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Rat Testicular Endogenous Steroids and Number of Leydig Cells Between the Fetal Period and Sexual Maturity

J. TAPANAINEN,[2] T. KUOPIO,[3] L. J. PELLINIEMI[4] and I. HUHTANIEMI[1,5]

Department of Clinical Chemistry[2]
University of Oulu
Oulu, Finland

Department of Anatomy,[3] *and Laboratory of Electron Microscopy*[4]
University of Turku
Turku, Finland
and
Departments of Clinical Chemistry, and Immunology and Bacteriology[5]
University of Helsinki
Helsinki, Finland

ABSTRACT

Endogenous androgens (androstenedione, testosterone, 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol), and some of their C21 precursors (pregnenolone, progesterone and 17-hydroxyprogesterone) were measured in rat testes between Day 18.5 of pregnancy and Day 64 postpartum, and correlated with numerical densities of Leydig cells. The latter parameter showed an early maximum on Day 19.5 of the fetal period, a nadir on Day 15 postpartum, and a gradual increase thereafter. The two dominating androgens, testosterone and 5α-androstane-3α,17β-diol, had similar levels until 15 days of age, but the 5α-diol predominated thereafter. The total steroid content per Leydig cell was highest on Day 18.5 of gestation (77 ng/10^6 cells). A decline started already in utero, and reached a nadir of 5 ng/10^6 cells on Day 29. Thereafter, a slight increase occurred with advancing age. It is concluded that: *1*) The fetal testis has highest Leydig cell and endogenous steroid concentrations. A nadir in these parameters is seen 2–4 wk postpartum. The Leydig cell concentration increases around puberty on Days 40–60, but only a slight concomitant increase occurs in steroids. *2*) A sharp decline in steroid content per Leydig cell occurs during the last fetal days, but the postnatal decline of testicular steroids is due to Leydig cell loss. *3*) The new Leydig cell generation after 15 days has a persistently low steroid concentration through puberty. *4*) The relative proportions of endogenous steroids are similar in the fetal and immature testes, but the pubertal increase in steroidogenesis is characterized by increased ratios of C19/C21 steroids, 5α-reduced/3-keto-4-ene androgens, and 17β-hydroxy/17-keto androgens.

INTRODUCTION

Testicular testosterone production is activated in the developing rat in utero, and reaches a maximum on Days 18–19 of gestation (Nomura et al., 1966; Warren et al., 1973). It decreases after birth and reaches a nadir at about 2 wk of postnatal age. Thereafter testosterone production rises again, and attains the high adult level between 40–60 days of age in connection with pubertal sexual maturation (Knorr et al., 1970; Podesta and Rivarola, 1974; Purvis et al., 1978; Corpechot et al., 1981; Huhtaniemi et al., 1982). The fetal-neonatal and pubertal-adult phases of testicular androgen-producing activity are known to be due to two distinct growth phases of Leydig cells, termed the fetal and adult Leydig cell populations (Roosen-Runge and Anderson, 1959; Lording and de Kretser, 1972). Some morphological differences are known between the two cell generations (Christensen and Chapman, 1959; Narbaitz and Adler, 1967; Lording and de Kretser, 1972). Huhtaniemi et al. (1981, 1982, 1983a,b) have recently observed further differences in their tropic regulation. Concerning steroidogenesis, certain qualitative changes occur during the postnatal development, the best characterized of them being the temporary predominance of 5α-reduced androgens in the immature rat testis (Nayfeh et al., 1966; Coffey et al., 1971; Ficher and Stein-

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[1] Reprint requests: Dr. Ilpo Huhtaniemi, Department of Clinical Chemistry, University of Helsinki, Meilahti Hospital, SF-00290 Helsinki, Finland

berger, 1971; Lacroix et al., 1975; Purvis et al., 1978). Possible special characteristics of the fetal Leydig cell steroidogenesis are, however, still unknown. The purpose of this study was to examine changes in the rat testicular steroidogenesis during development, as reflected by testicular endogenous steroid concentrations. As the Leydig cell number and numerical density vary greatly during development, a morphometric analysis of testicular Leydig cell concentrations at different ages was performed, to find out whether changes in the endogenous steroid content per Leydig cell also occur. Special emphasis was directed towards identifying qualitative and quantitative differences in the steroidogenesis of the fetal and adult populations of Leydig cells.

