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Postnatal Development of the Testis, Fighting Behavior, and Fertility in House Mice[1]

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Male house mice (*Mus musculus*) were killed between 10 and 100 days of age to establish the temporal relationships between the development of fighting behavior and fertility and the spermatogenic and steroidogenic functions of the testis. Testis growth was directly related to increases in the concentration of protein and carbohydrate and the ratio of RNA/DNA in testicular homogenates, with the most obvious changes being noted between 21 and 55 days of age. Plasma androgen concentrations increased 300% during that time period and then declined 50% by 100 days of age. Importantly, increases in plasma androgen concentration paralleled increments in the numbers of Leydig cells and step 7 spermatids. Spermatogenesis was completed qualitatively by 35 days of age, and spermatozoa were detected at the cauda epididymis–vas deferens juncture at 40 days of age. Isolation of [^{14}C] androgens following *in vitro* incubation of teased testis tissue with [1-^{14}C]acetate demonstrated that 80% of the radioactive products were associated with [^{14}C]androstenedione and 5-α-reduced androgens between 10 and 40 days of age, while 80% was associated with [^{14}C]testosterone after 55 days of age. Intermale aggression was first observed at 35 days in 90% of the individuals tested. In comparison, viable mating was first apparent at 40 days and reached adult levels by 55 days of age. It was concluded that the development of viable mating in males encompasses a relatively broad time span that was closely tied to the maturation of the spermatogenic and steroidogenic functions of the testis, while aggressive behavior had a sharp onset that coincided with the first evidence of heightened androgen secretion.

Numerous studies have linked aggressive and sexual behavior with testicular androgens in adult males of numerous mammalian species (Young *et al.*, 1964; Phoenix *et al.*, 1967; Bronson and Desjardins, 1971). Moreover, several lines of evidence strongly indicate that circulating androgens organize neural control systems associated with these behaviors very early during neonatal life (Bronson and Desjardins, 1971; Money and Ehrhardt, 1971). Typically, such conclusions were reached by either supplying exogenous hormones or by removing endogenous hormones, producing organization at various ages. It is surprising, considering the amount of interest in this area and the obvious pivotal role of the testis in modulating male social behavior, that almost no information is available concerning the normal interrelationships between fighting and mating

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behavior, on the one hand, and testis function during postnatal maturation, on the other.

The present study was designed to examine the ontogeny of fighting and mating as related to changes in the spermatogenic and androgenic functions of the testis during postnatal maturation in house mice. The results show that intermale aggression has a sharp onset and precedes the appearance of fertile mating in male house mice. Initiation of both behaviors, however, follows a marked increase in plasma androgens. Importantly, aggressiveness continues to increase beyond the peak noted in plasma androgens, while maximal incidence of fertile mating was coincident with peaks noted in plasma androgen levels, testosterone biosynthesis *in vitro*, and spermatogenic activity.

MATERIALS AND METHODS[4]

General

Laboratory-raised descendants of a wild stock of house mice (*Mus musculus*) were killed at 10, 21, 30, 35, 40, 45, 50, 55, 60, 75, or 100 days of age. Our breeding colony was developed from animals trapped originally in eastern Pennsylvania. Animals were mated at random in the colony, and wild males were introduced infrequently to reduce inbreeding. Ten- and twenty-one-day-old animals were removed directly from litters; other animals were weaned at 21 days of age and housed individually in 17.5 × 25 × 12.5 cm steel cages in a room maintained at 25°C with a daily light–dark cycle of 14:10. Litter size was standardized to a maximum of nine at 1–2 days of age by randomly removing littermates. Food (Rockland Mouse/Rat Diet) and water were provided in excess at all times. Where levels of statistical significance are presented, data were evaluated using analysis of variance to test for differences among treatment means. Correlations between age and indices of aggressiveness were evaluated using Kendall's rank-order correlation test.

[4] The following trivial names are used: androstenedione = androst-4-ene-3,17-dione; testosterone = 17β-hydroxy-androst-4-en-3-one; androstanedione = 5α-androstane-3,17-dione; androsterone = 3α-hydroxy-5α-androstan-17-one; androstanediol = 3α,17β-dihydroxy-5α-androstane; dihydrostestosterone = 17β-hydroxy-5α-androstane-3-one.

Tissue Collection

Males were weighed, decapitated, and trunk blood was collected in tubes containing 0.1 ml of 20% sodium oxalate. Immediately after collection, blood was cooled to 5°C, centrifuged, and the supernatant plasma stored at −20°C until analyzed for total androgens. Testes were weighed and placed in ice cold 0.154 M KCl or Zenker-formol fixative. Seminal vesicles were removed after grasping the base of each gland with forceps to prevent loss of secretory material, trimmed, placed directly on Dry Ice, and then weighed. The glands and their entire contents were homogenized and an aliquot used for fructose determination (Davis and Gander, 1967). The body was eviscerated and placed in 10% neutral buffered formalin prior to weighing the adrenal, thymus, and preputial glands.

Histological Analysis

Zenker-formol fixed testes, epididymides, and vas deferens were dehydrated, embedded in paraffin, cut at 4 μm, and stained with periodic acid-Schiff and counter-stained with hematoxylin (Humason, 1962). Testes from 21-, 30-, 35-, 40-, 50-, 60-, and 100-day-old mice were studied in detail. The number of type A spermatogonia, preleptotene and pachytene spermatocytes, step 7 spermatids, and Sertoli nuclei (containing a nucleolus) present at stage VII of the cycle of the seminiferous epithelium (Oakberg, 1956) were counted in 20 round tubular cross-sections from five mice per age category. All cell counts were corrected for differences in nuclear diameter by applying Abercrombie's formula (Abercrombie, 1946). Cell counts made at stage VII of the cycle of the seminiferous epithelium were considered to be representative of spermatogenesis as a whole. Estimates of Leydig cell numbers were obtained by counting the number of cells (at a magnification of 200×) in 20 randomly selected fields and multiplying this number (n) by weight of the testis (w) for five animals per age category (Clegg, 1966). Sections of tissue prepared from the medial portion of the vas deferens and from the cauda epididymis–vas deferens juncture were examined for the presence or absence of spermatozoa.

Behavioral Observations

Animals 30 days of age or older were paired in a neutral cage for 15 min under white light between 6 and 9 PM. Latency to attack, total fights, and time spent fighting were recorded (Cat-

lett, 1961). All mice were returned to their home cages after fighting and then killed between 8 and 11 AM the following day. Previous data (McKinney and Desjardins, unpublished) indicate that aggressive behavior during a single 15-min pairing period has no effect on androgenic functions of the testis as measured in this study.

