Age Related Changes in the Production and Metabolism of Testicular Steroids in the Rat

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AGE RELATED CHANGES IN THE PRODUCTION AND METABOLISM OF TESTICULAR STEROIDS IN THE RAT.

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by

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ABSTRACT

Age-related changes in the production and metabolism of testicular steroids were studied by chronic in vivo hCG stimulation of 4 month (Y) and 18 month (O) rats, followed by in vitro hCG stimulation of the chronically stimulated testis tissue. The O showed a reduced initial response to hCG with significantly lower levels of T on D1 and D2. Although on D4-D10 the peripheral levels of T were not significantly different in Y and O, at D10 the Y had returned to basal levels and the O were still at 3x basal. The D10 endogenous levels of T were also significantly higher in the O. In vitro stimulation of the chronically stimulated testis tissue showed both Y and O unresponsive to further stimulation, but the O produced more T both with and without hCG stimulation. These facts may indicate some failure in the desensitization process in the O. Another difference between Y and O was significantly higher levels of endogenous 20α hydroxyprogesterone in the O, suggesting possible differences in local control mechanisms.

Neither chronic in vivo hCG stimulation nor in vitro aromatization of testosterone (T) to estradiol (E2) resulted in any significant differences between Y and O in production or utilization of estradiol. However, during chronic hCG stimulation there were age-related differences in the ratios of T/E2 suggesting the possibility of an extra-testicular role for E2 in age-related changes.

Aromatization of T resulted in lower levels of E1 and of Δ4D in the O as compared to the Y. Also, when stimulated with hCG the Y, but not the O, exhibited a significant decrease in production of Δ4D. Other metabolites of T such as 7α-hydroxytestosterone (7α-OHT) and 7α hydroxyandrostendione (7α-OH Δ4D) did not show age-related differences.
Comparision of the clearance of T in acutely castrated O and Y rats showed that the O clear T more slowly than the Y. When injected with supraphysiological doses of T, both Y and O castrated rats cleared T faster than after acute castration. The O still showed slower t 1/2α and t 1/2β after these injections but the amount of T cleared from the blood calculated as MCR (liters of blood cleared/ kg of body wt/ day) was not significantly different in the Y and O.
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I. INTRODUCTION - DEFINITIONS AND THEORIES OF AGING

Aging is a complex multifaceted process which has been defined in many ways. Strehler (1977) summed up the process by calling aging an increase in the likelihood of an individual's death. He elaborated on the process by describing two classes of factors influencing aging: the determinant factors, inherent genetic events, intrinsic to the species; and stochastic factors, unpredictable, accidental, environmental happenings which are subject to modification. Other researchers have classified age related events according to the level of organization at which they occur. For example, Bart and Turturro (1983) described changes that lead to senescence as occurring at the level of the cell, in the individual organ or organ systems, and in populations. Shock (1977) categorized elements of aging as genetic, nongenetic, and physiological. Using Shock's categories as a general outline, recognizing that these categories are interrelated and sometimes overlapping, we will consider some of the important theories of aging.

A. Genetic Theories of Aging

In genetic theories age-related changes are attributed to changes in the genome itself, its transcription or translation. The changes may be intrinsically programmed or may be caused by external factors. An example of a theory of programmed senescence is Hayflick's (1961, 1965) classic work on the limited in vitro life span of cultured fibroblasts in which he found that normal human embryonic tissue, grown under the most favorable of conditions, underwent about fifty population doublings and then died. His conclusion was that death of cultured normal cells was an
inherent property of the cells themselves. The fact that the number of divisions a cell can undergo is directly proportional to the age of the donor also lends credence to this theory (Martin et al. 1970). However, the relationship of this in vitro phenomenon to in vivo aging is still controversial since it is clear that most animals do not die as a result of a limited number of cell divisions (Hayflick 1976). Moreover, there is some evidence that loss of potential to divide in vitro may, in fact, be a sign of differentiation, and not of aging (Bell et al. 1978). In his codon restriction theory Strehler (1977) suggests that differentiation is the cause of aging - as cells become differentiated they lose their ability to translate some of their genetic material either because of programmed autoinhibition or because of changes in programming during development and aging. He also allows for possibility of operon-like repressors and derepressors as program moderators, but considers them more important in coordination of chemical and neural adaptation responses than in specific ontological and aging events.

Rothstein (1982) summarized many papers investigating RNA changes during aging and concluded that although there are a number of reports of decreased RNA synthesis in old animals these decreases do not significantly compromise translation. In a similar survey of research on DNA, he also stated: "the fidelity of DNA polymerase is unchanged with age." However, recent work by Robberson et al. (1982) has demonstrated that rearrangement of the genome may be important in the production of the senescent phenotype in human diploid fibroblasts. Some studies (Strehler et al. 1979, Strehler and Chang 1979, Shmookler et al. 1980) have also suggested that senescence in human cell cultures is associated with a decrease in repetitive sequence DNA.
Changes in non-DNA nuclear proteins have also been investigated. Medvedev et al. (1977) claimed that DNA-histone binding patterns changed with age, masking and making unavailable critical areas of information coded in the DNA molecules. Stein (1975) and Sakagami et al. (1979) also found changes in non-histone nuclear binding proteins. These studies indicate the likelihood of nuclear control of the development of senescence in cultured human diploid fibroblasts.

Two genetic theories of aging that stress changes caused by chance are the somatic mutation theory and the error-catastrophe theory. According to somatic mutation theories (Failla 1958, Szilard 1959, Curtis 1963) spontaneous and/or radiation induced mutations cause or accelerate aging, or accumulate to a point where the organism is no longer viable. As ongoing research elucidates DNA repair mechanisms (Hart and Setlow 1974, and Wheeler and Lett 1974) and describes the common presence of repetitive gene sequences that could compensate for altered genetic material, it becomes more probable that mutations are minor determinants of aging (Strehler 1977).

The proponents of error theories, of whom Medvedev (1964) was the first, state that aging and death are the result of errors that occur during information transfer and lead to the formation of nonfunctional enzymes or structural proteins. Orgel (1963) claimed that although the initial frequency of transcriptional and translational errors in a cell may be low, these increase exponentially in nondividing cells. The cumulative effects of these errors lead to an "error catastrophe" and the death of the cell. Support for this theory varies; and Orgel himself first modified (1970), and then withdrew support for his original thesis (1973).
The error theories, however, led to research on different aspects of altered proteins and enzymes, for example, changes in their activity and three dimensional conformation. In a survey of 413 experiments dealing with dozens of enzymes in humans, rats, mice, horses, guinea pigs, and cows, 169 decreased in activity, 91 increased, and another 153 either remained unchanged or increased at some ages and decreased at others (Shock 1981). Other enzymes become more heat labile (Holliday and Tarrant 1972, Reiss and Rothstein 1975), or underwent structural (Gershon and Gershon 1970, 1973) or conformational changes (Sharma and Rothstein 1980).

B. Nongenetic Theories of Aging

Nongenetic theories of aging are focused on the functional and structural changes that cellular components undergo with the passage of time. Although these changes may be related to genetically programmed events, their observed expression is at other levels of organization. An important theory in this category is the free radical theory, first postulated by Harman (1956, 1968, 1972). Free radicals are highly reactive chemicals produced both enzymatically and nonenzymatically as transient intermediates in normal metabolic processes. Alternately they may be the result of spontaneous UV or ionizing radiation.

Biological systems are normally protected from the damaging effects of free radicals by naturally occurring antioxidants such as Vitamin E, or by enzyme systems such as superoxide dismutase and peroxidase which inactivate superoxide radicals and peroxides respectively. This protection is not complete, however, as evidenced by the gradual widespread accumulation of lipofuscin particles in a variety of aging tissues (Strehler 1964). These highly insoluble pigments are the result
of protein crosslinking caused by a product of lipid peroxidation, malonaldehyde (Rothstein 1982). Since there is no direct evidence that this so-called "age pigment" causes impaired function in cells it may be a marker rather than a cause of aging changes. However, the role of free radicals in aging and their interaction with intrinsic protective mechanisms is a continuing field of research.

Crosslinking of other macromolecules, however, is considered to be an important factor in aging by many researchers (Bjorksten 1968). Crosslinking in collagen and elastin are well documented (Kohn 1978) and similar crosslinks probably occur in enzyme proteins and in DNA (Bjorksten 1974). It is likely that crosslinkage is a substantial contributor to age related debilities (Strehler 1979).

Other nongenetic senescent changes have been related to general metabolic functions such as basal oxygen consumption (Sohol 1976), relationships among metabolism, brain weight, and body size (Sacher 1978), response to environmental influences such as exposure to cold (Finch 1969), and nutrition (Berg and Simms 1960).

C. Physiological Theories of Aging

Physiological theories of aging are used to explain failures of individual organ systems, or the failures or impairments of physiological control mechanisms. Many studies have shown an age-related decline in immune responses in both men and mice (Walford 1974). Studies have linked thymic involution, which begins shortly after puberty, with immunological aging and aging in general (Yunis et al., 1971, Yunis and Greenberg, 1974, and Good and Yunis 1974). Moreover, many diseases associated with aging such as arthritis, renal disease, and autoimmune diseases have been linked to decreased or altered immune function and

Interestingly, lowered body temperature and diet restriction can prolong life span, and it appears that this effect is realized through the immune system. Diet appears to have a powerful influence on thymus and peripheral lymphoid tissues and their immune functions (Kenny et al. 1968). Diet can also affect the tempo of immunologic aging and the onset and progression of major diseases of aging (Ross and Brass 1971, 1973, Jose and Good 1973, Fernandes and Good 1981).

An important aspect of aging in the immune system is the decline in T cell function. T cell response to antigens is decreased, the T cell helper function in the interaction with B cells decreases (which may explain decline in primary antibody response), T cell graft rejection and graft-versus-host reactions decrease, and the actions of T suppressor cells decline (Adler 1975). This decline in T suppressor cells is probably related to the rise in autoimmune diseases of aging. In fact, the incidence of autoantibodies increases with age (Goidl et al. 1981) and the increase of autoantibodies is associated with decreased survival (Hooper et al. 1972).

A consideration of physiological control systems must include a review of the aging nervous system. The neuron itself has been the subject of much research, not only because it is the functional unit of the brain but also because it is an example of a postmitotic (i.e., fully differentiated) cell. This makes it an especially good subject for aging research, because, as mentioned previously, aging is often defined as being the result of differentiation. A wide spectrum of age related changes in the brain, such as neuronal loss, lipofuscin accumulation, and structural changes in dendrites and axons are localized in individual
cells rather than occurring generally over an area.

A survey of neurotransmitters by Finch (1977) and neuroenzymes by Rothstein (1977) showed no consistent pattern of change with aging. Receptor levels generally decrease or remain unchanged during aging (Rothstein 1977). McNeill (1983) summed up neuronal changes at the cellular level during aging as an "integration of progressive and regressive cell changes specific to brain region, cell type, and species of study".

Other functions partially or entirely controlled by the nervous system, including breathing capacity, muscular activity, and regulation of blood pressure and heart rate also decline with age (Shock 1981). Brain mass loss and transient ischemic episodes are also observed in human aging, but these may or may not have a great effect on the mental and physiological capabilities of the individual.

Symptoms such as mental confusion, personality changes, memory loss, and general degeneration of mental functions, traditionally associated with normal aging, have now been recognized as a specific disease, Alzheimer's disease. Neurofibrillary tangles and amyloid plaques, characteristic of this disease, occur to some extent in most aging individuals, especially by the seventh to ninth decades. However, this quantitative relationship does not prove that such changes are inevitable aspects of aging. In fact, modern studies increasingly stress that age-related diseases of the nervous system are, in fact, specific and selective, and not simply the natural deterioration of brain function (Liss 1979).

The endocrine system, especially the hypothalamus/pituitary axis, has been espoused as a biological or aging clock by Everitt (1976). The
pituitary plays a major regulatory role in body functions through its control of other endocrine organs and its regulation of growth, reproduction, and metabolism. The hypothalamus controls many pituitary functions, and acts generally as a receptor and processor for a wide variety of internal and external stimuli. Everitt (1976) stated that the primary control of aging resides in the genes, and their expression is programmed as a function of time. The rate of aging and actual duration of life is a result of the interaction of genetic program with the environment and this interaction is mediated by the hypothalamus-pituitary endocrine system. He pointed out that many hormones, although necessary for life, also accelerate physiological aging or increase the incidence of age-related pathology. He claimed that endocrine function may be programmed by a hypothalamus "clock" that measures function over a period of time, turning on or off various endocrine events.

Specific age-related neuroendocrine changes noted by Everitt include changes in hypothalamic sensitivity to negative feedback, a general decrease in levels of target gland-hormones, changes in gonadotropin levels, decreases in levels of reproductive steroids, and a decrease in glucose tolerance. Hormones are also a factor in the aging of collagen and the skeletal systems and various organs in the cardiovascular, renal and reproductive systems.
A. Testicular Steroidogenesis

The male reproductive steroid hormone, testosterone (T) is produced primarily by the testes and to some extent by the adrenals. The site of T production in the testis is the Leydig cell, which is also an important site of aromatization of T to estradiol (E2). The androgens, including T, and the estrogens, including E2, are produced in a series of enzyme catalyzed reactions which use cholesterol as substrate.

The cholesterol may come from preformed stores in the cell, it may be produced de novo by the testis (Martin 1985), but most of it is carried to the cells as plasma cholesterol by lipoprotein carriers (Freeman and Ascoli 1982). Azhar and Menson (1981) showed that in vivo administration of inhibitors of lipoprotein secretion caused a decrease in plasma cholesterol and a decrease in plasma T. Leydig cell suspensions from these rats exhibited a decreased steroidogenic response both basally and after trophic hormone stimulation. In most mammalian species low density lipoproteins (LDL) deliver cholesterol to the testis (Hadley 1984). The rat, however, may preferentially utilize heavy density lipoproteins (HDL) as indicated by the presence of HDL receptors on Leydig cell membranes (Chen et al. 1980, Azhar and Menson 1981).

