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TESTICULAR CARNITINE ACETYLTRANSFERASE
ACTIVITY AND SERUM TESTOSTERONE LEVELS
IN DEVELOPMENTAL STAGES OF THE RAT AND RAM

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the
Graduate School of The Ohio State University

By

Bruce Dee Schanbacher, B. S., M. S.

****

The Ohio State University

1973

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INTRODUCTION

The functional importance and complexity of events taking place within the seminiferous tubules of mammals have recently renewed the curiosity and interest of numerous biologists. As the various germ cells, i.e., the spermatogonia, spermatocytes and spermatids, were found to be spatially arranged in well-defined cellular associations, the "cycle of the seminiferous epithelium" emerged (Regaud, 1909; Leblond and Clermont, 1952; Clermont and Perey, 1957; Fawcett et al., 1959).

To understand the differentiation process in the developing testis, one must keep in mind its two functions: 1) production of viable, fertile spermatozoa and 2) secretion of male sex hormones (androgens).

The first function involves the process of spermatogenesis, i.e., the process by which a spermatogonial stem cell gives rise to spermatozoa. Clermont (1972) has divided this process into three distinct phases. The first phase concerns the spermatogonia, which proliferate to give rise to spermatocytes and simultaneously maintain their numbers by renewal. The second phase involves the primary and secondary spermatocytes, which go through the process of the reduction or meiotic divisions leading to the formation of haploid cells, the
spermatids. The third phase concerns a complex series of cytological transformations of the spermatids leading to the production of spermatozoa. Numerous reviews on mammalian spermatogenesis have been published recently (Albert, 1961; Roosen-Runge, 1962; Courrot et al., 1970; Clermont, 1972).

The second function involves the process of steroidogenesis, i.e., the biosynthesis and secretion of the male sex hormones. The primary hormone, testosterone, is secreted by the Leydig cells and secondarily by the adrenal and, possibly, by the seminiferous tubules. It is basically responsible for the maintenance of spermatogenesis, control of gonadotrophin secretion by a feedback mechanism on the hypothalamus and pituitary and for the expression of libido, male behavior, and sexual differentiation. A comprehensive review of testicular androgens has been made by Eik-Nes (1970).

In a recent review, Bishop (1969) advanced the concept that an analysis of changing enzyme patterns in spermatogonia, spermatocytes, spermatids and spermatozoa would prove useful in the understanding of germ cell differentiation. Such identification of nonmorphological characteristics of each cell type was referred to as "finger printing" the testis. As shown by Marquis and Fritz (1965) and Vernon et al., (1971), carnitine acetyltransferase was an enzyme which could be used to "finger print" spermatogenesis in the rat. A comprehensive review of the germ cell related enzymes has been made by Gomes and VanDemark (1974).
The purpose of this investigation was to confirm the results of Vernon et al. (1971) for the rat and to determine if this enzyme could be used in "fingerprinting" spermatogenesis in the ram. Since androgens secreted by Leydig cells are known to influence the maturation and metabolism of the germinal epithelium and influences of testosterone on testicular carnitine acetyltransferase are of interest, serum testosterone levels were also measured.

The research described in this thesis was undertaken to determine developmental changes in rat and ram carnitine acetyltransferase and to relate these to hormonal changes. It is hoped that the data will better establish the pubertal changes which take place in these species.
Establishment of Spermatogenesis in the Rat

The growth curve of rat testis is sigmoid in shape. At first, there is a slow growth rate followed by a more rapid one when spermatogenesis commences. By 60 days of age, testicular growth decreases and closely parallels changes in body weight.

Clermont and Perey (1957) found that sex cords of rats at birth displayed supporting cells and gonocytes. Though many of the gonocytes disappear during the first week after birth, a sufficient number are maintained to give rise to future spermatogonia. Soon a spurt in tubular growth is seen with an increase in the number of interstitial cells. The supporting cells have differentiated by 18 days and 2 generations of spermatogonia are present. Testes taken from 20-day-old rats contain proleptotene and leptotene spermatocytes while late pachytene spermatocytes appear about day 26. Cap stage spermatids appear shortly thereafter and elongated spermatids are found in 40-day-old rats. Increasing numbers of testicular sperm are seen in the seminiferous tubular lumen after 50 days (Knorr et al., 1970 and Clermont, 1972) while complete spermatogenesis has been reported by 63 days of age (Swerdloff, 1972).
Endocrine Aspects in the Rat

Normal spermatogenesis requires the presence of hormones, however their role in regulation of the germinal epithelium remains unknown. A recent review of this topic has been made by Steinberger (1971).

Lostroh (1963) showed regression of the germinal epithelium immediately following hypophysectomy. Clermont and Morgentaler (1955), however, maintained the epithelium with androgen therapy and Hall and Eik-Nes (1962) found interstitial cell stimulating hormone (ICSH) to give similar results by stimulating the production of androgens by Leydig cells. Subsequent studies involving gonadotrophins (Lostroh et al., 1963) resulted in 3 conclusions: 1) follicle stimulating hormone (FSH) stimulates the early stages of germ cell development (spermatogonia and primary spermatocytes); 2) FSH stimulates Sertoli cells; and 3) both FSH and ICSH (i.e., androgen production) are necessary for meiosis and the development of spermatids.

These conclusions are questioned when hormones are injected within 5 days of hypophysectomy. Under these conditions, the germinal epithelium is stimulated qualitatively and quantitatively to the same extent by testosterone, ICSH, and FSH (Clermont and Harvey, 1967).

