AGING CHANGES IN TESTICULAR RESPONSIVITY TO GONADOTROPIN STIMULATION IN RATS.

BY

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THESIS

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This study examined the effects of aging on testicular endocrine function and response to gonadotropin stimulation in male rats. Sprague-Dawley rats of 4 and 18 months were used throughout. Peripheral blood, testicular venous blood and endogenous testicular production were quantified for testosterone (T), 20α-dihydroxyprogesterone (20αOH P), progesterone (P) and estradiol-17β (E2). The levels of both P and E2 were similar in both age groups but significantly less T and 20αOH P were found in testicular venous plasma and testis of aged rats. However, serum LH as quantified by RIA showed no age-related difference. In vitro incubations of both decapsulated testicular tissue and isolated Leydig cells with hCG (0-100 mIU) showed significantly less T production by the aged rats at basal levels and at all doses of hCG. Two groups of 4 and 18 month rats were injected i.v. with 50 IU hCG to study the in vivo response to gonadotropin stimulation. Peripheral blood was drawn at 0, 1 and 5 hours after hCG injection. Testicular venous (TV) blood and endogenous testicular production (EP) were determined 3 hours after hCG. T, P, E2 and 20αOH P were quantified in all samples. Results indicate no age-related difference in P or E2 production before or after hCG. T and 20αOH P levels were significantly lower in TV blood and EP 3 Hr after injection. Peripheral blood concentrations of T showed
significantly higher levels in 4M rats at 0 and 1 hours, however, at 3 hours post-hCG, peripheral serum T levels were found to be similar in both age groups. This similarity in peripheral T levels did not reflect secretory differences as demonstrated by TV and EP levels of T 3 hours after hCG, but was more likely due to differences in the metabolic clearance rates (MCR) of T. We therefore conclude that reproductive aging in rats is in part due to an inherent defect in testicular function. This defect may be a lesion in the steroidogenic enzymes leading to the formation of T which lies distal to LH binding, cAMP production and P formation. Testicular E2 may not play a major role in reproductive senescence in rats.
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INTRODUCTION:

Identification of the physiological center(s) which control aging has always been a goal for gerontologists. Identification of the cause of physiologic aging is made difficult since aging may be accompanied by age-related diseases which mask the aging process. Aging, as measured by the increase of the likelihood of an individual's death, may originate from two distinct loci. The first locus is intracellular and termed determinant while the second locus is extracellular and termed stochastic. Determinant processes are those which occur in all animals irregardless of environmental influences. Stochastic processes, on the other hand, are the results of environmental influences and not due to any occurrences within the cell (Stechler 1977).

While it has been demonstrated that environmental influences such as low caloric diets and lower temperatures increase the lifespan of animals, this effect may merely reflect a reduced growth rate and not a slower rate of cellular aging (Berg and Simms 1960; Finch 1969).

While studying the lifespan of cultured cells, (Hayflick 1965) observed that normal diploid human fibroblasts had a finite ability to divide in vitro and that the population-doubling potential was inversely related to the age of the donor. For example, while cells obtained from fetal tissue could replicate 50±10 times, cells obtained from mature
animals were only able to undergo $30 \times 10$ replications before death.

Orgel (1963) proposed a theory for cellular aging based on the accumulation of errors in protein synthesis. Briefly, if mutations occurred in protein(s) involved with replication; transcription, translation or DNA repair, these errors would be magnified with each successive division eventually resulting in death. Orgel also subscribed to the theory where positive feedback occurs between macromolecular constituents and the cell itself. For example, the greater the number of errors which have accumulated in the macromolecular portion of the cell, the greater the increase of errors accumulating in the cell and subsequently the tissue.

While genetic control of aging is a popular concept, a more modern approach to the problem involves the immune system. It has been shown in mice and men that involution of the thymus occurs during aging (Weksler 1981). A decrease in T cell function accompanies thymic involution as demonstrated by decline in rosette formation, delayed hypersensitivity and tissue graft rejection tests (Adler 1975). It is now known that B cells require the presence of mature T cells during their maturation and development. Using cell transfer experiments, immature B cells transplanted into cultures containing T cells obtained from aged animals were unable to produce normal amounts of antibodies. Antibody response in vivo as well as resting antibody titer was also
shown to be impaired in aged animals.

Suppressor T cells are specialized cells which control B cell production of autoantibodies. The suppressor cells exhibit a decreased function with increasing age. This situation eventually leads to the formation of greater than normal numbers of autoantibodies in aged animals. A rise in circulating immune complexes accompany the increase in autoantibodies. It may be that chronic tissue damage by autoantibodies and circulating immune complexes may be in part, responsible for the physical degeneration of aged individuals.

Another line of investigation is in the study of the relationship between endocrine function and physiologic aging. Everitt (1973) proposed that the aging process is dependent on the lack of essential pituitary hormones. The hypothalamus has been described as the "aging clock" since this organ controls a wide variety of physiologic functions. According to Clemens and Meites (1971) the hypothalamus may contribute to aging by increasing serotonin output and decreasing its catecholamine secretion. The net effect is an increased prolactin secretion and decreased gonadotropin output by the pituitary. The mechanisms responsible for aging of endocrine organs involved in reproduction are still unclear. While the female possess a physiologic clock indicating the reproductive status (cyclicity) the male has no obvious means of monitoring its reproductive status. Testicular function in terms of endocrine function and/or sperm production, is the most likely model for examining reproductive aging in the
male. This thesis deals directly with testicular endocrine function as it changes with age using the rat as a model. The following is a review of topics important to this subject.

LITERATURE REVIEW:

Leydig Cell and Sertoli Cell Morphology:

The testes consists of 82.4% seminiferous tubules, 1.9% capsule, and 15.7% interstitial tissue by volume (Mori, Hiroshi and Christensen 1980). The interstitial tissues encompass blood and lymph vessels, fibroblasts, myoid cells, white blood cells and Leydig cells. The Leydig cells composes only 2.7% of the entire volume of the testes (Mori et al 1980). The Leydig cells are the major steroid producing cells of testes although the Sertoli cell has been shown to produce androgens in vitro (Hall, Irby, deKretser 1969; Steinberger, Tcholakian, Steinberger 1979). In the Leydig cell, the most abundant organelles are the smooth E.R. and mitochondria. These organelles together with the microsomes are the major site of steroidogenic enzymes. Cholesterol side chain cleavage enzymes seem to be located within the mitochondria (Bloom and Fawcett 1975). Lipid droplets are also found in great quantities and are thought to be storage of steroids. These organelles are typical of all steroid producing cells (Martin 1976). Sertoli cells, in addition to containing much smooth endoplasmic reticuli, golgi bodies and mitochondria also possess an extensive network of mitotubules.
The organelles and nuclei are concentrated toward the basal end of the cell. The function of the Sertoli cell were thought to be maintainence of the blood-testes barrier and selective transport of materials in and out of the seminiferous tubules. The Sertoli cell has also been found to produce a transport protein, androgen binding protein (ABP) (French 1973). Recently, Bardin has shown that the Sertoli cell is active not only in synthesizing ABP, but also transferrin and many other proteins that are involved with transport.

Chemical Nature of Steroid Hormones:
Steroid hormones all contain a 5 carbon ring (cyclopentane ring) fused to a saturated phenanthrene molecule, thus forming a cyclopentanoperhydrophenanthrene structure. Numbering of the carbon atoms and letters assigned to the 4 rings are indicated below.

