Elevated Urinary Testosterone and Androstanediol in Precocious Adrenarche

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Extract

Using a newly devised radioligand method for the simultaneous determination of urinary testosterone (T) and androstanediol (Adiol) nine girls with precocious adrenarche were evaluated. In the base-line state average urinary T excretion (1.29 $\mu\text{g}/24$ hr) and Adiol excretion (1.33 $\mu\text{g}/24$ hr) were significantly elevated when compared with 15 age-matched controls (0.30 and 0.33 $\mu\text{g}/24$ hr, respectively, $P < 0.001$ for both). Adrenocorticotrophic hormone (ACTH) infusion performed in five patients with precocious adrenarche produced at least a 50% increase in urinary T excretion in all and a similar increase in Adiol excretion in four of five patients. Dexamethasone administration in the same five patients produced a 25% fall in urinary T excretion in all and a comparable fall in Adiol in four.

Speculation

This study documents elevated urinary T and Adiol excretion in precocious adrenarche in the base-line state. An adrenal source for these androgens is suggested by the response to ACTH stimulation and dexamethasone suppression.

Pubic or axillary hair before age 8 in girls or age 9 in boys, without the appearance of other secondary sex characteristics, has been called precocious adrenarche (20). Silverman *et al.* (18) found that girls greatly outnumber boys in development of this characteristic (28 out of 29) and that there was a high incidence of mental deficiency (7 out of 29). An abnormal electroencephalogram was noted in 5 of 10 patients studied by Liu and coworkers (9). Height age and bone age are slightly advanced when compared with the chronologic age. The onset of true puberty occurs at the usual age. Urinary excretion of 17-ketosteroids has been reported to be elevated by most investigators (4, 18, 22) but not all (1, 2). Individual urinary 17-ketosteroids, androsterone, etiocholanolone, and dehydroepiandrosterone have been shown to be elevated (2, 22, 23), although urinary T has been normal (2, 22). Because these ketosteroids arise from the adrenals, an adrenal cause for precocious adrenarche has been postulated.

We have studied girls with precocious adrenarche in the base-line state, after ACTH stimulation, and after dexamethasone suppression to explore further the role of the adrenals in this condition. Since single plasma values of androgens are influenced by diurnal variation and episodic secretion, the study utilized 24-hr urine collections to more nearly represent the daily "integrated" value of adrenal secretion. Urinary T and Adiol were measured using a newly devised technique of high sensitivity and specificity.

EXPERIMENTAL PROCEDURE

MATERIALS

[1,2,6,7-³H]T, [4-¹⁴C]T, [1,2-³H]androsterone (24), and unlabeled steroids (25) were purified on paper chromatography before

use. [1,2-³H]Adiol was prepared from [1,2-³H]androsterone by NaBH₄ reduction and purified on paper chromatography (12). The sex hormone binding globulin was obtained from third trimester pregnant women, pooled, and stored in aliquots at -20° before use. Methanol and methylene chloride (distilled in glass (26)) were used as purchased. Cyclohexane, benzene, and mesitylene were reagent grade and purified on silica gel columns (11).

CHROMATOGRAPHY

Two systems of paper chromatography using Whatman no. 1 paper were used for purification of the urine extract. *System 1* (cyclohexane-benzene-methanol-water, 10:4:10:2) was a straight phase system that separated T from Adiol and other 17 β -OH androgens, whereas *system 2* (mesitylene-methanol-water, 3:2:1) was a reverse phase system that also separated T from Adiol. Textile dyes were used as markers: K & K blue in *system 1* (F-9 (Plastoviolet MR (28)) and Isatin (2,3-diketoinoline (29)) in *system 2*. Tritium standards for T and Adiol were run on a separate page in each tank along with the textile dyes and then located by radiochromatogram scanner (model 7201 (30)).