In order to better establish these pituitary-gonadal interrelationships, Deboljuk et al. (1973) selectively depleted spermatogonia, spermatocytes or young spermatids with busulfan and assayed subsequent gonadotrophins. Their findings suggest
immature spermatids, and perhaps to a lesser degree primary spermatocytes, may be involved in a feedback regulation of LH and FSH secretion. Gomes et al. (1973) have conducted a similar study. The exact mechanisms by which gonadotrophins control spermatogenesis remain a problem of current investigation. Blizzard et al. (1972) have recently discussed the pubertal pituitary-gonadal interrelationships.

Since androgens have been shown essential in the process of spermatogenesis, especially meiotic divisions and spermiogenesis, it would be interesting to know the level of these hormones and when biosynthesis and secretion occurs.

The measurement of testosterone secretion by the fetal rat testis has not been possible for technical reasons, however, androgens have been isolated from the plasma of neonates. Røsko et al. (1968) found 0.27 ng of testosterone per ml of plasma and 328 ng/g (wet weight) of testis on the first day of life. Thereafter levels of testosterone fell until the age of 30 days after which they rose gradually to adult levels of 2 ng/ml. The postnatal decline in plasma levels of testosterone is reflected by histochemical changes in Leydig cells. Niemi and Ikonen (1962) have suggested that two distinct generations of Leydig cells exist in the rat and that the appearance of the second generation coincides with the prepuberal surge in gonadotrophins.

The postnatal period of testosterone regression is also reflected in the conversion of progesterone and cholesterol to
androgens in vitro by testes of neonatal and mature rats but not in 20-day-old rats (Steinberger and Fischer, 1968). More recently, Steinberger and Fischer (1971) found conversions by 20-day-old rat testes of both testosterone and androstenedione to 5α-reduced androgens. No speculation, however, concerning the role of these androgens in the process of spermatogenesis in immature rat testes or in the regulation of pituitary gonadotrophins was given.

Near day 25, rat testis is highly sensitive to gonadotrophic stimulation and a subsequent increase in serum testosterone follows (Hall, 1970).

Establishment of Spermatogenesis in the Ram

As in the rat, the growth curve of ram testis is sigmoid in appearance (Courot, 1962 and Skinner et al., 1968). During the first 2 or 3 months after birth, only two types of cells are present in the sex cords: the supporting cells, which have small, highly stainable nuclei and are located along the basement membrane, and the gonocytes, whose large and lightly stained nuclei are found in the central part of the sex cords (Ortavant et al., 1969).

During 4 to 6 months after birth, the supporting cells proliferate and take on the aspect of Sertoli cells after they stop dividing. Meanwhile the number of gonocytes increases progressively giving rise to A-type spermatogonia. By the 105th day of age, primary spermatocytes are observed, while 120 days pass before spermatids are seen. During this period there is a marked acceleration in the rate of growth of all
testicular elements including Leydig cells (Carmon and Green, 1952 and Gier and Marion, 1970). The last stages of the cycle of the seminiferous epithelium occur only toward the 140-150th day (Courot, 1962 and Sapsford, 1962).

No significant differences between the weights and volumes of the left and right testis and epididymi were found in the Clun Forest ram (Colyer, 1971). This study agrees with those of Courot (1962) and Skinner et al. (1968) that a marked increase in testis weight occurs after body weight has reached 20 kg (about 12 weeks of age). Epididymal weight showed a similar but less dramatic increase.

The date of establishment of spermatogenesis in the ram varies between reports because of two primary factors: initiation of spermatogenesis is dependent more on the development of the animal than on its age (Kibler et al., 1963 and Skinner and Rowson, 1968), and the timing of birth is important since reproductive potential is related to season in this species (Lodge and Salisbury, 1970).

Decreased fertility during the summer may be due to increased photoperiod or increased temperature. Experiments designed to determine the causative agent generally fail since either 16 hours of daylight or artificially increased scrotal temperature result in destruction of late spermatocytes and spermatids (Dutt, 1960, Ortavant, 1962 and Voglmayr et al., 1971).

Johnson et al. (1969), after exposing yearling rams to
32 C for 14 days, found an increase in in vitro incorporation of sodium acetate-1-14C into esterified cholesterol. A subsequent study (Gomes et al., 1971) showed rams to have smaller testes and decreased testosterone levels in testis tissue and in plasma from the spermatic vein. This finding is interesting since a current study indicates summer testosterone levels in the ram may be elevated (Gomes and Joyce, 1973).

Nevertheless these observations may only have quantitative bearing on the establishment of spermatogenesis. Additional studies related to seasonal reproduction are necessary before both quantitative and qualitative aspects are discerned.

Several experiments have dealt with quantitating spermatogenesis in the ram while under different physiological conditions. Intratesticular injections of cadmium or spermatic vascular ligations result in nonreversible destruction of both spermatogenic and interstitial cells (Ball et al., 1972).

Hemicastration in the adult ram results in compensatory hypertrophy of the remaining testis (Voglmayr and Mattner, 1968). Both testis weight and flow of rete fluid were increased over control testes whereas the concentration of spermatozoa in the fluid remained constant. It was suggested that the increase in the diameter of the seminiferous tubules could account almost entirely for the increase in testis weight.

In order to accurately quantitate the germinal epithelium in rams of different physiological states, Lino (1971) has
determined appropriate correction factors involving Sertoli cell nuclear volume and seminiferous tubular radii to be used in cell counts.

Endocrine Aspects in the Ram

The supporting cells of the impuberal ram testis are under gonadotrophic control, whereas the gonocytes are more independent. Injections of ICSH into the hypophysectomized lamb increases testis weight and the total number of supporting cells (Courto et al., 1970). Without gonadotrophic administration, spermatogenesis is halted with the presence of spermatogonia.