Cholesterol side-chain cleavage, the initiating and rate limiting step in the production of testicular steroids (Bakker et al. 1982), takes place in the mitochondria, and the remaining steps of the conversion of pregnenolone (Pr) to T or E2 occur in the smooth endoplasmic reticulum (SER) (Zirkin et al. 1982). Figure 1 shows the pathways of steroidogenesis in the testis, figure 2 the important testicular enzymes,
and figure 3 the structural formulae of the steroids formed. Note that there are two possible in vivo pathways for the production of T. The $\Delta^4$ pathway predominates in rats and the $\Delta^5$ pathway in humans.

B. Role of Luteninizing Hormone

Steroidogenesis of T is stimulated both acutely and chronically by lutenizing hormone (LH) (Rommerts and Brinkman 1980). Binding of LH (or human chorionic gonadotropin, hCG) to Leydig cell membrane receptors results in activation of adenyl cyclase, increased levels of cAMP, protein kinase stimulation of phosphorylation of at least six proteins, and ultimately results in increased production of T. The steroidogenic activities of LH include induction of protein formation and cAMP action enhancement of cholesterol uptake and entrance into the mitochondria, and stimulation of a number of steroidogenic enzymes $\Delta^5-3\beta$ hydroxysteroid dehydrogenase, C_{17-20} lyase, 17\alpha hydroxylase, and 5\alpha reductase. LH promotes release of a number of steroids and stimulates prostaglandin (PG) generation (Martin 1985). The prostaglandins, in turn, amplify LH stimulation and contribute to regulation of LH receptor numbers (Martin 1985), and modulate prolactin (Prl) effects (Rillima 1980). Trophic effects of LH include maintenance of structure, function, and numbers of Leydig cells, support of synthesis of SER, and steroidogenic enzymes and receptors (Martin 1985).

C. Role of Calcium

Ca$^{2+}$ and calmodulin may also be involved in this activation of steroidogenesis (Hall et al. 1981). Cooke et al. (1985) proposed that Ca$^{2+}$ is necessary for the release of arachidonic acid (a cyclic nucleotide precursor) from membrane phospholipids. They also proposed that a lipoxygenase product of arachidonic acid metabolism stimulates the
transport of cholesterol or cholesterol esters to the mitochondria. Lin (1985) also claimed that there are Ca\(^{2+}\)-dependent protein kinases, both Ca\(^{2+}\)-phospholipid dependent, and Ca\(^{2+}\)-calmodulin dependent, which function as steroidogenic modulators separate from the classical cAMP dependent pathways. In this schema Ca\(^{2+}\)-dependent hormones and neurotransmitters hydrolyze membrane phospholipids to diacylglycerol and inositol triphosphate which then act as second messengers to initiate a signal cascade which results in mobilization of Ca\(^{2+}\)-dependent protein kinase phosphorylation and subsequent physiological response.

D. Down Regulation and Desensitization

Prolonged stimulation with hCG, LH, or cAMP, after initial enhancement of steroid production, causes Leydig cells to show a dose dependent loss of receptors and a subsequent defect in steroidogenesis that prevents maximum production of T in subsequent stimulation (Chen and Payne 1977, Cigorraga et al. 1978, Sharpe 1976, Moger 1980, Haour et al. 1982). Although the terms "down regulation" and "desensitization" are sometimes used interchangeably to describe this phenomenon, a distinction can be made between the two. Down regulation involves a decrease in receptor numbers (Tsuruhara et al. 1977, Cigorraga et al. 1978) and their eventual internalization and degradation (Conn et al. 1978, Rajaniemi 1979). Desensitization involves a decreased in vitro maximum pregnenolone and testosterone response to hCG in isolated Leydig cells (Dufau et al. 1977, Hsueh et al. 1977, Tsuruhara et al. 1977). High doses of gonadotropin administered in vivo cause an "early" steroidogenic defect prior to pregnenolone formation that is caused by a mitochondrial protein factor able to inhibit the cleavage of the cholesterol side chain (Hattori 1982). Treatment with lower doses of gonadotropin causes a
"late" steroidogenic lesion with reduction of the activities of the microsomal enzymes 17α-hydroxylase and 17-20 desmolase (Hsueh and Erickson 1979, Cigorraga et al. 1978). Estradiol appears to be a modulator of this defect (Cigorraga et al. 1980, Aquilano and Dufau 1983) inducing a protein which precedes the lesion and is presumed to be an inhibitor of enzymatic activities of the microsomal cytochrome P-450 (Nozu 1981a, 1981b, 1982).

Estrogen has been implicated as a modulator of desensitization in a number of works, several of which will be discussed under the Role of Estradiol. One recent study, for example, which found no evidence of gonadotropin induced desensitization in neonatal testes, correlated this with a lack of E2 receptors and the presence of αfetoprotein which strongly binds E2, thereby preventing its inhibition of enzymatic activity (Huhtaniemi et al. 1983).

Another important aspect of receptor down regulation and desensitization is that they are "uncoupled" reactions. Loss of 2/3 of Leydig cell receptor sites and a marked desensitization of cAMP response was shown to cause no change in the maximum in vitro response of T production to hCG stimulation (Hsueh et al. 1977). This study also suggested a two step desensitization process: first, occupancy of a small fraction of receptor sites by hCG causes initial activation and then inhibition of cAMP responses; second, an actual loss of receptor sites after 1-3 days, with gradual replacement of sites to a normal number after 5-10 days. Receptor loss is an important factor in the mechanism of sustained Leydig cell desensitization (Tsuruhara et al. 1977), but not in the acute response to LH/hCG stimulation (Amsterdam et al. 1981).

Testicular LH/hCG receptors appear to be internalized by a method
different from the coated pits and vesicles that are often associated
with receptor-ligand internalization. Amsterdam et al. (1981)
characterized LH receptor action in some detail. According to their
studies the rate of dissociation of receptor-hormone complex was very
slow. They suggested that the receptors were associated with
cytoskeletal microfilaments. Laws et al. (1984) did show evidence of
direct involvement of microtubules and microfilaments in the down
regulation of LH/hCG receptors. Amsterdam et al. also noted that uptake
of the receptor-hormone complexes after binding was not essential for the
acute cellular response to the hormone (desensitization of the adenylate
cyclase system preceded any appreciable internalization of
receptor-hormone complexes), but the uptake may serve as a mechanism for
metabolizing the bound hormone and recycling the receptor molecules.
Amsterdam et al. also indicated that coated pits and vesicles were
probably not the major cellular vehicles for receptor aggregation and
internalization since their electron micrographs showed localized
clustering of hormone-receptor complexes on microvilli and membrane
ruffles.

In summary, modulators of desensitization are many and are complexly
interrelated. One example is that the steroidogenic lesion in down
regulation at cholesteral side chain cleavage may be initiated not only
by LH/hCG, but also by E₂, gonadotropin releasing hormone (GnRH),
prolactin (Prl), glucocorticoids, epidermal growth factor, and arginine
Welsh et al. 1982, and Adashi and Hsueh 1982). This melange of hormone
modulators and the numerous other factors mentioned above, such as
synthesis of new proteins, involvement of cytoskeletal elements, and
changes in the action of the cytochrome P-450 system, are indications
that age related changes in cellular metabolism are very likely to be
reflected by changes in the densensitization process.

E. The Role of Estradiol in Steroidogenesis

Estradiol is involved in a number of testicular control mechanisms
(Catt et al. 1980), and when administered exogenously it inhibits
testicular androgen production (Kalla 1980, Bartke et al. 1977, Melner
and Abby 1980, Hsueh et al. 1978). It is secreted by both rat and human
testes (Kelch et al. 1972, de Jong et al. 1973), and is aromatized from T
in Leydig cells of mature and immature rats and humans (Valladares and
Payne 1979a, 1979b, Canick et al. 1978). Although Sertoli cells are also
a site of aromatization in immature rats (Dorrington and Armstrong 1975)
this function decreases in Sertoli cells and increases in Leydig cells
during maturation (Tsai-Morris et al. 1985). Aromatization of T to \( E_2 \)
is stimulated by treatment with LH or hCG both in vivo (Canick et al. 1979)
and in vitro (Valladares and Payne 1979a, 1979b).

The presence of \( E_2 \) receptors in rat Leydig cells (Abney 1976,
Brinkman et al. 1972) and in human testes (Murphy et al. 1980) suggests
that \( E_2 \) action on the testis is modulated via \( E_2 \) receptor mechanisms. In
LH/hCG treated Leydig cells there was an acute elevation of testicular \( E_2 \)
followed in 3-6 hours by desensitization of steroidogenesis and loss of \( E \)
receptors. Replenishment of \( E_2 \) receptors in five days was accompanied by
return of response to hCG (Melner and Abby 1980, Mogur 1980, Nozu et al.
1981a, 1981b, Aquilano et al. 1983). A number of other studies also
showed a decrease in receptor numbers when \( E_2 \) receptors were exposed to
densensitizing doses of hCG (Lin et al. 1982, Payne et al. 1982,
O'Shaughnesy et al. (1981).

Also linking E₂ action with desensitization is the fact that E₂ inhibits the activity of 17α-hydroxylase, C₁₇,₂₀-lyase, and 17α hydroxysteroid dehydrogenase (Brinkman et al. 1980, Danutra et al. 1973, Chowdhury et al. 1974). Both 17α-hydroxylase and 17-20 desmolase are inhibited in desensitization (Cigorraga et al. 1978, Chasalow et al. 1979). The fact that aromatase activity is stimulated by gonadotropins (Canick et al. 1978, Valladares and Payne 1979a, 1979b, Payne et al. 1976) is also indicative of the interaction of E₂ with the steroidogenic process.

Mediation by E₂ in the desensitizing process is further supported by a series of events involving translocation of E₂ receptors to the nucleus (Nozu et al. 1981a, 1981b), the E₂-dependent activation of RNA polymerase (Aquilano and Dufau 1983), and the synthesis of an estrogen dependent protein in the Leydig cell after treatment with hCG (Nozu et al. 1981b), or treatment with E₂ (Ciocca and Winters 1985). The appearance of the E₂ dependent protein precedes desensitization of microsomal enzymes. Electron microscopy studies show that protein is located mainly in the cytoplasmic matrix close to the cisternae of the smooth and rough endoplasmic reticula. The fact that it is not found in association with secretory granules suggests a role in local control (Ciocca and Winters 1985).

Some studies, however, indicate that mechanisms other than receptors link E₂ with desensitization. For example, Brinkman et al. (1982) point out that E₂ receptor translocation does not always result in inhibition of testicular enzymes, and that gonadotropin induced inhibition of T synthesis without significant translocation of E₂ receptors has been
observed. In their own study on rat testicular homogenates, which had come from rats that had been subjected to in vivo E₂ treatment, they concluded that the hCG induced depression of C₁₇-₂₀ lyase occurred much faster than the E₂ induced depression of that enzyme. They also demonstrated that rapid E₂ induced depletion of E₂ receptors does not necessarily result in a subsequent immediate inhibition of the C₁₇-₂₀ lyase. In a recent work studying in vivo and in vitro aromatization in rats, Tsai-Morris et al. (1985) suggest that the post hCG, E₂ -mediated densensitization of Leydig cell steroidogenesis is initiated by an early activation of aromatase activity and is subsequently magnified by a significant rise in E₂ formation due to increased substrate availability.

The availability of E₂ as a substrate for further metabolism may allow other control mechanisms. Leinonen (1982) showed that the human testis actively metabolizes E₂ to estrone (E₁). E₁ is biologically less active than E₂, but it and estriol (E₃) compete with E₂ for receptor binding sites (Abney 1976). Conversion of E₂ to E₁ is dependent on the amount of E₂ present, is catalyzed by 17β hydroxysteroid dehydrogenase, and is not inhibited by high testicular T concentrations. This suggests that in humans the effects of E₂ on testicular steroidogenesis may be at least partly modulated by its intratesticular metabolism. Grotjan et al. (1980) did not find metabolism of E₂ by rat Leydig cells. However, since the Δ₄ pathway in rats metabolizes E₁ from androstenedione as well as from E₂ the possibility of a role for E₂ involvement in local control cannot be discarded.

The presence of E₂ receptors in the pituitary indicate that E₂ plays more than a local role in the control of steroidogenesis (Singh and Muldoon 1982). In humans estrogens have been cited as important feedback
regulators of gonadotropin secretion through their action on the pituitary and hypothalamus (Winters 1985). In fact, the binding capacity (measured as moles of steroid bound/mg cytosol protein) of E2 receptors in the pituitary is 10x that of the testis, and the hypothalamus has binding capacity equal to that of the testis. The depletion and replenishment patterns are similar to those in the testis (Cidlowski and Muldoon 1974). Ellinwood et al. (1982) showed that injection of an aromatase inhibitor into men or nonhuman primates (resulting in blocking of peripheral aromatase) caused an increase in LH secretion due to curtailing of the negative feedback effect of E2. The hypothalamus and pituitary also aromatize T to E2 (Singh and Muldoon 1982). GnRH increases the pituitary sensitivity to E2, and E2 in turn sensitizes the pituitary to subsequent stimulation by GnRH. Both in vivo and in vitro GnRH causes an increase in the E2 nuclear receptors of the anterior pituitary and an accompanying decrease in cytoplasmic E2 receptors (Singh and Muldoon 1982).

Binding of estrogen hormones in neuroendocrine tissue of male rats is significantly higher than that of androgens, and the half life (T 1/2) of E2 is significantly prolonged in the pituitary and hypothalamus (Henderson et al. 1979) suggesting that E2 may in fact play an important role in control at this level.

