The Developmental Changes in Plasma Adrenal Androgens during Infancy and Adrenarche Are Associated with Changing Activities of Adrenal Microsomal 17-Hydroxylase and 17,20-Desmolase

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ABSTRACT The plasma concentrations of dehydroepiandrosterone, androstenedione, and dehydroepiandrosterone sulfate decrease during the first year of life, remain low during childhood, and then increase during adrenarche. To determine whether alterations in adrenal enzyme activity might explain the changing secretory pattern of the adrenal androgens, we measured human adrenal microsomal 3β-hydroxysteroid dehydrogenase-isomerase, 17,20-desmolase, 17-hydroxylase, and 21-hydroxylase activities. 12 adrenals from individuals aged 3 mo to 60 yr were studied. The patients were divided into three groups based upon the age of the patient when the adrenal glands were obtained: group 1, infants aged 3–8 mo ($n = 3$); group 2, preadrenarchal or early adrenarchal children aged 2–9 yr ($n = 4$); and group 3, adults aged 20–60 yr ($n = 5$). The mean activity of the 17,20-desmolase, 17-hydroxylase, and 21-hydroxylase fell by 50% and that of 3β-hydroxysteroid dehydrogenase-isomerase activity rose 80% from group 1 to group 2. A fourfold increase in 17,20-desmolase ($P < 0.002$) and 17-hydroxylase ($P < 0.001$) activity and a doubling in 21-hydroxylase activity ($P < 0.005$) occurred between groups 2 and 3. We conclude that the decline in plasma adrenal androgens after birth appears to be associated with a rise in 3β-hydroxysteroid dehydrogenase-isomerase and a fall in 17,20-desmolase and 17-hydroxylase activity. The subsequent increase in plasma adrenal androgen concentration during adrenarche is coincident with a rise in 17,20-desmolase and 17-hydroxylase activity.

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INTRODUCTION

Albright (1), after observing pubic and axillary hair growth in patients with gonadal dysgenesis, postulated that the adrenal gland may secrete an androgen near the time of puberty. He named this process "adrenarche" (2). Subsequent studies have shown that adrenarche is associated with a measurable rise in plasma adrenal androgens, dehydroepiandrosterone (DHA),[1] dehydroepiandrosterone sulfate (DHAS), and androstenedione (Δ), by about 7 years of age (3–14). These plasma steroids reach adult values late in the second decade of life. Plasma cortisol concentration (15) and cortisol secretory rate corrected for body size (16) remain constant during this developmental period, suggesting that a shift in the pattern of adrenal steroidogenesis has occurred. This metabolic change is also associated with the development of the adrenal reticular zone, which usually first appears between the ages of 3 and 7 (17, 18).

The altered pattern of adrenal steroid secretion has long been thought to reflect a change in adrenal enzyme activities. Warne et al. (19) have suggested

[1] *Abbreviations used in this paper*: Δ, androstenedione (4-androsten-3,17-dione); DHA, dehydroepiandrosterone (5-androsten-3β-ol-17-one); DHAS, dehydroepiandrosterone sulfate (5-androsten-3β-yl sulfate-17-one); HSD, 3β-hydroxysteroid dehydrogenase-isomerase; TLC, thin-layer chromatography.

that 3β-hydroxysteroid dehydrogenase-isomerase (HSD) activity decreases during adrenarche. This hypothesis could account for the rising plasma levels of the Δ^5-steroids, DHA and DHAS. The hypothesis is supported by experiments showing that estrogen, a noncompetitive inhibitor of HSD activity in vitro (20), increases the urinary Δ^5-pregnenetriol/pregnanetriol ratio in girls with gonadal dysgenesis treated with estrogen (21). Further support of this theory comes from the recent demonstration that ACTH stimulation increases the ratio of plasma 17-hydroxypregnenolone/17-hydroxyprogesterone and DHA/Δ during adrenarche, observations compatible with a fall in HSD activity (22). This same study also showed a decline in the ratio of plasma 17-hydroxypregnenolone/DHA, suggesting an increase in 17,20-desmolase activity during adrenarche. Thus, based on urine and plasma steroid measurements, two hypotheses have been proposed to explain the rise in adrenal androgen secretion during adrenarche: first, a decrease in HSD activity and, second, an increase in 17,20-desmolase activity. We have attempted to examine these hypotheses by measuring the activity of the four microsomal enzymes in the cortisol and androgen biosynthetic pathways in human adrenal glands obtained from patients between the ages of 3 mo and 60 yr.