The interrelationships of pituitary and gonad in the ram have received the attention of few investigators.

To determine the effect of testosterone on LH secretion and release in the castrated ram, Pelletier (1970) studied the mode of action of a single intramuscular injection of this androgen. From his study, he concluded that testosterone inhibited the synthesis of LH releasing factor in the hypothalamus since large amounts of LH were found in the hypophysis but not in the general circulation.

Crim and Geschwind (1972) using radioimmunoassay have shown increases in serum FSH and LH in castrated rams 30 to 150 days of age. As expected, testosterone propionate markedly suppressed these gonadotrophin titers. Gonadotrophin concentrations in the pituitaries of developing rams were determined in a study by Skinner et al. (1968).
As suggested for the rat, maintenance of spermatogenesis and production of viable spermatozoa with ICSH may involve stimulation of Leydig cells to synthesize and secrete testosterone.

Whether testosterone is the major inhibitor of gonadotrophin release is yet to be determined. Testosterone is the dominant androgen of the fetal sheep testis (Attal, 1969). It also has been shown to be the primary androgen secreted into the spermatic vein of young as well as adult rams (Grim and Geschwind, 1972). Androstenedione and dehydroepiandrosterone were undetectable in their study, however maximum sensitivity was only 10 ng/ml. Testosterone secretion in adult testes with normal spermatogenesis was 42-160 ng/ml of spermatic venous blood. These levels agree with those reported by Setchell et al. (1965), i.e. 25-95 ng/ml, and those by Comes et al. (1971), i.e. 8.2 ± 4.4 μg/100ml.

Peripheral levels of androgens, including testosterone, have yet to be measured in the ram.

Carnitine Acetyltransferase and Testicular Metabolism

The role of carnitine acetyltransferase (EC 2.3.1.7) has received only limited attention since 1965 when it was found in the testis. It has been shown by Fritz (1963) and other investigators that the action of this enzyme is to catalyze the transfer of an acetyl group from its thioester linkage with extramitochondrial CoA to an oxygen ester linkage with the hydroxyl group of carnitine. Located in the inner membrane of the mitochondrion, it transfers acetate groups to intramitochondrial CoA thereby keeping separate the extra- and
intramitochondrial CoA. The acetyl CoA is then used as substrate by the oxidative Krebs cycle, which occurs in the inner matrix compartment. In addition to energy transfer, carnitine acetyltransferase may catalyze the formation of acetyl carnitine which then serves as a store of "active" acetyl groups (Geer and Newburgh, 1970).

Carnitine acetyltransferase functioning in the translocation of acetyl groups across barriers which are impermeable to CoA has also been suggested (Fritz and Yue, 1964). However, the precise function of this enzyme and its necessity in intermediary metabolism are not clear (Lin and Fritz, 1972).

The presence of testicular carnitine acetyltransferase was first observed by Marquis and Fritz (1965a). The high levels of this enzyme in testis extracts were discussed in an accompanying paper in which they reported that spermatozoa have the highest specific activities of carnitine acetyltransferase of the tissues examined, and that these cells probably contribute a major portion to the enzyme activity found in testes (Marquis and Fritz, 1965b). Levels of enzyme were analyzed in the reproductive tissues obtained from male rats of varying ages and following cryptorchidism. Transferase levels in rat testes increased more than 10-fold during the interval between 2 and 5 weeks of age, a period during which the germinal epithelium is rapidly proliferating. In the same study, 18-day cryptorchid testes contained transferase levels only slightly greater than those
of normal 14-day-old animals. It was also noted that androgens were required to permit maintenance of normal carnitine and carnitine acetyltransferase levels in testes and epididymides of cryptorchid rats.

A later report by Vernon et al. (1971) indicated levels of carnitine acetyltransferase in testes of neonatal rats to be approximately 5% of those obtained in testes of adult rats. By using the sedimentation velocity technique of Lam et al. (1970), Vernon's group was able to separate the germinal cells of the rat testis and compare enzyme levels in relatively homogeneous cell populations. Changing activities measured during development indicated that maximal rates of increase were achieved when primary spermatocytes were produced. Diplotene primary spermatocytes had highest activity (6.1 units/10⁸ cells) while spermatogonia had lowest activity (0.16 units/10⁸ cells). Such results demonstrated the usefulness of the "marker enzyme", carnitine acetyltransferase, for the investigation of spermatogenesis, especially the transition of spermatogonia into spermatocytes.

In the same study, testicular carnitine acetyltransferase levels decreased significantly 14 days after hypophysectomy and then continued to decrease until 35 days after the operation.

Carnitine acetyltransferase measurements in testes of hypophysectomized rats provided ancillary evidence consistent with the conclusion that either FSH, LH, or testosterone was required for the normal restoration of pachytene-diplotene
spermatocyte formation. However, production of spermatids and normal levels of carnitine acetyltransferase required FSH plus LH, or FSH plus testosterone (Go et al., 1971).

Geer and Newburgh (1970) recently studied carnitine acetyltransferase in the germ cells of Drosophila melanogaster. The activity of this enzyme in mature sperm was high, however, it was extremely low in spermatogonia, spermatocytes, and spermatids. Electron microscopy indicated that the appearance of high carnitine acetyltransferase activity during spermiogenesis in the fly is dependent upon the development of the mitochondrion and its association with the axial filament complex.