The sterane structure contains asymmetric carbon atoms at positions 5, 8, 9, 10, 13 and 14. Progesterone and the androgens contain methyl groups attached to carbon atoms 13, 14 and are known as androstane derivatives. Androgens have a total of 19 carbon atoms while estrogens lack carbon
atoms 19, 20 and 21 and are estrane derivatives. Progestosterone has 21 carbon atoms with C-20 attached to C-17. Progestins and androgens contain a double bond between carbons 4-5 or 5-6 indicated by $\Delta^4$ or $\Delta^5$ while the A ring of estrogen is aromatized (Martin 1976).

**Androgens and Estrogen Secretion in Males:**

Androgens and estrogens may be formed in a number of tissues provided the proper substrates and enzymes are present.

The two major steroid secreting organs in the male are the testes and the adrenal cortex, both of which follow the same general synthetic pathway as illustrated. (see figure 1) The Leydig cells of the testes are the major site of synthesis in the male. Limited steroid secretion may take place within the Sertoli cell but is dependent on intermediates furnished by the Leydig cells (Hall, Irby and deKretser 1969). Leydig cells are able to use either plasma derived fatty acids or cholesterol transported via blood lipoproteins for their supply of cholesterol. At least one study has suggested that HDL serves as a major source of cholesterol for Leydig cell steroidogenesis in rats (Quinn, Dombrausky, Payne and Chen 1981) but this may not be entirely correct since crude membrane fractions obtained from rat testes yield specific HDL and LDL binding sites (Hwang, Rajendran, Johnston and Menon 1981). Other studies indicate LDL and not HDL cholesterol as the primary source of cholesterol used by pig Leydig
cells (Benhamed, Haour and Saez 1981). HDL may increase the number of hCG binding sites as well as stimulate cell multiplication while LDL stimulates androgen synthesis (Chen, Cheung, Kramer and Reaven 1981). Chen et al (1981) also found that HDL and LDL may stimulate T synthesis and suggests the effects of both may be enhanced by hCG treatment. It may be that both LDL/HDL and hCG receptors modulate each other's activity (Chen et al 1981). Once cholesterol has been either delivered to the Leydig cell or produced within the mitochondria and acted upon by CSCC enzymes it is transported to microsomal enzymes for further conversion to various steroids. The metabolites are packaged in the smooth E.R. and are eventually secreted by the golgi apparatus (Christenson and Mason 1965).

Androgen Production in Development:

The steroid secretion by testes of rats are found to undergo distinct phases both quantitative and qualitative aspects. Testosterone concentrations in plasma are 0.6ng/ml in male fetuses after 18 days of gestation. This level drops to 0.3 ng/ml at birth where it remains until the 4th week when it begins to rise again (Corpechot, Baulieu and Robel 1981). Testosterone is the major product of the testes until day 10 when the concentrations of the 5α-reduced metabolites of T begin to exceed the concentration of T in plasma and testes (Nayfeh, Barefoot and Baggett 1966). This situation
persists until day 30-40 when T again becomes the major secretory product and the concentration of the 5α-metabolites become barely detectable in plasma (Nayfeh et al 1966; Resko, Feder and Goy 1968). Testosterone increases to adult levels of 3ng/ml by the 8th week (Corpechot et al 1981).

According to Purvis (1980), the secretory changes associated with the developing rat may be due to a change in Leydig cell populations. He postulated that there exists two populations of Leydig cells, the first being developed in utero secreting T preferentially. These cells disappear by postnatal day 10 giving way to the second population appearing between days 10 and 20. The second population secretes primarily 5α-reduced steroids until day 30-40 when a change in pathways leads to increased T production (Purvis 1980). Payne et al (1980) using metrizimide gradient purification and low speed centrifugation confirmed the presence of two populations of Leydig cells in rat testes.

The change from 5α-reduced steroid to the physiologically active androgen testosterone is thought to be the stimulus for the onset of puberty in rats (Neyfeh, Coffey, Hausson and French 1975). This changeover phase is also accompanied by an increase in responsiveness by the Leydig cell to hCG stimulation although then sensitivity declines after puberty. The increased responsiveness translates to an increase in T response to a given stimulus while a decreased sensitivity produces a slower rate of response to a given stimulus.
In rabbits, \textit{in vitro} testicular conversion of $^{14}$C-progesterone (P) to T was demonstrated as early as the 18th day of gestation. This was before Leydig cells could be morphologically identified. Conversion to T decreases slowly after birth until adult levels of T are reached (Lipsett and Tullner 1965).

Testosterone production in mice follows a pattern similar to that observed in rats. Testosterone levels fall steadily after birth until puberty when they rise to peak values. After puberty, T levels slowly decrease to adult levels which are then maintained well into old age (Eleftheriou and Lucas 1974).

In guinea pigs, T levels were found to decline after birth and rose prior to puberty as in rats and mice. An increase in T production is observed during puberty which then declined until adult levels are reached (Rigaudier, Pelandy, Robert and Delost 1976).

In humans, T production begins as early as the 12th-18th week of gestation (Bloch 1964). Unlike the situation in rodents, T is not the primary steroid produced by the early post-natal testes. Dihydroprogesterone is the steroid in highest concentration in the testes until just prior to puberty (Bloch 1964). During puberty, 17αOH progesterone and T are produced in higher quantities with T becoming the predominant steroid in the adult human (Bloch 1964).
Hormonal Changes in Aging:

A decline in T production associated with aging has been found in bulls, rats, guinea pigs and rabbits (Collins, Inskeep, Preher, Tyler and Casida 1962; Ewing 1972; Ghanadian, Lewis and Chisholm 1975; Rigaudier et al 1976; Chan, Leathem and Esashi 1977; Miller and Riegle 1978). However, mice do not display a decrease in T production in old age and humans only show declining T levels after the 65th year (Vermuelen, Rubens and Verdonk 1972; Eleftheriou and Lucas 1974).

In those species with lower T production in old age, testicular weight loss may help explain the loss of testicular function. This has not proved to be the case in many strains of aged rats. Strains such as the Long-Evans (Chan et al 1977) Wistar (Miller and Riegle 1978) and Sprague-Dawley (Hasrmon, Danner and Roth 1978) exhibit no testicular weight loss during aging. However, the Fischer strain of rat has been found to have increased testicular weight due to the high incidence of spontaneous interstitial cell tumors in this strain (Bethea and Walker 1979). While comparison of testicular weight versus T production has proven inconclusive, other factors such as serum LH, LH receptors, enzymatic defects in the steroidogenic pathway as well as the Leydig cells' ability to respond to gonadotropin should be studied.

The crucial enzyme $\Delta^5-3\alpha$hydroxysteroid dehydrogenase has been found to have decreased activity in aged rats although it is not clear if this is due to a reduced enzyme level
Aged rabbit testes were shown to contain enzymes with reduced activities (Ewing 1967).

Pituitary responsiveness to releasing hormones may also change with age. Aged human males were found to secrete significantly more LH after injection of 200 ug LHRH than young males (Rubens, Dhont and Vermuelen 1974). In contrast to humans, a decreased responsiveness by the pituitary of aged Long-Evans male rats to 500 ng of LHRH injected i.v. was observed by Riegle and Meites (1976). In the same study, the old rats were found to produce less LH in response to both methyl dopa injections and stress than did young rats.

Serum LH levels have been found to decline in aged rats of both sexes (Huang, Marshall and Meites 1976). However, aged human males have been shown to exhibit increased serum LH levels with lower serum T levels (Rubens et al 1974). In those studies using aged rats, measurements of serum LH and T showed a decline of 15-30% for LH while serum T levels fell nearly 400% (Gray 1978; Pirke, Vogt and Geiss 1978). This suggests that declining LH values alone may not represent the cause of the depressed T production found in aged rats.

