnesota Hospitals was studied.

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Patient No. 1 was admitted at age 4 days with findings of female pseudohermaphrodism, including a urogenital sinus. Because of signs of impending salt-losing crisis (serum Na—122 meq/l, serum K—7.2 meq/l) DOCA therapy, 5 mg/day, was started on the 8th day of life. These physical and chemical signs were diagnostic for CAH. Adrenal suppression by I.M. Dexamethasone was started on the 12th day of life.

Patient No. 2 was an infant male admitted on the 2nd day of life with signs of intestinal obstruction. Jejunal atresia was found at surgery and his postoperative course was complicated only by 2 episodes of partial intestinal obstruction relieved by oral intubation. His subsequent course has shown him to be endocrinologically normal.

All urine samples were collected on ice and frozen at –20°C until processed. Each aliquot was adjusted to pH 4.6, 1/3 volume pH 4.6 acetate buffer and 350 units of commercial beta-glucuronidase (Ketodase®) per ml urine were added, and incubation at 37° was carried out for 48 hours. The beta-glucuronidase released steroids were recovered by extraction 3 $\times$ with 1 vol ethyl acetate. The ethyl acetate solvolysis procedure of Burstein and Lieberman(8) was then carried out on the urine residue, releasing the sulfate-conjugated steroids.

The unknown compounds, each subsequently identified as 16 OH pregnenolone, were isolated from the steroids released by ethyl acetate solvolysis of urine samples from patients 1 and 2. The compounds had migration rates in the following paper chromatography systems identical to an authentic sample of 16 OH pregnenolone: NB-1(9), NB-2 (9), cyclohexane-dioxane-MeOH-H_2O (4:4:2:1), benzene-MeOH-H_2O (4:2:1). Aliquots of the unknowns were acetylated in acetic anhydride:pyridine (1:1) and migration rates were identical to that of the authentic sample, acetylated in parallel, in the E-1(9) system. After

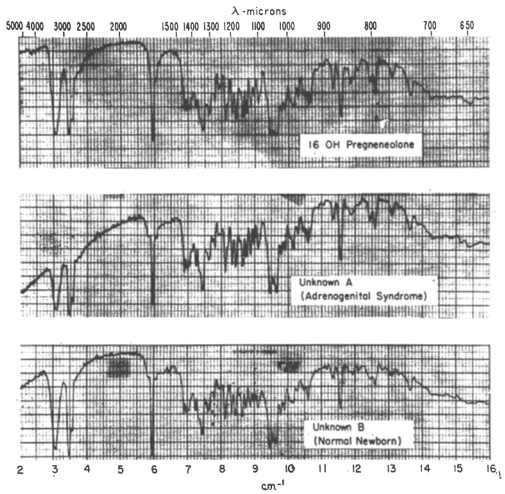

FIG. 1. Infra-red spectra of standard 16 OH pregnenolone and unknown compounds isolated from Patients 1 and 2.

purification on a silica gel column, aliquots of the unknown steroids were subjected to the Oertel reaction(10) and to concentrated H_2SO_4. Sharp 407 mμ peak absorptions were present with the Oertel reagent, indicating a Δ5-3β ol configuration. The chromogens formed with concentrated H_2SO_4 were identical to that formed with the authentic standard as recorded on a Beckman DK Recording Spectrophotometer.

Infra-red spectra were obtained on each of the samples that had been tentatively identified as 16 OH pregnenolone. Each sample was recrystallized twice from methanol prior to recording the spectra on a Beckman IR5A instrument using micro KBr pellets. The spectra of the samples and of standard 16 OH pregnenolone are shown in Fig. 1. Positive identification of 3 other steroids isolated from the urines of patient 1 were obtained by infra-red spectral analysis. These steroids were pregnane 3α, 17α, 20α triol (pregnanetriol), 3α,17α-Dihydroxypregnane-20-one (17 OH pregnaneolone), and 3β,16α-Dihydroxyandrost-5-ene-17-one (16 OH DHIA).