The importance of carnitine acetyltransferase in sheep and its role in relieving "acetyl pressure" in the CoA system have been discussed by Snoswell and Henderson (1970). These authors reported carnitine acetyltransferase activities in sheep liver mitochondria to be some 40-50 times that found in the rat liver mitochondria although activities for carnitine palmitoyltransferase were nearly identical. The high activity appears to be an intrinsic feature of the species and is not associated with active rumen formation since young animals (2 weeks old) had similar enzyme activities.

Although the physiological function of carnitine acetyltransferase is uncertain, the preceding authors have suggested that the presence of carnitine acetyltransferase in large amounts in sheep liver, and most likely other ruminant species, allows the "acetyl pressure" in the starved condition to be
shifted from the vital CoA system to the carnitine system. If however, carnitine is limiting and acetyl carnitine is very slowly oxidized as suggested, carnitine acetyltransferase in ruminants may play a minor role in acetate utilization.

Scott and Setchell (1968) have found palmitic acid (16:0) to be the major component in neutral lipids and phospholipids of ram testes. This fatty acid has also been suggested to be the primary energy source in ruminants (Palmquist, 1972). These findings may indicate a need to evaluate carnitine palmityltransferase in the developing ram testis.

Mann (1951) and Voglmayr et al. (1971) have found a preference by ejaculated and testicular spermatozoa respectively for exogenous glucose. This is perhaps unexpected, since spermatozoa are normally in a fluid medium which contains practically no glucose. The mechanisms of substrate transfer from the extracellular fluid to the germinal cells is still unknown but may involve the limiting membranes of the seminiferous tubule, Sertoli cells and/or the membranes of the germinal cells themselves. This limitation may be the functional role of carnitine acetyltransferase, i.e., the transfer of an energy source to the maturing spermatozoon.

Two previous observations made by Scott et al. (1967) may further help explain the role of carnitine acetyltransferase in ram spermatozoa. Decreases in phospholipid content with concomitant loss in acyl esters and reduction in the chain length of the fatty acids strongly suggests that lipids serve
as substrates for spermatozoa during their maturation in the epididymis; and, the capacity of testicular spermatozoa to synthesize lipids is greater than in ejaculated cells indicating active lipid metabolism may be important as a way of providing substrate for the cells during their storage in the epididymis.

Carnitine acetyltransferase activity has yet to be measured in the testes of the ram.
MATERIALS AND METHODS

Experimental Animals

All rats used in this study were of the Wistar strain either purchased from Lab Supply Co., Indianapolis, Indiana, or raised in the animal colony of the Animal Reproduction Teaching and Research Center, The Ohio State University. The animals were fed a diet of Purina Lab Chow and allowed free access to a supply of fresh water. Animals were maintained with air conditioning at 24°C and subjected to 12 hours of light and 12 hours of darkness daily.

To study developmental changes in testicular carnitine acetyltransferase, 21 prepuberal rats and 10 normal adult rats weighing 350 - 400 grams were used. The testes of these animals were surgically removed following ether anesthesia.

In order to determine the effects of abdominal temperatures on carnitine acetyltransferase activity, artificial cryptorchidism was produced by making a midventral incision in 40 ether-anesthetized rats. Both testes were retracted into the abdominal cavity and retained there with a loose ligature. At daily intervals thereafter, 4 cryptorchid animals were sacrificed and both testicular tissue and blood samples collected for analysis.

Seventy crossbred rams utilized in this study were obtained from flocks at The Ohio State University and The Ohio
Agricultural Research and Development Center. Testes from young lambs were obtained by castration while testes of older animals were collected at the time of slaughter.

**Histological Procedures**

Samples of testes from each experimental group were fixed in Bouin's solution, embedded in paraffin, sectioned at a thickness of 5 μ, and stained with hematoxylin and eosin. These preparations were used to follow the spermatogenic process.

**Enzyme Extraction**

After testes were collected, the tunica albuginea and testicular blood vessels were quickly removed. Tissue (0.5 g) was extracted twice in glass homogenizers with 10 to 20 ml per g (wet weight) of ice cold 0.1 M K₂HPO₄, buffered at pH 8.0, containing 0.01 M sodium ethylenediaminetetraacetic acid, and 0.1% sodium deoxycholate. After each extraction, the homogenates were centrifuged at 10,000 x g for 15 minutes and the supernatant fractions combined. The known volume of supernate was maintained at 0 - 2 C for 2 - 3 hours before being assayed.

**Enzyme Assay**

Aliquots of the testicular extracts were assayed for carnitine acetyltransferase activity by the method of Marquis and Fritz (1965). The coupled enzyme assay involves the following reactions:

1) \( \text{acetyl} \text{carnitine} + \text{CoASH} \xrightleftharpoons{\text{CAT}} \text{carnitine} + \text{acetyl-CoA} \)
2) \[ \text{acetyl-CoA + oxaloacetate} \xrightarrow{\text{GS}} \text{citrate + CoASH} \]

3) \[ \text{malate + NAD} \xrightarrow{\text{MD}} \text{oxaloacetate + NADH} \]

Overall reaction:

\[ \text{malate + acetylcarnitine + NAD} \xrightarrow{\text{carnitine acetyltransferase}} \text{citrate + carnitine + NADH} \]

The routine assay mixture contained 100 μmoles of Tris-HCl (pH 7.8), 2.5 μmoles of NAD, 0.17 mg of CoA, 10 μmoles of DL-sodium malate, 0.07 unit of malate dehydrogenase, 0.07 unit of crystalline pig heart citrate-condensing enzyme, 30 μmoles of DL-acetylcarnitine and 1 μmole of NaCN in a final volume of 1 ml. Continuous recordings of the increase in optical density at 340 nm (33 C) resulting from NAD reduction were made using a Beckman DB spectrophotometer coupled with a Sargent Model SR recorder. After a 10 minute preincubation period, reaction was initiated by the addition of the enzyme. One unit of carnitine acetyltransferase activity was defined as the amount of enzyme which transformed 1 μmole of substrate per minute at 33 C in the assay system used.