While studying LH receptors in aging Wistar rats, Tsitouras et al (1979) found a 27% reduction in the number of receptors in the aged rats. In the same study, cAMP levels were similar in both young and old rats. The authors suggest that the reduction of receptors, although significant, would not affect the steroidogenic output since only 20% of the
total number of receptors need be occupied to elicit the maximum hormone response (Catt and Dufau 1975). If the primary defect was in receptors, cAMP production should be reduced in the old rats but Tsitouras et al (1979) found that cAMP levels were similar in both young and old rats.

Still another line of investigation has proceeded from the findings that in the rat, diminished steroidogenic enzyme responses to LH/hCG stimulation has been demonstrated in vivo and in vitro using either cAMP or T production as the endpoints. Chan et al (1977) using 250 mg aliquots of testicular tissue incubated in Krebs-Hensleit buffer (KHB) demonstrated a decreased ability of aged Long-Evans rats to convert $7\alpha^-3^2$H-progesterone to T with or without hCG added in vitro. However, Pirke et al (1978) using 100 mg aliquots of testicular tissue incubated in Krebs-Ringers buffer (KRB) for 3 hours with various doses of hCG found identical dose-response curves for both young and old rats. This study used Wistar rats and strain differences may account for the disparity of results between Chan et al (1977) and Pirke et al (1978).

Pirke et al (1978) did observe a reduced response in old rats when using a NADPH generating system. This system, although unphysiologic, produces an exaggerated response as compared to the response accomplished using either cAMP or hCG (Pirke et al 1978).

When using metrizimide purified Leydig cells from 4 and 24 M Sprague-Dawley rats, Chen et al (1981) found no difference
in the ability to convert $5 \times 10^{-7}$M pregnenolone or progesterone to testosterone. However, the same cells incubated with LH produced different dose-response curves. The old rats produced significantly less T than did the young rats (Chen et al 1981). Bethea and Walker (1979) found no significant difference in T production after hCG stimulation when using isolated but not metrizimide purified Leydig cells from 5 and 12 month Fischer rats. However these results should not be used for comparison as the health status of the Fischer rats was not assessed.

Recent work by Sharpe and McNeilly (1980) illustrated a distinct difference between dispersed Leydig cells and intact testes in their sensitivity to LH/hCG stimulation. They found that dispersed cells were from 4-8 times more sensitive to hCG stimulation than intact testes. This may explain the difference in results between Pirke et al (1978) and Chen et al (1981). Pirke et al (1978) used 100 mg testes slices incubated with varying doses of hCG while Chen et al (1981) incubated metrizemide purified cell preparations with LH. An age related difference in favor of young rats was found in the response to LH using isolated cells while no apparent difference between ages was found using testicular tissue. Sharpe and McNeilly (1980) postulate that in pure cell preparations, many more receptor sites become available to LH/hCG interaction with an increase in sensitivity resulting. Tissue slices do not
offer the same amount of available receptor sites due to the interaction of Leydig cells with other testicular components. This reduces the chance that a molecule of LH/hCG will find an open binding site. It became obvious that because of the variables inherent to in vitro systems, similar in vivo experiments were required.

In vivo treatment of male animals with LH/hCG was shown to stimulate the production of progesterone, testosterone and estrogens (Steinberger 1976). However Longscope (1973) observed that in response to 3000 I.U. hCG i.v. for three days, aged men produced significantly less T than young men. Chan et al (1977) and Miller and Riegle (1978) also observed a reduced response in old rats after acute hCG treatment when testosterone was measured. Their young rats produced significantly more T than aged rats up to 1 hour after hCG injection. Harmon et al (1978) found that if they waited for 3 hours after hCG injection, the old rats would produce similar amounts of T as the young. They suggest that any age-related difference in T production would become insignificant 3 hours after hCG because the old rats have an intact, yet slower rate of response.

In these earlier in vivo studies, an assumption was made that the changes in serum T concentrations reflected changes in the secretory rates. This may not prove to be the case as a reduced MCR for T was reported in both aged men and rats and is a factor to be reckoned with (Vermuelen
et al 1972; Chan, McFarlin and Zummo 1981). Other factors may also play a role in the reduction of steroidogenic competency in aged animals. Eik-Nes (1970) found that an adequate flow of blood to the testes is essential for proper function. Pirke et al (1979) reported a reduction of capillary blood flow to the testes of aged rats. Leydig cell function was reduced due to the unavailability of oxygen and essential substrates. Damber et al (1981) attributed the loss of capillary permeability and blood flow to the testes of aged rats to the local effects of estrogens. Both Pirke et al (1979) and Damber et al (1981) were able to correct the reduced blood flow through hCG treatment. Setchell and Sharpe (1981) were able to enhance capillary permeability in the testes of 75 day old rats by 200 I.U. hCG injected i.v. They postulate that while hCG may not directly effect capillary permeability, a substance secreted by the testes in response to hCG may produce the effect.

Role of Estrogens in Testicular Aging:

Administration of estrogens to male animals may result in decreased androgen output (Tcholakian, Chowdhury, and Steinberger 1974), inhibition of gonadotropin secretion (Swerdloff, Grover, Jacobs and Bain 1973) and a refractive-ness to the stimulatory effects of gonadotropins (Saez, Haour, Loras, Sanchez and Catharid 1978). Cigorraga et al
(1980) found that Leydig cells obtained from rats 3 days after acute hCG treatment exhibited a lesion in the function of key steroidogenic enzymes which may be attributed to an increased intratesticular estrogen (E_2) level. They suggested that locally produced E_2 may function to reduce LH binding sites and regulate the activities of 17, 20 lyase and 17α-hydroxylase, two important enzymes in the pathway leading to T formation.

HCG treatment has been found to increase the activity of aromatase enzymes (Payne, Kelch, Musich and Halpern 1976; Valladares, Sarker and Payne 1978; and Canick, Makris Gunsalers and Ryan 1979).

Abney (1976) reported the presence of a cytoplasmic estradiol receptor in rat testes. It was suggested that hCG, by stimulating the production of various aromatizable substrates, may increase the amount of intratesticular E_2. Testicular estrogens may play a role in the loss of function while the E_2 receptor would control this effect through an "ultra-short" feedback mechanism within the Leydig cell (Moger 1980; Melner and Abney 1980).

Estrogen receptors have been determined in both young and old rats with no age-related difference observed in either the affinity constant (K_a) or the number of binding sites (Lin, Chen, Murant, Ostuman and Nankin 1981).

Differences between rats and humans are evident when serum E_2 is measured in aging. Serum levels of E_2 were
similar in young and old rats while serum $E_2$ levels in humans were found to be significantly higher in humans over 50 years of age (Lin et al 1981; Longscope 1973).
MATERIALS AND METHODS:

Animals:
Sprague-Dawley rats originally obtained from Charles Rivers Laboratory (Charles River, Mass.) were subsequen-
tially bred in our facilities to produce the animal colony
used in these experiments. They were fed Purina Laboratory
Chow and water ad libitum and housed in a 12h light, 12h
dark environment at a constant temperature of 25°C. Only
animals exhibiting no visible pathologies were used for
study.

Buffers and Solutions:
Buffer used in radioimmunoassays was phosphate-buff-
ered saline (PBS) with 1% gelatin added; pH was adjusted
to 7.4 (see Appendix).

A charcoal-dextran T-70 solution was prepared (500 mg
charcoal, 50 mg Dextran T-70 per 100 ml) in PBS to be used
for the separation of free from bound hormone. To insure
good separation, fresh charcoal-dextran solution was made
weekly and kept in suspension with a magnetic stirrer.