16 OH pregnenolone was quantitated in a series of urine samples collected from each patient. The steroid was isolated from the fraction released by ethyl acetate solvolysis following extraction of the beta glucuronidase released steroids. Following purification of the fraction by a modification of the Herrmann and Silverman florisil column procedure (11), the extract was chromatographed on paper in the NB-1(9) system for 24 hours. This single paper chromatographic step was found to give excellent separation of the 16 OH pregnenolone. The area corresponding to 16 OH pregnenolone was eluted, the residue was purified on a silica gel column modified from that described by Goldzieher(12), and the steroid was quantitated by the Oertel reaction(10) using authentic 16 OH pregnenolone as the standard.

Pregnanetriol and 17 OH pregnaneolone were quantitated in the series of urine samples collected from the patient with CAH. The steroids were isolated from the fraction released by beta glucuronidase. The extract was fractionated on florisil columns by a modification of the Herrmann and Silverman procedure(11) and the eluate containing the two steroids was chromatographed on paper in the NB-2 system(9). The area corresponding to pregnanetriol was eluted, fractionated on a silica gel column per the procedure described by Goldzieher(12) and quantitated by its H_2SO_4 chromogen using pregnanetriol as the standard. The area corresponding to 17 OH pregnaneolone was eluted, then reacted with potassium borohydride(13), the 17 OH pregnaneolone being converted to pregnane 3α,17α,20β triol. This latter compound was quantitated by its H_2SO_4 chromogen, using pregnane 3α,17α,20α triol as the standard.

Results. Fig. 1 shows the infra-red spectra of the solvolysis released steroids isolated from patient 1 (Unknown A) and patient 2 (Unknown B), and the authentic standard sample of 16 OH pregnenolone. The spectra of Unknowns A and B are essentially identical to that of the authentic 16 OH pregnenolone. The infra-red spectral identification of pregnanetriol and 17 OH pregnaneolone in urine of patient 1 and the quantitation of these steroids as shown in Table I identify this patient as having the C-21 hydroxylase deficiency type of CAH. Neither pregnanetriol nor 17 OH pregnaneolone could be found in the urine samples collected from patient 2.

Table I lists the quantities of 16 OH preg-

TABLE I. Urinary Excretion of Steroid Metabolites in mg/24 Hr.

Patient 1				Patient 2	
Age (days)	16 OH PG*	P'triol†	17 OH PG‡	Age (days)	16 OH PG*
7	8.1	.62	1.2	4	.81
8	3.7	.46	1.2	6	.21
9	7.4	.70	2.5	8	.23
10	5.0	.98	2.7	12	.25
11	6.5	.83	1.6	15	.36
15	.03	.00	.0		

* 16 OH PG = 16 OH pregnenolone.
† P'triol = pregnanetriol.
‡ 17 OH PG = 17 OH pregnaneolone.

nenolone excreted daily in the urine samples examined. The 16 OH pregnenolone was present in amounts varying from 3.7 to 8.1 mg/day before adrenal suppressive therapy in patient 1. It was by far the most abundant steroid in the steroid fraction released by ethyl acetate solvolysis in this patient. In the endocrinologically normal newborn, the peak excretion of 16 OH pregnenolone was 0.81 mg/day, 2 days after abdominal surgery. The excretion then dropped to levels of 0.21 to 0.36 mg/day over the next 9 days.

Discussion. The present paper contains the first reports of the identification of 16 OH pregnenolone in a normal newborn infant and in an infant with the C-21 hydroxylase deficiency type of CAH. This steroid was presumably excreted as a sulfate conjugate because it was recovered from the urine by ethyl acetate solvolysis, following extraction of beta-glucuronidase released steroids. Eberlein(7) has presented evidence that young infants with CAH excrete large amounts of sulfate conjugated steroids. He found in 3 infants less than 15 days of age an excretion of 2.7 to 14.7 mg/day of sulfate conjugated steroids measured by the Oertel reaction but not by the Zimmermann reaction. In our patient 1, 16 OH pregnenolone was by far the most abundant sulfate conjugated steroid and it was excreted in amounts varying from 3.7 to 8.1 mg/day. Thus it could make up much of the sulfate conjugated fraction quantitatively but not fully qualitatively defined by Eberlein(7).