MATERIALS AND METHODS

Animals

Male rats of the Wistar strain were used for the study. Conditions of light (14L:10D) and temperature (22°C) were controlled, and water and laboratory animal chow were available ad libitum. Male and female rats were placed in the same cage for 1 night, and separated in the following morning. If pregnancy ensued, the day after mating was taken as Day 0.5 of gestation. Groups of pregnant mothers were killed on Days 17.5, 18.5, 19.5, 20.5 and 21.5 of gestation, and testes of the fetuses were excised, weighed, and processed as described below.

After birth, litters of 8–10 male pups were kept with a mother in a nursing cage, and the weaning took place at the age of 20 days. Thereafter, 5 males were kept per cage. Groups of males were killed at the following ages: 1 (day of birth), 2, 3, 5, 7, 9, 11, 15, 19, 23, 29, 38, 44, 54 and 64 days. The testes were excised and weighed. One testis of each pair was snap-frozen in liquid nitrogen and stored at −20°C until used for steroid analyses. The other testis was used for morphometric analysis of the Leydig cell numerical densities and numbers, as described below. The steroid measurements and morphometry were done at different fetal ages, as seen in the details of the figures.

Steroid Analyses

Steroid standards and solvents were as described by Hammond et al. (1977). The extraction procedure and subsequent radioimmunoassays (RIAs) for measurements of endogenous tissue steroids have been described before using adult human testis and prostatic tissue (Hammond et al., 1978; Hammond, 1978). In short, testis tissue was homogenized in a Teflon-glass homogenizer in 10 volumes (wt/vol) or at least 300 μl of 50 mM phosphate-buffered 0.14 M NaCl (PBS; pH 7.4). The homogenizer was washed twice with 300 μl of PBS, and the combined buffer samples were extracted twice with 4 ml diethyl ether-ethyl acetate (9:1, vol/vol). The steroid recoveries in the extraction and subsequent chromatography were monitored by adding approximately 6000 dpm [^{3}H] testosterone to the tissue homogenates in PBS. Measured in this way, the recoveries were over 85%. Samples with lower recoveries were reanalyzed, or the results were discarded. Previous studies from this laboratory (Hammond et al., 1977) have shown that the recoveries of the different steroids measured were similar.

The extracts in diethyl ether-ethyl acetate were combined, dried under nitrogen, and subjected to chromatographic fractionation in Lipidex 5000 (Packard-Becker, B. V., Chemical Operations, Groningen, The Netherlands) microcolumns, as described by Hammond et al. (1977, 1978). The solvent system was light petroleum ether: chloroform (98:2, vol/vol). The fractions separated were analyzed by radioimmunoassays for pregnenolone, progesterone, 17-hydroxyprogesterone, androstenedione, testosterone, 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol with antisera characterized previously (Jänne et al., 1974; Apter et al., 1976; Hammond et al., 1977; Hammond, 1978). In each assay run, a control sample of human serum monitored assay quality.

Morphometric Analysis of Leydig Cell Numbers

The testes for morphometric analysis were cut into pieces which were fixed in 5% glutaraldehyde in 0.16 M 2, 4, 6-collidine-HCl buffer (pH 7.4). After 3 h the specimens were washed in the buffer and postfixed in 1.5% K-ferrocyanide – 1% osmium tetroxide for 2 h. The tissue blocks were dehydrated in series of ethanol, and embedded in Epon. Sections (1 μm) for light microscopy were cut with a Sorvall Porter-Blum ultramicrotome and stained with toluidine blue.

Morphometric analysis of the Leydig cell concentrations was done with a light microscope fitted with an eyepiece grid. Leydig cells were identified as described by Lording and de Kretser (1972). The number of Leydig cell nuclei per unit area (N_A) was counted from 6–12 grid fields. The numerical density (N_V), number of Leydig cells per unit volume, was calculated from N_A, from measured average nuclear diameter (D=7.1 μm) and from average thickness of the sections (T) utilizing the Floderus equation $N_V = N_A/(T + D - 2h)$, where h is the height of the smallest recognizable section of the nucleus (0.14 μm) (Mori and Christensen, 1980). The long and short axes of the nuclei were measured. The axial ratio was 1.27 and therefore the nuclei were assumed spherical (Mori and Christensen, 1980). The specific gravity of the testis tissue was measured using series of reference sucrose solutions in order to calculate the number of Leydig cells per unit mass (N_M). The total number of Leydig cells per testis was calculated from N_M and testis weight (Mori and Christensen, 1980). The calculations of N_A were performed using three to five animals per age group and three to five blocks per each animal.