In vitro incubations of isolated Leydig cells were
performed in 5 ml polyethylene tubes. The cells were in-
cubated in 1 ml culture medium TC-199 (Difco Labs, Detroit,
Mich.) with the addition of 0.1% bovine serum albumin (BSA)
(Sigma, St. Louis, Mo.) and adjusted to a pH of 7.4 with
10% NaHCO₃ solution. Incubations of decapsulated testicular tissue were performed in Krebs-Hensleit bicarbonate buffer (KHB) with 0.2% glucose and pH adjusted to 7.4 (see Appendix).

Buffer for radioimmunoassays of LH was 0.01 M PBS as recommended by the NIAMDD kit (see Appendix).

**Hormone Preparation:**

- 17β-Estradiol (2,4,6,7-³⁰H) (s.a. 115 Ci/m mole), testosterone (1,2,6,7-³⁰H) (s.a. 93.9 Ci/m mole), hydroxypregn-4-ene-3-one, 20α-(1,2,-³⁰H) (s.a. 50 Ci/m mole) and progesterone (1,2,6,7-³⁰H) (s.a. 95Ci/m mole) were purchased from New England Nuclear, Boston, Mass. Upon receipt, isotopes were brought to a volume of 5mls with a solution of benzene: ethanol (9:1 v/v) and stored at 4°C. For RIA use, isotopes were aliquoted, dried under N₂ and then redissolved in PBS to give a final working concentration of 8000 cpm/100 ul for estradiol, testosterone and progesterone and 4000 cpm/100 ul for 20α-hydroxypregn-4-ene-3-one.

- Unlabelled steroids were obtained from Sigma Chemical Company, St. Louis, Mo. and dissolved in absolute ethanol to be used as reference standards.

- Human Chorionic Gonadotropin (hCG-2500 I.U./mg) was purchased from Sigma Chem. Co., St. Louis, Mo.

- Rat luteinizing hormone (rLH, RP-1) was provided with the NIAMDD program kit.
Na$^{125}$I (2 mCi-s.a. 500 Ci/m mole) was purchased from New England Nuclear and used immediately to iodinate both hCG and rLH preparations.

**Iodination of LH/hCG:**

Iodination was accomplished using the chloramine-T oxidation method according to NIAMDD directions. The procedure was as follows: 25ul of 0.5 M $\text{PO}_4^-$ buffer, pH 7.6 was added to the 1 mCi Na$^{125}$I in the combi-vial provided, 20 ul of LH/hCG and 10 ul chloramine-T were then added and the vial was mixed gently for 60 seconds. Then 25ul of Na$_2$S$_2$O$_5$ was added to terminate the reaction. The resulting mixture was applied to a sephadex column for separation of the iodinated protein from the free $^{125}$I. The column consisted of a 10ml disposable serological pipette plugged at the tip with a small glass bead and just prior to packing, it was coated with 2 mls of 2% BSA-0.01 M PBS. It was then packed with either 3 gm G-75 sephadex for LH assay or G-100 sephadex for hCG assays. Immediately after transfer of the hormone - $^{125}$I mixture to the column, 100 ul of a rinse solution containing blue dextran (see Appendix) was used to rinse the combi-vial and added to the column. The column was then eluted with 0.01 M PBS and aliquots of 0.5 ml were collected until the blue dextran appeared in the elute, usually up to 21 ml. During the elution, two peaks would result with the initial 3-5 ml containing the iodinated
protein and the final 7-20 ml containing the free $^{125}$I.
The fraction on the trailing portion of the initial peak was used because this fraction contained the least damaged and most immunoreactive hormone.

**LH Radioimmunoassay:**

The procedure was as follows: The serum sample/standard was brought to 0.5 ml in 1% BSA-0.01 M PBS. Approximately 20,000 cpm of $^{125}$I-LH in 0.1 ml in 1% BSA-0.01 M PBS was added followed by anti-LH antiserum (1:5000) in 0.2 ml in 3% NRS-0.05 M EDTA-0.01 M PBS (see Appendix). Total volume was 0.8 ml. The mixture was incubated at room temperature for 18 hours after which 0.2 ml of goat anti-rabbit globulin solution (1:30) was added and vortexed lightly. The goat anti-rabbit globulin (Miles, USA) was made to a 1:30 dilution the maximum amount of bound $^{125}$I-LH/hCG could be precipitated. This mixture was further incubated at room temperature for 24 hours. After incubation, all samples were centrifuged at 1,000 x g for 30 minutes at 4°C. The supernatant was discarded and the resulting pellet was gently washed with 1 ml of 0.01 M PBS to remove unbound radioactivity and centrifuged at 1,000 x g for 30 minutes. The supernatant was then discarded. The washing procedure was repeated three times. The final washed pellet was counted using a Beckman auto-gamma counter (Courtesy of Dr. C. Sladek, University Rochester School of Medicine,
Dept. Anatomy).

**Extraction Procedure:**

Serum samples for steroid RIA were first extracted since they could not be assayed directly. Samples were made up to 200 ul with water and then vortexed after addition of 2 ml freshly opened diethyl ether (photorex grade, J.T. Baker Chem.). They were allowed to stand at room temperature for 30 minutes to reach equilibrium and then chilled to -70°C to freeze the aqueous layer. The organic layer was then decanted into glass culture tubes. The extraction procedure was repeated and the organic phase pooled. The organic phase was then evaporated under Nitrogen immediately.

When whole testes were quantified for their steroid contents, the procedure was modified. Testes were decapsulated and minced, then sonicated for 2-10 seconds with a Biosonik tissue disrupter (Bronwill, USA). The sonicated homogenates were then transferred to a 250 ml separatory funnel and shaken after the addition of 40 ml ether. The organic layer was separated and evaporated under vacuum to dryness using a rotary evaporator. The samples were transferred to 13 x 100 mm culture tubes and evaporated to dryness under N₂. The samples were processed for either RIA or further purified by Sephadex LH-20 column chromatography.
Sephadex LH-20 Column Chromatography:

Most samples were processed in the following manner: disposable 10 ml serological pipettes were cut off to 20 cm length and plugged with a small amount of glass wool at the tips. One gram of sephadex LH-20 (Sephadex, N. J.) was suspended in 5 ml of the slurry solvent (C$_6$H$_6$:MeOH, 9:1, v/v). The slurry mixture was then applied to the column at the same time a volume of slurry solvent was poured through forming a uniform packing. Once packed, another column-volume of slurry solvent was put through the column to ensure proper packing. Without letting the column run dry, the first solvent system was added (Iso-octane:C$_6$H$_6$:MeOH, 85:10:5, v/v/v). Care was taken not to disturb the packed column with undue force. Two full column volumes of the first solvent were run through the column for proper equalibretion before sample application. Previously dried samples were redissolved in 0.2 ml of the first solvent and let stand for at least 15 minutes before applying them to columns. Transfer of samples to columns was accomplished by applying the 0.2 ml to the top of the column bed when the sample front was just below the top of the column packing. The samples were then rinsed with another 0.2 ml of first solvent system and transferred to the column. Once applied, the samples were allowed to drain into the column before adding any solvent. Using the first solvent system, P was collected in fractions 1-7, 20%OH P
in 8-13 and T in 14-20. To elute E₂, a second solvent system was used but not before the first solvent had passed through the column. The second solvent (Iso-octane:C₆H₆:MeOH, 55:20:25, v/v/v) was added by running it down the sides of the column so as not to disturb the column bed. The next three ml of second solvent system were then discarded. Fractions 25-31 contained E₂. The fractions collected from the column were then washed down with absolute ethanol in preparation for RIA.