**Blood Collection**

Each rat was anesthetized with ether and blood collected via cardiac puncture. The animals were then sacrificed by cervical dislocation before testes were excised, trimmed and weighed. Blood was collected from the jugular vein of the consious ram.

Blood was allowed to clot and the serum removed following centrifugation (4 C) for 15 minutes at 3020 x g. The serum was frozen and stored at -20 C until thawed for testosterone assay.
Testosterone Assay

After thawing, an appropriate volume of serum was transferred to 15 ml conical centrifuge tubes. The serum was then extracted with 1 ml of cold diethyl ether on a vortex mixer for 30 seconds. The centrifuge tube was then placed in a dry ice:acetone mixture to freeze the serum and the ether was decanted off. This procedure was repeated twice and the pooled ether extract was dried under nitrogen.

The dried samples were suspended in 0.5 ml of a 0.1% gelatin assay buffer, 100 μl of diluted antiserum and mixed on a vortex mixer. Thirty minutes later, 2 x 10^6 cpm (ca 5.7 x 10^4 dpm, 164 pg) of 1,2-^3H-testosterone, in 100 μl of assay buffer, were added to each tube and mixed again.

After the tubes were incubated overnight at 4 C, 100 μl of a dextran-coated charcoal suspension was added to each tube, stirred and then incubated in an ice bath for 20 minutes. The assay tubes were centrifuged at 3020 x g for 20 minutes and the supernatant liquid decanted into counting vials. Ten ml of biosolve scintillation fluid were added to each vial and finally counted. Testosterone levels in these samples were estimated from a standard curve which was constructed from known concentrations of testosterone.

Statistical Analysis

Analysis of variance, students t-test, calculation of means, standard errors and F statistics were all accomplished using the computer programs of Dr. Russell V. Skavaril, Department of Genetics, The Ohio State University.
RESULTS AND DISCUSSION

The Developing Rat

Testicular Weight and Histology

Mean testicular weights in the developing rat at various ages from 16 to 61 days and in adults are given in Table 1. Initial weights of $0.08 \pm 0.002 \text{ g}$ (mean $\pm$ SE) increased to $1.68 \pm 0.04 \text{ g}$ in adult rats with a maximum growth rate occurring between 23 and 50 days of age. Weights were significantly smaller ($P < 0.01$) than those of adult rats until the age of 61 days. These data are supportive of the fact that the growth curve of the rat testis is sigmoid in shape.

Evaluation of the seminiferous epithelium in rats 16 days of age and older was made histologically in this study. The seminiferous tubules of the 16-day-old rats contained Sertoli cells and numerous spermatogonia (Plate I, Fig. a). Few interstitial cells were visible in animals of this age group. In 29-day-old rat testes, numerous interstitial cells were present and the germinal epithelium had advanced to the stage of pachytene spermatocytes (Plate I, Fig. b). Although seminiferous tubular diameters were not measured in this study, the greatest apparent increase in tubular diameter was observed between 23 and 29 days of age. This age span corresponds to greatest tubular growth (Clermont...
TABLE 1

Changes in Testicular Weights, Carnitine Acetyltransferase Activity and Serum Testosterone With Age in the Rat

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Testis Weight (g)</th>
<th>CAT Activity (μmoles NADH produced/min/g tissue)</th>
<th>Serum Testosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>0.08 ± 0.00*</td>
<td>1.20 ± 0.08*</td>
<td>0.28 ± 0.02*</td>
</tr>
<tr>
<td>23</td>
<td>0.13 ± 0.02*</td>
<td>3.43 ± 0.11*</td>
<td>0.70 ± 0.10*</td>
</tr>
<tr>
<td>29</td>
<td>0.30 ± 0.02*</td>
<td>4.58 ± 0.37*</td>
<td>0.45 ± 0.05*</td>
</tr>
<tr>
<td>33</td>
<td>0.44 ± 0.01*</td>
<td>4.55 ± 0.17*</td>
<td>0.68 ± 0.11*</td>
</tr>
<tr>
<td>40</td>
<td>0.93 ± 0.03*</td>
<td>6.24 ± 0.17</td>
<td>1.30 ± 0.15*</td>
</tr>
<tr>
<td>50</td>
<td>1.40 ± 0.02*</td>
<td>6.54 ± 0.16</td>
<td>0.83 ± 0.13*</td>
</tr>
<tr>
<td>61</td>
<td>1.60 ± 0.02</td>
<td>5.95 ± 0.08</td>
<td>1.63 ± 0.34</td>
</tr>
<tr>
<td>Adult</td>
<td>1.68 ± 0.04</td>
<td>6.21 ± 0.07</td>
<td>2.36 ± 0.41</td>
</tr>
</tbody>
</table>

* Significantly different from adult values (P < 0.01).
PLATE I

Photomicrographs of testis tissue from adult and prepuberal rats (all X 150).

Figure a . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 16 day old rat
Figure b . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 29 day old rat
Figure c . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 61 day old rat
Figure d . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . Normal adult rat
and Perey, 1957), a time when spermatocytes are being produced from spermatogonia. The tubules of 61-day-old rat testes (Plate I, Fig. c) were nearly adult size and contained all germinal cells, including testicular sperm. A section of normal adult testis is also shown (Plate I, Fig. d). Histological sections of other age groups follow the spermatogenic process as defined for the rat.