METHODS

Materials. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADPH, pyruvate, lactate dehydrogenase, NAD, and all unlabeled steroids were obtained from Sigma Chemical Co. (St. Louis, Mo.). Thin-layer chromatography plates, Silica Gel LK6DF, were obtained from Whatman, Inc. (Clifton, N. J.). [4-^{14}C]Progesterone (58.8 mCi/mmol) and 17α-[4-^{14}C]hydroxyprogesterone (60 mCi/mmol) were purchased from Amersham Corp. (Arlington Heights, Ill.). [4-^{14}C]Dehydroepiandrosterone (57.6 mCi/mmol), [1,2-^{3}H(*N*)]-androstenedione, 17α-[1,2]^{3}H(*N*)]hydroxyprogesterone, and 11-[1,2-^{3}H(*N*)]deoxycortisol were obtained from New England Nuclear (Boston, Mass.). The ^{14}C- and ^{3}H-labeled steroids were at least 97% pure, as determined by thin-layer chromatography.

Tissue. Adrenal glands were obtained during surgery or at autopsy and immediately placed on ice (Table I). Autopsy specimens were received between 5 and 6.5 h after death. The only abnormality of the glands to gross inspection was the paired adrenal weight of 5 g for the 3.5-yr-old child, almost double the normal adrenal weight for a child of this age (4). This is not an unusual finding, however, in children dying with central nervous system disease.

Microsomal preparation. Preparation of the microsomes was usually begun within an hour of adrenal gland removal. Microsomes were prepared at 4°C, as previously described (23). After the fat and connective tissue had been trimmed away, the adrenal glands were homogenized with a glass-Teflon homogenizer in 0.25 M sucrose. The homogenates were centrifuged for 10 min at each of three forces, 755, 8,700, and 16,000 *g*. The resulting 16,000-*g* supernatant solutions were centrifuged for 60 min at 105,000 *g*. The microsomal pellets were washed once by suspension in a Tris-KCl-EDTA buffer (0.02 M Tris buffer, pH 7.4, 0.15 M KCl, and 1 mM EDTA) and recentrifuged. The microsomes were stored at −70°C in the Tris-KCl-EDTA buffered solution until assayed. The final protein concentration of the microsomal fractions, determined by the method of Lowry et al. (24), ranged from 1.1 to 8.0 mg/ml.

Enzyme assays. Adrenal microsomal 3β-hydroxysteroid dehydrogenase-isomerase, 17-hydroxylase, 21-hydroxylase, and 17,20-desmolase activities were measured using a modification of previously described methods (23, 25, 26). Table II lists the contents of the reaction mixture used in each enzymatic assay. The substrate concentrations were chosen to ensure a maximum rate of product formation. Using an adult adrenal microsomal preparation, substrate concentrations were varied from 10 to 60 μM for each enzyme. The rate of product formation was independent of substrate concentration at the concentrations used for each assay. The cofactor concentrations were chosen to permit a maximum rate for each reaction. Each enzyme

TABLE I
Adrenal Glands

Age	Sex	Origin of adrenal tissue	Adrenal histology
3 mo	Female	Autopsy; death from intussusception	Fetal zone present
6 mo	Male	Autopsy; death from intussusception	Fetal zone present
8 mo	Male	Surgery; adrenal removed with Wilm's tumor	Fetal zone absent
2.5 yr	Female	Surgery; normal adrenal adjacent to feminizing adenoma	Reticular zone absent
3.5 yr	Male	Autopsy; spastic quadraplegia, seizure disorder; death from pneumonia	Reticular zone absent
6 yr	Female	Surgery; adrenal removed with Wilm's tumor	Reticular zone absent
9 yr	Male	Surgery; renal transplant donor; brain death from head injury; received glucocorticoids for 3 d before surgery	Reticular zone present
20 yr	Male	Surgery; renal transplant donor; brain death from head injury	Reticular zone present
31 yr	Female	Surgery; renal transplant donor; brain death from SAH*	Reticular zone present
43 yr	Female	Surgery; renal transplant donor; brain death from SAH*	Reticular zone present
56 yr	Female	Surgery; renal transplant donor; brain death from SAH*	Reticular zone present
60 yr	Female	Surgery; adrenalectomy for breast cancer	Reticular zone present

* SAH, subarachnoid hemorrhage.