F. 7α-Hydroxytestosterone and 7α-Hydroxyandrostenedione

7α-hydroxytestosterone (7α-OHT) and 7α-hydroxyandrostenedione (7α-Δ4D) are produced in vivo from T or androstenedione (Δ4D) by the microsomal fractions of mature rat testis (Inano et al. 1970). The 7α-hydroxylase activity related to their production is found in the smooth endoplasmic reticulum and shows a distribution similar to other enzymes
involved in the conversion of pregnenolone (Pr) to T (Inano and Tamaki 1971a, Inano et al. 1970). Lecroix et al. (1975) found that exogenously or endogenously T is metabolized mainly to $7\alpha$OHT in adult male rats.

Since $7\alpha$OHT and $7\alpha$OH$\Delta4$D do not have direct systemic androgenic or anabolic effects and yet are present in significant amounts in adult rat testis (Rosness 1977, van der Molen and Rommerts 1981, Inano et al. 1973) they and their $7\alpha$hydroxylase enzyme may be significant local regulators of testicular steroid metabolism (Mittler et al. 1984). There is a reasonable amount of evidence that this is the case. $7\alpha$OHT inhibits $5\alpha$ reduction of T in cell extracts from mature rats and also inhibits $3\beta$ hydroxysteroid dehydrogenase, unlike T which inhibits both $3\alpha$ and $3\beta$ hydroxysteroid dehydrogenase (Rosness et al. 1977, Inano et al. 1973). $7\alpha$OHT and $7\alpha$OH$\Delta4$D also inhibit activities of $\Delta5$-$\Delta3\beta$-hydroxysteroid dehydrogenase and $\Delta4$-$\Delta5$ isomerases. Their action is competitive, with $7\alpha$OH$\Delta4$D being the stronger inhibitor at comparable concentrations. $7\alpha$OH inhibits $7\alpha$hydroxylase activity and $7\alpha$OH$\Delta4$D inhibits $7\alpha$hydroxylase and $17\beta$ hydroxysteroid dehydrogenase. Interestingly, $7\alpha$hydroxylase activity in vitro is also inhibited by prior in vivo hOG and T injections. When $7\alpha$OHT is injected in vivo to castrated male rats the levels of serum LH do not change significantly, but $7\alpha$OH$\Delta4$D causes a significant reduction in serum LH. LH in the anterior pituitary is not changed by either treatment (Inano et al. 1973). Incubation of $7\alpha$OH$\Delta4$D with subcellular fractions of rat testes yields only $7\alpha$OHT. Enzymes tested but not affected by the $7\alpha$OH compounds include $17\alpha$hydroxylase, $C_{17-20}$ lyase, and $20\alpha$hydroxysteroid dehydrogenase (Inano and Tamaoki 1971b).

In addition to the interactions with T and hOG mentioned there is
some indication that the \( 7\alpha\text{OH} \) compounds may interact with other local control factors. Snider et al. (1982) reported that \( 7\alpha\text{OH} \) substituted androstenediones were potent competitive inhibitors of biosynthesis of estrogens. Acting at secondary binding sites they greatly enhanced the affinity of aromatizing enzymes for synthetic inhibitors. \( E_2 \) and \( 5\alpha \) dehydrotestosterone (DHT) are the two main peripheral metabolites of T in humans. \( 7\alpha\text{OH}\Delta^4\text{D} \) inhibits \( 5\alpha \) reductase, and may exert some control over \( E_2 \). It is possible, therefore, that \( 7\alpha\text{OH}\Delta^4\text{D} \) may have effects beyond the local testicular level.

G. Metabolic Clearance of Testosterone

In addition to production, interactions, and circulating levels, another aspect of hormone activity that must be considered is the length of time a given hormone remains in peripheral circulation. The peripheral circulation level of a hormone is dependent both on its production rate and its rate of clearance from circulation. This relationship is usually described by the term metabolic clearance rate (MCR) which is generally defined as "the volume of blood from which the steroid hormone is completely and irreversibly removed in unit time. The term metabolic includes any form of irreversible removal such as formation of metabolites in the tissues or blood or excretion of the free hormones by the kidney" (Tait and Burstein 1964). Metabolic clearance rate can be measured after a rapid single injection or a continuous infusion of steroid.

It has been shown that the disappearance curve of an injected steroid, expressed as fraction of injected dose/liter of blood sampled and represented by \( x \), can be represented by two exponentials:

\[
x = A e^{-at} + B e^{-bt}
\]
If \( \ln x \) is plotted against time on semi-log paper it will yield two straight lines of slopes \( \alpha \) and \( \beta \), and corresponding y intercepts of A and B, where \( t = \) the sampling time in minutes after injection. Each line represents one component of a two-compartment model of steroid clearance, a faster component represented by the line equation \( y = A e^{-\alpha t} \) and a slower component represented by \( y = B e^{-\beta t} \). B is determined by linear regression using the "peel off" method of Gurpide (1975) and the values for the fast component are determined by subtracting the values of the slower component from the combined curve. Half lives of the hormone in the blood are calculated as follows:

\[
\frac{t \alpha}{2} = \frac{\ln 2}{\alpha}
\]

\[
\frac{t \beta}{2} = \frac{\ln 2}{\beta}
\]

the metabolic clearance rate (MCR) is calculated by the equation:

\[
MCR = \frac{\alpha \beta}{A\beta + B\alpha}
\]


The inner pool or faster clearing component, is generally considered to be in rapid equilibrium with the plasma pool of the subject and the outer pool or slower clearing component is thought to be distributed in the tissue and/or tightly bound in such a way as to not be readily accessible to the plasma pool (Tait and Burstein 1964).

An understanding of steroid binding to serum proteins is an essential aspect of understanding MCR. Although there is much variation in the metabolic clearance rate of T in men there is a significant correlation between free T and MCR (Vermeulen et al. 1969), with free T
clearing much more quickly than bound T (Vermuelen et al., 1971). Plasma proteins that bind T include sex steroid binding globulins (SSBG), also called T-E binding globulins (TeBG), albumin (Alb), and testicular androgen binding protein (ABP). In men 44% of circulating T is bound to TeBG, 50% to albumen, 4% to other proteins, and only about 2% is in free form. Other androgens that are bound with high affinity include DHT, androstenediol, and androstanediol, but not Δ4D (Martin 1985), which explains the very fast clearance rate of Δ4D (Dunn et al. 1981).

The binding proteins serve several functions. In the Leydig cell they create a steep Leydig cell/plasma gradient which allows the bound steroid to be secreted. Once in the plasma they provide protection from degradation for the steroids, and a reserve supply of androgen, since specifically bound protein is not active. However, hepatic extraction of androgens is much greater than the free fraction would indicate, therefore some of the Alb non-specifically bound steroid must be metabolized. Alb is a reservoir for excess hormone. Although it has low affinity receptors it provides many binding sites, so only a massive injection of steroid would raise free hormone levels (Martin 1985). E2 enhances production of TeGB, while androgens retard production (Martin 1985), which may be a factor in the faster MCR seen in men compared to women (Vermeullen et al., 1969). TeGB levels in normal men respond to 5 days of hCG treatment by decreasing, probably in response to the increase in systemic androgen levels (both total and free) and the decrease in TeBG binding capacity (Dunn and Clark 1982).
III. Statement of Purpose

In order to investigate the effects of aging on desensitization, the role of E in aging, and changes in the metabolism of T, the following experiments were designed.

To compare the effects of desensitization of steroidogenesis in young and old, rats were chronically stimulated in vivo with hCG for 10 days. Serum levels of T, E$_2$, progesterone (P) and 20\(\alpha\) hydroxyprogesterone (20\(\alpha\) OHP) were monitored during and at the end of the 10 days. In addition, testicular venous blood and endogenous levels of the testicular hormones were measured, and testicular in vitro response to further stimulation was measured at the end of the stimulation period.

In order to determine if age related changes occurred in E$_2$ receptors in the testis an experiment was planned to compare receptors in dispersed Leydig cells and in cytosol preparations in young (4 month) and old (18 month) rats. In order to investigate local testicular control mechanisms and steroidogenic responses to gonadotropin stimulation an experiment using both dispersed Leydig cells and testis tissue was carried out to determine if there were age-related changes in aromatization of T to E$_2$, and also to E$_1$, E$_3$, 7\(\alpha\)OHT and 7\(\alpha\)OH\(\Delta 4\)D, and \(\Delta 4\)D, with and without stimulation by hCG.

In order to investigate differences between Y and O in the clearance of T from circulation, young and old were castrated and their T clearance was monitored (Experiment A). This experiment had sampling times from 15 minutes to 18 hours. A second experiment (Experiment B) focused on early clearance times using sampling times of 5 minutes to 3 hours. In a third
experiment (Experiment C) 2 groups of castrates, young and old, were injected with T and its clearance was monitored. The same animals were used for experiment B and C whenever possible. Clearance of T was calculated both as simple disappearance and as metabolic clearance rate (MCR).
IV. MATERIALS

A. Animals

Male rats of the Sprague-Dawley strain from our own breeding colony were raised on Purina Rat Chow and tap water ad libitum. They were housed under conditions of fourteen hours artificial lighting, ten hours darkness, at a temperature of 24°-26°C. Only animals with no obvious pathologies were used in experiments.

B. Radioisotopes and Unlabeled Hormones

Radioisotopes were purchased from New England Nuclear, Boston, Ma and included \([2,4,6,7-^3\text{H}]\) estradiol (s.a. 115 Ci/m mole), \([1,2,6,7-^3\text{H}]\) testosterone (s.a. 93.9 Ci/m mole), \([1,2-^3\text{H}]\) 20α-hydroxyprog-4-ene-3-one (s.a. 50 Ci/m mole), \([1,2,6,7-^3\text{H}]\) progesterone (s.a. 95 Ci/m mole), \([7-\alpha ^3\text{H}]\) testosterone (s.a. 25 Ci/m mole), \([^{14}\text{C}]-17\alpha\text{-estradiol (s.a. 57 mCi/m mole)}\), \([^{14}\text{C}]\text{ progesterone (s.a. 57.2 mCi/m mole)}\), and \([^{14}\text{C}]-\text{testosterone (s.a. 51.9 mCi/m mole). Newly purchased isotopes were brought to a volume of five mls with benzene: ethanol (9:1 v/v) and were stored at 4° C.}

Non-labeled steroid hormones (Sigma Chemical Co, St. Louis, Mo) used as reference standards were stored at 4°C in ethanol, as were \(7\alpha\) hydroxytestosterone and \(7\alpha\) hydroxyandrostenedione (M.C.R. Steroid Collection, U.K.). Human chorionic gonadotropin (hCG-2500 IU/mg) (Sigma Chemical Company), was stored lyophilized or dissolved in aliquots of saline at -20°C.

C. Buffers, Reagents, and Other Materials

Collagenase (Worthington Millipore Co., Freehold, NJ) was stored dry
at -20°C. Tissue Culture Medium 199 (Difco Laboratories, Detroit, Mi) and Krebs-Henslet buffer with glucose (see appendix) were used for testes tissue incubations. Estrogen receptor incubations were carried out in a tris-EDTA-molybdate buffer (TEMPb) (see appendix) and radioimmunoassays (RIA) were done in phosphate buffered saline (PBS) with 0.1% gelatin (see appendix).

Dextran T-70 (Pharmacia, Upsala, Sweden) charcoal solutions were prepared by using 20 times washed activated charcoal to make a 0.3% charcoal/0.03% Dextran solution in TEMPb for the estrogen receptor assay and a 0.5% charcoal/0.05% Dextran solution in phosphate buffered saline for RIA procedures. Charcoal-Dextran solutions were kept for 1 week at 4°C.

Antisera for progesterone (P), and testosterone (T), prepared by Dr. S.W.C. Chan of our laboratory, showed high specificity and low cross reactivity with competing steroids. Antisera for estradiol (E₂) and 20α progesterone (20α-OH-P) (Miles-Yeda, Kankakee, Ill) were diluted 1:15 with PBS. Other antisera were diluted with PBS as follows: APS-2 (progesterone antisera) 1:2000, ATS-1 (T antisera) 1:2000, and CTs-1 (T antisera) 1:25,000. Stock antisera were stored at -20°C and when diluted with buffer were stored for up to one month at 4°C.

Anhydrous ether (Baker, Photorex grade) was used for extractions and chromatography. Organic solvents used for thin layer and column chromatography were Fisher Certified ACS grade (Fisher Science, NJ) or Baker Analyzed Reagent grade (Baker Chemical, Phillipsburg, NY).

Columns for column chromotography were made by cutting the mouthpiece ends from 10 ml disposable glass pipettes (Corning Glass Co., Corning NY) and plugging their tips with glass wool.
The glass 20 cm x 20 cm plates (Alltech Assoc., Deerfield, Il) used for thin layer chromatography were coated in our laboratory with silica gel (Silicar TLC 76F Mallinckrodt, St. Louis, Mo) (31 g gel + 60 ml distilled water) and spread to a thickness of 2.5 mm using a Shandon Southern Unoplan spreader and plate holder (Shandon Southern, Eng.). Plates, left to air dry for several hours or overnight, were charged for one hour at 90°-110° C. After cooling to room temperature they were stored in a desiccator until used. If the plates were not used within twenty four hours of preparation they were dried for 15-30 minutes at 85°-110° C before use.

Commercially prepared plastic backed silica gel plates (Silica Gel 60 F254 0.2 mm MC/B Manufacturing Chem. Inc. Cincinnati, Oh) were used for some isotope purifications. Developing tanks were lined with paper (Whatman chromatography 1, W and K Bolston Std., Eng.). The color developing spray used to identify TLC reference standards was anisaldehyde/ sulfuric acid/ acetic acid (see appendix).