Fertility was examined by pairing males (other than those used in evaluation of aggression) 30, 35, 40, 45, 50, 55, or 100 days of age with sexually naive females 75 to 80 days of age. Females were weaned and placed 4–5 per cage at 21 days of age. Two days prior to pairing with males, females were removed from groups and caged singly. Males of a given age then were paired with a female for 24 hr and removed from the cage. Previous work indicated this procedure results in a peak in the proportion of females in estrus during the 24-hr-pairing period in this stock of mice (McKinney, 1972). This finding was confirmed by examining for vaginal plugs (external observation) and determining the stage of the estrous cycle (Bronson *et al.*, 1966) by using vaginal lavage on the morning following pairing with a male. Incidence of pregnancy among sexually receptive females (those having plugs or in estrus) was determined by autopsy 7–10 days after pairing. The proportion of females categorized as sexually receptive during pairing and subsequently found to be pregnant at autopsy provided an index of fertility in males of different ages.

Plasma Androgens

Total plasma androgens were determined by the competitive protein-binding procedure of Murphy (1967). The procedure adopted for mouse blood consisted of extracting 0.5–1.0 ml of blood plasma, equilibrated with 2000 dpm [1,2-^{3}H]testosterone (45 Ci/mM), two times with 10 ml of freshly distilled diethyl ether. The combined ether extracts were washed with 1.0 ml of 0.1 N NaOH and 1.0 ml of 0.001 *M* ethylenedinitrilotetraacetic acid for 0.5 min, respectively, and concentrated to 3.0 ml by evaporation under nitrogen at 45°C. Aliquots (0.5 ml) of extract were used to determine the amount of [1,2-^{3}H]testosterone lost during extraction (all samples were corrected for 100% recovery). Two aliquots (0.75 and 1.25 ml) of the ether extract from each sample were assayed for total androgens. Ether extracts were evaporated under nitrogen, and 1.0 ml of "protein-tracer solution" (prepared by adding 0.4 ml of ethanolic [1,2-^{3}H]testosterone (10 μCi/ml; 45 Ci/mM) to 100 ml of 0.2 M sodium phosphate buffer, pH 6.0, containing 1% third trimester human pregnancy plasma) was added to each sample. Samples were incubated at 45°C for 5 min, remixed, and placed at 4°C for an additional 30 min. Following addition of 50 mg of Florisil (60–100 mesh; extensively washed to remove fines and activated at 100°C for 1 h before use), each tube was agitated for 2 min at 25°C. Tubes were maintained at 4°C for 10 min before placing a 0.5-ml aliquot of the supernatant in a scintillation vial with 12 ml of toluene-based scintillation fluid (0.73% 2,5-diphenyloxazole) to determine the amount of radioactive testosterone bound. Standard curves containing various amounts of testosterone (0–10 ng per tube) were run in duplicate for each assay. The precision of the procedure was checked by repeatedly assaying samples from a large pool of blood plasma obtained from adult males. The coefficient of variation for 18 duplicate determinations was less than 4.5%. Moreover, addition of 1, 5, and 10 ng of testosterone to 1 ml samples (five determinations per unit mass) of blood plasma from castrated mice resulted in 1.2 ± 0.2, 5.4 ± 0.3, and 11.0 ± 0.5 ng/ml, respectively. Since the present procedure is not specific for testosterone (Frick and Kincl, 1969), five samples of pooled blood plasma from intact adult mice were assayed using the above procedure and that described by Brownie *et al.* (1964) as modified by Kirschner and Coffman (1968). Values averaged (±SEM) 6.7 ± 0.5 ng/ml for the present procedure and 5.2 ± 0.4 ng/ml for that of Kirschner and Coffman (1968), which has been extensively validated in this laboratory (Desjardins and Ewing, 1971; Johnson and Ewing, 1971). Thus, approximately 80% of the total androgen present in the plasma of adult males may be attributed to testosterone.

Biochemical Composition and Androgen Biosynthesis

Teased testis tissue was prepared (Umbriet *et al.*, 1953), in an ice-cold Petri dish containing a few drops of 0.154 M KCl. Testis tubules (75–100 mg fresh wt) were placed in 10 ml of 95% ethanol and assayed for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Tucker, 1964). Testis tubules were homogenized (10% w/v) in 0.154 M KCl using an all-glass Ten-Broeck homogenizer. Aliquots of the homogenate were analyzed for carbohydrate (Carroll *et al.*, 1956) and protein (Lowry *et al.*, 1951).

Testis tissue from two or more animals of the same age was pooled to provide adequate tissue for androgen biosynthesis. This pooling was accomplished by incubating 200 to 250 mg of tissue for 2 h under 95% O_2:5% CO_2 at 34°C in a 25 ml Erlenmeyer flask containing 2.8 ml of Krebs-Ringer bicarbonate buffer (pH 7.4), 0.1 ml of sodium [1-^{14}C]acetate (25 μCi; 7.5 mM), and 0.1 ml D-glucose (10 mM) (Desjardins *et al.*, 1971). The reaction was stopped by freezing each flask

on Dry Ice. The levels of acetate present in the incubation flasks were saturating, since increased concentrations of [1-^{14}C]acetate did not increase the amount of ^{14}C incorporated into [^{14}C] androgens isolated in the present study.