Radioimmunoassays for Steroids:

Radioimmunoassays of steroids were performed after ether extraction and Sephadex LH-20 column chromatography. Recoveries were determined through the addition of ¹⁴C-steroids as internal standards. Assays were performed in triplicates with interassay and intra-assay variations found to be within ± 10%. Water blank values were 5-25 pg for 20αOH P and P and 5-10 pg for T and E₂.

Antisera for T, P and E₂ were prepared by Dr. S.W.C. Chan in our laboratory and exhibited high specificity and low cross-reactivity with competing steroids. Some antisera for E₂ and 20αOH P were obtained from Miles-Yeda (Kankakee, Ill.) and were highly specific with low cross-reactivity.

The radioimmunoassay procedure was as follows: tubes containing standards/samples were dried under N₂ and then redissolved in 100 ul of PBS with 0.1% gelatin. All samples
then received 100 ul each of $^3$H-steroid and antiserum. The samples were incubated either for 1 hour at 37°C or for 18 hours at 4°C. They were then placed in ice for 15 minutes before the addition of 0.3 ml of charcoal/dextran solution. Samples were briefly vortexed before being set in ice for 10 minutes. The samples were then centrifuged at 1000 x g at 4°C for 10 minutes. Aliquots were removed from the supernatant and counted in a counting fluid containing omnifluor and triton-x (see appendix) using a Packard Tri-carb liquid scintillation counter-model 3320 with a counting efficiency of 58% for $^3$H and 85% for $^{14}$C.

In Vitro Incubations:

Six animals each of 4 and 18 months with no visible pathological signs were used for all experiments. The animals were decapitated and trunk blood was collected. Their testes were then removed and placed in ice-cold saline until weighed. After weighing, the testes were decapsulated and 250 mg aliquots removed. These tissue aliquots were lightly teased with fine forceps to dissociate the interstitial cells from the tubules. The teased tissue was then placed into 25 ml erlenmeyer flasks containing 5 ml of KHB; pH 7.4 with glucose and with 0-100 mIU hCG/flask. The flasks were then placed in a Dubnoff metabolic shaking incubator for 2 hours at 37°C under an atmosphere of 95% O$_2$-5% CO$_2$. The incubation was terminated by rapidly freezing the
samples at -70°C. Testosterone RIA was then performed without prior extraction or chromatography to determine the T production in response to hCG stimulation.

Dispersed Leydig cells were prepared according to the method of Dufau, Mendelson and Catt (1974) using the collagenase digestion method. Collagenase (Type II, Sigma) was used at a concentration of 0.25 mg/ml. Dispersion was accomplished in 25 ml polypropylene centrifuge tubes (Corning Glass, Corning, N.Y.) containing 1-2 pair of testes/10 ml KHB buffer with collagenase. The tubes containing the testes were then incubated in a Dubnoff metabolic shaking incubator for 15 minutes at 34°C until the testes were slightly dissociated. The tubes were then allowed to stand at room temperature for 5 minutes before the supernatant containing the cells was poured through a millipore filter unit using only the plastic support without the filter (Millipore Filter Corp., Bedford, Ma.). The tubules were rinsed with 10 ml of KHB and filtered through the same millipore filter unit as before. The cells were then spun at 800 x g for 15 minutes at 4°C in 15 ml polypropylene centrifuge tubes. After centrifugation, the supernatant was poured off leaving a pellet of interstitial cells. This was then resuspended in 0.2 ml of tissue culture media TC-199 (Difco) containing 0.1% BSA. A cell count was established using a hemocytometer and the cells were diluted in TC-199 to give a final concentration of approximately 200,000 cells/tube.
Incubations were performed in 12 x 75 mm polyethylene tubes containing the cells, varying doses of hCG from 0-100 mIU and TC-199 to balance the volume to 0.3ml. The samples were incubated in a Dubnoff metabolic shaking incubator at 34°C for 3 hours under an atmosphere of 95% O₂-5% CO₂. The incubations were terminated by rapidly freezing the samples at -70°C. Aliquots were removed for direct RIA of T as described previously without prior extraction.

**In Vivo hCG Stimulation:**

To study the effects of hCG stimulation on testicular steroid production, six rats each of 4 and 18 months were used. Peripheral blood was removed via the tail vein prior to the start of the experiment. The animals were then injected intracardiacally with 50 IU hCG dissolved in 0.2 ml of saline (0.9%). Peripheral blood samples were collected at 1 and 3 hours after hCG injection. After each blood sampling, an equal amount of saline was injected i.p. to replenish lost fluids. Testicular venous blood was collected using a modified method of Bardin and Petersen (1967) at 3 hours post-injection. To expose the testes, the tunica and scrotal sac were slit longitudinally. The testes were then drawn out and placed in a heparinized glass funnel leading to a 0.5 ml polypropylene centrifuge tube. A loop of the testicular vein was cut and blood allowed to flow into the funnel and collected.
The rats were then sacrificed by decapitation and trunk blood collected. The testes were removed and placed into 20 ml vials containing 10 ml of cold saline and frozen in dry ice-acetone for later analysis.

Blood samples were allowed to clot overnight at 4°C then centrifuged at 5,000 x g for 10 minutes at 4°C to separate serum, and frozen until processed.

**Statistical Tests:**

Statistics were performed according to the students "t" test with significance recognized at P<0.05.
RESULTS:

Testicular Weights:
Testicular weights were determined to be not significantly different between young and old rats with weights of 3.52 ± 0.13 g and 3.51 ± 0.13 g respectively. Testicular weights are expressed as mean ± SEM g, N = 30.

Serum LH Levels:
Serum LH levels in 4 and 18 month rats were determined to be 16.9 ± 1.67 ng/ml and 20.76 ± 1.76 ng/ml respectively; (N = 25). These values were found to be not significantly different between the two age groups.

In Vitro Response to hCG using Decapsulated Testicular Tissue:
Basal testosterone production was 29.9 ± 3.2 ng/100 mg/2 hr for the young rats as compared to 4.55 ± 0.59/100 mg/2 hr for the old. Testosterone production by the 4 month rat testis was thus more than 6 times higher than that of the 18 month rats' (<0.001). Significant stimulation over basal levels was first obtained with 100 mIU hCG dose in young rat tissue while the tissue from older rats needed only 10 mIU (P<0.05). Maximal stimulation of T production was achieved at 100 mIU hCG dose in both age groups. At all doses of hCG, significantly more T (P<0.001 at all doses)
was produced by the 4 M rats as compared to the 18 M rats (Figure 3 and 4).

**In Vitro Response to hCG Using Isolated Leydig Cells:**

At basal stimulation, Leydig cells isolated from 4 M rats produced $1.37 \pm 0.45$ ng T/10$^6$ cells/3 hr compared with $0.411 \pm 0.006$ ng T/10$^6$ cells/3 hr produced by the 18 M rats. These values were determined to be nor significantly different. Significant stimulation over basal levels was first obtained at 5 mIU hCG dose for both age groups ($P<0.05$). Maximal stimulation was achieved at 10 mIU hCG for the 4 M rats and 18 M rats. Cells of 4 M rats produced significantly more T than the aged rats at all doses of hCG except basal ($P<0.005$ for all doses except basal; Figure 5 and 6).

**In Vivo Response to hCG:**

**Progesterone:**

In control animals, progesterone (P) levels in peripheral serum were found to be similar in both age groups. At 1 hour post-hCG injection, serum P levels rose to similar levels in both young and old rats. Three hours after hCG treatment, serum P levels in both 4 M and 18 M rats had fallen below those levels observed at 1 hour, yet were still 3 times as much as basal levels. No age related
differences in serum P concentrations were found after 3 hours of hCG (Figure 7).