D. Counter and Fluors

All radioactive samples were counted in a Packard Tri-Carb liquid scintillation counter, model 3320 with an efficiency of 57% for 3H and 91% for 14C. Counting vials included: 1) bags (Nalgene filmware bags, Nalgene, Rochester, NY) used with plastic or 20 ml glass carrier vials; 2) 5 ml glass vials used with twenty ml glass carrier vials; 3) twenty ml glass carrier vials used alone. Aqueous samples were counted in Omni-fluor with Triton X (see appendix) (New England Nuclear, Boston, Ma) and nonaqueous samples were counted in PPO/POPOP (see appendix) (New England Nuclear).
V. METHODS

A. Serum Samples

Rats were anesthetized and the blood sample was collected by snipping off the tip of the tail. Alternatively, trunk blood was collected from decapitated rats. Blood samples were stored on ice and then refrigerated until clotted. Serum was separated by centrifugation for five minutes at 13,000 rpm in a Microfuge B (Beckman, Palo Alto, Ca) and was stored at -20°C for later assay.

B. Extractions

Serum or incubation aliquots of cell or tissue incubation solutions were extracted with ten times their volume of appropriate organic solvent (ethyl ether, petroleum ether, methylene chloride). Very small samples were brought to two hundred ul with water before extraction. The general extraction procedure using either ethyl ether or petroleum ether was as follows. The solvent was added to the sample and was mixed by gentle inversion and shaking, or by vortexing, taking care to avoid formation of emulsions. The mixture was equilibrated for at least forty five minutes, and then was frozen at -70°C until the aqueous layer solidified (10-20 minutes). The organic layer was decanted and the sample was extracted a second time. Organic layers were pooled and dried under N2 at 37°C. Before use, samples were washed down repeatedly with ethanol or acetone, were redissolved in appropriate solvents, and aliquots were removed. Methylene chloride extractions (used primarily for T) followed the same basic procedure except that the organic layer was removed by pipetting it from under the frozen aqueous layer. Freezing facilitated the removal of the organic layer.
When whole testes were quantitated for endogenous hormones the procedure was as follows. Testes were thawed, decapsulated, minced with scissors, and sonicated in 10 ml of saline, using two bursts of eight to ten seconds each on setting 8.5 of the Biosonik Tissue Disrupter (Bromwell). Sonicated homogenates were extracted twice in a 250 ml separatory funnels with 50 ml ethyl ether. Organic layers were pooled and evaporated in a rotary evaporator. After repeated washings samples were transferred to 8 ml glass tubes and dried in a 37°C dry bath under N₂.

The efficiency of recovery procedures was determined by adding ¹¹C labeled hormone to a tritiated hormone and calculating recovery values against an unextracted total count tube using double label counter setting. Extraction recovery routinely exceeded 95%, or appropriate adjustments were made in the calculations. Water blanks were included in extraction and RIA procedures to allow subtractions of reagent backgrounds when necessary.

C. Radioimmunoassay (RIA)

To quantify steroids the following RIA procedure was followed. First a standard curve RIA using unlabeled steroids of known concentration was prepared (Fig 4). Tritum labeled hormones of about 40-50 pg per 0.1 ml concentration (for hormone labeled in four positions, such as T and E₂ about 8,000 -10,000 cpm per 0.1 ml and for isotopes labeled in two positions, such as P and 20αOH P, about 3,000-4,000 cpm per 0.1 ml) were incubated with antiserum diluted to give approximately 50% bind of the radioisotope when no competing unlabeled hormone was used.

Dried standards and samples (extracted and sometimes separated by Sephadex LH-20 chromatography) were redissolved in 0.1 ml PBS with 0.1%
gelatin. Antiserum and radioisotope, 0.1 ml of each, were added to the sample and standard and the samples were incubated for one hour at 37°C, or for 18 hours at 4°C. If incubation was at 37°C the reaction was stopped by placing the samples in an ice water mixture for 15 minutes. Bound hormone was separated from free by the addition of 0.3 ml Dextran coated charcoal. Samples were vortexed and allowed to settle for 10 minutes after which they were centrifuged at 1000 x g at 4°C for 10 minutes. Aliquots (0.3 ml) of the supernatant were removed and counted in 3 ml Omnifluor-Triton X.

Hormone values were determined by comparing samples to the standard curve. Standards were always run in duplicate, and whenever sample volume permitted, samples were run in duplicate and assays were also duplicated. Appropriate adjustments for reagent (water) blanks and extraction losses were made when necessary.

D. Tissue Incubations

Testis tissue incubations were carried out using 250 mg of decapsulated, slightly teased tissue segments suspended in 5 ml KRB with glucose in 25 ml Erlenmeyer flasks, or using 100 mg tissue in 2.5 ml KRB under the same conditions. Incubations were at 37°C or 34°C under an atmosphere of 95% O₂ - 5% CO₂ for 2-4 hours with gentle shaking. At the end of the incubations tissue was strained out by pouring the incubation mixture through fiberglass plugged funnels and the solutions were stored at -20°C for later assay.

E. Leydig Cell Dispersal

Leydig cells were prepared for incubation after the method of Dufau, Mendelson, and Catt (1974). Each decapsulated testis was placed in a 15 ml polypropylene centrifuge tube (Corning Glass) with 5.0 ml of
collagenase in Med. 199 (0.25 mg collagenase/ml) and the mixture was incubated at 34°C with moderate shaking. When the seminiferous tubules were visibly dissociated (15-20 min) the tubes were allowed to remain upright at room temperature for 10 minutes to allow settling of the tubules. The Med. 199 was then poured off through the plastic support of millipore filter (Millipore Filter Corp., Bedford, Ma). The tubules were rinsed with 5 ml of Med. 199, allowed to settle for 5 minutes, and then poured through the filter. The cells were spun at 800 x g for 15 minutes at 4°C in 15 ml polypropylene tubes. After centrifugation the supernatant was discarded and the cell pellet was gently resuspended in 1 ml Med. 199, centrifuged as before, and the supernatant again was discarded. The pellet was resuspended in 0.5-2 mls of Med. 199, and cell concentrations were determined by doing cell counts with a hemacytometer. Leydig cells were identified using a modification of the 3-α hydroxysteroid dehydrogenase method of Weibe (1976) (see appendix) which stains Leydig cells blue. Leydig cell counts were usually about 30-35% of the total cells.

Cells were incubated in T.C. Med. 199 with 0.1% BSA in 5 ml plastic test tubes - using a total incubation volume of 0.5 ml or 1.0 ml. Incubation was at 34°C under a constant atmosphere of 95% O₂ - 5% CO₂ with gentle shaking. Incubations were 2-4 hours, at the end of which time the cell media mixture was quickly frozen and stored at -20°C. For assay the cells were thawed, spun down at 800 x g for 10 minutes and aliquots of the supernatants were removed.

F. Chromatography Techniques

1. Column Chromatography

Column chromatography using Sephadex LH-20 (Pharmacia, Piscataway,
NJ) was used to separate \( P, 20\alpha\text{OHP}, E_2, \) and \( T \) from sera and extracted testes. Columns were packed using 1 gram of Sephadex suspended in 5 ml of slurry mixture (benzene: methanol, 9:1, v/v). One column length (10 ml) of slurry mixture was run through the column to complete packing of Sephadex and then two column lengths (20 ml) of solvent system I (iso-octane: benzene: methanol, 85:10:5 v/v/v) were eluted through the column to equilibrate it. Previously extracted and dried samples were redissolved in 0.2 ml of solvent mixture I and the sample was loaded on the column. Using solvent system I as described above, \( P \) was eluted in the 2-8 ml fraction, \( 20\alpha\text{OHP} \) in the 8-13 ml fraction, \( T \) in the 14-22 ml fraction. At 24 ml solvent system II (iso-octane: benzene: methanol, 55:20:25 v/v/v) was applied to the column, and \( E_2 \) was collected in the 25-31 ml fraction.

Eluates were dried down at 37\(^\circ\)C under \( \text{N}_2 \). Test tubes containing samples were washed several times with ethanol and stored dry at 4\(^\circ\)C.

Before samples were chromatographed in any experiment an elution profile was determined by chromatographing tritiated hormones in individual columns, collecting 1 ml fractions and plotting fractions vs. cpm. Adding all cpm under a given peak and comparing to original total count cpm also gave general recovery values for each hormone. A combined elution profile of \( P, 20\alpha\text{OHP}, T, \) and \( E_2 \) in systems I and II described above is shown in figure 9.

2. Thin Layer Chromatography

Test tubes containing extracted, dried Leydig cell or testis tissue samples were washed down at least twice with ethanol and dried. Samples were then plated using acetone and a 10, 8, 6, 4, 2 drops wash procedure. Reference standards were plated in the two outside lanes of the plate and
two samples were plated in the two center lanes about 2 cm from the bottom of the plate. Development continued until the sample reached approximately 3 cm from the top of the plate. After development in appropriate solvent system (see figure 16) the plates were placed in a hood and allowed to dry thoroughly. Reference standards were located by viewing the plate under UV light and by use of anisaldehyde color developing spray. Color development required a 10-30 minute incubation in a drying oven at 85°-100°C.

Samples were removed from the gel as follows. Using the reference standards as guides an area of 1 cm x 4 cm was scraped from the plate for each hormone of each sample. The gel was scraped into a funnel tightly plugged with cotton and the hormones were eluted with 3-7 ml of methanol or ethanol. Samples were usually left to air dry overnight and drying was completed at 37°C under N₂. Overlapping or poorly separated samples were eluted together and were then replated and developed in a system designed to separate them.

After several washdowns, samples were redissolved in 1 ml ethanol, an aliquot was removed and dried down in a 20 ml glass counting vial, and counting was carried out in 10 mls PPO/POPOP. Adjustments were made for procedural loses by calculating individual recoveries from radioisotopes added before extraction. Purification of radioisotopes was carried out on glass and precoated plastic backed silica gel plates. The developing system used for the glass plates was chloroform ethanol (95:5) and for the precoated plastic backed plates benzene:MeOH (85:15).

G. Counter Calibration

Calibration of the counter was carried out to maximize counting efficiency. Calibration curves using machine standards (³H and ¹⁴C)
determined the following settings and efficiencies: Single label (\(^3\)H) settings - windows in both red (\(^3\)H) and green (\(^14\)C) channels 50-1000, red gain 44%, green gain 7.25%. These settings gave counting efficiencies of 57% for \(^3\)H and 91% for \(^14\)C.

The above settings gave a counting efficiency for \(^3\)H of 30% for Omnifluor - Triton X and 47% for PPO/POPOP.

For double label readings (\(^3\)H + \(^14\)C) windows were adjusted to virtually eliminate forward spill (\(^3\)H - \(^14\)C = << .1%) and to minimize backward spill (\(^14\)C - \(^3\)H = 9%). Gain settings were adjusted to maximize efficiency and to maintain equal recoveries for both \(^14\)C and \(^3\)H channels. Double label settings were: \(^3\)H window (red channel) 50-250, red gain 44%; \(^14\)C window (green channel) 250-1000, green gain 8.55%. Efficiencies were 26.4% for \(^3\)H and 27.7% for \(^14\)C. An average of 27% was used in double label calculations.

VI. METHODS: INDIVIDUAL EXPERIMENTS

A. Chronic hCG Stimulation by Human Chorionic Gonadotropin

Four month (4 m) and eighteen month (18 m) male rats were injected intraperitoneally (i.p.) for 10 days with 5 IU human chorionic gonadotropin (hCG) dissolved in saline. At one hour post injection on days 1, 2, 4, 7, (D1, D2, D4, D7) peripheral blood samples of about 2 ml were drawn from the tail vein of each rat. Fluid was replaced by i.p. saline injection. Blood samples were handled as previously described and serum samples were stored at -20°C.

On day 10 at one hour post hCG injection the rats were anesthetized with ether, the scrotum was cleansed with 70% ethanol, hair was trimmed
from the area and the scrotum was opened to expose the testis. The large testicular vein was cut and 0.5-1.0 ml of blood was collected in small tubes. This blood sample was called testicular venous (TV) blood. This testis was removed, and kept in saline on ice until assayed. The second testis was removed and quickly frozen and stored at -70°C and was stored until used for determination of endogenous hormones. After decapitation, terminal peripheral or trunk blood (TP or D10) was collected and the serum was stored for assay.

Incubations of tissue and Leydig cells were carried out as described. Testis tissue samples (250 mg in 5 ml kRB) were incubated for 2 hrs at 34°C with 0, 20, 50, and 100 mIU hCG. Leydig cells were incubated for 3 hrs at 34°C using 2 x 10^5 cells per test tube in a total of 0.3 ml Med. 199 with 0, 2.5, 5, 10, and 100 mIU hCG. At the end of the incubations all samples were stored at -20°C until assayed.

Testosterone was quantitated by RIA using extracted aliquots from the tissue and cell incubations. Endogenous hormone levels in the contralateral testis were determined by RIA of extracted and column chromatographed aliquots of testicular homogenate (see general methods).

Basal values of T, E_2, P, and 20αOHP were determined by RIA after petroleum ether extraction of sera. Serum samples from D1, D2, D4, D7, D10 (TP) and TV were extracted with ether, subjected to column chromatography and RIA.

B. Estrogen Receptors

Estrogen receptor assays were done according to the method of Abney (1976) and were performed using both dispersed Leydig cells and cytosol preparation from testis tissue. Rats were decapitated and testes were quickly removed to ice cold TEMD buffer. Testes were weighed and
decapsulated - one was minced for preparation of cytosol receptors ($R_c$) and one was used to obtain dispersed Leydig cells.