Statistical Analysis of the Results

Statistical comparisons of age-groups were performed using Duncan's new multiple range tests for least significant differences. $P<0.05$ was selected as the limit of statistical significance.

RESULTS

The Leydig cells were divided into fetal and adult populations on the basis of their morphological appearance at different ages (Figs. 1–4).

The testicular endogenous steroid concentrations in different age groups are presented in Table 1, and the total steroid contents and concentrations are shown graphically in Fig. 5. Due to the limited number of measurements in certain ages, some of the adjacent age groups were pooled in the table. The levels of each steroid were highest in the fetal testes. A clear decline was seen in every steroid perinatally, and the decline continued until the age of 20–30 days. Testosterone, adrostenedione and 5α-androstane-3α,17β-diol were the three dominating steroids in fetal testes, and during the first days postpartum, but from Days 10–15 onwards, 5α-androstane-3α,17β-diol became gradually the dominating steroid. The concentrations of all C21 steroids continued declining with advancing age.

Figure 6 shows the concentration of the two predominating androgens, testosterone and 5α-androstane-3α,17β-diol, throughout the age range studied. Despite large variation at some individual ages, especially during the sharp perinatal decline in steroid concentrations, it is evident that the concentrations of the two steroids are roughly equal until 15 days of age, whereafter the ratio shifts to great favor of the 5α-diol. This shift in the 5α-androstane-3α, 17β-diol:testosterone ratio is the earliest clear qualitative change observed in testicular steroids before the pubertal activation of androgen production.

Another clear shift with development was observed in the C19:C21 steroid ratio (Fig. 7). It was about 9 on Day 18.5 in utero, declined then to 2–4 by Day 5 of postnatal age, and rose slowly towards the age of 30 days. Thereafter it increased sharply in the oldest age groups studied. A similar shift after 30 days was seen in the ratios of 5α-reduced:3-keto-4-ene androgens and 17β-hydroxy:17-keto androgens (Fig. 7). Furthermore, it is noteworthy that there was a sharp decrease in the 5α-reduced:3-keto-4-ene androgen ratio from 44 to 64 days of age.