After thawing, the contents of each flask were homogenized in a glass grinding vessel with a motor driven Teflon pestle until pieces of tissue were no longer visible. The homogenate and 0.154 M KCl used to rinse the flask and homogenizer were transferred to an extraction tube containing 50 μg (and about 4000 dpm tritium) of each of the following androgens: androstenedione, testosterone, androstanedione, androsterone, and androstanediol. This mixture was extracted three times with 30 ml of redistilled dichloromethane and the combined extracts evaporated under nitrogen at 45°C. The residue was dissolved in 15 ml of petroleum ether (bp 30–60°C) and partitioned five times between 15 ml of aqueous 70% methanol. The combined methanolic extracts were evaporated to the aqueous phase under nitrogen at 45°C and extracted with 20 ml of redistilled dichloromethane three times. The combined dichloromethane extracts were evaporated under nitrogen and the residue chromatographed on a thin-layer plate (20 × 20 cm, coated with 250 μm of silica gel) in chloroform–methanol (98.5:1.5). A reference lane on each thin-layer plate was sprayed with a 0.01% solution of morin and visualized under ultraviolet light after air drying. The areas corresponding in chromatographic mobility to authentic androstenedione, testosterone, androstanedione, androsterone, and androstanediol were identified, individually scraped, and eluted from the silica gel with warm chloroform–methanol (2:1) into a conical test tube. Eluates were dried under nitrogen, concentrated, and the residue rechromatographed as outlined above, except that the following solvent systems were used: testosterone and androsterone; benzene–ethyl acetate (65:35), androstanediol (includes trace amounts of 3β-androstanediol); dichloromethane–acetone (80:20), androstenedione and androstanedione; benzene ethyl acetate (50:50). Following chromatography, authentic steroids were identified, and compounds with similar chromatographic mobility were eluted as noted above. Eluates were placed into scintillation vials after which 12 ml of toluene-based scintillation fluid {0.3% dimethyl 1,4-bis[2-(4-methyl-5-phenyl-oxazolyl)]benzene and 0.5% 2,5-diphenyloxazole} was added to determine the radioactivity associated with each compound isolated. All radioactivity values were corrected to 100% recovery by determining the amount of tritiated steroid remaining after extraction and chromatographic isolation in each sample. In some experiments, the identity of the radioactive products (i.e., [^{14}C]androstenedione, [^{14}C]testosterone, [^{14}C]androstanedione, [^{14}C]androsterone, and [^{14}C]androstanediol) isolated from testes incubated in the presence of [1-^{14}C]acetate was checked by recrystallization with 50 mg of the appropriate authentic steroid in aqueous methanol, acetone, and ethanol, respectively. In all instances, the specific activity of the crystals associated with a specific radioactive product was similar (i.e., did not differ by more than 2%) after isolation from these three different solvent systems. The high degree of radiochemical homogeneity demonstrated by recrystallization studies indicates that material subjected to quantification by liquid scintillation spectrometry was similar to the respective authentic steroid. In some initial experiments, the formation of [^{14}C]dihydrotestosterone was examined according to the same protocol used for other [^{14}C] androgens. [^{14}C]Dihydrotestosterone was isolated, but it accounted for less than 2% of the total radioactivity regardless of the age category studied; consequently, it was not isolated in subsequent experiments.

Measurement of Radioactivity

Radioactivity was measured in a Packard Tri-Carb (Model 3365) liquid scintillation spectrometer equipped with automatic external standardization. In all cases, sufficient radioactivity was allowed to accumulate to give a probable error of 2% or less. All radioactivity values were corrected to 100% efficiency (dpm) by counting each sample against a ^{137}Cs external standard and comparing the value obtained to a standard correlation curve (count rate of external standard vs the percent efficiency of samples of known radioactivity containing varying amounts of a quenching additive for either ^{14}C or ^{3}H). The counting efficiency for ^{14}C and ^{3}H was 70 ± 2% and 40 ± 2%, respectively.

Chemicals

Isotopically labeled [1,2-^{3}H]testosterone (45 Ci/mmole), [1,2-^{3}H]androstenedione (45 Ci/mmole, [1,2-^{3}H]androsterone (35 Ci/mmole), and sodium [1-^{14}C]acetate (56.9 mCi/mmole) were purchased from New England Nuclear, Boston, Mass. [^{3}H]Androstanedione and [^{3}H]androstanediol were biosynthesized and provided through the courtesy of Dr. J. D. Wilson, The University of Texas Southwestern Medical School, Dallas, Texas. Radioactive steroids were repurified before use by either paper or thin-layer chromatography or both in several solvent systems. Reagents used to prepare scintillation fluids were obtained from Packard Instrument Co., Inc., Downers Grove, Ill. Silica gel (Silicar TLC-7GF) and solvents (nanograde quality) used to isolate androgens were purchased

from Mallinckrodt Chemical Co., St. Louis, Mo. Carrier steroids or those used for thin-layer chromatography were supplied by Steraloids, Inc., Pawling, N.Y., and recrystallized before use.

RESULTS

Organ and Body Weights

Body growth was greatest prior to 40 days of age, but continued through 100 days (Fig. 1). Marked increases in paired testis weight occurred between 10 and 55 days of age (Fig. 2). At 55 days, paired testis weight was 95% of that noted in 100-day-old mice. Seminal vesicle growth followed a sigmoid pattern with no increase occurring between 10 and 21 days and the most rapid growth occurring from 21 to 55 days of age (Fig. 3). Preputial gland weights paralleled seminal vesicle weights, but the rate of growth was maximal between 21 and 45 days (Fig. 4). Adrenal glands more than tripled in weight (200% increase) from 10 to 21 days and were maximal by 40 days (Fig. 5). Thymus gland weight increased 49% from 10 to 21 days, then decreased to an apparent baseline by 60 days (Fig. 6).

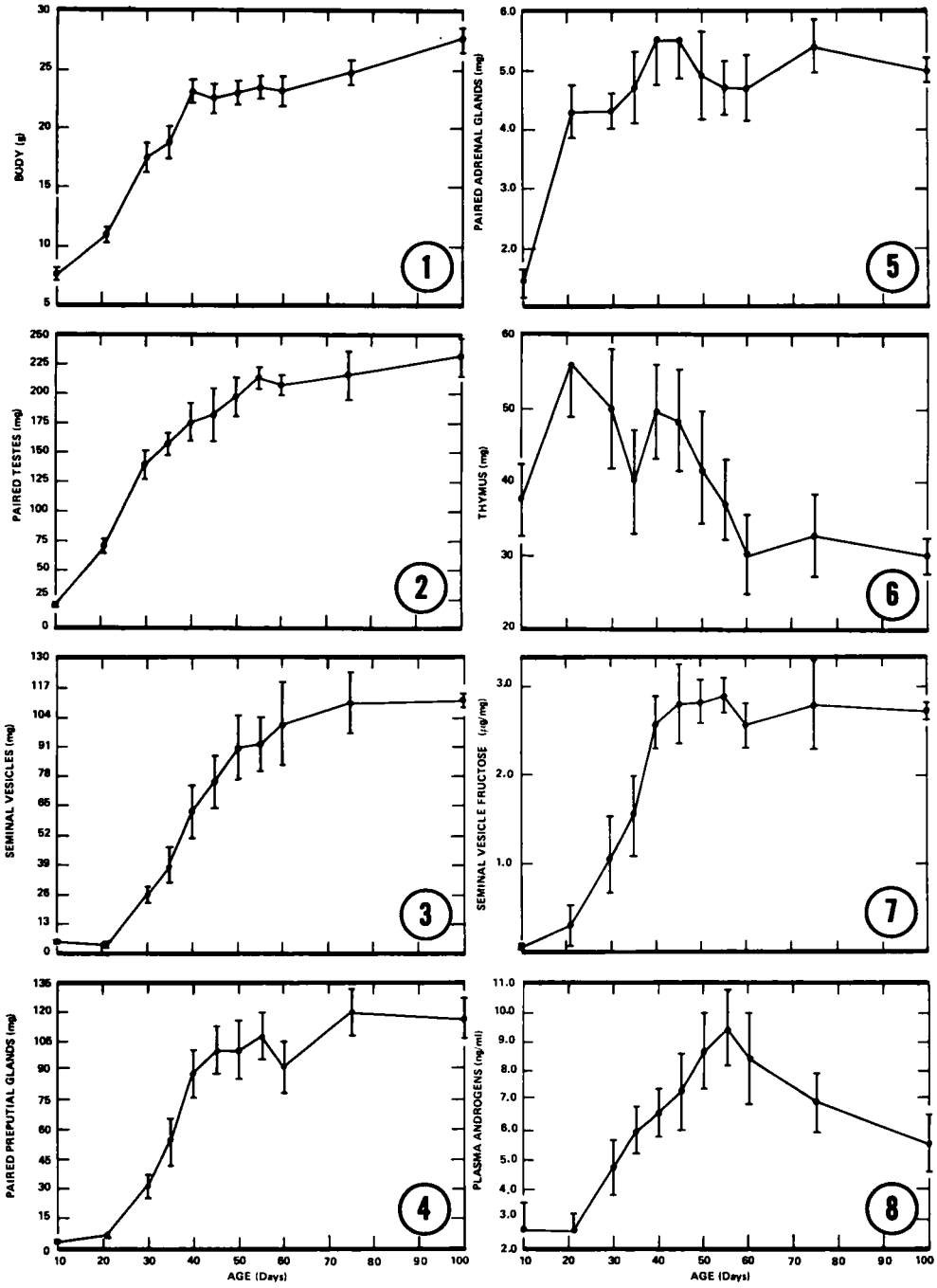
Total Testis Protein, Carbohydrate, RNA, and DNA