For cytosol preparation the minced testis was homogenized under ice cold conditions using 3-4 strokes of a Teflon-glass Potter-Elvehjem homogenizer (1 g tissue/ 4 ml TEM buffer) and was then centrifuged in polyallomer tubes at 17,000 x g for 10 min at 4°C (Sorvall RC2- B centrifuge with a 55-34 rotor). The supernatant was transferred into Oak Ridge centrifuge tubes and spun at 105,000 x g 1 hr at 4°C using a Beckman ultracentrifuge with a Ti 50 rotor. After centrifugation the S100 fraction was measured for protein using the BioRad assay. Values obtained were 4-8 g protein/ml cytosol. The assay for receptor binding was set up using enough S100 fraction to equal 1 mg protein per test tube in a total incubation volume of 1 ml. Tritiated estradiol ($^3$H-E$_2$) was used in concentrations of 0.005 pm to 1.0 pm and competing ligand - both E$_2$ and diethylstilbestrol (DES) were used in concentrations from 100 to 1000 x the $^3$H-E concentrations. An assay using varying amounts of cytosol was also run. Incubations were carried out in plastic tubes overnight (16-18 hrs) and were terminated by adding 1 ml Dextran coated charcoal to each tube. Samples were allowed to set for 15 min (during which time they were gently vortexed several times) and were then centrifuged at 1500 x g. Supernatants were decanted into 20 ml glass counting vials, and 10 ml aqueous Fluor was added for counting.

Leydig cells were collagenase dispersed, and were used in concentrations of 2 x 10$^5$ to 1 x 10$^6$ Leydig cells/ 1 ml Med. 199. HE concentrations used ranged from .01 - 0.1 p moles with 100 x concentration of DES, and incubations were at 37°C for one half hr, and at 4°C for 18 hr. Incubations were terminated by adding 1.0 ml ice cold
TEM buffer, and centrifuging at 4°C at 800 x g for 10 min. After
discarding the supernatant, the cells were washed 3 x with TEM buffer
and were extracted by adding one ml ethanol to each tube. After a ten
minute incubation at 37°C, the extract was decanted into a counting vial
and was counted in 10 mls of aqueous fluor.

C. Aromatase Activity and 7 α OH Compounds

Two sets of experiments were carried out to determine if there were
age-related differences in the aromatization of T to E2 or in the
production of other T metabolites, specifically 7α hydroxytestosterone (7α
OHT) and 7α hydroxyandrostenedione (7α OH Δ4D), and in the production of
other testicular steroids - estrone (E1), estriol (E3), and
androstenedione (Δ4D). In aromatase experiment A (Arom A) the testes
of 4 m and 18 m rats were removed, weighed, and decapsulated. 250 mg
samples from one testis of each pair and dispersed Leydig cells from the
contralateral testis (5 x 10⁵ Leydig cells per 0.5 ml Med. 199. in plastic
tubes) were preincubated for 30 min at 34°C under 95% O₂ / 5% CO to
remove endogenous hormone. At the end of the preincubation period the
tissue samples were strained through glass funnels tightly plugged with
fiberglass and the tissue and fiberglass plugs were placed in fresh
flasks containing 5 mls KRB, 0.3 n moles [7⁻³H] testosterone (7α⁻³H)T
and with and without 50 mIU hCG. They were incubated as previously
described for 2 hrs at 34°C. At the end of the preincubation period the
Leydig cell tubes were spun at 800 x g for 10 min at 4°C, the supernatant
was poured off, the cells were gently resuspended in Med. 199 and were
transferred to tubes containing 0.3 n moles [7α⁻³H]T, with and without
25 mIU hCG. They were incubated as described for 2 hrs at 34°C in a
total incubation volume of 0.5 mls. The amounts of [7α⁻³H] T and hCG
used were based on experiments by Valladares and Payne (1979) and by earlier pilot experiments in our laboratory (personal communication S.W.C. Chan).

Before removal of an aliquot for extraction the Leydig cell samples were spun at 800 x g for 10 min at 4°C to spin out all cells. Aliquots of Leydig cell and tissue incubates were extracted 2x using 10 times their volume of ether. After appropriate wash and drying procedures the extracts were subjected to thin layer chromatography (TLC) to separate the following hormones - $E_1$, $E_2$, $E_3$, androstenedione, $7\alpha$OH-T and $7\alpha$OH$\Delta$4D. Chloroform:ethanol (95:5) was used for the initial separation, with chloroform ethanol (92:8) used to separate $7\alpha$OH-T from $E_3$, and ethyl ether:chloroform (4:1) used to separate $7\alpha$OH$\Delta$4D from $E_2$, and $E_1$ from $T$. See figure 16 for a flow chart summarizing the systems. Samples were eluted with 1 ml of methanol followed by 2 mls of ethanol, and were dried down and counted as described previously.

Aromatase experiment B (Arom B) followed the same protocol as Arom A except for the following differences: (1) Rats used were 4 m and 24 m rather than 4 m and 18 m; (2) there was no preincubation period; (3) Leydig cells (1 x 10^6 / 1.0 ml Med. 199 with 0.6 n moles [7$\alpha$-3H]T with and without 50 mIU hCG) were incubated for 2 hrs, and tissue samples (250 mg in 5 ml KRB with 0.6 n moles [7$\alpha$-3H]T, with and without 50 mIU hCG) were incubated for 4 hrs; (4) samples were eluted directly into counting vials and the whole sample was counted.

D. Disappearance of Testosterone from Circulation in Castrated Young and Old Male Rats.

Each rat was anesthetized with ether, weighed, and approximately 0.5 ml of blood was removed from the tail. After this and each subsequent
blood sampling, the rat was injected i.p. with an amount of saline equal to the amount of blood withdrawn. The scrotum was opened and both testes were isolated from surrounding tissue. Thread was tied around each spermatochord, cutting off blood supply to and from the testes. This was considered to be time 0. The testes were quickly removed and the incision was closed with stitches and metal wound clips. At intervals of 5, 10, 15, 30, 45, 60, and 120 min blood samples of 0.5-1.0 ml were drawn from the tail. In some early experiments samplings were continued for up to 6 or 18 hrs. Hemocrit capillary tubes were also filled at each sampling time for determination of hematocrit values. During the first 15-30 min the rats remained under ether anesthesia. After this time light ether anesthesia was administered as needed to allow blood samples to be drawn. Blood samples were kept on ice or were refrigerated until clotting occurred. Serum was separated and extracted as previously described. Methylene chloride was used for the extractions in this series of experiments. T was quantified by RIA.

In addition to following the disappearance of T immediately after castration, previously castrated rats were injected via cardiac puncture with 100 ug T dissolved in saline. Samples were drawn and treated as above.
VII. RESULTS

A. Chronic Stimulation by Human Chorionic Gonadotropin

1. In Vivo

Serum samples taken 1 hr post hCG injection on D1, D2, D4, D7, and D10 were quantified by RIA for T, E2, P, 20α-OHP. D10 testicular venous blood was also quantified for testicular production of the four hormones, and testis tissue was quantified for endogenous hormone levels. The results are shown in tables 1-5, and figures 10-13.

a. Testosterone

T levels of both young (Y) and old (O) (see table 1, figure 10) rose from basal levels to a peak on D2. Although non-stimulated basal levels were higher in the young than in the old, the difference was not significant (other groups of animals in this laboratory have shown significant basal differences in T levels between Y and O). By D1 the Y values were significantly higher than the O, both in actual ng/ml and in ng/ml change from basal. After reaching a peak on D2 both Y and O levels started to decline. The O reached a peak of 6.02 ± 0.91 ng/ml serum while the Y reached a peak of 10.5 ± 1.46 ng/ml serum, but if comparisons are made on the basis of change from basal (in ng/ml serum) both Y and O show no significant differences in response after D1. However, if change from basal is compared to basal levels the O show a slower return to basal. Testicular venous blood had higher levels of T in the O than in the Y, but the difference was significant only at P<0.1. The endogenous levels of T, however, showed a significant difference, with the level of T in the O almost 4x the level in the Y.

b. Estradiol
Estradiol levels (table 2, figure 11) in the Y and the O rats were not significantly different in endogenous, testicular venous, or in serum levels except at D1 when Y was significantly higher than O and D2 when the O were significantly higher in serum E2 than the Y.

In comparing the ratio of T to E2 there are some differences. Table 3 shows that at basal levels the Y have a higher T/E2 ratio than the O and this higher ratio is maintained through the 10 days of hCG stimulation, except for D7 when the O have a slightly higher ratio. In the D10 TV samples the O have a slightly higher T/E2 ratio than the Y and the endogenous levels of T and E2 show a considerably higher T/E2 ratio in the O.

c. Progesterone

Progesterone (table 4, figure 12) showed no significant differences in serum levels except at D7 when the Y were higher. When compared as changes from basal there were no significant differences in serum levels. Testicular venous and endogenous levels also showed no significant differences between Y and O.

d. 20α hydroxyprogesterone

20α hydroxyprogesterone (table 5, figure 13) showed no significant age related differences in serum levels, including testicular venous D10 blood. However, there was a very significant difference in the endogenous levels, with the O having substantially higher levels of 20 αOH.

2. In Vitro Treatment with hCG after Chronic In Vivo Stimulation

Tissue samples from one testis of each animal treated in vivo were incubated with 0-100 mIU hCG to test the in vitro response of chronically
hCG stimulated testes. The results are shown in figure 14. In the control animals basal values were 30.0 ng T/100 mg tissue/2 hr incubation for the Y and 4.5 ng for the O. The Y showed a response to hCG stimulation at 20 mIU hCG and a significantly greater response at 100 mIU hCG. The O showed a significant response at 20 mIU but did not show a greater response at higher dosage. In the chronically treated samples, however, the response was very different. With no hCG stimulation the O produced 17.6 ng T/100 mg tissue/2 hr incubation while the Y produced only 6.33 ng. Treatment with 20, 50, or 100 mIU hCG caused slight declines in the response of the Y, while the O stayed the same.

B. Role of Estradiol in Aging Male Rats

1. Estradiol Receptors

Since specific binding sites (i.e. receptors) have some finite number it is possible to saturate binding activity with their specific ligand. To measure E_2 receptors the prepared receptors are exposed to various concentrations of ^3H-E_2 both with and without a 100x molar excess of an unlabeled competitive ligand (DES). The DES should occupy essentially all the high affinity sites, but not interfere appreciably with the binding of ^3H-E_2 to low affinity, nonspecific, nonsaturable sites. The bound and free fractions of the incubates are separated by charcoal absorption and a saturation curve is plotted showing bound vs. free. The specific binding is calculated by subtracting nonspecific (represented by the ^3H-E_2 + DES incubates) from the total bind (^3H-E_2 only incubates). Non specific binding should be relatively low compared to specific binding. In our experiments both dispersed Leydig cells and cytosol receptor preparations showed very high nonspecific binding. Figure 15a shows the best ratio of specific to nonspecific binding we
were able to obtain, approximately a 1:1 ratio. Figure 16b represents
the Scatchard analysis of the same data, showing receptors to be present
in a concentration of 3.05 fm/mg protein and the $K_a$ to be $2.54 \times 10^{10}$ M$^{-1}$.
Preparation of uterine cytosol receptors showed the expected high ratio
of specific to nonspecific binding indicating that the high nonspecific
binding was peculiar to the testis $E_2$ receptor preparations. Since we
were not able to overcome this procedural problem no valid comparison of
$Y$ and $O$ testicular $E_2$ receptors could be made.

2. Aromatization of Testosterone to Estrone, Estradiol, Estriol

Testis tissue and dispersed Leydig cells of $Y$ and $O$ rats were
incubated with $^3$H to compare aromatization of $T$ to $E_2$. Tables 7a and 7b
show the aromatization of $[7\alpha-^3H]T$ to $E_1$, $E_2$, and $E_3$. The dispersed
Leydig cells showed age-related differences only in the amount of $E_1$
produced, with the $Y$ producing significantly more $E_1$ than the $O$ both with
and without hCG stimulation. Although the changes in $E_2$ production after
hCG stimulation were not statistically significant there was consistency
in the effect of hCG. Three of four of the $O$ animals showed a decrease
in $E$ production after hCG stimulation and four of four of the $Y$ showed
an increase. The $O$ declined an average of 29% and the $Y$ increased an
average of 31%. Leydig cell results were calculated only from the A
experiment (see Methods) since thin layer chromatographing resulted in
very low recoveries for experiment B. Table 7b shows the results of
tissue incubations. There were no significant differences between $Y$ and
$O$ with or without hCG in the tissue incubations. Both tissue
experiments, A and B, gave comparable results except that very low
recoveries prevented calculation of $E$ in experiment B.
C. Metabolism of Testosterone to 7α hydroxytestosterone and 7α hydroxyandrostenedione and Androstenedione

In the preceding experiment of aromatization of \([7\alpha-^3H]T\) to \(E_1\), \(E_2\), and \(E_3\) incubates were also quantified for 7αOHT, 7αOHΔ4D, and androstenedione (Δ4D). Tables 8a and 8b summarize the results. 7αOHT showed no significant differences in Y or O with or without hOG. Leydig cells from the Y rats showed significantly higher conversion of \([7\alpha-^3H]T\) to Δ4D with and without hOG stimulation when compared to Leydig cells from O rats. However, stimulation of the Leydig cells of Y animals by hOG caused a significant depression of conversion of T to 7αOHΔ4D compared to non-stimulated values.

D. Clearance of Testosterone from Circulation in Young and Old Rats

Experiment A, which compared the disappearance of T from the circulation of O as compared to Y rats is summarized in figure 17 and table 9a. Both Y and O rats showed an initial rapid drop in T (measured as T remaining in ng/ml serum) with the Y clearing 1/2 the basal amount of T in 15 min. The O, however, took 2 hr to clear 1/2 their basal amount of T. The difference in clearance is even more dramatic if actual amounts of T cleared are compared. In 2 hr the O cleared only about 0.2 ng while the Y cleared about 3.0 ng. After 6 hr the Y had 3% of their basal values of T remaining while the O still had 30%. At 18 hr post castration the values in Y and O were similar, 0.083 ng/ml for the old, and 0.106 ng/ml for the Y, representing 20% and 4% of basal respectively.