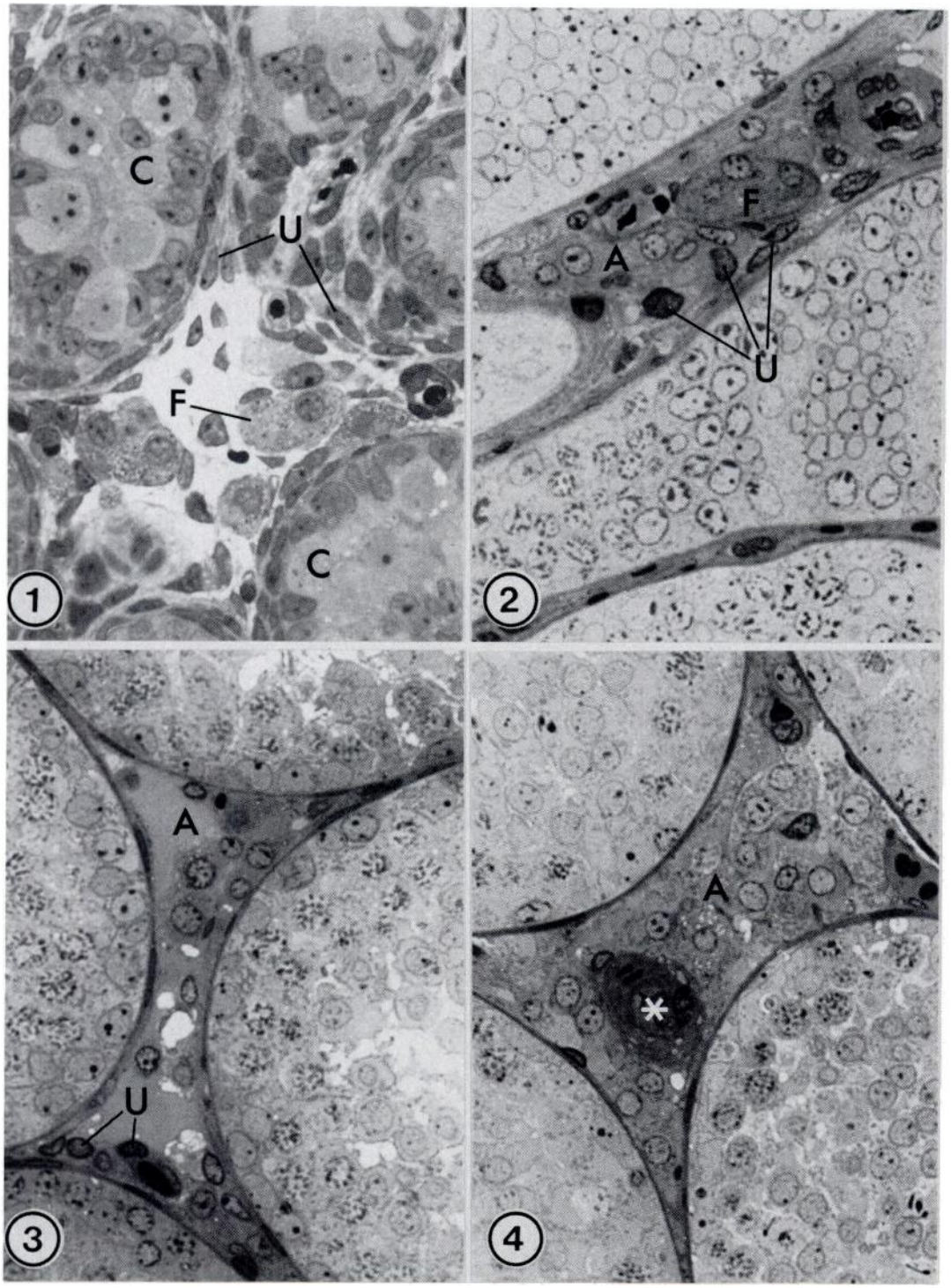
Figure 8 shows the number of Leydig cells per unit mass, and number per testis at the different ages. The cell number per testis has an early maximum on Day 19.5 of fetal age. A decline takes place during the first days after birth, and thereafter there is a steady increase with age until Day 54. A biphasic curve is very clearly observed in the Leydig cell concentrations. After the fetal peak on Day 19.5 (68×10^6 cells/g), there is a decrease until Day 15 of postnatal age, and thereafter, the Leydig cell concentration increases clearly, but is still only one third of the peak concentration of the fetal period.

When the total endogenous steroid content per Leydig cell is plotted from the data presented in Table 1 and Figs. 5 and 8, it is highest in the fetal testes (Fig. 9). It decreases by about 50% during the last 2 days of pregnancy ($P<0.05$), and stays at this level during the first 2 wk of postnatal life. A further sharp decline occurs around 20–30 days of age ($P<0.05$). Thereafter, the steroid concentration per Leydig cell increases about 2-fold in 54- to 64-day-old rats ($P<0.05$), but does not reach levels measured in the fetal and neonatal testes.

DISCUSSION

Highest endogenous steroid levels were detected in the fetal testes, which corroborates earlier measurements of high fetal testicular testosterone (Warren et al., 1973; Slob et al., 1980; Paz et al., 1980). In our measurements, testosterone represents only 40% of the total androgens of fetal testes, and measurement of only testosterone thus underestimates their total androgen-producing capacity. The insufficiency of testosterone in monitoring testicular androgen pools is even clearer in older age groups (over 15 days postpartum) where 5α-androstane-3α,17β-diol is the main endogenous androgen. Also, androstenedione is an important constituent of the immature testis androgens, since this steroid was detected at some ages (18–45 days) in concentrations exceeding those of testosterone. Androsterone, not measured in our study, is likewise a significant constituent of the immature testis steroid pool (Moger, 1979). The C19 steroids represented at each age the bulk of the endogenous steroids measured.

A pronounced decline was seen in the total steroid concentration and content per Leydig cell during the last days of fetal life. The reason for this abrupt decline is not clear. The time range of the decline is obviously too narrow to be explained by appearance of a new, steroidogenically less active population of Leydig cells. The fetal testis can use extratesticular steroid precursors (from the mother, placenta or other fetal tissues) for androgen production since incubation studies have shown that exogenous



FIGS. 1–4. Light micrographs of developing postnatal rat testicular interstitial tissue. Epon sections (1 μm) stained with toluidine blue. ×670.