The average concentration of protein in testicular homogenates decreased about 10% between 10 and 21 days (Table 1). After this initial decrease, testis protein concentrations averaged approximately 104 mg/g through 35 days, then increased gradually up to 113 mg/g by 75 days of age (Table 1). The carbohydrate concentration of the testis increased steadily from 1.5 mg/g at 10 days to 2.6 mg/g at 75 days of age (Table 1). Testicular RNA concentrations declined about 25% between 10 and 40 days, but increased from 5.3 to 10.1 mg/g between 40 and 60 days of age (Table 1). As RNA, testis DNA concentrations initially decreased through 35 days, then increased steadily from 5.7 to 8.7 mg/g between 35 and 60 days of age. Beyond 60 days of age, the concentrations of both nucleic acids tended to decline but these changes were not significant ($P > 0.25$). Despite the early decrease in testicular RNA and DNA concentrations, the ratio of RNA–DNA (Table 1) increased successively from 0.52 to 1.15 between 10 and 60 days of age, but after 60 days fluctuations in the RNA–DNA ratio were not significant ($P > 0.25$).

Seminal Vesicle Fructose, Plasma Androgens, and in Vitro *Synthesis of Testicular Androgens*

Seminal vesicle fructose concentrations increased most rapidly from 21 to 50 days of age. At 21 days, fructose concentrations averaged 0.2 μg/mg and reached 2.8 μg/mg by 50 days of age (Fig. 7). Between 55 and 100 days of age, no measurable changes were observed in the concentration of seminal vesicle fructose ($P > 0.25$), resulting in a plateau beyond 55 days. Plasma androgen concentrations (Fig. 8) were similar at 10 to 21 days of age ($P > 0.25$), but the level increased linearly from 2.3 to 9.1 ng/ml between 21 and 55 days. Following the peak noted at 55 days, plasma androgen concentrations declined consistently to 5.7 ng/ml at 100 days.

In vitro incubation of testis tubules with [1-^{14}C]acetate resulted in measurable radioactivity associated with androstenedione, testosterone, androstanedione, androsterone, and androstanediol regardless of the age category studied (Table 2). In general, the total amount of radioactivity associated with [^{14}C] androgens synthesized from [1-^{14}C]acetate *in vitro* was three to four times greater in testes obtained from 10-, 21-, and 30-day-old mice than those obtained from 60-, 75-, and 100-day-old mice. Moreover, at 10 and 21 days of age, approximately 2% of the [^{14}C] androgens isolated were associated with [^{14}C]-testosterone. During this period of development, the radioactivity attributed to other [^{14}C] androgens averaged about 15% for [^{14}C]androstenedione, 17% for [^{14}C]an-



FIGS. 1–8. Changes in body and organ weights, seminal vesicle fructose, and plasma androgen concentrations between 10 and 100 days of age in house mice. All data are shown as mean ±1 standard deviations. Sample sizes range from 20 to 24 animals for body and organ weight values and seminal vesicle fructose concentrations except the 10-day-old group (n = 10). Pooling of blood plasma resulted in a sample size of 10 in each age category for total androgen concentration.

TABLE 1

AVERAGE CONCENTRATION OF PROTEIN, CARBOHYDRATE, RNA, AND DNA IN TESTICULAR HOMOGENATES DURING POSTNATAL MATURATION IN HOUSE MICE[a]

Days of age	Sample size	Protein	Carbohydrate	RNA	DNA	
		(mg/g Testis)				RNA/DNA
10	9	121.4 ± 42.4	1.5 ± 0.1	7.1 ± 0.4	12.7 ± 0.9	0.52 ± 0.02
21	11	104.3 ± 3.8	1.6 ± 0.1	6.5 ± 0.3	8.5 ± 0.9	0.89 ± 0.12
30	11	104.2 ± 3.3	1.7 ± 0.1	6.1 ± 0.4	7.1 ± 0.4	0.89 ± 0.06
35	11	104.6 ± 1.5	1.8 ± 0.2	5.8 ± 0.3	5.7 ± 0.5	1.01 ± 0.11
40	11	106.0 ± 2.5	1.9 ± 0.1	5.3 ± 0.3	6.7 ± 0.6	0.98 ± 0.18
45	12	105.6 ± 3.3	2.0 ± 0.1	5.6 ± 0.1	6.3 ± 0.4	0.96 ± 0.09
50	12	105.3 ± 2.9	2.4 ± 0.1	5.8 ± 0.2	6.9 ± 0.4	0.97 ± 0.19
55	20	110.2 ± 2.0	2.3 ± 0.2	6.7 ± 0.1	6.4 ± 0.2	1.08 ± 0.04
60	10	112.0 ± 1.2	2.5 ± 0.2	10.1 ± 0.8	8.7 ± 0.3	1.15 ± 0.05
75	11	113.3 ± 1.4	2.6 ± 0.1	8.3 ± 0.7	7.6 ± 0.2	1.09 ± 0.09
100	10	113.8 ± 0.5	2.6 ± 0.2	8.9 ± 0.3	7.8 ± 0.2	1.13 ± 0.02

[a] Each value represents the mean ± standard error of the indicated number of observations.