In experiment B, Y and O animals were monitored for T clearance concentrating on earlier sampling times. The results are shown in figure 18 and table 9a. The Y again showed a drop to 1/2 basal levels in about
15 min., while the O took more than 1 hr to reach 1/2 basal levels. In fact, at 3 hr the O showed values only slightly below 1/2 basal levels while the Y at 3 hr were at 15% basal.

Figure 19 and table 9b show the results of experiment C in which 4m and 18m rats were injected with 100 ug of T and its clearance was monitored. In this experiment Y and O showed similar clearance of T. Although the Y still cleared 1/2 the basal amount faster than the O (about 8 min. compared to about 20 min.) the difference was much less than when clearance was monitored in acutely castrated rats. The O however, did have slightly more T left at each sampling time and by 3 hours the difference had become significant with the O having 4.61 ng T/ml serum left compared to the Y value of 1.50 ng/ml.

The same data used to determine disappearance of T was used to calculate MCR. Figure 20 shows the line equations for the MCR calculations and tables 10 and 11 show the MCR values. Figure 21a shows the MCR of a 4m rat after castration. The mean MCR for 4m rats was 15.9 l/d or 30.0 l/kg/d, with a t 1/2 early of 8.2 min and a t 1/2 late of 164.2 min. Figure 21b shows the pattern of T clearance in an 18m rat after castration. Because of the fluctuating values it was not possible to calculate MCR.

Injection with 100 ug T resulted in a MCR in the 4m rats of 41.8 l/d or 90.8 l/kg/d and a t 1/2 early of 5.0 min and a t 1/2 late of 44.9 min. The 18m rats showed a MCR of 39.1 l/d or 82.7 l/kg/d with a t 1/2 early of 11.5 and a t 1/2 late of 92.7 (see tables 10 and 11).
VIII. DISCUSSION

A. Chronic Stimulation by Human Chorionic Gonadotropin

A variety of age-related changes in steroidogenic responses have been reported. Most authors agreed that serum levels of T decrease with age in rats (Chan et al. 1977, Chan and McFarlin, 1981, Pirke et al. 1978) and in men (Takahashi et al. 1983, Warner et al. 1984). This decrease in circulating T is expected considering the many reports of decreased responsiveness to hCG in aged men (Chan et al. 1982, Harmon et al. 1978, Meites 1979). Declines in Leydig cell numbers have been reported for rats and men (Kaler and Neaves 1978, Warner et al. 1984, Neaves et al. 1985).

In old men LH levels often rise (Davidson et al. 1983, Meites 1979, Baker et al. 1976, Takahashi et al. 1983, Warner et al. 1984, Harmon and Tsitouras 1980) and qualitative changes also are observed (Warner et al. 1984). In rats, some investigators have found decreased serum LH levels (Grey 1978, Pirke et al. 1978), and decreased numbers of LH receptors (Tsitouras et al. 1979). Others have not found differences in either serum LH levels or receptor numbers (Chan and McFarlin, 1981, Kaler and Neaves, 1981). Even in those studies which found lowered LH levels and/or lower numbers of receptors the differences are not in themselves adequate explanation for the significant differences between Y and O with respect to circulating T and responsiveness to gonadotropin stimulation. Other age-related changes include decreased levels of pituitary LH (Riegle et al. 1977), reduced LH release in response to stress (Euher et al. 1975, Riegle and Meites 1976), decreased amplitude of pulsatile secretions (Dorsa et al. 1982, Miller and Riegle 1978), and changes in
structure of LH (Conn et al., 1980).

In our experiments testosterone production was initially stimulated and then depressed by in vivo chronic hCG treatment in both O and Y rats. The Y showed a pattern of early response followed by a return to basal T levels by D10. The levels of T in the O showed a similar pattern but on D1 and D2 the O had significantly less T in circulation than the Y and although there were no significant differences on D4-D10 in peripheral serum levels, by D10 the O were still at 3x their basal level (Table 1). From D2-D4 the Y showed a sharp decrease in serum T levels, with a leveling off on D4-D7 and then a decrease from D7-D10. The O showed no significant changes between D2 and D7, then from D7-D10 they showed decreases in levels of T similar to the Y (figure 10).

One interesting finding was that the D10 endogenous levels of T in O were nearly 3x the levels in the Y. Since androgen receptors have been found in Leydig cells (Isomaa et al., 1985, Gulizia et al., 1983, Verhoeven 1980) and a number of studies have suggested autoregulatory actions of androgens on testis (Adashi and Hsueh 1981, Tsai and Sanborn 1982, Damey and Ewing 1981), it appears that there may be differences in the androgen induced autoregulatory apparatus of the Y and O rats. T is an inhibitor of 17\(^\alpha\) hydroxylase (Quinn and Payne 1985) and should therefore autoregulate when high levels of T are present, but this obviously is not occurring in the O. Another question is, what is the fate of this high level of endogenous T? Endogenous E\(_2\) is not significantly higher in the O so the T is not being aromatized to E\(_2\). TV levels of T are not significantly higher in the O so it is not being secreted. Perhaps it is bound by androgen binding protein (ABP) and concentrated in the sertoli cells (Martin 1985).
Progesterone levels showed increases on D1 which then gradually declined, with the only significant difference between Y and O occurring on D7. 20α-hydroxyprogesterone showed no age related differences in serum levels, but the O levels of endogenous 20α-OHP were significantly higher than the Y. 20α-hydroxy steroids have been implicated as regulators of androgen synthesis, although they are produced in the Sertoli cells they may travel to the Leydig cells (Martin 1985) and have some local regulation effect there. Since 20α-OHP has also been reported to increase levels of ABP (Friedl 1984) it provides a means of moving to the Sertoli cells the large amounts of endogenous T found in the testes on D10 (Martin, 1985).

In vitro stimulation of testis tissue from the chronically stimulated animals caused no additional response by the 18m tissue although basally it produced more T than the Y. The 4m tissue not only had lower basal T levels when incubated with no hCG but decreased production further when incubated with 20-100 mIU hCG. This is the expected response since desensitization by definition makes the testes nonresponsive to further stimulation. These results were in contrast to Risbridger et al. (1982) who showed hyperresponsiveness to hCG stimulation in vitro after 1 day to 3 weeks of in vivo hCG treatment. However, they used much higher doses of hCG than we did both for chronically stimulating and for subsequent in vitro assays.

Risbridger et al. also demonstrated the presence of antibodies to hCG in plasma of rats treated 14 days or more days. The hCG antibodies were able to neutralize effects of exogenous hCG and return plasma T levels to normal. This may also explain why in some long term studies chronic stimulation by hCG first caused the expected desensitization and
then after the initial period of refractory caused increased maximal steroidogenic response. Long term treatment (several weeks) also results in an increased number of Leydig cells (Rommerts and Brinkiman 1980) which may account for long term increase in maximal response.

Whether the reason, antibody response or desensitization, there is a very significant difference in the in vitro response of the O and the Y testis on D10 of chronic low level hCG stimulation. Although both Y and O were non-responsive to further hCG stimulation, basally the Y were producing much less and the O much more than their normal basal (i.e. not chronically stimulated) levels, indicating some defect in the regulatory mechanism of the O.

B. Changes in Estrogens During Aging

There are conflicting reports on serum E₂ levels in aging humans, with some reporting significantly higher levels in older men (Longcope 1973, Pirke and Doerr 1975) and others reporting that E₁ and E₂ levels remain constant during aging (Davidson et al. 1983, Harmen and Tsitouras 1980). Hensell et al. (1974) reported an increase in aromatase activity in older men and an increase in the fractional conversion of circulating androstenedione to E₂. Baker et al. (1976) reported an increase in urinary E₂ due to peripheral aromatization of androstenedione.

Because of the procedural problems mentioned we were unable to draw conclusions regarding the role of E₂ receptors during aging. However, Lin et al. (1981) and Winters and Takahashi (1983) observed that there were no significant age related changes in concentration or affinity of E binding sites in rat testis or kidney.

This laboratory and others (Chan et al. 1981, Chan and McFarlin 1981, Lin et al. 1981, Winters and Takahashi 1983) have shown that serum
and testicular $E_2$ levels do not rise significantly in aged rats. Saksena and Lau (1979) observed that although $E_2$ levels remained relatively constant in rats aged 2 to 15 months, $E_1$ levels dropped somewhat, and the ratio of total T ($T + 5\text{DHT}$) to total E ($E_1 + E_2$) decreased drastically. We also found that the O had lower serum T to $E_2$ ratio than the Y both at basal levels and on D1, D2, D4, or D10 of chronic in vivo hCG stimulation (see table 3). Even though the O were secreting $E_2$ above their normal basal levels for the 10 days, only on D7 was the $T/E_2$ ratio somewhat higher in the O. On D10 TV ratios of $T/E_2$ are slightly higher in the O than the Y and endogenous $T/E_2$ ratios are 25% higher in the O than in the Y. $E_2$ has been implicated as a local modulator of steroidogenesis and desensitization. Shifting of the relative amounts of T and $E_2$ both in circulation and in the testis may be either a reflection or a cause of changing control patterns during aging. However, it appears that $E_2$, considered by itself, is not an intratesticular modulator in aging.

There is evidence for extra-testicular changes in $E_2$ action with age. The ability of the pituitary to aromatize androgens to $E_2$ indicates possible $E_2$ action beyond the testicular level (Naftolin et al. 1972, Callard et al. 1978). Haji et al. (1981) reported that $E_2$ binding to cytosol receptors in the hypothalamus and pituitary were reduced in aged male rats. This is potentially significant considering that in humans estrogens have been cited as important gonadotropin regulators through their action on the pituitary and hypothalamus (Winters 1985) and in rats they also appear to have a role in neuro-endocrine control (Henderson et al. 1979). Possibly the shift in $T/E_2$ ratios we observed has extra-testicular implications.
We showed no age-related *in vitro* changes in testicular aromatization of T to E$_2$ by dispersed Leydig cells or teased decapsulated testis tissue (see table 7a and 7b). Levels of E$_2$ in the Leydig cell incubations were much higher than those reported by Valladares and Payne, (1979a). There was also a large variation of E$_2$ produced in replicate incubations. It is likely that these E$_2$ values are inflated by contamination with $^3$H-T substrate, which is suggested by the relatively high E$_2$ recoveries (64%) compared to relatively low T recoveries (31%).

In the tissue incubations, where the E$_2$ levels were less variable and in a more believable range the recoveries of E$_2$ were more characteristic at 31% and 23% although the T recoveries were still low at 29% and 34%.

Valladares and Payne also showed significant stimulation of aromatase activity by hCG which we did not see. However, if stimulation was present in the Leydig cell incubations it was probably masked by the high H-T contamination. The tissue incubations showed no significant stimulation by hCG, but other studies (Chan *et al.* 1981, Sharpe and McNielly, 1980) have shown that intact testis and decapsulated testis tissue are less responsive to hCG stimulation than are dispersed Leydig cells, so it is possible that the conditions were not satisfactory for aromatization by the tissues.

Total substrate recovery as calculated by combining the amount of metabolized products and non metabolized [7α-$^3$H]T was 87% for the Leydig cells, 69% for tissue incubation A and 43% for tissue incubation B.

One significant difference we found was much higher levels of estrone (E$_1$) in Y rats compared to O, both before and after hCG stimulation. This is in agreement with Saksena and Lau's (1979)
previously quoted statement that total T (T + OHT) to total E (E₁ + E₂) ratios decline in aged rats. Their work also reported decreased E₁ levels in O rats. Since E₁ binds competitively with E₂ receptors but is not as biologically active as E₂, it may act as an E₂ modulator. This control may be lessened in the O rat if the ratio of E₁ to E₂ declines along with the total E, which it did in the Saksena and Lau study.

C. Age-related Changes in 7α Hydroxytestosterone and 7α Hydroxyandrostenedione

Although there have been few studies of age-related changes in the actions of 7α OH compounds, it has been shown that they are involved in the sexual maturation of rats. Lecroix et al. (1975) found 7α OHT in testis of sexually mature but not in immature rats. In fact, he showed levels of 7α OHT as high as the levels of T in the mature animals, with both T and 7α OHT showing parallel endogenous increases at about 50 days of age. As the 7α hydroxylase enzyme system gained activity there was a corresponding decrease in 5α reductase activity. Saksena and Lau (1979) showed a significant rise in serum T starting at about 42 days, which is compatible with inhibition of 5α reductase activity. Proportionately less T is converted to DHT so T levels rise.

Since 7α OHT and 7α OHΔ4D appear to be significantly involved in control of steroidogenesis during the changing patterns of sexual maturation it is logical to predict some involvement in the changing patterns of steroidogenesis associated with aging. Chan et al. 1977 isolated a significant amount of a polar compound, tentatively identified as 7α OHT, in incubations of testis of old, but not the testes of young rats.
In the present experiments we did not find elevated levels of \(7\alpha\text{OH}T\) in O rats either before or after hCG stimulation (tables 8a and 8b). One reason for this may be that even in the 4 hr tissue incubation 32% of the \([7\alpha\text{-}^{3}\text{H}]T\) substrate was not metabolized, and in the 2 hr tissue Leydig cell incubation 64% was not metabolized. A factor which may have made a difference when compared to the Chan et al. results is that in their experiment the substrate used was progesterone rather than \([7\alpha\text{-}^{3}\text{H}]T\). It is possible that using T as substrate inhibited formation of \(7\alpha\text{OH}T\) since T has been shown to inhibit \(7\alpha\text{hydroxylase activity in vivo}\) (Inano et al., 1973). Also it is possible that our recoveries were very low, since we had no means of calculating recoveries of \(7\alpha\text{ OH} metabolites.