FIG. 1. Newborn rat. Differentiated fetal-type Leydig cell clusters (*F*), and undifferentiated spindle-shaped mesenchymal cells (*U*) between the testicular cords (*C*).

FIG. 2. Age 18 days. Fetal-type Leydig cell cluster (*F*), adult-type Leydig cells (*A*) and spindle-shaped undifferentiated cells (*U*) with dark nucleus.

FIG. 3. Age 28 days. Adult-type Leydig cells (*A*) and undifferentiated cells (*U*).

FIG. 4. Age 43 days. Large confluent areas of adult-type Leydig cells (*A*). An arteriole is marked with an *asterisk*.

C19 and C21 steroid substrates are actively converted to testosterone in fetal testes and result in up to 50-fold higher testosterone production than from the endogenous substrate (Sanyal and Villee, 1977). Therefore the pronounced drop of maternal serum progesterone during the last days of gestation (Wiest, 1970; Tapanainen et al., 1979) could result in a clear drop of testicular androgen levels. A change in the gonadotropic stimulation is not a likely explanation for the declining steroid levels of the near-term fetal testes (Corbier et al., 1978; Slob et al., 1980; Picon and Habert, 1981).

The decline in steroid concentrations during the first 2 wk of life can be explained by the decrease of testicular Leydig cells, since the steroid concentration per Leydig cell did not change significantly at the same time. A further drop in steroids per cell occurred after Day 20 of age, and another low level of this parameter was attained after Day 30. Intriguingly, this latter decline was paralleled by the replacement of the fetal Leydig cells by the adult population,

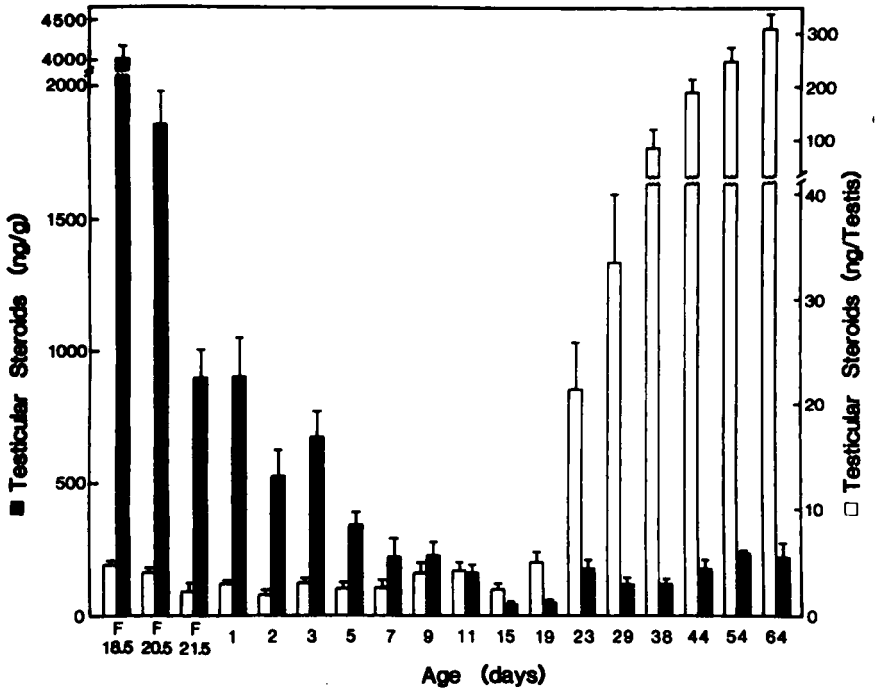


FIG. 5. Testicular content and concentration of endogenous steroids (sum of pregnenolone, progesterone, 17-hydroxyprogesterone, androstenedione, testosterone, 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol) between Day 18.5 of fetal life (*F*) and Day 64 postpartum. Each bar is the mean ± SEM of 3–7 individual measurements.