TABLE 2

In Vitro INCORPORATION OF [1-^{14}C]ACETATE INTO [^{14}C]ANDROGENS BY MOUSE TESTES DURING POSTNATAL MATURATION

Product isolated[a]	Days of age										
	10	21	30	35	40	45	50	55	60	75	100
[^{14}C]Androstenedione	1,958[b] ± 172	1,431 ± 129	408 ± 79	651 ± 81	312 ± 49	289 ± 67	287 ± 83	150 ± 71	182 ± 88	177 ± 69	123 ± 37
[^{14}C]Testosterone	250 ± 37	200 ± 41	301 ± 32	600 ± 54	642 ± 39	705 ± 47	1,321 ± 109	2,896 ± 165	3,660 ± 220	3,533 ± 196	3,409 ± 204
[^{14}C]Androstanedione	3,185 ± 244	2,553 ± 209	1,367 ± 212	1,922 ± 419	699 ± 88	531 ± 59	463 ± 41	315 ± 39	300 ± 51	282 ± 44	288 ± 41
[^{14}C]Androsterone	4,330 ± 285	3,105 ± 272	2,605 ± 263	2,328 ± 281	951 ± 77	858 ± 73	991 ± 81	862 ± 98	747 ± 69	453 ± 71	478 ± 64
[^{14}C]Androstanediol	1,660 ± 168	1,906 ± 177	1,729 ± 171	1,400 ± 193	444 ± 59	516 ± 56	460 ± 47	332 ± 51	339 ± 65	782 ± 73	282 ± 80

[a] Each value represents the mean ± standard error of five replicates. [^{14}C]Androgens synthesized by teased testis tubules (200–250 mg) incubated at 34°C for 2 h in KRB buffer (pH 7.4) containing sodium [1-^{14}C]acetate (25 μCi; 2.5 mM) and glucose (10 mM).

[b] dpm/100 mg testis.

drostanediol, 28% for [^{14}C]androstanedione, and 35% for [^{14}C]androsterone. By 40 days of age the distribution of radioactivity changed markedly among the [^{14}C] androgens isolated. The magnitude of this change consisted of a threefold increase in [^{14}C]-testosterone formation and nearly a fivefold drop in [^{14}C]androstenedione synthesis. In addition, the radioactivity associated with 5α-reduced androgens declined at least threefold in the case of [^{14}C]androstanediol and fourfold in the case of [^{14}C]androstanedione and [^{14}C]androsterone, respectively. Although the greatest decline in [^{14}C]androstenedione and 5α-reduced [^{14}C] androgens occurred primarily between 21 and 40 days of age, the production of these testicular androgens *in vitro* continued to diminish through 55 days of age. After this period, only minor fluctuations in radioactivity were detected. Reduced formation of [^{14}C]androstenedione and 5α-reduced androgens by testis incubates coincided with steady increments in [^{14}C]testosterone production. Thus, between 40 and 60 days of age, [^{14}C]testosterone increased from 642 to 3660 dpm/100 mg testis. Following this six-

TABLE 3

INDICES OF AGGRESSIVENESS AS RELATED TO AGE IN MALE HOUSE MICE[a]

Age	Incidence of fighting among pairs	Total fights per paired encounter	Latency to attack (sec)	Duration of fighting (sec)
30	0/12	0.0	—	—
35	9/10	6.1 ± 1.4	274 ± 40	34 ± 11
40	11/11	10.4 ± 1.2	198 ± 34	124 ± 24
45	12/12	9.2 ± 1.2	227 ± 56	79 ± 16
50	12/12	10.7 ± 0.9	152 ± 27	138 ± 18
55	20/24	9.8 ± 1.3	121 ± 22	96 ± 17
60	11/11	11.5 ± 1.8	167 ± 39	127 ± 23
75	10/11	11.0 ± 1.5	83 ± 26	146 ± 31
100	10/10	15.0 ± 1.1	90 ± 9	184 ± 26

[a] Each value represents the mean ± standard error.

TABLE 4

INCIDENCE OF PREGNANCY AMONG FEMALES 7–10 DAYS FOLLOWING PAIRING WITH A MALE

Age of male	Number of females in estrus during pairing	Percent pregnancies among females in estrus during pairing
35	17	0
40	15	20.0
45	13	30.7
50	14	64.3
55	17	88.2
100	36	91.6

TABLE 5

RELATIONSHIP BETWEEN AGE AND APPEARANCE OF SPERMATOZOA AT THE JUNCTURE OF THE CAUDA EPIDIDYMIS–VAS DEFERENS AND IN THE MEDIAL VAS DEFERENS[a]

Age (days)	Anatomical location	
	Cauda epididymis–vas deferens juncture	Medial vas deferens
30	0/8	0/8
35	0/8	0/8
40	10/10	0/10
45	8/8	2/8
50	8/8	2/8
55	6/6	2/6
60	9/9	3/9

[a] Data are presented as the number of sperm positive cases per number of males examined.

fold increase in [^{14}C]testosterone, the radioactivity associated with testosterone plateaued and accounted for approximately 75 to 80% of the total [^{14}C] androgens isolated after *in vitro* incubation of testes with [1-^{14}C]acetate.

Fighting and Mating Behavior

The first incidence of spontaneous intermale aggression was observed in 35-day-old mice (Table 3). Latency to attack and its correlate, cumulative attack time, increased with age and with time in isolation, as indicated by a downward trend in latency to attack, an upward trend in total fights, and duration of fighting per observation period (Table 3). These indices of aggressiveness were significantly correlated with age ($P < 0.01$ in each case), suggesting that the development of intermale aggression in isolated house mice may be only partially dependent on androgens, since indices of aggressiveness continued to increase despite evidence indicating that testicular androgenic functions were maximal by 55–60 days of age. No evidence of successful mating was observed in 35-day-old males, but mating efficiency increased from 20% at 40 days of age and approached adult levels by 55 days of age (Table 4). Onset of successful breeding was coincident with first appearance of spermatozoa (40 days of age) at the juncture of the cauda epididymis and vas deferens, but preceded evidence of sperm in the medial portion of the vas deferens (Table 5).