The isolation of 16 OH pregnenolone from urine excreted by the endocrinologically normal newborn infant is consistent with an active 16 hydroxylation system in fetal adrenal glands(1) and with a relative deficiency in the fetal adrenal 3β hydroxy steroid dehydrogenase system as indicated by the prominent labeling of Δ5-3β ol steroids after incubation of fetal adrenals with acetate-^{14}C(5). The finding of much greater amounts of 16 OH pregnenolone in the patient with CAH than in the normal newborn male may be the result of the marked ACTH stimulation of the adrenal cortex that occurs in CAH and thus may be considered an exaggeration of the normal neonatal pattern of steroid excretion. Studies are in progress to define further the changes in 16 OH pregnenolone excretion that occur with increasing age and with adrenal cortical stimulation in normal newborn infants and in patients with CAH.

Summary. Isolation and identification of 3β,16α-Dihydroxypregn-5-ene-20-one (16 OH pregnenolone) in the urine of 2 young infants are described. Patient 1, a female pseudohermaphrodite with CAH, excreted 3.7-8.1 mg/day prior to adrenal suppression therapy. Patient 2, an endocrinologically normal newborn male with intestinal obstruction, excreted 0.21-0.81 mg/day. It is concluded that the high excretion of 16 OH pregnenolone in the patient with CAH is an exaggeration of the normal neonatal pattern secondary to the marked adrenal ACTH stimulation in untreated patients with CAH.

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Effects of Chlorothiazide on Renal Ammonia Production, Plasma Electrolytes and Acid Base Balance in Acute Renal Failure. (28551)

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The metabolism of the anuric kidney has not been studied systematically. It has been postulated that the anuric kidney is in a state of hibernation, the low oxygen consumption being accounted for by metabolic processes unrelated to glomerulo-tuberlar metabolism (1). Recent investigations have suggested that the major part of renal oxygen consumption is needed for reabsorption of filtered sodium(2,3). If this is correct and oxygen consumption by the anuric kidney is very low it is necessary to assume that filtration must have ceased in the anuric kidney. More recently higher values of oxygen consumption by the anuric kidney have been reported(4) and the hypothesis of the 'resting' state has been questioned. Therefore, it was decided to see if the anuric kidney produced ammonia and to determine the effect of chlorothiazide in renal ammonia production, plasma electrolytes and acid base balance in patients with acute renal failure. Measurements were made either before or after treatment by haemodialysis and the same intravascular catheters were used for metabolic determination and for haemodialysis.

Methods. Five patients with acute oliguric renal failure were studied during the first week of the onset of oliguria. Endogenous creatinine clearances were less than 1 ml per minute on the day of the study and no urine was voided during the study or for the subsequent 16 hours. Renal blood flow was measured using a constant infusion of indocyanine green (IG) into the renal artery(5) following its catheterisation under fluoroscopic control by a percutaneous femoral arterial radiopaque teflon catheter(6) and the catheterisation of the corresponding renal vein following femoral vein puncture. A loading dose of IG 0.3 mg per kg body weight was given intravenously and it was then infused at 0.5 mg per minute (in a volume of 1 ml) into the renal artery catheter by a Sigma constant infusion pump. After a 20-minute equilibration period, samples were obtained anaerobically from the renal vein and femoral arterial catheters (allowing for dead space sampling times). Two control samples were obtained (minus 10 minutes and 0 time), then 1000 mg of chlorothiazide were administered intravenously in a volume of 50 ml. Subsequent samples were obtained at 10 and 20 minutes after chlorothiazide administration. Arterial and renal venous blood were analysed for IG(7), ammonia (NH_3)(8), oxygen content(9) and arterial blood for pH, bicarbonate and pCO_2 (10) and plasma sodium and potassium using an E.E.L. flame photometer. Renal blood flow (RBF) was calculated for one kidney from the renal venous-arterial IG difference and infusion rate of IG using the indirect Fick principle(5). The renal venous-arterial NH_3 difference and RBF were used to calculate renal vein NH_3 release ($\mu g/min/1.73M^2$) and renal oxygen consumption (ROC) was obtained from RBF values and arterial-renal venous oxygen content difference $(A\text{-}RV)O_2$.