TABLE 1. Concentrations of pregnenolone (*Preg*), progesterone (*Prog*), 17-hydroxyprogesterone (*17-OH-prog*), androstenedione (*A-dione*), testosterone (*T*), 5α-dihydrotestosterone (*5α-DHT*) and 5α-androstane-3α,17β-diol (*5α-diol*) in the rat testis tissue between ages of Day 18.5 of gestation and Day 64 postpartum. Measurements of certain age groups were compiled as indicated in the table. The concentrations are given in ng/g wet tissue (mean ± SEM).

Age (days)	n[a]	Testis weight (mg)	Preg	Prog	17-OH-prog	A-dione	T	5α-DHT	5α-A-diol	TOTAL
F 18.5[b]	4	1.4 ± 0.08	127 ± 35	197 ± 13	95 ± 12	897 ± 153	1620 ± 190	48 ± 7.5	1050 ± 220	4030 ± 170
F 20.5	3	2.3 ± 0.1	82 ± 31	256 ± 58	158 ± 18	434 ± 51	549 ± 144	26 ± 0.91	352 ± 189	1860 ± 120
F 21.5	7	3.2 ± 0.2	32 ± 8.0	118 ± 33	51 ± 10	128 ± 52	141 ± 50	15 ± 2.0	333 ± 83	818 ± 215
1–3	16	4.0 ± 0.3	63 ± 14	107 ± 30	27 ± 4.6	112 ± 18	137 ± 34	22 ± 3.2	238 ± 55	706 ± 84
5–9	15	12 ± 1.8	21 ± 4.1	35 ± 7.5	8.8 ± 1.1	42 ± 14	73 ± 25	7.0 ± 0.9	84 ± 13	271 ± 56
11–15	10	43 ± 13	6.8 ± 1.6	10.4 ± 3.5	3.4 ± 0.7	23 ± 7.4	50 ± 17	4.0 ± 0.6	59 ± 13	157 ± 30
19–23	12	106 ± 3.1	6.9 ± 1.6	4.3 ± 0.8	5.3 ± 1.8	41 ± 11	7.7 ± 1.5	6.4 ± 0.8	69 ± 15	141 ± 22
29	4	288 ± 15	3.1 ± 1.0	2.1 ± 0.5	6.2 ± 2.5	32 ± 11	7.0 ± 3.6	5.4 ± 0.9	49 ± 9.9	105 ± 31
38–44	9	672 ± 16	0.90 ± 0.15	1.2 ± 0.25	2.3 ± 0.7	11 ± 3.5	8.1 ± 2.7	2.1 ± 0.5	119 ± 24	145 ± 19
54	5	1350 ± 24	0.94 ± 0.009	1.2 ± 0.22	0.82 ± 0.12	11.7 ± 2.0	18.7 ± 5.9	3.7 ± 1.4	210 ± 42	247 ± 47
64	5	1480 ± 40	1.26 ± 0.17	1.28 ± 0.07	0.70 ± 0.13	17.3 ± 4.1	35 ± 12	3.7 ± 1.4	162 ± 28	221 ± 27

[a] Number of individual testes analyzed.
[b] Gestational age of the fetuses (*F*).

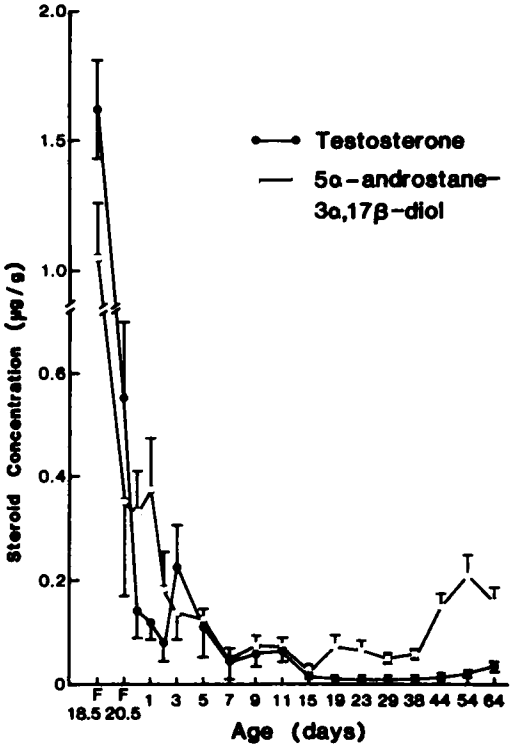


FIG. 6. Concentrations of testosterone and 5α-androstane-3α,17β-diol in the rat testis tissue between Day 18.5 of fetal life and Day 64 postpartum. Each point is the mean ± SEM (n=3–7).

and could thus represent a difference in the steroidogenic capacity of the two cell populations. This would timewise corroborate our previous observations on differences in the regulation of luteinizing hormone receptors and steroidogenesis in the fetal and adult Leydig cell populations (Huhtaniemi et al., 1981, 1982). A shift from the neonatal to the adult-type responses also occurs in these functions at about 20 days of age.