Spermatogenesis and Interstitial Tissue

At 10 days of age, the seminiferous epithelium contained Sertoli cells, all classes of spermatogonia, and primary spermatocytes through the mid-pachytene phase of meiosis I. Spermatogonia and spermatocytes were observed in specific cell associations, making it possible to distinguish four categories of tubules in 10-day-old mice. These tubules were identified by: (1) the presence of three or four type A and numerous intermediate spermatogonia, (2) several type A and 10–12 type B spermatogonia, (3) mainly preleptotene and a few pachytene spermatocytes along with type A spermatogonia, and (4) only type A spermatogonia plus leptotene and zygotene spermatocytes. The relative distribution of these four cell associations was determined by classifying 50 tubules from each of five mice. Based on these criteria, 8.7, 12.4, 26.3, and 52.6% of the tubules were in categories 1, 2, 3, and 4, respectively. Although spermatids could be distinguished by 21 days of age, this same classification system was used to compare the relative distribution of germ cells in testes from 10- and 21-day-old mice. The number of tubules in categories 1, 2, 3, and 4 was 7.0, 13.9, 30.1, and 89.0%, respectively, at 21 days of age. The similarity in the frequency of appearance of these stages for mice in both age groups suggested the cycle of the seminiferous epithelium was established by 10 days of age. After 21 days of age, the acrosomic system of developing spermatids was used to determine the relative distribution of the stages of the cycle of the seminiferous epithelium in 21-, 30-, 35-, and 100-day-old mice. The results presented in Table 6 indicate that spermatogenesis had advanced through step 8 of spermiogenesis by 21 days, and that the first 12 steps in this process were evident by 30 days of age. Although the complete array of germ cells was not noted until 35 days of age (i.e., stage VIII tubules contained step 16 spermatids), the data in Table 6 demonstrate that the relative distribution of the various stages of the cycle of the seminiferous epithelium was independent of age, since the frequency of appearance of the stages was markedly similar in immature and adult mice.

TABLE 6

PERCENTAGE OF TUBULAR CROSS SECTIONS AT VARIOUS STAGES OF THE CYCLE OF THE SEMINIFEROUS EPITHELIUM[a]

Stage of cycle	Days of age			
	21	30	35	100
I	9.8	10.9	11.3	10.7
II	5.9	5.8	5.4	5.6
III	4.0	3.8	3.3	3.5
IV	7.1	8.6	9.1	8.8
V	6.8	8.2	8.8	7.9
VI	5.9	6.4	6.6	6.3
VII	17.2	16.1	15.9	16.5
VIII	7.8	8.4	8.1	8.0
IX	27.1	6.9	7.2	7.1
X	27.1	4.7	4.7	5.5
XI	27.1	10.1	9.9	10.6
XII	8.4	10.0	9.7	9.5

[a] Based on determining the stage of the cycle of the seminiferous epithelium of 1000 tubules (200 tubules from each of five mice) at indicated age.

The numbers of type A spermatogonia, preleptotene, and pachytene spermatocytes and step 7 spermatids per tubular cross section at stage VII of the cycle of the seminiferous epithelium increased in a stepwise manner between 21 and 40 days of age (Table 7). By 50 days, the number of germ cells counted at stage VII was similar ($P > 0.25$) to the number observed in testes of 60- or 100-day-old animals (Table 7). During the period that germ cell numbers were increasing, the ratio of type A spermatogonia to pachytene spermatocytes and the ratio of preleptotene to pachytene spermatocytes remained relatively constant (Table 8). However, the ratio of pachytene spermatocytes to step 7 spermatids increased steadily from 1:0.24 at 21 days to 1:3.3 at 50 days and remained at this

TABLE 7

AVERAGE NUMBER OF CELLS IN ROUND TUBULAR CROSS SECTIONS OF MOUSE TESTES AT STAGE VII OF THE CYCLE OF THE SEMINIFEROUS EPITHELIUM DURING DEVELOPMENT[a]

Days of age	Germ cell classification				Sertoli cells
	Type A spermatogonia	Preleptotene spermatocytes	Pachytene spermatocytes	Step 7 spermatids	
21	1.68 ± 0.10	14.4 ± 0.4	12.7 ± 0.5	4.4 ± 1.0	4.2 ± 0.1
30	0.76 ± 0.10	16.6 ± 0.3	15.0 ± 0.5	39.4 ± 1.3	3.7 ± 0.2
35	0.83 ± 0.09	17.9 ± 0.4	17.7 ± 0.4	40.7 ± 1.4	3.0 ± 0.2
40	0.92 ± 0.07	18.5 ± 0.5	17.5 ± 0.3	44.6 ± 1.0	3.1 ± 0.2
50	0.88 ± 0.08	18.1 ± 0.3	17.0 ± 0.3	46.3 ± 1.2	2.8 ± 0.1
60	0.88 ± 0.10	18.3 ± 0.4	17.2 ± 0.4	56.9 ± 1.3	2.8 ± 0.3
100	0.90 ± 0.09	18.0 ± 0.4	17.5 ± 0.5	57.8 ± 1.1	2.8 ± 0.2

[a] Each value represents the average ± standard error of five mice (20 tubules at stage VII of the cycle of the seminiferous epithelium were counted per mouse). All cell counts were corrected for differences in nuclear diameter by Abercrombie's formula.

TABLE 8

RATIO OF GERM CELLS IN THE SEMINIFEROUS EPITHELIUM (STAGE VII) OF MICE DURING TESTICULAR DEVELOPMENT[a]

Days of age	Type A sptg.[b] / Preleptotene sptc.[c]	Preleptotene sptc. / Pachytene sptc.	Pachytene sptc. / Step 7 sptd.[d]
21	1:21.2 ± 0.7	1:0.88 ± 0.07	1:0.24 ± 0.03
30	1:21.8 ± 0.9	1:0.95 ± 0.05	1:2.63 ± 0.08
35	1:21.5 ± 0.8	1:0.94 ± 0.06	1:2.30 ± 0.10
40	1:20.1 ± 0.9	1:0.95 ± 0.05	1:2.55 ± 0.09
50	1:20.6 ± 0.8	1:0.94 ± 0.05	1:3.31 ± 0.07
60	1:20.8 ± 0.9	1:0.94 ± 0.06	1:3.31 ± 0.08
100	1:20.0 ± 0.9	1:0.97 ± 0.06	1:3.30 ± 0.07

[a] Each value represents the average ± standard error of five mice. Ratios were calculated after correcting cell counts for differences in nuclear diameter by Abercrombie's formula.

[b] sptg. = spermatogonia.

[c] sptc. = spermatocyte.

[d] sptd. = spermatid.

level through 100 days of age (Table 8). These results suggest that increases in spermatids were proportionally greater than increases in either spermatogonia or spermatocytes during testis maturation. In contrast to germ cells, the number of Sertoli cells per tubular cross section decreased from an average of 4.2 at 21 days to 2.8 at 50 days, indicating that seminiferous tubules continued to elongate through 50 days. Sertoli cell counts made at 60 and 100 days of age were equivalent to those noted at 50 days, implying that tubular growth was complete and coincident with the attainment of optimal germ cell numbers (Table 7).

Leydig cells were present in testes of 10-day-old mice, and indices of Leydig cell numbers increased steadily from 10 through 60 days of age (Table 9). The number of Leydig cells per testis was maximal at 60 days and declined about 35% by 100 days of age. Thus, Leydig cell numbers increased prior to and for at least 25 days following the completion of spermatogenesis.