TABLE II
Enzyme Assay Conditions (Volume, 400 μl)

	17,20-Desmolase
3β-Hydroxysteroid dehydrogenase-isomerase	
Propylene glycol, 10 μl	Propylene glycol, 10 μl
[^{14}C]Dehydroepiandrosterone, 50 μM	17-[^{14}C]Hydroxyprogesterone, 25–60 μM
NAD, 2 mM	NADPH, 600 μM
Pyruvate, 5 mM	Glucose-6-phosphate, 10 mM
Lactate dehydrogenase, 1 U/ml	Glucose-6-phosphate dehydrogenase, 1.5 U/ml
Sodium phosphate buffer (pH 7.4), 50 mM	$MgCl_2$, 5 mM
Microsomes containing 25 μg of protein	Tris buffer (pH 7.4), 50 mM
	Microsomes containing 150 μg of protein
21-Hydroxylase	17-Hydroxylase
Propylene glycol, 10 μl	Propylene glycol, 10 μl
17-[^{14}C]Hydroxyprogesterone, 50 μM	[^{14}C]Progesterone, 45 μM
NADPH, 600 μM	NADPH, 600 μM
Glucose 6-phosphate, 10 mM	Glucose 6-phosphate, 10 mM
Glucose 6-phosphate dehydrogenase, 1.5 U/ml	Glucose 6-phosphate dehydrogenase, 1.5 U/ml
$MgCl_2$, 5 mM	$MgCl_2$, 5 mM
Sodium phosphate buffer (pH 7.4), 50 mM	Sodium phosphate buffer (pH 7.4), 50 mM
Microsomes containing 35 μg of protein	Microsomes containing 35 μg of protein

assay was initiated by the addition of microsomes and incubated at 37°C in air in a Dubnoff metabolic incubator. At predetermined times, each enzyme reaction was terminated by the addition of 1.6 ml of methanol containing 50 μg each of 17-hydroxyprogesterone, androstenedione, and 11-deoxycortisol. The activity of each enzyme was determined at three to five time points, each time point measured in triplicate. The activity of intact microsomes at zero time and of boiled microsomes were determined with each assay as a control. The amount of product formed by each enzyme varied linearly with respect to time (Fig. 1). The procedure was varied only for the 8-mo-old and

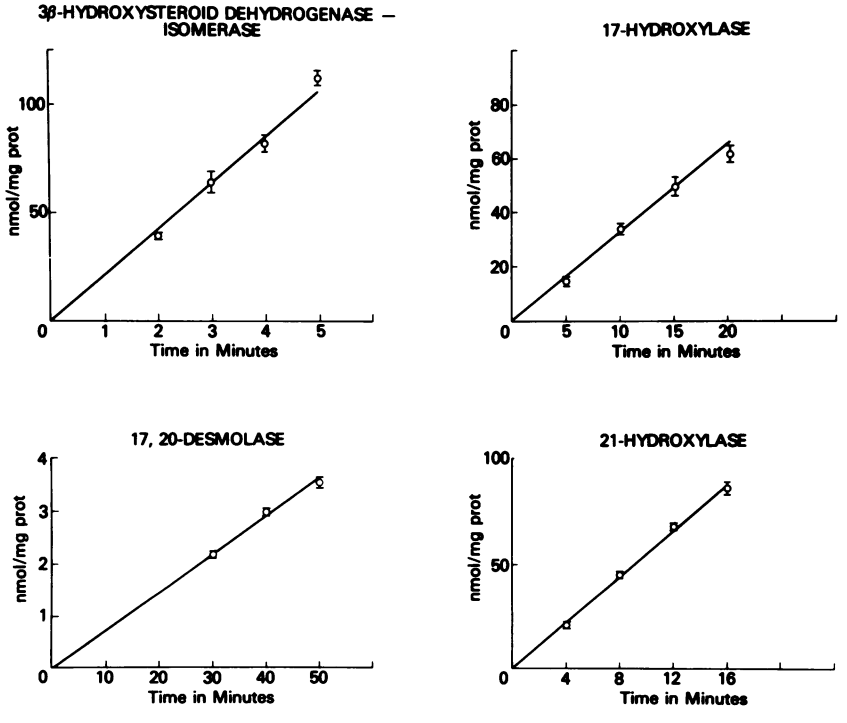

FIGURE 1 Amount of product formed as a function of time (mean±SD). A demonstration of the linear rate of reaction for each adrenal microsomal enzyme as measured in the 3.5-yr-old child.

the 2.5-yr-old adrenals, where each time point for the 17,20-desmolase assay was performed in duplicate with 75 μg of microsomal protein, a step necessitated by the small amount of available tissue.