PREPARATION OF URINE FOR RADIOLIGAND ASSAY

Collected urine was acidified with glacial acetic acid and stored frozen. In each assay, a urine pool from prepubertal children and distilled water for a method blank were assayed. To 25 ml prepubertal urine (5 ml adult urine), 4,500 cpm (26 pg) [1,2-³H]Adiol and 4,500 cpm (12 pg) [1,2,6,7-³H]T were added for recovery. One thousand Fishman units of β -glucuronidase (Keto-dase (31))/ml urine were added for hydrolysis and incubated at pH 5.0 for 24 hr. Six volumes of methylene chloride were used for extraction; this was then washed successively with 1/10 volume 0.1 N NaOH, 0.1 M acetic acid, and distilled water. The dried extract was run in *system 1* for 20 hr; T and Adiol were each then developed in *system 2* for 30 hr. After elution with methanol, dried eluates were dissolved in 1.0 ml 0.1 M sodium phosphate buffer (PBS), pH 7.0. A 0.2-ml aliquot was used to estimate recovery and 0.1-ml and 0.2-ml aliquots for radioligand assay.

RADIOLIGAND PROCEDURE

T and Adiol were assayed in separate radioligand assays. Cold steroid was dissolved in ethanol (10 ng/ml) and pipetted in duplicate to make a standard curve from 0.1 to 5.0 ng. PBS was added to the dried standards and samples to a volume of 0.2 ml and then [³H]T or [³H]Adiol and sex hormone binding globulin were added to bring the total volume to 0.5 ml. The final dilution of the binding globulin was 1:350. After overnight incubation at 4°, the separation of free from bound steroid was achieved with 0.5 ml 0.20 g/100 ml charcoal in PBS for T and 0.25 g/100 ml in PBS for Adiol. The tubes were centrifuged at 3,000 rpm after incubation for 15 min at 4° for 20 min, and the bound steroid was decanted into a counting vial with 10 ml counting fluid (Liquiflor (24)) and counted in a liquid scintillation counter (Packard Tri-Carb model 3375 (30)). A desk-top calculator was used for the computations

using the best linear fit for a logit-logarithmic plot of the standard curve. Water blanks were consistently below the sensitivity of the standard curve and were not subtracted from the value read from the standard curve.

SUBJECTS

Nine girls with precocious adrenarche were evaluated with informed consent as outpatients; five of these were later admitted to the Pediatric Clinical Research Center of The New York Hospital-Cornell Medical Center for further study. In the five patients admitted, after a base-line period, 40 units of ACTH (Acthar (32)) were given by continuous infusion over 8-hr. Dexamethasone, 0.5 mg every 6-hr (1.0 mg every 6 hr for *SM*), was begun the following day for 4 days (7 days for *PS*). Twenty-four-hour urine collections were obtained in the base-line state on the day of ACTH infusion and *day 4* of dexamethasone administration (*day 7* for *PS*). 17-Hydroxysteroids and 17-ketosteroids were determined by previously described laboratory procedures (13, 14). Bone age was read according to the standards of Greulich and Pyle (5). Twenty-four-hour urine collections obtained from 15 age-matched prepubertal children in the base-line state served as controls. Four additional children without adrenal disease as listed in Figure 1 also received ACTH and dexamethasone as part of their endocrine evaluation as inpatients to the Pediatric Clinical Research Center of The New York Hospital-Cornell Medical Center and served as control subjects.

RESULTS

EVALUATION OF METHOD

The specificity of the method depended on the chromatography systems and the sex hormone binding globulin. This protein binds preferentially to C_{19} steroids in the 5α -configuration with a sterically unhindered 17β -hydroxy group and not to C_{21} steroids (6, 8). *System 1* separated T from Adiol and other potentially interfering steroids (3β -androstenediol, $\Delta 5$ -androstenediol, and estradiol). To verify that T and Adiol were effectively separated, [3H]Adiol and [^{14}C]T were added to five urine samples and then processed routinely. After *system 2*, less than 0.5% of either labeled steroid remained unseparated. The sensitivity of the standard curve, based on 10% displacement, was 0.100 ng for T and 0.050 ng for Adiol. This allows detection of 0.02 μg T and 0.01 μg Adiol in a 24-hr urine, assuming the total volume is 500 cc and the recovery 40%. An adrenalectomized prepubertal child had values of 0.08 $\mu g/24$ hr for T and 0.04 $\mu g/24$ hr for Adiol, indicative of the high specificity of the method.