DISCUSSION

The present results directly correlate, for the first time, the interrelationships between fighting and mating behavior and the spermatogenic and steroidogenic activity of the testis during postnatal maturation in house mice. From the standpoint of the steroidogenic function of the testis, the results demonstrate that the concentration of circulating androgen increased 300% between 21 and 55 days of age and declined 50% between 55 and 100 days. The relationship between age and peripheral androgen concentrations noted in developing male house mice generally parallels changes noted in plasma testosterone concentrations during maturation in the rat (Resko *et al.*, 1968; Knorr *et al.*, 1970; Grota, 1971). However, the present results indicate that the concentration of circulating androgens noted during early sexual development (i.e., 10–30 days of age) was appreciably greater than the concentration of testosterone found during the comparable period of development in rats (Resko *et al.*, 1968). Since the procedure used to determine plasma androgens (i.e., competitive protein-binding) did not discriminate between testosterone and other androgens capable of displacing radiolabeled testosterone from the binding protein, it is possible that androgens other than testosterone may play an important role in either organizing androgen-dependent responses or stimulating androgen-dependent tissues or both during postnatal development in mice. Furthermore, it is suggested that increases in plasma androgen concentrations noted in mice may in part be due to an increased secretion of these steroids by the testes, since Knorr *et al.* (1970) observed that increases in plasma testosterone levels were associated with corresponding increases in the concentration of testosterone in the spermatic venous effluent through 40 days of age.

Growth of the accessory sex glands and increases in seminal vesicle fructose concentrations coincided with increases in plasma androgen concentrations through 55 days of age. Beyond 55 days, accessory sex gland weights and seminal vesicle fructose concentrations plateaued despite the consistent and gradual decline in peripheral androgen concentrations. Maintenance of these androgen-dependent tissues at adult levels in the face of declining androgen titers suggests that the relative sensitivity of these tissues to circulating androgens differs pre- and postpubertally as has been postulated previously on the basis of the response of the seminal vesicles to exogenous testosterone (Hooker, 1942). In contrast to the direct relationship observed between accessory sex organ weights and plasma androgen concentrations, no association was noted between adrenal gland growth and any index of testis function. However, cessation of adrenal gland development coincided with increases in plasma androgen levels, possibly reflecting X-zone involution between 30 and 40 days of age (Brain and Nowell, 1969). Similarly thymic involution, as indicated by weight, was associated with the appearance of increased levels of plasma androgens.

TABLE 9

INDEX OF LEYDIG CELL NUMBERS DURING TESTICULAR MATURATION

Days of age	Leydig cell index (n × w)[a]	Spermatogenic stage[b]
10	2.1 ± 0.2	Pachytene spermatocytes[c]
21	9.5 ± 1.3	Step 8 spermatids
30	36.1 ± 3.8	Step 13 spermatids
35	38.9 ± 3.2	Complete spermatogenesis
40	42.4 ± 4.4	Complete spermatogenesis
50	60.6 ± 4.1	Complete spermatogenesis
60	65.9 ± 4.7	Complete spermatogenesis
100	42.9 ± 3.2	Complete spermatogenesis

[a] Each value represents the mean ± standard error of five mice. Leydig cells were counted in 20 fields, selected at random, at a magnification of 200× for each mouse.

[b] Spermatogenic stage refers to the most advanced germ cell noted in the seminiferous epithelium.

[c] Mid-pachytene stage.

Studies pertaining to the capacity of the testis to synthesize androgens *in vitro* from [1-^{14}C]acetate demonstrated that the formation of [^{14}C]testosterone accounted for approximately 2% of the total [^{14}C] androgens synthesized at 10 and 21 days of age. At this time, nearly 85% of the [^{14}C] androgens isolated after *in vitro* incubation with [1-^{14}C]acetate were associated with 5-α-reduced androgens, particularly [^{14}C]androsterone and [^{14}C]androstanediol. The incorporation of [1-^{14}C]acetate into [^{14}C]testosterone became more apparent with successive increments in age, so that between 60 and 100 days approximately 75 to 80% of the [^{14}C] androgens isolated was attributed to [^{14}C]testosterone. The transitory increase in testosterone formation during sexual development was accompanied by a gradual but consistent decrease in radioactivity associated with [^{14}C]androstenedione and 5-α-reduced androgens ([^{14}C]androstanedione, [^{14}C]androsterone, and [^{14}C]androstanediol). Present observations agree with those of Steinberger and Ficher (1969), who demonstrated that radiolabeled progesterone was converted to androstenedione and 5-α-reduced androgens rather than testosterone by rat testes *in vitro* between 20 and 40 days of age. However, beyond 40 days, testosterone formation was apparent and occurred as the chief radioactive metabolite of [^{14}C]progesterone. Additionally, these investigators and others (Steinberger and Ficher, 1971; Coffey *et al.*, 1971) suggested that the accumulation of 5-α-reduced androgens following incubation of immature (20 days of age) rat testes with appropriate steroid precursors (e.g., progesterone, androstenedione, and testosterone) *in vitro* could be attributed to testosterone catabolism rather than the lack of testosterone formation. On the basis of these observations and the present finding that steroidogenesis in mice testes parallels that of rats during postnatal development, it is suggested that the accumulation of 5-α-reduced androgens following incubation of mouse testes *in vitro* may result from testosterone catabolism rather than the inability to synthesize testosterone. Thus, it would appear that activation and/or suppression of testicular 5-α-reductases play a significant role in the capacity of mouse testicular tissue to accumulate testosterone *in vitro* during sexual development. However, one should not lose sight of the fact that the steroidogenic pattern noted during postnatal development was based on *in vitro* observations and may not resemble steroidogenesis *in vivo*. Nevertheless, the results show that the capacity of the testis to synthesize [^{14}C]testosterone from [1-^{14}C]acetate *in vitro* generally paralleled increases in plasma androgen concentrations during development.

The important observation made in these studies is that mouse testes were capable of utilizing [1-^{14}C]acetate to form [^{14}C] androgens as early as 10 days of age. Inherent in these observations is the implication that testes contained all the enzymes required for *de novo* androgen synthesis at 10 days, and that such steroids may be secreted since circulating androgens were detected at this time period. Demonstration of *de novo* androgen formation also suggests that circulating gonadotropins must have been present in sufficient quantity to stimulate steroidogenesis at 10 days, since the biosynthetic pathways leading to testicular androgen synthesis depend on these hormones (Menon *et al.*, 1967; Hall, 1970). In support of this speculation, Ojeda and Ramirez (1972) and Swerdloff *et al.* (1971) have shown that significant concentrations of radioimmunoassayable luteinizing and follicle stimulating hormone are present in the peripheral circulation of male rats from 5 through 50 days of age. Additionally, the comparatively higher synthesis of [^{14}C] androgens by incubates of immature mouse testes may be due to the presence of proportionately greater numbers of Leydig cells per unit mass of tissue, since Baillie (1961) calculated that testes of albino mice contained

about three to four times more intertubular tissue during early neonatal development as compared to adults.