Controls. A single, pooled cynomolgus macaque adrenal microsomal preparation was run with each enzyme assay to control for intra- and interassay variability. The intra- and interassay coefficients of variation were: HSD, 7% and 7%; 17-hydroxylase, 6% and 7%; 21-hydroxylase, 5% and 8%; and 17,20-desmolase, 5% and 10%, respectively. An adrenal was minced and divided into two portions, and the microsomes from each portion were prepared separately as a test for possible variability in enzyme activity due to differences in microsome preparation. The activity of each enzyme was then measured and no significant difference was found between the two preparations. Possible loss of microsomal enzyme activity with storage at −70°C was also evaluated. The longest period of time from microsomal preparation to assay was 9 mo. Each of the four microsomal enzymes was assayed seven times from 1 wk to 13 mo after preparation, using an adult human adrenal gland. No significant change in activity of the four enzymes was observed over this period of time. To ensure that the endogenous steroid content of the microsomes would not significantly decrease the specific activity of the substrates and thus falsely lower the observed activities, the microsomal preparation from the 6-yr-old was assayed for dehydroepiandrosterone, 17-hydroxyprogesterone, and progesterone, using previously described radioimmunoassays (27–29). The maximal endogenous steroid concentration did not exceed 0.6% of the substrate concentration for any assay.

Thin-layer chromatography (TLC). Products were isolated by TLC. 400 μl of the 2-ml reaction mixture was added to 100 μl of ethanol containing an amount of tritiated product which resulted in a ^{3}H:^{14}C ratio (dpm) between 3 and 15. A volume of 50 μl of this 500-μl mixture was chromatographed. The 3β-hydroxysteroid dehydrogenase-isomerase and 21-hydroxylase products were chromatographed twice in chloroform:acetone (8:1). The 17-hydroxylase products were chromatographed once in benzene:acetone:ethyl acetate (10:1:1) followed by a single run in cyclohexane: ethyl acetate (45:55). The 17,20-desmolase reaction mixture was chromatographed twice in chloroform:ethanol (98:2). Recovery ranged from 80 to 90%. The products were detected with a short-wave ultraviolet light, scraped from the plate into scintillation vials, and measured for radioactivity by liquid scintillation spectrometry after the addition of 10 ml of Aquasol (New England Nuclear).

Derivative formation. The identity of each product was confirmed by derivative formation using the product formed after the longest incubation. The remainder of the 500-μl sample prepared for TLC was chromatographed in the TLC systems described above. The products androstenedione and 17-hydroxyprogesterone were reduced with sodium borohydride in methanol (30) and then acetylated. The product 11-deoxycortisol was acetylated and then similarly reduced. Each derivative was purified by TLC using chloroform: acetone (8:2 or 8:3). The ^{3}H:^{14}C dpm ratio after each set of derivatizations did not increase by >11% of the original ratio.

Calculations. Enzyme activities are expressed as mol/min mg^{-1} microsomal protein corrected for recovery. Comparison of enzyme activities was made with Student's *t* test.

RESULTS

The patients were divided into three groups based upon the age of the patient at the time the adrenal glands were obtained: group 1, infants aged 3–8 mo; group 2, preadrenarchal or early adrenarchal children aged 2–9 yr, and group 3, adults aged 20–60 yr. The adrenal microsomal enzyme activities of the HSD, 17,20-desmolase, 17-hydroxylase, and 21-hydroxylase are presented in Table III. No significant difference in the activity of the HSD enzyme was observed among the three groups, although the mean HSD activity rose 80% from group 1 to group 2 and fell 24% from group 2 to group 3. A fourfold increase in 17,20-desmolase ($P < 0.002$) and 17-hydroxylase ($P < 0.001$) activity was observed after adrenarche (group 3 compared with group 2). A twofold increase in 21-hydroxylase activity ($P < 0.005$) was also seen in group 3 when compared with group 2.