Progesterone levels in testicular venous (TV) blood of control rats were found to be similar with \(7.68 \pm 2.19\) ng/ml in 4 M rats and \(6.77 \pm 1.22\) ng/ml in 18 M. Three hours after hCG, P levels in TV blood rose to \(8.47 \pm 1.62\) ng/ml in 4 M rats and \(9.88 \pm 2.09\) ng/ml in 18 M rats with no significant difference between age groups observed.

Endogenous production of P by the testis at basal stimulation was 20 ng/testis for both 4 and 18 M rats. HCG treatment for 3 hours produced significant stimulation (\(P<0.005\)) in both 4 and 18 M rats, however, no significant difference between age groups was observed.

\(20\alpha\)-Hydroxy - 4 - Pregnane - 3, One (20\(\alpha\)OH P):

At basal stimulation, 20\(\alpha\)OH P levels in peripheral sera were similar in 4 and 18 M animals at 0.1 ng/ml. At 1 hour post hCG injection, serum levels rose sharply to peak values of 3.4 ng/ml in both age groups with slightly higher values observed in the young animals. At 3 hours post-injection, serum levels fell to below those observed at 1 hr while remaining up to 5 times higher than basal levels. Significantly higher 20\(\alpha\)OH P levels were found in the 4 M animals as compared to the 18 M animals 3 hours after hCG (\(P<0.005\)).

20\(\alpha\)OH P levels in testicular venous blood of control
animals were found to be significantly higher in young rats (406 ± 14 pg/ml) as compared to old rats (216 ± 13 pg/ml). At 3 hours post-hCG injection, 20\(\alpha\)OH P levels rose to nearly 5 times the control values in both age groups with the young rats producing significantly more 20\(\alpha\)OH P than old (P<0.025; Figure 8).

Endogenous testicular production in control rats showed significantly higher 20\(\alpha\)OH P production in the 4 M rats as compared to the 18 M rats (P<0.005). Three hours after hCG injection, 20\(\alpha\)OH P levels were significantly higher than control levels in both age groups. Significantly more 20\(\alpha\)OH P was produced by the 4 M rats as compared to the 18 M animals (P<0.025).

**Testosterone (T):**

Testosterone levels in peripheral serum of control animals were significantly higher (P<0.001) in 4 M rats as compared to values obtained for the 18 M animals. Maximum stimulation in both age groups occurred 1 hour after hCG treatment with significantly greater T production in the 4 M rats (15.2 ± 1.7 ng/ml) as compared with the old rats (8.05 ± 1.5 ng/ml; P<0.05). Comparable T concentrations were found in both age groups 3 hours post hCG injection (Figure 9).

Testicular venous blood levels of T in control animals showed significantly higher T concentration in the young
(7.75 ± 1.9 ng/ml) as compared to old rats (2.05 ± 0.25 ng/ml; P<0.025). At 3 hours post-hCG injection, a ten-fold increase over basal levels was observed in both age groups with young rats producing significantly more than the old (P<0.025).

Endogenous testicular production of T in control animals was found to be significantly higher in the 4 M rats as compared to the 18 M animals (P<0.005). At 3 hours post-injection, T levels rose significantly in both age groups over basal levels. Significantly higher levels were observed in the young rats (13.6 ± 1.4 ng/testis) as compared to the old rats (7.22 ± 1.65 ng/testis); (P<0.025).

**Estradiol - 17 (E₂):**

Peripheral serum levels of E₂ in control animals were similar at 28.6 pg/ml for both age groups. At 1 hour post-hCG injection, both age groups exhibited maximum stimulation over basal levels, however, no significant difference between age groups was observed. Three hours after hCG treatment, E₂ serum levels were not appreciably different for the two age groups (Figure 10).

Estradiol in TV blood of control animals was significantly higher (P 0.001) in 4 M rats (76 ± 5.6 pg/ml) as compared to 18 M rats (57 ± 3 pg/ml). E₂ levels in TV blood 3 hours after hCG injections were not significantly different
between age groups.

Endogenous testicular production of $E_2$ in control animals showed similar levels in both age groups. At 3 hours post-hCG injection, an increase of approximately 4 times over basal values was observed in both age groups. No significant difference in testicular $E_2$ concentrations was found between the two age groups after hCG treatment.
DISCUSSION:

In general, most strains of laboratory rats do not experience testicular weight loss during aging. (Chan et al 1977; Tsitouras et al 1977; Lin et al 1980). However, an increased testicular weight was found in the Fischer strain of rat (Bethea and Walker 1971). This may be explained by the fact that spontaneous interstitial cell tumors occur frequently in these aged rats. Although this same study also reported a decreased Leydig cell mass in aged Fischer rats, their conclusion was based solely on the number of Leydig cells recovered after collagenase dispersion and not on quantitative morphometric study. Fischer rats, because of their incidence of tumors, should therefore be considered as an exception.

The present study finds no age-related loss of testicular weight in the Sprague-Dawley strain and is agreement with the findings of Lin et al (1980).

It has been suggested that declining LH secretion by the pituitary of aged rats may chronically hypostimulate the testes resulting in decreased steroid production (Riegle and Meites 1976). Declining serum LH concentrations in aging rats have been reported by Pirke et al (1978) and Gray (1971). The percent reduction of serum LH in the old rats was 29% in the study by Gray (1971) while only as 18% reduction was observed by Pirke et al (1978).
Kaler and Neaves (1981) found higher LH levels and lower T concentrations in sera of aging rats. In the same study, they also found an increased total blood volume in the aged rats and suggested this might cause a dilution of both LH and T. While the blood volume was increased in the old rats, they were still able to maintain higher than normal levels of LH per unit volume than the young rats. The present study shows no age-related difference in serum LH while a large decline of serum T concentration is found in the aged rats.

In those studies reporting both decreased serum LH and T in aged rats, the percentage decline of LH (15-30%) has been much less than the corresponding decline in T (200-400%). In both the present study and the study of Kaler and Neaves (1981), circulating gonadotropin levels are normal or increased. Hypostimulation by LH alone therefore could not explain the large decrease in serum T concentration in the aged rats. In fact, Kaler and Neaves data suggested that the testes may actually become less responsive to LH stimulation with age since the old rats were able to maintain normal T concentrations in blood with increased serum LH.

While serum LH levels in aged Sprague-Dawley rats remain normal, a different situation exists in humans. Men over the age of 65 experience an increase of serum LH while serum T levels do not appear to be significantly decreased
until after the 70th year (Vermuelen, Rubens and Verdonek 1972; Rubens, Dhont and Vermuelen 1974). Pituitary responsiveness to LHRH appears to be augmented in aged men unlike the situation in rats (Rubens et al 1974; Riegle and Meites 1976). In both studies, the subjects were injected with a dose of LHRH i.v. while LH levels in blood were monitored at time intervals up to 2 hours. Although aged rats produced less LH in response to LHRH injection, old men produced significantly more LH than did young men 1 and 2 hours after treatment. In view of the increased LH levels in sera of aged men, Rubens et al (1974) concluded that decreased T secretion had a primary testicular origin. A similar situation may exist in aged rats as suggested by the findings of the present study and that of Kaler and Neaves (1981).