**Testicular Carnitine Acetyltransferase**

As shown in Table 1 and Figure 1, carnitine acetyltransferase activity in the developing rat testis was $1.20 \pm 0.08$ units at 16 days of age and increased to a value of $6.21 \pm 0.07$ units in adult animals. The marked increase in activity at 23 and 29 days of age coincides with a rapid proliferation of the germinal epithelium, at which time primary spermatocytes become prominent. Activities were significantly lower ($P < 0.01$) in testes of rats 16 to 33 days of age compared to adults. These data confirm earlier reports that carnitine acetyltransferase levels in testes of young rats were found to be much lower than those in mature animals (Marquis and Fritz, 1965).

Geer and Newburgh (1970) have examined carnitine acetyltransferase levels in testes of *Drosophila melanogaster*. Testes from sterile flies were observed to have low carnitine acetyltransferase activities. These authors inferred from their study that testicular carnitine acetyltransferase synthesis in fertile flies is dependent on the maturation of late spermatids and spermatozoa. However, in an attempt to locate the activity of this enzyme in the germinal cells of rats, Vernon et al. (1971), using a cell separation technique, found high levels in primary spermatocytes, particularly
Changes in testicular carnitine acetyltransferase activity with age in the rat
Figure 1

[Graph showing the production of NADH in relation to age (days) from 16 to 61 days and an adult stage.]
diplotene spermatocytes. In contrast, very low levels were found in spermatogonia, and late spermatids had one-third to one-fourth the activity found in diplotene spermatocytes. If synthesis and degradation of carnitine acetyltransferase were to cease prior to the first meiosis, it would be anticipated that one-fourth of the enzyme in primary spermatocytes before diakinesis should be distributed to the four spermatids which would have been generated. However, this possibility remains to be examined.

The presence of particular cell types as observed in histological sections and the enzyme activities found in this study indicate that carnitine acetyltransferase is synthesized in primary spermatocytes. Based on this information, the enzyme carnitine acetyltransferase should be a useful "marker enzyme" to study differentiation of the germinal epithelium. This conclusion was also made in the studies of Vernon et al. (1971).

**Serum Testosterone Levels**

The mean serum testosterone level determined by radioimmunoassay in normal adult rats was 2.36 ± 0.41 ng/ml. Low levels in rats 16 days old (Table 1 and Figure 2) were quickly elevated at a time when gonadotrophin titers were expected to increase, i.e., 23 days. Rat serum from 16-day-old animals contained approximately 10% of the adult testosterone level, after which levels increased as a function of age. These levels remained significantly lower than adult levels until the age of 61 days (P < 0.01).

The increases in serum testosterone reported here closely
Figure 2

Changes in serum testosterone levels with age in the rat
parallel increases in carnitine acetyltransferase activity
\( r = 0.59, P < 0.01 \). Whether this enzyme is under the influence
of testosterone or the gonadotrophins is yet to be determined.

Jain (1972) has recently evaluated the radioimmunoassay
method for testosterone and has determined peripheral levels in
the male rat. In his study, testosterone levels reported for
adult male rats were 2.02 to 3.85 ng/ml depending on experimental
conditions. These values are in agreement with those reported in
the present study.

The Cryptorchid Rat

Testicular Weight and Histology

Mean testicular weights for control and cryptorchid rats
(1 - 10 days) are reported in Table 2. The large testis weights
observed in cryptorchid rats are best explained by the older
animals used in this study. Nevertheless, weight decreases
were noticeable after the third day. Similar weight decreases have
been reported by other investigators (Lloyd, 1972 and Jain, 1972).

In addition to decreases in testicular weights, seminiferous
tubular diameters also decreased. However, before this decrease,
an apparent tubular swelling was observed in 1 day cryptorchids.
This swelling is probably the result of increased tubular secretion
since lumina appear greatly enlarged (Plate II, Fig. a). By day
4, tubules appeared smaller, sperm number was diminished and the
appearance of multinucleated cells was evident (Plate II, Fig. b).
Further reduction in tubular diameters was apparent in animals
TABLE 2

Changes in Testicular Weights, Carnitine Acetyltransferase Activity and Serum Testosterone Following Cryptorchidism in the Rat

<table>
<thead>
<tr>
<th>Days Following Cryptorchidism</th>
<th>Testis Weight (g)</th>
<th>CAT Activity (μmoles NADH produced/min/g tissue)</th>
<th>Serum Testosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.68 ± 0.04</td>
<td>6.21 ± 0.07</td>
<td>2.36 ± 0.41</td>
</tr>
<tr>
<td>1</td>
<td>2.25 ± 0.12*</td>
<td>3.24 ± 0.22*</td>
<td>0.62 ± 0.12</td>
</tr>
<tr>
<td>2</td>
<td>2.45 ± 0.15*</td>
<td>2.29 ± 0.13*</td>
<td>1.75 ± 0.75</td>
</tr>
<tr>
<td>3</td>
<td>2.47 ± 0.10*</td>
<td>2.03 ± 0.12*</td>
<td>0.85 ± 0.15</td>
</tr>
<tr>
<td>4</td>
<td>1.63 ± 0.16</td>
<td>1.84 ± 0.13*</td>
<td>3.18 ± 0.65</td>
</tr>
<tr>
<td>5</td>
<td>1.18 ± 0.10*</td>
<td>1.80 ± 0.19*</td>
<td>1.78 ± 0.93</td>
</tr>
<tr>
<td>6</td>
<td>1.20 ± 0.09*</td>
<td>1.03 ± 0.18*</td>
<td>0.80 ± 0.05</td>
</tr>
<tr>
<td>7</td>
<td>1.49 ± 0.15</td>
<td>1.46 ± 0.18*</td>
<td>1.12 ± 0.93</td>
</tr>
<tr>
<td>8</td>
<td>1.22 ± 0.10*</td>
<td>1.21 ± 0.17*</td>
<td>1.48 ± 0.52</td>
</tr>
<tr>
<td>9</td>
<td>1.05 ± 0.10*</td>
<td>1.18 ± 0.14*</td>
<td>2.12 ± 1.28</td>
</tr>
<tr>
<td>10</td>
<td>0.99 ± 0.11*</td>
<td>0.91 ± 0.07*</td>
<td>2.45 ± 0.65</td>
</tr>
</tbody>
</table>

* Significantly different from control values (P < 0.01).
PLATE II

Photomicrographs of testis tissue from control and cryptorchid rats (all X 150).