Indices of Leydig cell numbers increased from 10 through 60 days of age and paralleled increases in seminal vesicle development, plasma androgen concentrations, and the number of step 7 spermatids between 21 and 50 days of age. Moreover, the 50% reduction in plasma androgens noted between 55 and 100 days of age coincided with a 30% reduction of Leydig cell numbers. Similarly, others reported a significant reduction in Leydig cell numbers in mice (Baillie, 1961) and rats (Clegg, 1966 and Knorr *et al.*, 1970) beyond 60 days of age.

Biochemical analysis of the testis made during postnatal maturation revealed that the major alterations in protein, carbohydrate, RNA, and DNA concentrations occurred between 10 and 60 days of age. Slight but consistent increases in protein and carbohydrate concentrations were measurable between 21 and 55 days of age. In contrast, the concentrations of both RNA and DNA declined during this same time period. Despite the decline in nucleic acid concentration, the ratio of RNA/DNA increased from less than unity at 10 days to greater than unity at 60 days, suggesting a continual increase in protein synthetic activity. Importantly, the increases in protein and carbohydrate concentrations and the ratio of RNA/DNA paralleled the development of spermatogenic activity and reached maximum levels during the same period that the seminiferous epithelium contained maximum numbers of step 7 spermatids.

Examination of the seminiferous epithelium during development showed that all classes of spermatogonia and mid-pachytene spermatocytes were present as early as 10 days of age. Since the time required for the appearance of mid-pachytene spermatocytes exceeds one cycle of the seminiferous epithelium (i.e., 8.8 days, Clermont and Trott, 1969), it may be concluded that type A spermatogonia must have begun dividing about 9 days earlier. On this basis, it appears that the cycle of the seminiferous epithelium started soon after birth in the present study. Indeed, by 10 days of age, it was possible to group specific types of spermatogonia and/or spermatocytes to encompass all stages of the cycle of the seminiferous epithelium observed in adults (Oakberg, 1956). The frequency of appearance of these groups of cells paralleled that noted in older animals. Moreover, when it became possible to identify the stages of the cycle of the seminiferous epithelium on the basis of spermatid development, the frequency of appearance of the 12-cell associations or stages were equivalent in 21-, 30-, 35-, and 100-day-old mice. These observations are in keeping with the concept that the cycle of the seminiferous epithelium is a phenomenon of fixed duration in all species studied thus far (Clermont, 1972). Germ cell counts made at stage VII of the cycle of the seminiferous epithelium demonstrated that the growth of the seminiferous epithelium was due primarily to increases in the number of spermatids rather than type A spermatogonia and spermatocytes. In keeping with this observation, only minor fluctuations were noted in the ratios of type A spermatogonia to preleptotene spermatocytes and preleptotene to pachytene spermatocytes between 21 and 100 days of age (Table 8). However, during this same time span the ratio of pachytene spermatocytes to step 7 spermatids increased roughly twelvefold. Interestingly, the pattern of germ cell development noted in house mice in this study closely corresponds with that observed in other strains (CF-1 and CD-1) of laboratory mice (Nebel *et al.*, 1961; Bartke, 1970) and rats as well (Clermont and Perey, 1957). Importantly, the increase in the ratio of pachytene spermatocytes to step 7 spermatids was related directly to increased concentrations of plasma androgens and the capacity of the testes to synthesize [^{14}C]testosterone *in vitro* from [1-^{14}C]acetate. The

present findings relating increases in testosterone to the differentiation of spermatocytes in developing mice coincides with the evidence indicating that testosterone plays a pivotal role in the completion of the late stages of the meiotic division of primary spermatocytes and the formation and maturation of spermatids in laboratory rats (Lostroh, 1969; Steinberger, 1971).

Although the seminiferous epithelium contained step 16 spermatids (stage VIII of the cycle of the seminiferous epithelium) at 35 days of age, spermatozoa were not observed in the cauda epididymis–vas deferens juncture until 40 days of age. Even though sperm were present in 40-day-old animals, the incidence of fertile matings increased steadily from 40 through 55 days of age. The significance of this finding resides in the fact that the development of fertile mating lagged the physical capability for reproduction in terms of spermatozoan production and availability for transmission to the female. Moreover, the results indicate that initial attainment of maximum reproductive efficiency was linked with peaks noted in plasma androgen concentration, *in vitro* synthesis of [^{14}C]testosterone from [1-^{14}C]acetate, and the numbers of step 7 spermatids and Leydig cells. Once attained, reproductive efficiency apparently remained maximal even though there was a substantial decrease in the levels of androgens in the plasma.

In contrast to the onset (i.e., 40 days) and time (i.e., 55 days) required for the attainment of adult fertility levels, fighting was suddenly and fully mature at 35 days of age. Within this context, it should be recognized that the results pertaining to intermale aggression may be biased in the sense that only one member of the pair must be aggressive for both animals to be scored positive for fighting. Nevertheless, the changes seen in aggression after 35 days of age were not associated with an increase in frequency but rather of latency to attack and its direct correlates, cumulative attack time, and incidence of fighting. Moreover, latency to attack and cumulative attack time have been shown in mice to increase with time spent in isolation (Valzelli, 1969). Consequently, the present observations suggest that the onset and development of fighting and mating behavior are independent and most probably involve separate "neural substrates" within the central nervous sytem. Furthermore, based on differences noted in plasma androgens and other indices of testicular steroidogenic activity measured during the development of fighting and mating behavior, it seems reasonable to propose that the neural centers regulating aggression may be activated with proportionally smaller amounts of androgen than that required for sex behavior. In keeping with this hypothesis, Beeman (1947) observed that the development of aggressiveness preceded any increase in the growth of the accessory sex glands of castrate mice receiving testosterone propionate implants.

We consider puberty to represent the period of postnatal development during which the capacity for sexual reproduction is achieved (Critchlow and Bar-Sela, 1967). On the basis of our results, puberty occurred between 40 and 55 days of age in the present stock of house mice. Aggressiveness and sex behavior (as indicated by fertility) appeared as separate behavioral systems having different developmental periods but whose onset corresponded to increased elaboration of testicular androgens. Termination of the pubertal period as indicated by maximal reproductive performance and the attainment of adult spermatogenic activity was intimately related to peaks in plasma androgen concentration, *in vitro* synthesis of testosterone, and a plateau in testicular and body growth and accessory sex organ development.

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