Recovery before radioligand assay was 40–50% of added radiolabeled steroid. Accuracy for samples ranging in content from 2.5–40 ng had a correlation coefficient of 0.999 for T and 0.998 for Adiol. The interassay variation for the prepubertal urine pool was 11.1% for T ($n = 7$) and 9.8% for Adiol ($n = 8$), whereas the interassay variation was 14.5% ($n = 9$) and 15.6% ($n = 10$), respectively.

NORMAL SUBJECTS

In the control group of 15 age-matched prepubertal children ranging in age from $4\frac{1}{2}$ to $7\frac{1}{2}$ years, with an average of $6\frac{1}{2}$ years of age, the daily urinary excretion of T was $0.30 \pm 0.18 \mu g$ (SD) and of Adiol was $0.33 \pm 0.21 \mu g$ (SD), with no significant differences observed in male or female subjects.

PRECOCIOUS ADRENARCHE SUBJECTS

Clinical data for the patients with precocious adrenarche are presented in Table 1. The age range was $4\frac{3}{4}$ to $8\frac{3}{4}$ years and average age, $6\frac{1}{2}$ years, which compared favorably with the control subjects. The bone age was not advanced significantly in any of the patients.

In *patient MP*, height was significantly advanced and in three patients weight was increased (19). In the patients with precocious

adrenarche, urinary T and Adiol were elevated to $1.29 \pm 0.40 \mu g/24$ hr and $1.33 \pm 0.57 \mu g/24$ hr, respectively, both significantly different from the normal controls at $P < 0.001$. Urinary 17-ketosteroids were also increased in the patients with precocious adrenarche to $2.2 \pm 0.8 mg/24$ hr, compared with 15 normal subjects who excreted $1.2 \pm 0.4 mg/24$ hr ($P < 0.005$) while urinary 17-hydroxysteroids were normal (Table 1).

Response to stimulation with ACTH and suppression with dexamethasone in the precocious adrenarche patients along with four control subjects is depicted in Figure 1. In each of the control subjects ACTH increased the 17-ketosteroid, T, and Adiol excretion by at least 50%. Dexamethasone had no effect on the 17-ketosteroids, although T decreased by 25% in three of four of the controls and Adiol in two of four. In precocious adrenarche the base-line values for T and Adiol excretion are all higher than those for the control subjects, and ACTH increased the 17-ketosteroids, T, and Adiol (except Adiol in *LD*), by at least 50% as in the control subjects. Dexamethasone regularly suppressed urinary T and Adiol excretion (T five out of five, Adiol four out of five) but not urinary 17-ketosteroids (one out of five).

DISCUSSION

Urinary T excretion has been measured by many methods but only gas-liquid chromatography, double isotope dilution derivative, and radioligand assay are sufficiently sensitive for use in prepubertal children. Previous investigations have produced results similar to those reported here. A few representative results are reviewed here: Vestergaard *et al.* (21) achieved a sensitivity of 0.02 μg /sample with a gas-liquid chromatography technique that used the entire 24-hr urine. In 10 children, 4–8 years of age, he reported a range of 0.1–0.8 $\mu g/24$ hr. Also employing gas-liquid chromatography, McRoberts *et al.* (10) found values of 0.3–3.9 μg in older children who ranged in age from 7–10 $\frac{1}{2}$ years. Degenhart *et al.* (3) utilized a double isotope dilution derivative method capable of detecting 0.01 μg /sample and obtained values of 0.3–4.2 μg from children ranging in age from 5 $\frac{1}{2}$ to 8 $\frac{1}{2}$ years. A range of 0–2.9 μg

Fig. 1. Hormonal response in patients with precocious adrenarche and control subjects during base-line (B), ACTH (A), and dexamethasone (D) periods.