Relative proportions of the different steroids changed only slightly up to the age of 29 days. After this age, however, a clear increase took place in ratios of C19:C21 steroids, 5α-reduced:3-keto-4-ene androgens and 17β-hydroxy:17-keto androgens, indicating activation of C17–20 lyase, 5α-reductase and 17β-hydroxysteroid dehydrogenase enzymes. Interestingly, no parallel increase in the total steroid content per Leydig cell occurred, which indicates that the pubertal activation of androgen production is not due to general activation of steroidogenesis, but merely represents a qualita-

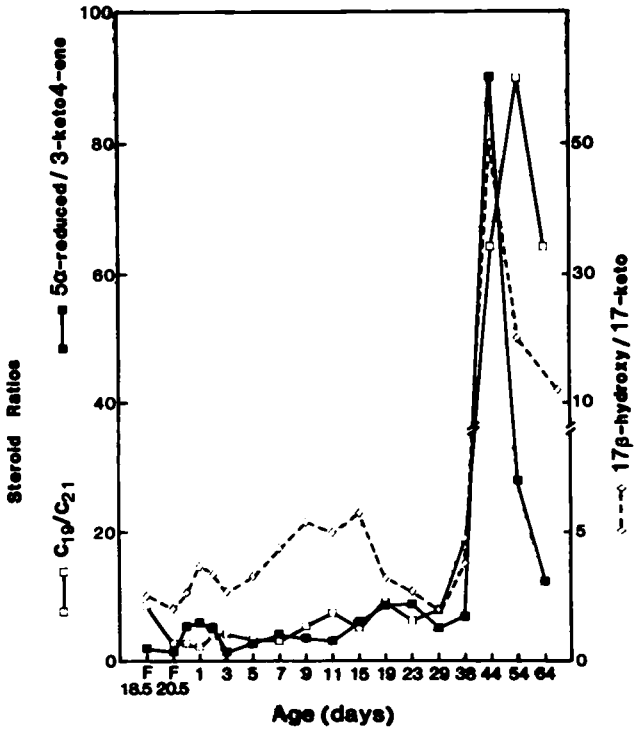


FIG. 7. Relative changes in the ratios of testicular endogenous concentrations of C19:C21 steroids, 5α-reduce:3-keto-4-ene androgens and 17β-hydroxy:17-keto androgens between Day 18.5 of fetal life (*F*) and Day 64 postpartum. Each point is the mean of 3–7 observations. The SEM bars were omitted for the sake of clarity. All the steroid ratios on Days 44 and 54 (and the C19:C21 steroid ratio on Day 64) were significantly higher ($P<0.01$–0.05, Duncan's test) than those measured in animals of 38 days of age and younger.