In our work stimulation by hCG caused significant depression of conversion of T to \(7\alpha\text{OH}\Delta 4D\) by the Y, but not the O Leydig cells. There were no other age-related changes in \(7\alpha\text{OH}\Delta 4D\). Although hCG had no effect on Y or O, we did see significantly higher \(\Delta 4D\) production in the Y than in the O (table 8a).

**D. Disappearance of Testosterone from Circulation**

Aging male rats and aging men show a decreased response to gonadotropin stimulation which is reflected in lowered serum T levels in the aged. In Harmon et al. (1978) postulated that O rats when compared to Y had a similar, but slower, response to gonadotropin stimulation. His study showed that although at 1 hr post hCG injection young rats showed higher levels of serum T, by 3 hr post injection the serum T levels for young and old were similar. Another possibility proposed by our laboratory was that aged rats clear (i.e. metabolize) T from their circulation more slowly (Chan et al., 1981, 1982).
Figures 17, 18, 21a, and 21b show that after castration O rats (18-24m) clear T from their systems more slowly than Y rats whether T clearance is calculated as simple disappearance or as MCR. Figures 19, 22a, and 22b show that when castrated rats are injected with a supraphysiological dose of T the O rats are able to clear T in amounts and at rates more similar to the Y. This indicates that although the O normally clear T slower than the Y they have the ability to increase their rate of metabolism.

Figure 17 shows a peculiar feature of the clearance of T in O rats, an increase in T levels at about 45 minutes after castration, which is seen only in castrated animals, not in the castrated, then injected animals. This type of transient increase has been observed after administration of anesthetic. However, ether, which was used in our experiments, was found to show this response the least of the anesthetics tested (Free et al. 1980). It is possible, however, that it is an anesthetic related phenomenon. Possibly the adrenals react to the stress of the castration surgery by being stimulated to produce more T, but there is no obvious mechanism that would allow this to happen. Adrenal secretion of T has been documented by Bardin and Peterson (1967), who found increased concentrations of both T and Δ4D in adrenal venous blood after orchietomy. This transient increase is not seen in the Y, probably because of the relatively greater amounts being cleared. If there is such a surge it is masked by the higher levels of T.

In experiment B (figure 18) additional early sampling times show that the clearance of T in the O is a fluctuating clearance rather than the straight forward disappearance seen in the Y. This may indicate extra testicular production of T, probably of adrenal origin. The time
at which the Y had cleared 1/2 of the basal amount of T was less than 15 min in this group also, while the O still took over 1 hr to clear 1/2 of their basal levels.

Figure 19 shows the results of experiment C which follows the disappearance of 100 ug T which was injected into 4m and 18m castrates. Although the time to reach 1/2 of basal is still longer in the O (20 min vs 8 min) the difference is much less than that seen after castration. In addition to simple disappearance curves, metabolic clearance rates (MCR) were also calculated to illustrate the clearance of T in the Y and O animals in experiment C.

Our comparison of acutely castrated 4m rats and 4m castrated rats injected with supraphysiological doses of T showed an increase in disappearance rate and MCR, accompanied by a decrease t1/2α and t1/2β, occurring after injection (figures 17, 19, 21a, 22a, and tables 9a, 9b, 11). These experiments show that the Y and O testis can be stimulated to metabolize T at levels higher than they normally do. This observation is in agreement with the work of Vermeulen et al. (1969) who showed an increased MCR after injection of T into hypogonadal men, and the work of Southren et al. (1968) who demonstrated increased MCR after chronic doses of testosterone propionate. The Y and O patterns of clearance were also different (figures 19, 22a, 22b). The young showed a two compartment model of clearance and the old showed fluctuating levels of T during clearance. MCR could not be calculated for the O because of these fluctuating values.

We also compared MCR after injection of T in rats that had been castrated less than 1m previously with MCR in rats that had been castrated 12m to 14m previously. No significant differences were found
in clearance rates.

A consideration of the relationship between androgen binding and MCR is also important when studying age related changes in steroid metabolism. Vermeulen and Ando (1979) found a highly significant relationship between free T and MCR in humans.

There have been a number of reports of decrease in free T and an increase in bound T in old men (Vermeulen et al. 1969, Baker et al. 1976, Davidson et al. 1983, Pirke and Doerr 1975, Warner et al. 1984), although some recent studies have suggested that some healthy subgroups of older men may maintain a free T/bound T ratio similar to young men (Warner et al. 1984, Harmon and Tsitouras 1980). A slower MCR has been reported in old men compared to young, and a lower blood production rate (production rate = concentration of steroid x MCR) has also been reported (Vermeulen et al. 1971). This slower MCR may reflect higher levels of bound vs free T. Dunn and Clark (1982) showed that with 5 days of chronic hCG treatment, total and free T levels increased and TeBG binding capacity decreased by 32% in humans. This decrease in TeBG after hCG stimulation was attributed to an increase in systemic androgen levels which has an inhibitory effect on TeBG production.

Although rats do not have TeBG, binding of T to Alb may be a factor in steroid clearance. We observed that T injection in the Y resulted in decreased t 1/2\alpha and t 1/2\beta. Although both were significantly shortened, the t 1/2\alpha decreased by 39% and the t 1/2\beta decreased by 73%. The t 1/2\beta represents hormone not readily available for metabolism, which includes bound hormone. The greater decrease in t 1/2\beta may be indicative of low affinity binding of much of the steroid to albumin. Since this binding is non-specific and of low affinity it is easily
broken and the T is made available for metabolism. Decreased protein binding does not account for all the increased MCR after chronic T stimulation so other factors must be at work also (Mahoudeau et al. 1971).

The clearance of T from the O castrates in our experiment was not as clear cut a situation as the Y (figure 21b). When experiment B showed fluctuating clearance values for the O rats, especially a rise at 3 hrs, the values from experiment A were compared to the B values. Although more than 1/2 of the rats in experiment A did not have a sampling time of 3 hr, nevertheless, a substantial number of them did show an increase in T levels sometime between 1 and 6 hr. Measurement of T in 18m castrated animals showed a basal value of 0.154 ng/ml of serum, which may represent T produced by the adrenals. Experiment A substantiated this value (table 9a) by showing that at 5 to 6 hr post castration 0.145 ng T per ml of serum remained in the O rats and at 18 hr post castration 0.083 ng T/ml serum still remained. It is possible that the fluctuations shown by the O in clearance of T is due to adrenal production of T. When the clearance of T was followed beyond 3 hrs to 6 or more hours there was a sharp drop to the basal levels mentioned above (.083-.145 ng T per ml of serum) at about 4 hours. Adrenal secretion of T has been documented and increased concentrations of both T and Δ4D have been measured in adrenal venous blood after orchiectomy (Bardin and Peterson 1967). It appears that in the O rat about 20% of the circulating T may be of adrenal origin. Alternatively, the adrenals may be able to produce this much T after castration, at least for short periods of time (figures 17, 18, 21b).

To summarize the differences between clearance and MCR in Y and O rats: O rats clear T much differently from Y rats at normal
physiological levels with Y rats showing a typical 2 pool clearance model, while the O show a much slower, fluctuating clearance model, reflecting some extra gonadal contribution of T, possibly from the adrenals. When injected with supraphysiological doses of T, both Y and O rats show a typical 2 compartment clearance model with similar MCR's although the O still have somewhat slower t 1/2α and t 1/2β.
Table 1  Testosterone Levels After Chronic hCG Treatment

<table>
<thead>
<tr>
<th>Peripheral Serum Levels</th>
<th>Change From Basal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/ml serum</td>
</tr>
<tr>
<td></td>
<td>4m</td>
</tr>
<tr>
<td>Basal</td>
<td>1.33 ± 0.26 (7)</td>
</tr>
<tr>
<td>D1</td>
<td>4.12 ± 0.94 (7)</td>
</tr>
<tr>
<td>D2</td>
<td>10.50 ± 1.46 (7)</td>
</tr>
<tr>
<td>D4</td>
<td>5.34 ± 1.63 (7)</td>
</tr>
<tr>
<td>D7</td>
<td>4.81 ± 1.00 (7)</td>
</tr>
<tr>
<td>D10 (TP)</td>
<td>1.84 ± 0.95 (5)</td>
</tr>
<tr>
<td>TV</td>
<td>15.0 ± 3.41 (7)</td>
</tr>
<tr>
<td>Endogenous</td>
<td>403.8 ± 118.8 (7)</td>
</tr>
</tbody>
</table>

Statistical significance:  * P < 0.05; ** P < 0.02; *** P < 0.01
all values = mean ± SEM
(n) = number of animals in each group
Dn = Day of treatment
TP = Terminal Peripheral
TV = Testicular venous
<table>
<thead>
<tr>
<th>Dn</th>
<th>Peripheral Serum Levels</th>
<th>Change From Basal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg/ml serum</td>
<td>pg/ml serum</td>
</tr>
<tr>
<td></td>
<td>4m</td>
<td>18m</td>
</tr>
<tr>
<td>Basal</td>
<td>28.6 ± 5.2 (6)</td>
<td>28.6 ± 7.3 (6)</td>
</tr>
<tr>
<td>D1</td>
<td>70.0 ± 14.4 (7)</td>
<td>36.5 ± 8.3 (5)</td>
</tr>
<tr>
<td>D2</td>
<td>44.0 ± 12.1 (6) —** — 78.7 ± 13.1 (5)</td>
<td>48.1 ± 17.4</td>
</tr>
<tr>
<td>D4</td>
<td>52.7 ± 17.0 (6)</td>
<td>76.7 ± 17.4 (5)</td>
</tr>
<tr>
<td>D7</td>
<td>100.8 ± 11.5 (6)</td>
<td>83.0 ± 8.3 (6)</td>
</tr>
<tr>
<td>D10 (TP)</td>
<td>58.6 ± 6.5 (7)</td>
<td>116.4 ± 36.9 (6)</td>
</tr>
<tr>
<td>TV</td>
<td>155.0 ± 45.9 (7)</td>
<td>253.3 ± 46.3 (6)</td>
</tr>
<tr>
<td>Endogenous</td>
<td>109.6 ± 24.0 (7)</td>
<td>254.0 ± 68.2 (6)</td>
</tr>
</tbody>
</table>

Statistical significance: * P < 0.05; ** P < 0.02; *** P < 0.01

all values = mean ± SEM

(n) = numbers of animals in each group

Dn = Day of treatment

TP = Terminal Peripheral

TV = Testicular venous
Chronic Stimulation by Human Chorionic Gonadotropin

Table 3  Ratio of Testosterone to Estradiol

<table>
<thead>
<tr>
<th></th>
<th>4m</th>
<th>18m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>46</td>
<td>28</td>
</tr>
<tr>
<td>D1</td>
<td>59</td>
<td>28</td>
</tr>
<tr>
<td>D2</td>
<td>239</td>
<td>76</td>
</tr>
<tr>
<td>D4</td>
<td>101</td>
<td>61</td>
</tr>
<tr>
<td>D7</td>
<td>48</td>
<td>61</td>
</tr>
<tr>
<td>D10</td>
<td>31</td>
<td>24</td>
</tr>
<tr>
<td>TV</td>
<td>97</td>
<td>128</td>
</tr>
<tr>
<td>Endogenous</td>
<td>3684</td>
<td>4629</td>
</tr>
<tr>
<td></td>
<td>Peripheral Serum Levels</td>
<td>Change From Basal</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td></td>
<td>ng/ml serum</td>
<td>ng/ml serum</td>
</tr>
<tr>
<td></td>
<td>4m</td>
<td>18m</td>
</tr>
<tr>
<td>Basal</td>
<td>1.86 ± 0.50 (8)</td>
<td>1.47 ± 0.38 (6)</td>
</tr>
<tr>
<td>D1</td>
<td>5.02 ± 0.56 (7)</td>
<td>4.55 ± 0.99 (6)</td>
</tr>
<tr>
<td>D2</td>
<td>5.54 ± 1.05 (7)</td>
<td>5.98 ± 1.43 (6)</td>
</tr>
<tr>
<td>D4</td>
<td>3.12 ± 0.64 (7)</td>
<td>1.73 ± 0.24 (5)</td>
</tr>
<tr>
<td>D7</td>
<td>3.19 ± 0.40 (7)</td>
<td>1.80 ± 0.21 (6)</td>
</tr>
<tr>
<td>D10 (TP)</td>
<td>3.48 ± 0.31 (7)</td>
<td>2.95 ± 0.73 (6)</td>
</tr>
<tr>
<td>TV</td>
<td>4.75 ± 0.47 (7)</td>
<td>4.07 ± 0.68 (6)</td>
</tr>
<tr>
<td>Endogenous</td>
<td>28.30 ± 2.76 (7)</td>
<td>31.80 ± 1.56 (6)</td>
</tr>
</tbody>
</table>

Statistical significance: * P < 0.05; ** P < 0.02; *** P < 0.01
all values = mean ± SEM
(n) = number of animals in each group
Dn = Day of treatment
TP = Terminal Peripheral
TV = Testicular venous
Table 5  20α Hydroxyprogesterone Levels After Chronic hCG Treatment

<table>
<thead>
<tr>
<th></th>
<th>Peripheral Serum Levels</th>
<th>Change From Basal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/ml serum</td>
<td>ng/ml serum</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4m</td>
</tr>
<tr>
<td>Basal</td>
<td>0.109 ± 0.006</td>
<td>** ***</td>
</tr>
<tr>
<td>D1</td>
<td>6.40 ± 1.86 (7)</td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>9.03 ± 2.93 (7)</td>
<td></td>
</tr>
<tr>
<td>D4</td>
<td>1.47 ± 0.17 (7)</td>
<td></td>
</tr>
<tr>
<td>D7</td>
<td>1.45 ± 0.15 (7)</td>
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</tr>
<tr>
<td>D10 (TP)</td>
<td>2.20 ± 0.41 (7)</td>
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</tr>
<tr>
<td>TV</td>
<td>3.62 ± 0.23 (7)</td>
<td></td>
</tr>
<tr>
<td>Endogenous</td>
<td>3.50 ± 0.56 (7)</td>
<td>** ***</td>
</tr>
</tbody>
</table>