Figure a .............................................................. 1 day cryptorchid
Figure b .............................................................. 4 day cryptorchid
Figure c .............................................................. 8 day cryptorchid
Figure d ..................................................  , , . Control
cryptorchid for 8 days as shown in Plate II, Fig. c. Increased
damage to the germinal epithelium, absence of spermatozoa and
mature spermatids, and an increased number of multinucleated cells
were evident in these animals. A section of control testis has
been presented in Plate II, Fig. d.

**Testicular Carnitine Acetyltransferase**

Levels of carnitine acetyltransferase in control and crypt-
orchid rat testes are reported in Table 2 and Figure 3. Control
levels of 6.21 ± 0.07 units compare with considerably lower
levels (0.91 ± 0.07 units) after 10 days of cryptorchidism.
Activity of carnitine acetyltransferase in all cryptorchids
was significantly reduced from control values (P < 0.01).

Carnitine acetyltransferase activities in the cryptorchid rat
testis were reported by Marquis and Fritz (1965). At 18 days
following cryptorchidism, these investigators found activities
to be less than one-seventh of those found in normal adults.
Since the germinal epithelium was known to regress dramatically
by this time, the reduced activity was explained by a lack of
testicular sperm which were suggested to be largely responsible
for testicular activity.

In the present study, 10 day cryptorchid testis contained
activities of carnitine acetyltransferase nearly one-sixth of
those reported for controls. Therefore, an evaluation of the
germinal epithelium of 10 day cryptorchid testis would result
in a conclusion similar to that made by Marquis and Fritz (1965).

However, the rapid loss of enzymatic activity following
Figure 3

Changes in testicular carnitine acetyltransferase activity following cryptorchidism in the rat
Figure 3

DAYS FOLLOWING CRYPTORCHIDISM

MICROMOLES NADH PRODUCED/MIN/G TISSUE
1 day cryptorchidism could not be explained solely by the loss of sperm or spermatids from the germinal epithelium. Therefore, degradation of the enzyme or an isozyme by abdominal temperatures may be a plausible explanation for its reduced activity.

Ewing and Schanbacher (1970) studied several glycolytic enzymes located in the 2, 4, 8, 12, 24 and 48 hour cryptorchid testis. With the exception of phosphofructokinase, their study showed little if any effects of abdominal temperature on enzymatic activities. Rapid mobilization of glycogen in the cryptorchid testis has suggested activation of a phosphorylase (Harkonen and Kormano, 1971) as a result of the exposure to elevated temperatures.

Serum Testosterone Levels

The average testosterone levels in control and cryptorchid rats have been reported in Table 2 and Figure 4. The level for control animals (2.36 ± 0.41 ng/ml) compared with a low level of 0.62 ± 0.12 and a high level of 3.18 ± 0.65 on the first and fourth day, respectively. These cryptorchid levels were not statistically tested against the control levels since only 2 blood samples were assayed for each group of cryptorchids.

The fluctuations of serum testosterone found in this study were not unexpected since other investigators have obtained similar results. Jain (1972) showed an initial decrease in serum testosterone which might be caused by ether anesthesia or surgical stress. Thereafter, levels returned to normal or above normal, i.e., 2 to 3 ng/ml.

Swing et al. (1973), studying the perfused rabbit testes,
Changes in serum testosterone levels following cryptorchidism in the rat
Figure 4

SERUM TESTOSTERONE (NG/ML)

DAYS FOLLOWING CRYPTORCHIDISM

0 1 2 3 4 5 6 7 8 9 10
found testosterone secretion by 18 day cryptorchid testes to be significantly higher than testes of sham operated animals during the fourth and fifth hours of perfusion.

The trend of serum testosterone in the present study may indicate an initial decrease followed by a return to normal at 10 days. Although carnitine acetyltransferase levels were found to decrease rapidly in the cryptorchid testis, no conclusion regarding the influence of testosterone on this enzyme could be made.

**The Developing Ram**

**Testicular Weight and Histology**

Testis weight in the crossbred ram (mostly Targhee crosses) increased from an initial birth weight of approximately 1 g to an adult weight of more than 250 g. As illustrated in Figure 5, the growth curve of the ram testis is sigmoid in shape with a maximum rate of growth occurring between 70 and 120 days of age. The variation in testicular weight among animals of a similar age is most likely due to genetic differences; however, health and environment may be contributing factors.

Histological sections from the developing ram showed neonatal ram testes to contain few Leydig cells and small inactive seminiferous tubules (Plate III, Fig. a). Except for a slight increase in tubular diameter, the seminiferous tubules remained quiescent in a majority of the lambs with only gonocytes and supporting cells present until the age of 60 days. Spermatogonia
Figure 5

Testis weight as a function of age in the ram
PLATE III

Photomicrographs of testis tissue from adult and prepuberal rams (all X 150).