Table 1. *Clinical and laboratory findings in 9 patients with precocious adrenarche and 15 age-matched control subjects¹*

Subject	Age, yr	Bone age, yr	Height, cm	Weight, kg	24-hr urinary			
					17-Hydroxy-steroids, mg/m ² /24 hr	17-Keto-steroids, mg/24 hr	Testos-terone, μg/24 hr	Andros-tanediol, μg/24 hr
<i>Patients</i>								
<i>LG</i>	4½ ₁₂	5½ ₁₂	108.5 (75) ²	19.7 (90) ²	3.3	1.7	1.12	1.23
<i>SM</i>	4½ ₁₂	4½ ₁₂ –5½ ₁₂	112.7 (75)	28.0 (>97)	1.7	0.7	1.03	0.72
<i>MP</i>	5½ ₁₂	5–5½ ₁₂	120.0 (97)	23.8 (97)	2.9	2.0	1.48	1.98
<i>LC</i>	5½ ₁₂	6½ ₁₂	121.5 (90)	26.5 (>97)	2.2	1.9	1.30	1.55
<i>SR</i>	5½ ₁₂	5½ ₁₂ –6½ ₁₂	119.0 (75)	20.4 (50)	2.3	1.9	0.78	0.65
<i>LD</i>	6½ ₁₂	6½ ₁₂ –7½ ₁₂	119.1 (75)	21.5 (50)	4.4	2.6	2.05	1.63
<i>OL</i>	7½ ₁₂	7½ ₁₂ –8½ ₁₂	128.6 (75)	28.1 (90)	3.2	3.5	1.11	1.20
<i>LK</i>	8½ ₁₂	7½ ₁₂ –8½ ₁₂	125.3 (25)	26.3 (25)	2.4	3.0	1.76	2.24
<i>PS</i>	8½ ₁₂	7½ ₁₂	123.7 (25)	24.1 (10)	2.7	2.1	1.00	0.77
Patients ³	6½ ₁₂				2.8 ± 0.8	2.2 ± 0.8	1.29 ± 0.40	1.33 ± 0.57
Normal subjects ⁴	6½ ₁₂				3.1 ± 1.1 ⁵	1.2 ± 0.4	0.30 ± 0.18	0.33 ± 0.21
	7½ ₁₂							

¹ Average values given ± 1 SD.² Percentile given in parentheses according to work of Stuart (18).³ Average values in precocious adrenarche (*n* = 9).⁴ Average values in normal prepubertal children (*n* = 15).⁵ Normal range for 17-hydroxysteroids.

was found in prepubertal normal children from 4-8 years by Rudd *et al.* (17), who employed sex hormone binding globulin in a sensitive radioligand assay.

The radioligand method described in the present study achieved a sensitivity similar to those methods described above and was more sensitive than most of the previously described techniques for measuring urinary T. The method allows the simultaneous measurement of Adiol and other urinary androgens (Δ^5 -androstenediol) that bind to sex hormone binding globulin. Androgens that can be easily converted to an androgen that binds with sex hormone binding globulin can also be measured, for example, androsterone reduced to Adiol by NaBH₄ as described under *Experimental Procedures*. Because only two chromatographic steps are required the method is relatively simple.

The results in the present work agree with previous work with few exceptions. Higher values reported by some authors (3, 10) may result from the more advanced pubertal status present in older patients. Urinary Adiol has not been previously measured in children.

Individual plasma 17-ketosteroids along with testosterone and dihydrotestosterone (7, 16) have been demonstrated to be elevated in this disorder. This has been confirmed by increased individual ketosteroids (2, 22, 23), and total 17-ketosteroids excretion in the urine. The fourfold increase in urinary T and Adiol found in this study in precocious adrenarche, when compared with values for control subjects, is greater than the twofold increase in plasma T observed by Rosenfeld (16) and Korth-Schutz *et al.* (15). This demonstrates the usefulness of the determination of 24-hr excretions of urinary androgens in detecting conditions of androgen excess.