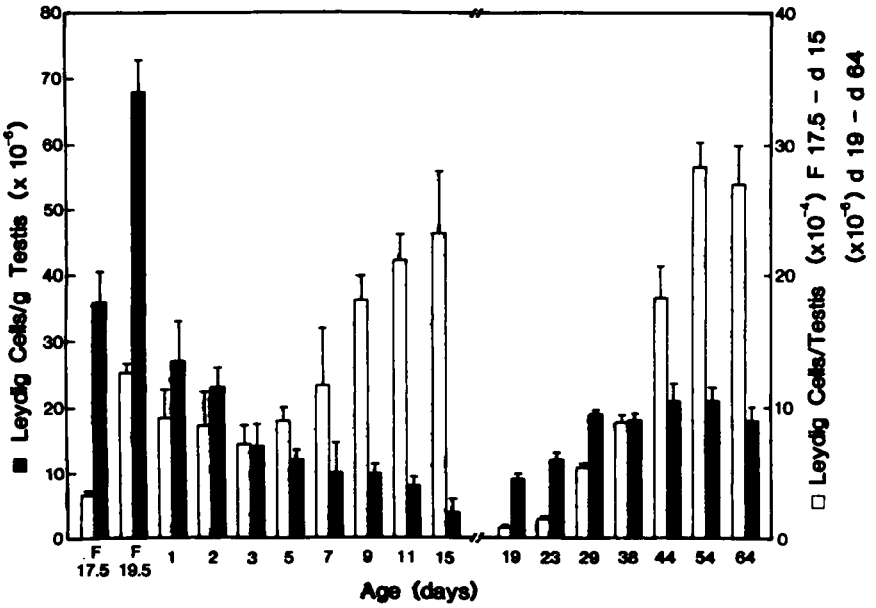


FIG. 8. Testicular content and concentration of Leydig cells between Day 18.5 of fetal age and Day 64 postpartum. Each bar is the mean ± SEM (n=3–5).

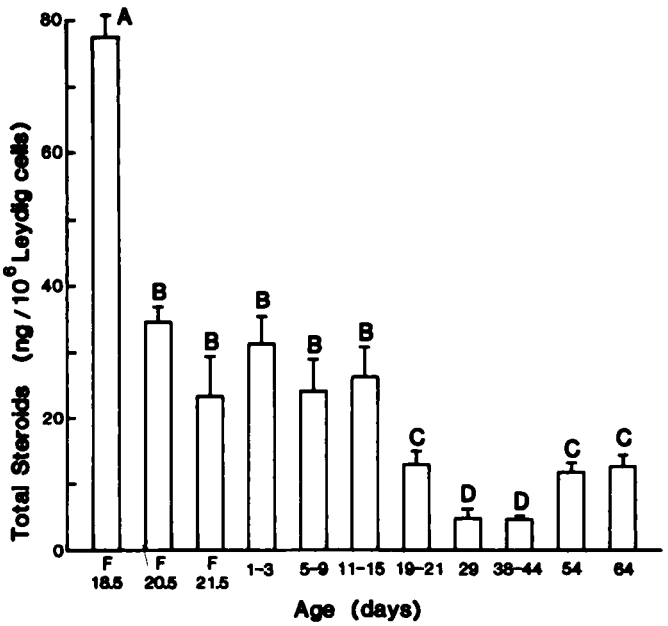


FIG. 9. Mean steroid concentration per Leydig cell from Day 18.5 of fetal life to Day 64 postpartum. Statistically significant difference was observed between the groups indicated by different *letters* ($P<0.05$, Duncan's test). Each bar is the mean ± SEM of 3–7 measurements. Since the steroids and cell numbers were assessed in different ages of fetuses, the numbers of Leydig cells per fetal testes were extrapolated from the data in Fig. 8 for fetal Days 17.5 and 19.5 and Day 1 postpartum, by assuming a linear change of cell numbers between these ages.

tive change in relative ratios of the steroids produced. As previously shown (Nayfeh et al., 1966; Lacroix et al., 1975; Purvis et al., 1978; Moger, 1979), 5α-androstane-3α,17β-diol is quantitatively the major androgen of the immature testis. A sharp peak in the proportion of the 5α-reduced androgens is seen around Day 40 of age. Others have shown previously that the peripubertal increase in testicular testosterone is accompanied by a reciprocal decline in proportion of the 5α-reduced androgens. There is, however, considerable variation in the timing of this change between individual reports, and the age when testicular testosterone concentration exceeds that of 5α-androstane-3α,17β-diol has been shown to vary from 30 to 90 days (Lacroix et al., 1975; Purvis et al., 1978; Moger, 1979; Corpechot et al., 1981). This stage was not reached by our animals by Day 64 of age, but the increase in testosterone and decrease in 5α-androstane-3α,17β-diol levels in the oldest age groups implied that our animals were approaching this phase of testicular development. Most importantly, however, there is good agreement in the sequence of these steroid changes between our and previous observations.

In conclusion, this study demonstrates that the steroid content of the fetal population of Leydig cells is clearly higher than that of the adult population. The fetal population of Leydig cells loses part of its steroidogenic activity already in utero, but the subsequent postnatal decline in testicular steroids is mainly due to decrease in the number per unit mass of Leydig cells. 5α-Reduced androgens predominate in the immature testis and the increase of testicular endogenous testosterone in late puberty is largely due to a shift in the relative proportion of 3-keto-4-ene to 5α-reduced androgens, without gross changes in total steroid concentrations.

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