Figure a .................................................... 15 day old ram
Figure b .................................................... 35 day old ram
Figure c .................................................... 60 day old ram
Figure d .................................................... 96 day old ram
Figure e .................................................... 120 day old ram
Figure f .................................................... 228 day old ram
appeared shortly thereafter (Plate III, Fig. c) and primary spermatocytes were much in evidence at 69 days of age. After 90 days, lumen formation had begun (Plate III, Fig. d), mitotic activity was more prevalent and tubular diameters were greatly increased. Elongated spermatid and an occasional spermatozoon were observed in tubules from rams 120 days of age (Plate III, Fig. e). Normal spermatogenesis was evident in most rams aged 160 days or older. The histological data of the present study are in close agreement with the findings of Carmon and Green (1952), Courot (1962) and Skinner et al. (1968).

**Testicular Carnitine Acetyltransferase**

The activity of carnitine acetyltransferase in the developing ram testis is shown in Figure 6. Low levels of this enzyme activity at birth (0.4 units) increased rather slowly to adult levels of approximately 1.0 unit. No peak activities in carnitine acetyltransferase were found in this study indicating that if the enzyme is associated with a particular cell type, these cells must not be generated at any particular age.

This study showed ram testes to have one-sixth the activity found in rat testes. Assuming germ cell populations in rat and ram testis are similar and the carnitine acetyltransferase assay to be optimal for both species, the activity of carnitine acetyltransferase reported in this study would imply that the germinal cells of the ram have less requirement for the carnitine-dependent transport system. This conclusion, however, requires the determination of enzyme activities in isolated cell populations.
Figure 6

Changes in testicular carnitine acetyltransferase activity with age in the ram
from ram testes. From these observations, it was concluded that carnitine acetyltransferase would be unsatisfactory as a "marker enzyme" in studying differentiation of the germinal epithelium of the ram.

The requirement for this enzyme in intermediary metabolism has been debated by several authors (Snoswell and Henderson, 1970, Johnson et al., 1972, and Lin and Fritz, 1972). The conclusion that carnitine acetyltransferase in ruminants probably plays a minor role in acetate utilization may help to explain the relatively low activities in the ram reported in this study.

**Serum Testosterone Levels**

Figure 7 is a scattergram of testosterone levels in the developing ram. Serum testosterone levels of approximately 0.1 ng/ml in neonatal lambs compare with approximately 2 ng/ml in 6 month old rams. The trend in the levels of this androgen appears to be a low level postnatally followed by an increase, corresponding to the commencement of spermatogenesis, after about 70 days of age.

Skinner et al. (1968) found androstenedione to be the major testicular androgen at birth, however, testosterone soon became predominant. Their data suggested that maximum production of testosterone in the young ram occurs during the growth period, i.e., 42 to 168 days of age. Although peripheral androgens were not measured, their activities were estimated by weight of accessory glands and their content of fructose and citric acid. The results indicated androgenic activity began at an early age.
Figure 7

Changes in serum testosterone levels with age in the ram
SERUM TESTOSTERONE (NG/ML)
in this species, i.e., 42 days of age.

In addition to the effects of androgens on sexual behavior and the accessory sex glands, Voglmayr et al. (1970) have suggested their role may be to preserve the endogenous nutritive reserves in spermatozoa by inhibiting oxidative metabolism as they pass through the epididymis. In this regard, testosterone or its metabolites may become increasingly inhibitory as the spermatozoa develop their capacity for motility.

Although serum testosterone levels and carnitine acetyltransferase activities are related in the ram \( r = 0.75, P < 0.01 \), no definite conclusion regarding their relationship can be inferred from the results of this study.
SUMMARY

Male Wistar rats and Targhee crossbred rams were used to determine spermatogenic and steroidogenic function of the pre-puberal rat and ram testis. By assaying the enzyme, carnitine acetyltransferase, and qualitatively evaluating the germinal epithelium of the developing testis, an attempt was made to identify the particular germ cells with which the enzyme is associated. In addition, serum testosterone levels were measured so that puberal changes could be more appropriately described.

As shown by previous investigators, carnitine acetyltransferase appeared to be closely associated with primary spermatocytes of the rat since activities increased markedly when primary spermatocytes were proliferating. For this reason, this enzyme could be an important "marker enzyme" to study the differentiation of germinal cells in this species.

Rats with experimental cryptorchid testes were found to have drastically reduced testicular carnitine acetyltransferase activities. Degradation of the enzyme by abdominal temperatures or a related factor was suggested as a plausible explanation for its reduced activity. Whether this enzyme is the "temperature-sensitive component" of the cryptorchid testis remains to be determined.

Serum testosterone levels in the rat closely parallel the
increased spermatogenic activity observed after 23 days of age. Low levels of testosterone in immature rats increased to adult levels of $2.36 \pm 0.41 \text{ ng/ml}$. An initial decrease in the testosterone levels of the cryptorchid rat has also been suggested; however, no explanation was given to relate this finding to decreased carnitine acetyltransferase activities in the same animals.

Carnitine acetyltransferase could not be associated with any particular cell population of the ram testis even though tissue activities increased as a function of age. The importance of this enzyme in ruminant intermediary metabolism has been questioned. This study indicates that carnitine acetyltransferase is probably unsatisfactory as a "marker enzyme" in studying spermatogenesis of the ram.

Serum testosterone levels in the developing ram have also been reported. Although large variations were observed between animals, testosterone levels increased after 70 days of age to approximately 2 ng/ml in sexually mature animals. The results of the present investigation, however incomplete, provide some measure of the pubertal changes which occur in these species.
BIBLIOGRAPHY