Previous in vitro studies have shown that aged rats demonstrated a decreased testicular responsivity to LH/hCG stimulation. Chan et al (1977) incubated slices of testicular tissue with $[{}^{3}H]$-progesterone and measured its conversion to various products after 4 hours while in the presence of hCG added in vitro. Their results show that tissue from old rats was much less responsive to hCG stimulation than tissue from young rats when testosterone was quantified. The young rats were able to convert 2.5 times more $^{3}H$-P to T than the old rats. Isolated Leydig cell preparations from aged rats have also been shown to have
a decreased reponsivity to LH stimulation (Lin et al 1980; Chan et al 1981). Lin et al (1980) reported that a dose of 1 mIU LH caused maximal stimulation of T production. At this dose, cells from aged rats produced only 17 ng T/10^6 cells as opposed to 42 ng T/10^6 cells produced by the young rats.

In our in vitro studies, both decapsulated testicular tissue and isolated Leydig cells were incubated with varying doses of hCG to determine the responsivity of young and old rats. Our results show that both tissue preparations from 4 M rats were more responsive to hCG stimulation than those of 18 M rats at all dosage levels. The young rats also appeared to be more sensitive to hCG as compared to the old rats since the rates of increase in T was much greater in the young rats.

Previously, Pirke et al (1978) reported no age-related difference in dose-response curves when measuring T production after either hCG or cAMP. In their study, testicular tissue from 3 and 26-28 month Wister rats were pre-incubated for 30 minutes at 33°C before being transferred to vials containing KRB buffer with hCG. They were then incubated at 33°C under 95% O_2-5% CO_2 for three hours. After the second incubation, the medium was decanted and testosterone assayed. Their results showed no difference in either sensitivity or responsivity between young and old rats (Pirke et al 1978). Bethea and Walker (1979)
found no difference in response between 4 and 24 M Fischer rats when equal numbers of Leydig cells were measured. However, any comparison with our data is made difficult since the health status of the Fischer rats was not fully evaluated. Strain differences may account for the disparity of results between Pirke et al (1978) and our current study.

Sharpe and McNielley (1980) demonstrated that dispersed Leydig cells were between 4-8 times more sensitive to hCG stimulation than were intact hemi-testis from the same rat. In our study, isolated Leydig cells were also more sensitive to hCG stimulation than were the decapsulated testicular slices. Sharpe and McNielley (1980) suggested that dispersed cells have more exposed receptor sites than intact hemitestis due to enzymatic impurities in the collagenase which may unmask "hidden" receptors. An alternative explanation may be that receptors which have normally been covered by adjacent cells or tissues become exposed after dispersion. This is consistent with the differences in sensitivities between the decapsulated testicular tissue and isolated Leydig cells in our study.

The present in vivo study clearly demonstrates an unimpaired ability of the aged rat testes to produce progesterone both before and after hCG treatment. A decline in enzymic activity of $\Delta^5$-3$\beta$ hydroxysteroid dehydrogenase has been demonstrated in aged male rats but it remains un-
established if this was due to a change in enzyme concentration or specific activity (Leathem and Albrecht 1975). It has also been shown that serum levels of 20α-OH progesterone decline in aged female rats (Chan and Leathem 1979). The present study suggests a decreased enzyme activity leading to the formation of 20α-OH P in testicular venous blood (TV) and testicular production of the 18 M rats both before and after hCG treatment were observed. Previous in vitro studies on changes of testicular responsivity have shown equivocal results. Chan et al (1977) reported significantly less T production by 18 month old Long-Evans rats in response to acute hCG treatment (50 IU hCG - i.v.) for up to 45 minutes post-injection. Miller and Riegle (1978) extended this study to 150 minutes and observed similar results. In both studies, the amount of T produced in response to hCG treatment was much greater in young rats as compared to the old rats. However, Harmon et al (1978) found the response of the old rats to be comparable to that of young rats 3 hours after hCG injection. They proposed that the aged rats in their study exhibited a delayed response to hCG stimulation but their overall steroidogenic competency was not impaired. These previous in vivo studies utilized only peripheral serum T as the sole measurement of testicular function. The present study monitored the secretion of four different hormones not only in peripheral serum but also testicular venous
plasma and endogenous testicular production. These measurements were made at 1 and 3 hours after an i.v. injection of hCG. Monitoring the secretion of four hormones at the testicular venous and endogenous testicular production is necessary to provide a clear understanding of the actual production and storage in the testes as well as how much is secreted into the testicular vein and circulation. Peripheral serum levels of T may not necessarily reflect testicular T production because of extra-gonadal contributions. Factors such as change in the blood volume or the metabolic clearance rate may also affect circulating concentrations of T (Vermuelen et al 1972; Kaler and Neaves 1981).

Our results indicate that young rats have significantly more T in peripheral circulation at basal stimulation and 1 hour after hCG than old rats. However, by 3 hours, these levels became comparable in both age groups. Testosterone concentrations as measured in TV blood and in testes at 3 hours post-injection of hCG illustrated much greater T production in the young rats as compared with the old rats. These findings suggest that while T concentrations in peripheral serum 3 hours after hCG may be similar, actual T production is much less in old rats as compared to the young. Thus, T concentrations as measured in serum do not truly reflect the actual production or secretion of T in aged rats.
A decrease in the MCR of T has been reported in old men (Vermuelen et al. 1972) and aged Sprague-Dawley rats (Chan et al. 1981). Indeed, the metabolic clearance rates of T showed an age-related decrease and therefore could explain the observed similarity in T levels 3 hours after hCG since this would maintain blood levels of T for longer periods in the aged rats as compared to the young rats. The decreased MCR of T in old rats combined with the fact that 1 hour after hCG produced peak levels of T in sera of both age groups lead to an extended period of high T in blood of old rats while the young rats were able to clear their circulation of much of the T produced 1 hour after hCG. This would result in the old rats possessing similar amounts of T in their circulation as the young rats after 3 hours. Without considering changes in the MCR and basing observations on peripheral T levels, it would appear as though the old rats exhibit a delayed, yet similar response to hCG stimulation as compared to the young rats 3 hours after hCG.

Aged humans as well as rats show a decreases ability to produce T in response to in vivo hCG treatment. Long-scope (1973) reported that after 3 days of hCG treatment, men older than 65 years produced significantly less T than did younger men. Rubens et al. (1974) treated young and old men with 1500 IU hCG/day for 3 days and observed similar results. They found the old men could produce only 50%
or less T than the younger men.

It appears that testicular function in rats decreases with age. A factor which may play a role in the decline of testicular function is the maintenance of blood flow to the testes.

Eik-Nes (1964) has shown that an adequate flow of blood to the testes must be maintained to insure a supply of O₂ and substrates. Pirke et al. (1979) reported a decrease in blood flow to the testes of aged rats. While in vitro injections of hCG were able to correct this situation, they were unsure of the mechanism of action. Setchell et al. (1981) using 55 day-old Wister rats injected with hCG, increased blood and lymph flow to the testes as well as capillary permeability. Although the exact mechanism affecting these changes was uncertain, they postulated that substances secreted by the testes in response to hCG was responsible and not the hCG itself. Testicular blood flow was not measured in our studies and may play a role in the observed decrease in testicular function of aged rats in vivo.

In contrast to the situation in aged humans, serum levels of estradiol do not appear to increase in aged rats (Longscope 1973; Chan et al.; Lin et al 1981). Cytoplasmic estradiol receptors in testes of Sprague-Dawley rats were also found to be similar (Lin et al 1981). Our present study indicates no age-related difference in E₂ production by the testes both before and after hCG. We therefore
suggest that testicular estradiol may not be an important factor influencing the declining reproduction status in the aging rat.