DISCUSSION

Our study does not support the hypothesis that adrenarche is the result of a decrease in HSD activity (19–21). Because the number of observations is small, however, further measurements are needed before definite conclusions about the role of changes in HSD activity on the adrenarchal process are drawn. On the other hand, the findings do suggest that the rise in plasma DHA, DHAS, and Δ during adrenarche may be the result of an increase in 17-hydroxylase and 17,20-desmolase activity. This hypothesis has the additional attribute of explaining the adrenarchal rise in plasma Δ, whereas the HSD hypothesis does not.

The apparent decreases in 17-hydroxylase and 17,20-desmolase activity between infancy and childhood were reversed between childhood and the post-adrenarchal adult. Thus, the apparent decline in 17-hydroxylase and 17,20-desmolase activity may explain the known fall in plasma DHA (8), DHAS (16), and Δ (29) concentrations from birth to 1 yr of age.

The activity of 21-hydroxylase appears to decline from groups 1 to 2 and then increases after adrenarche. Although an increase in 21-hydroxylase activity might be predicted to decrease the synthesis of adrenal androgens relative to cortisol, the magnitude of such an effect is not possible to predict without more knowledge of the kinetics of the androgen and cortisol pathways in vivo and of the degree of compartmentalization of the two pathways.

Three of the adrenal glands included in this study were obtained at autopsy, which may have affected the observed enzymatic activities. However, delayed tissue processing in these three glands does not appear to have caused a major decrease in enzyme activity, because the observed enzymatic activities in these glands were comparable to those measured in other subjects in a similar age group.

The current study does not identify the intra-adrenal site of the observed enzymatic changes. The

TABLE III
Microsomal Enzyme Activities

Group	Age	3β-Hydroxysteroid dehydrogenase-isomerase	17,20-Desmolase	17-Hydroxylase	21-Hydroxylase
		nmol/min mg prot^{-1}	*pmol/min mg prot*$^{-1}$	*nmol/min mg prot*$^{-1}$	*nmol/min mg prot*$^{-1}$
1	3 mo	10.8±1.1*	148±7	4.77±0.23	5.96±0.29
	6 mo	7.7±0.3	69±2	2.61±0.03	6.37±0.16
	8 mo	6.4±0.3	29±1	2.53±0.14	6.06±0.24
Mean±SE		8.3±1.3	82±35	3.30±0.73	6.13±0.12
2	2.5 yr	14.2±1.1	26±2	0.96±0.11	2.69±0.28
	3.5 yr	21.2±1.3	72±2	3.31±0.26	5.49±0.20
	6 yr	11.8±0.8	36±2	0.83±0.06	3.07±0.08
	9 yr	12.5±0.8	46±3	0.91±0.06	1.18±0.09
Mean±SE		14.9±2.15	45±10	1.50±0.60	3.11±0.90
3	20 yr	15.5±0.8	194±10	4.65±0.26	8.48±0.36
	31 yr	8.1±0.6	230±9	6.74±0.11	5.17±0.26
	43 yr	14.5±1.0	141±8	6.63±0.26	7.69±0.25
	56 yr	6.8±0.5	125±5	5.14±0.30	7.14±0.19
	60 yr	11.8±0.8	257±10	7.40±0.67	8.57±0.35
Mean±SE		11.3±1.7	189±25‡	6.11±0.52§	7.41±0.62‖

* Mean±SD.
‡ $P < 0.002$ compared with group 2.
§ $P < 0.001$ compared with group 2.
‖ $P < 0.005$ compared with group 2.

apparent fall in 17-hydroxylase and 17,20-desmolase activity between the 3-mo-old and 6-mo-old infants coincides with the period of rapid regression of the fetal zone (31, 33), which appears to be the principal site of DHAS secretion in the fetus (32). The increase in 17-hydroxylase, 17,20-desmolase, and 21-hydroxylase activity between groups 2 and 3 coincides with the development of the reticular zone (4), which is postulated to be the principal source of plasma adrenal androgens in adults (34, 35). Whether the changing enzymatic activities observed in this study are localized to the fetal and reticular zones remains an important question for further investigation.

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