An adrenal cause for this condition has been suspected because (1) in the prepubertal child these steroids are mainly of adrenal origin and (2) the administration of dexamethasone in a few patients resulted in decreased plasma and urine androgens (2, 15, 16). This study demonstrates elevated urinary T and Adiol in the base-line state in nine patients and further confirms the evidence for an adrenal cause in precocious adrenarche by demonstrating ACTH dependence. The urinary excretion of these androgens provides a diagnostic index for precocious adrenarche and suggests the possibility that the precursors of urinary T and Adiol excretion may play a role in the growth of sexual hair.

SUMMARY

Urinary T and Adiol excretion has been measured by a new radioligand method in nine patients with precocious adrenarche. Significant increase in the base-line excretion of both androgens was found when compared with age-matched controls. Administration of ACTH to five patients resulted in a large increase in the urinary excretion of T (five out of five patients) and Adiol (four out of five), whereas dexamethasone produced a significant fall in both T (all five), and Adiol (four out of five). This work supports the hypothesis that the adrenal gland is the source of the excess androgens in precocious adrenarche.

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Lung
plethysmography
premature infants

rebreathing
thoracic gas volume

An Analysis of a Rebreathing Method for Measuring Lung Volume in the Premature Infant

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Extract

Functional residual capacity (FRC) and thoracic gas volume (TGV) were measured in 20 infants, of whom 11 were healthy preterm infants, 5 were recovering from the respiratory distress syndrome (RDS), and 4 had other pulmonary problems. In addition, some of the theoretical aspects of rebreathing techniques, including the lung to bag N_2 difference at equilibrium, were studied by constructing a simple digital computer model of the system.

In both the normal preterm infants and the post-RDS group, the TGV was significantly greater than the FRC ($0.02 > P > 0.01$), indicating the presence of trapped gas (Fig. 2). The mean time taken to reach equilibrium during rebreathing was 44 sec in the normal infants and 52 sec in the post-RDS group (Table 2). In one infant with generalized cystic lung disease, equilibrium was not achieved even after 3 min of rebreathing (Fig. 1).

The computer-generated analysis of rebreathing for a normal 3-kg infant is shown in Figure 3, with an *in vivo* curve for comparison. The rapid equilibration of N_2 was completed within 55 sec but, as in the *in vivo* experiments, there was a change in slope of the line after 1.7 min. It can be seen from Table 3 that, if a small initial bag volume (100 ml) is used, 30 sec of rebreathing is adequate for equilibration only when FRC and dead space are normal, and errors would occur if either were enlarged.

The computer study showed that the gradient for N_2 between bag and lung at equilibrium is of the order of 0.2-0.37% which would

cause a negligible error when calculating FRC and assuming that lung N_2 is equal to the measured bag N_2 .

The combined *in vivo* and computer studies served to validate the rebreathing technique as a method for measuring FRC, and have enabled it to be modified for use even in small or very sick infants with poor ventilation. The most useful procedure to employ would seem to be to use an initial bag volume of 150-200 ml and to continue the rebreathing for 1.5-2 min, with samples being taken for analysis at approximately 20-sec intervals.

Speculation

Computer simulation of rebreathing by infants could be used to explore gas mixing under various conditions. The pattern of mixing might reflect maturation of lung function in the preterm or sick neonate.

Lung volume in infancy can be measured readily by standard procedures which are used for older children and adults, providing that the equipment is suitably scaled down, as has been shown for the inert gas dilution technique (12), for nitrogen washout (14), and for whole-body plethysmography (1). However, these techniques require considerable instrumentation and are rarely suitable for studying very small or seriously ill infants or for general use where the necessary apparatus and expertise are not available, and