In conclusion, the decline in steroidogenic function in aged rats is, at least in part, due to a decreased responsivity of the Leydig cell to gonadotropin stimulation. Considering the present evidence, an enzymic lesion may exist in the steroidogenic pathway leading to the formation of testosterone. This lesion lies distal to LH binding, cAMP formation and progesterone production. Testicular estrogens do not appear to play a major role in the decreased steroidogenic function in aged Sprague-Dawley rats.
figure 1.
Steroid hormone synthesis in the testis

Plasma fatty acids
Acetyl CoA

Sulfated androgens

Pregnenolone sulfate

Dehydroepiandrosterone (DHA, DHEA)

17a-OH pregnenolone

Cholesterol

Pregnenolone

17a-OH pregnenolone

Dihydrotestosterone (DHT)

20α-OH progesterone

17a-OH progesterone

Progesterone

Androstan-3,17-dione

20α-OH progesterone

17α-OH progesterone

Androstan-3,17-dione

3β-Androstene-3,17-diol

Dihydrotestosterone (DHT)

17β-Estradiol

Testosterone
Figure 2. Steroid purification by Sephadex LH-20 column chromatography. Steroids collected (ml) are plotted versus $^3$H-CPM. Progesterone, 20$\alpha$OH P, and T; fractions 1-3 respectively, were eluted using chromatographic system 1 (85:10:5 v/v/v iso-octane:benzene:methanol) with E$_2$; fraction 4, eluted using system 2 (55:20:25 v/v/v iso-octane:benzene:methanol).
Figure 3. Endogenous production \textit{in vitro} by decapsulated testicular tissue. Dose-response curves are plotted as testosterone (T) production versus mIU hCG dose. Each point represents the mean ± SEM; n=6. * - indicates significant stimulation above basal levels. P<0.05, using the students "t" test.
Figure 4. Endogenous production in vitro of testosterone by decapsulated testicular tissue. Dose-response curves plotted as T production versus mIU hCG dose. Basal values were subtracted from each point to illustrate the difference in percent increase between age groups. ** - Indicates significant difference in % increase over basal. P<0.05, students "t" test.
Figure 4.

ng T / 100 MG TISSUE / 2Hr

4M

18M

mIU hCG
Figure 5. Endogenous production in vitro of T by isolated Leydig cells. Dose-response curves are plotted as T production versus mIU hCG dose. Each point represents mean ± SEM; n=3. * - Indicates significant stimulation above basal values. P<0.05, students "t" test.
Figure 5

ng T / 10^6 CELLS / 3 Hr
Figure 6. Endogenous production in vitro of T by isolated Leydig cells. Dose-response curves are plotted as T production versus mlU hCG dose. Basal values were subtracted from each point to illustrate the difference in percent increase between age groups. ** - Indicates significant difference in % increase over basal. P<0.05, students "t" test.
Figure 6.

ng T/10^6 CELLS / 3 Hr

mlU hCG
Figure 7. Influence of acute hCG stimulation in vivo on progesterone (P) production by 4M ■ and 18M □ rats. Peripheral serum (TP) was quantified at basal and at 1 and 3 hours post-hCG injection. Testicular venous blood (TV) and endogenous testicular production (EP) were quantified both at basal and 3 hours post-hCG. Each point represents mean ng/ml ± SEM; n=6. - indicates significant age difference in steroid levels. P<0.05, students "t" test.
Figure 8. Influence of acute hCG stimulation in vivo on 20α-Hydroxy - 4 - Pregnan-3, one (20αOH P) production by 4M ■ and 18M □ rats. Peripheral serum (TP) was quantified at basal and at 1 and 3 hours post-hCG injection. Testicular venous blood (TV) and endogenous testicular production (EP) were quantified both at basal and 3 hours post-hCG. Each point represents mean ng/ml ± SEM; n=6. ★ - indicates significant age difference in steroid levels. P<0.05, students "t"test.
<table>
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<tr>
<th>BASAL</th>
<th>1 Hr</th>
<th>3 Hr</th>
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**20αP**

<table>
<thead>
<tr>
<th></th>
<th>TP</th>
<th>TV</th>
<th>EP</th>
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*Figure 8.*

**ng/ML**

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<th>10</th>
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<tbody>
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<td></td>
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<td>20</td>
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<tr>
<td>1</td>
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</table>
Figure 9. Influence of acute hCG stimulation in vivo on testosterone (T) production by 4M □ and 18M ○ rats. Peripheral serum (TP) was quantified at basal and at 1 and 3 hours post-hCG injection. Testicular venous blood (TV) and endogenous testicular production (EP) were quantified both at basal and 3 hours post-hCG. Each point represents mean ng/ml ± SEM; n=6. ★ - indicates significant age difference in steroid levels. P<0.05, students "t" test.
Figure 9.

The chart illustrates the concentration of T (testosterone) in ng/ML at different time points: Basal, 1 hour, and 3 hours. The Y-axis represents the concentration levels, while the X-axis shows the time points. The data points are marked with stars.
Figure 10. Influence of acute hCG stimulation in vivo on estradiol-17β (E2) production by 4M ■ and 18M □ rats. Peripheral serum (TP) was quantified at basal and at 1 and 3 hours post-hCG injection. Testicular venous blood (TV) and endogenous testicular production (EP) were quantified both at basal and 3 hours post-hCG. Each point represents mean pg/ml ± SEM; n=6. ⭐ - indicates significant age difference in steroid levels. P<0.05, students "t" test.
APPENDIX

PBS - for steroid RIA only; pH 7.4
0.87% Na₂HPO₄ anhydrous
0.54% NaH₂PO₄·H₂O
0.1% Na - azide
0.9% NaCl
0.1% gelatin (heat and stir to dissolve)

Charcoal/Dextran solution:
0.5% 20 times washed charcoal
0.05% Dextran T-70
make up in PBS with 0.1% gel - good for 1 week then discard

0.01 M PBS - LH RIA only; pH 7.6
0.6% NaCl
0.1235% Na₂HPO₄ anhydrous
0.0179% NaH₂PO₄·4H₂O

hCG/LH Iodination solution:
0.5 M PO₄--; pH 7.6
6.12% Na₂HPO₄ anhydrous
0.95% NaHPO₄·4H₂O
store at room temperature to avoid crystallization
Chloramine T:
10 mg Chloramine T
10 ml 0.01 M PBS

Sodium Bisulfate:
25 mg Na\textsubscript{2}S\textsubscript{2}O\textsubscript{5}
10 mls 0.01 M PBS

Rinse Solution:
100 mg KI
800 mg Sucrose
1 mg Bromophenol blue
10 mls distilled H\textsubscript{2}O

KHB - Krebs Henslent buffer; pH 7.5 - for tissue incubation
make:
4.5% NaCl
5.75% KCl
6.10% CaCl\textsubscript{2}
10.55% KH\textsubscript{2}PO\textsubscript{4} (anhydrous)
19.10% MgSO\textsubscript{4}·7H\textsubscript{2}O
5.2% NaHCO\textsubscript{3} made fresh

use:
20 mls
0.8 ml
0.6 ml
0.2 ml
0.2 ml
5.25 mls
27.05 mls

add:
102.95 mls distilled H\textsubscript{2}O

* For testicular incubations
  add 0.2% glucose

130 mls total volume
Scintillation Counting Fluids:
for primarily aqueous samples:
8 g. Omnifluor
1 l. Triton X-100
2 l. Toluene
stir until homogenous-store in the dark

for primarily non-aqueous samples:
4 g. PPO
0.1 g. POPOP
4 l. Toluene
stir until homogenous-store in the dark
BIBLIOGRAPHY


Benahmed, M., Hauor, F. and Saez, J. M. (1981). Opposite effects of low density lipoprotein (LDL) and high density (HDL) on cultured pig leydig cell functions. The Endocrine Society Meeting. Abst. 59


CORRIGENDUM