Statistical significance: * P < 0.05; ** P < 0.02; *** P < 0.01
all values = mean ± SEM
(n) = number of animals in each group
Dn = Day of treatment
TP = Terminal Peripheral
TV = Testicular venous
Table 6  *In Vitro* Stimulation of Testis Tissue by hCG

<table>
<thead>
<tr>
<th>mIU hCG</th>
<th>No Prior Treatment</th>
<th>Chronic <em>in vivo</em> hCG Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4m</td>
<td>18m</td>
</tr>
<tr>
<td>Basal/0</td>
<td>29.9 ± 3.2</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>20</td>
<td>35.0 ± 2.5</td>
<td>11.0 ± 2.4</td>
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<tr>
<td>50</td>
<td>36.0 ± 5.0</td>
<td>10.0 ± 2.5</td>
</tr>
<tr>
<td>100</td>
<td>48.0 ± 5.0</td>
<td>11.5 ± 1.8</td>
</tr>
</tbody>
</table>

*Statistical significance: * P \( < 0.05; ** P \( < 0.02; *** P \( < 0.01

N = 6 for each group
<table>
<thead>
<tr>
<th></th>
<th>ng/1x10⁶ Leydig cells/2 hr</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-hCG</td>
<td>+hCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4m</td>
<td>18m</td>
<td>4m</td>
</tr>
<tr>
<td>E₁</td>
<td>5.080 ± 0.500***</td>
<td>0.588 ± 0.168</td>
<td>3.867 ± 1.089*</td>
</tr>
<tr>
<td>E₂</td>
<td>24.51 ± 10.50</td>
<td>25.51 ± 18.46</td>
<td>32.10 ± 14.10</td>
</tr>
<tr>
<td>E₃</td>
<td>0.029 ± 0.009</td>
<td>0.049 ± 0.011</td>
<td>0.055 ± 0.016</td>
</tr>
</tbody>
</table>

Statistical significance: * P < 0.05; *** P < 0.02; ** P < 0.01
All values = mean ± SEM
Table 7b Aromatization of $^3$HT to $E_1$, $E_2$, $E_3$ by Testis Tissue

<table>
<thead>
<tr>
<th></th>
<th>-hCG</th>
<th>+hCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4m</td>
<td>18m</td>
</tr>
<tr>
<td><strong>Exp. A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_1$</td>
<td>0.457 ± 0.224</td>
<td>0.481 ± 0.087</td>
</tr>
<tr>
<td>$E_2$</td>
<td>1.609 ± 0.220</td>
<td>1.639 ± 0.362</td>
</tr>
<tr>
<td>$E_3$</td>
<td>0.244 ± 0.060</td>
<td>0.276 ± 0.440</td>
</tr>
<tr>
<td><strong>Exp. B</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_1$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_2$</td>
<td>1.81 ± .478</td>
<td>2.00 ± .206</td>
</tr>
<tr>
<td>$E_3$</td>
<td>.092 ± .032</td>
<td>.150 ± .031</td>
</tr>
</tbody>
</table>

Statistical significance: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$

All values = mean ± SEM
Table 8a Metabolism of $^{3}$HT to 7α-Hydroxytestosterone, 7α-Hydroxyandrostanedione, and Androstenedione by Leydig Cells

<table>
<thead>
<tr>
<th></th>
<th>-hCG</th>
<th>+hCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4m</td>
<td>18m</td>
</tr>
<tr>
<td>7α-OHT</td>
<td>0.242 ± 0.66</td>
<td>0.214 ± 0.068</td>
</tr>
<tr>
<td>7α-OH 4D</td>
<td>0.854 ± 0.111</td>
<td>0.905 ± 0.315</td>
</tr>
<tr>
<td>Δ4D</td>
<td>14.01 ± 2.60  **3.71 ± 1.01</td>
<td>14.63 ± 3.75  **3.76 ± 0.713</td>
</tr>
</tbody>
</table>

Statistical significance: * P < 0.05; ** P < 0.02; *** P < 0.01
All values = mean ± SEM
Table 8b Metabolism of $^3$H-T to 7α-hydroxytestosterone and 7α-hydroxyandrostenedione and Androstenedione by Testis Tissue

<table>
<thead>
<tr>
<th></th>
<th>-hCG</th>
<th>+hCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4m</td>
<td>18m</td>
</tr>
<tr>
<td>7αOHT</td>
<td>.456 ± .055</td>
<td>.495 ± .057</td>
</tr>
<tr>
<td>7αOHΔ4D</td>
<td>.244 ± .034</td>
<td>.255 ± .030</td>
</tr>
<tr>
<td>Δ4D</td>
<td>1.003 ± .156</td>
<td>.812 ± .238</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>4m</th>
<th>24m</th>
<th>4m</th>
<th>24m</th>
</tr>
</thead>
<tbody>
<tr>
<td>7αOHT</td>
<td>.228 ± .069</td>
<td>.243 ± .054</td>
<td>.243 ± .083</td>
<td>.273 ± .075</td>
</tr>
<tr>
<td>7αOHΔ4D</td>
<td>.137 ± .063</td>
<td>.109 ± .024</td>
<td>.100 ± .029</td>
<td>.139 ± .031</td>
</tr>
<tr>
<td>Δ4D</td>
<td>.604 ± .191</td>
<td>.604 ± .191</td>
<td>.710 ± 2.40</td>
<td>.540 ± .093</td>
</tr>
</tbody>
</table>

Statistical significance: * P < 0.05; ** P < 0.02; *** P < 0.01
All values = ± SEM
<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>5min</th>
<th>10min</th>
<th>15min</th>
<th>20min</th>
<th>30min</th>
<th>45min</th>
<th>1hr</th>
<th>2hr</th>
<th>4hr</th>
<th>5hr</th>
<th>6hr</th>
<th>8hr</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Exp A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4m 18m</td>
<td>2.75</td>
<td>± .505</td>
<td></td>
<td>1.23</td>
<td>1.04</td>
<td>± .138</td>
<td>.863</td>
<td>.706</td>
<td>.289</td>
<td>.137</td>
<td>.089</td>
<td>.106</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18m</td>
<td>.420</td>
<td>± .091</td>
<td>.245</td>
<td>.264</td>
<td>± .072</td>
<td>.326</td>
<td>.296</td>
<td>.204</td>
<td>.147</td>
<td>.143</td>
<td>.083</td>
<td></td>
</tr>
<tr>
<td><strong>Exp B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4m 18m</td>
<td>1.85</td>
<td>± .296</td>
<td>1.28</td>
<td>1.077</td>
<td>.788</td>
<td>± .134</td>
<td>.691</td>
<td>.538</td>
<td>.514</td>
<td>.352</td>
<td>.277</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18m</td>
<td>.465</td>
<td>± .203</td>
<td>.495</td>
<td>.382</td>
<td>± .229</td>
<td>.450</td>
<td>.471</td>
<td>.298</td>
<td>.217</td>
<td>.283</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All values = mean ± SEM

(n) = number of animals
Table 9b Disappearance of Testosterone after Injection of Young and Old Chronically Castrated Rats. ng T/ml serum

<table>
<thead>
<tr>
<th>min</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp C 4m (6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>243.2</td>
<td>113.9</td>
<td>68.73</td>
<td>53.17</td>
<td>36.42</td>
<td>27.73</td>
<td>9.57</td>
<td>4.36</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>± 37.6</td>
<td>± 10.9</td>
<td>± 10.2</td>
<td>± 5.86</td>
<td>± 3.65</td>
<td>± 2.51</td>
<td>± 1.14</td>
<td>± 1.51</td>
<td>± .376</td>
</tr>
<tr>
<td>18m (8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>191.6</td>
<td>119.4</td>
<td>85.6</td>
<td>77.68</td>
<td>46.39</td>
<td>39.61</td>
<td>19.73</td>
<td>12.09</td>
<td>4.61</td>
</tr>
<tr>
<td></td>
<td>± 42.5</td>
<td>± 24.4</td>
<td>± 22.6</td>
<td>± 21.4</td>
<td>± 11.5</td>
<td>± 10.4</td>
<td>± 4.87</td>
<td>± 2.95</td>
<td>± .134</td>
</tr>
</tbody>
</table>

All values = mean ± SEM
(n) = number of animals
### Table 10  MCR Values Young vs Old

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>α</th>
<th>β</th>
</tr>
</thead>
<tbody>
<tr>
<td>4m (9)</td>
<td>0.71838 ± 0.215</td>
<td>0.36751 ± 0.0285</td>
<td>-0.10517 ± 0.022</td>
<td>-0.00430 ± 0.0002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>4m (6)</td>
<td>1.23884 ± 0.255</td>
<td>0.44109 ± 0.078</td>
<td>-0.14483 ± 0.0132</td>
<td>-0.01632 ± 0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>18m (7)</td>
<td></td>
<td></td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>18m (8)</td>
<td>0.6658 ± 0.164</td>
<td>0.32315 ± 0.084</td>
<td>-0.07979 ± 0.020</td>
<td>-0.2009 ± 0.013</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A and B represent the Y intercept of the lines showing early and late times respectively, and α and β represent the slope of the lines of the early and late time clearances.

Statistical significance: * P < 0.05; ** P < 0.02; *** P < 0.01

All values = ± SEM
Table 11  Clearance of Testosterone in Young vs Old Rats

<table>
<thead>
<tr>
<th>Treatment - age</th>
<th>1/day</th>
<th>1/kg/day</th>
<th>t_{1/2} min (early)</th>
<th>t_{1/2} min (late)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4m (9)</td>
<td>15.8 ± 1.2</td>
<td>30.0 ± 5.0</td>
<td>8.2 ± 1.2</td>
<td>164.2 ± 8.6</td>
</tr>
<tr>
<td>18m (7)</td>
<td>***</td>
<td>***</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td>4m (6) inject 100 ug T</td>
<td>41.8 ± 2.8</td>
<td>90.8 ± 6.3</td>
<td>5.0 ± 0.5</td>
<td>44.9 ± 5.6</td>
</tr>
<tr>
<td>18m (8) inject 100 ug T</td>
<td>39.1 ± 7.1</td>
<td>82.7 ± 10.3</td>
<td>11.5 ± 2.2</td>
<td>92.7 ± 14.6</td>
</tr>
</tbody>
</table>

Statistical significance:  * P < 0.05;  ** P < 0.02;  *** P < 0.01
All data are expressed as mean ± SEM (n) = number of animals in each group
Figure 1  Steroid Biosynthetic Pathways - Testes

**Δ^4 Pathway**

- Cholesterol
  - Pregnenolone
    - Progesterone
      - 17α-OH progesterone
        - Δ^4-androstene-3,17,dione
          - Estrone
            - Testosterone
              - Estradiol
                - Estriol
              - 5α-dihydrotestosterone
                - 5α-androstanedione
                  - 5α-androstane-3,17β diol
                  - and
                    - Androsterone
                  - 5β-androstan-3α,17β diol

**Δ^5 Pathway**

- 17α-OH pregnenolone
  - Dehydroepiandrosterone (DHA)
    - Δ^5-androstene-3,17,dione
      - Testosterone
        - Estradiol
          - Estriol
Figure 2 Steroid Biosynthesis - Enzymes

\[ \Delta^4 \text{ pathway} \]

\[ \text{cholesterol} \]

\[ \Delta^5 \text{ pathway} \]

\[ \text{cholesterol side chain cleavage (CSCC)} \]

 pregnenolone

\[ \Delta^5_3\alpha\text{-OH steroid dehydrogenase} \]

+ \[ \Delta^4_ \text{,3-ketosteroid isomerase} \]

progesterone

\[ 17\alpha\text{-hydroxylase} \]

17\alpha\text{-OH progesterone}

\[ \text{C}_{17}, \text{C}_{20} \text{ lyase} \]

\[ \Delta^4\text{-androstene-3, 17-,dione} \]

\[ 17\beta\text{-hydroxysteroid dehydrogenase} \]

testosterone

\[ 5\alpha\text{-reductase} \]

5\alpha\text{-dihydrotestosterone (DHT)}

\[ 17\beta\text{-hydroxysteroid dehydrogenase} \]

3\beta\text{-or 3\alpha-ketoreductase} \]

5\alpha\text{-androstane-3, 17\beta diol or} 5\beta\text{-androstane-3\alpha, 17\beta diol}

\[ 17\beta\text{-hydroxysteroid dehydrogenase} \]

androsterone
PROGESTERONE

17α-OH-PROGESTERONE

Δ4-ANDROSTENE-3, 17-DIONE

TESTOSTERONE

CHOLESTEROL → PREGNENOLONE

PREGNENOLONE → PROGESTERONE

17α-OH-PROGESTERONE → 17α-OH-PREGNENOLONE

17α-OH-PREGNENOLONE → DEHYDROEPIANDROSTERONE (DHA)

DEHYDROEPIANDROSTERONE (DHA) → Δ3-ANDROSTENE-3, 17-DIONE

Δ4-ANDROSTENE-3, 17-DIONE

TESTOSTERONE

TESTICULAR STEROIDS
FIGURE 4
E<sub>2</sub> Receptor Preparation

**Rat Testes**

- **Cytosol**
  - 250 mg tissue
  - homogenize 3-4 strokes
  - dilute 1gm tissue/4ml TEMb buffer
  - centrifuge 17,000 xg 10 min at 4°C
  - centrifuge supernatant 105,000 xg 1 hr at 4°C
  - BioRad Assay
  - incubation 16-18 hr at 4°C
  - add charcoal-let set 15 min
  - centrifuge 1500 xg 10 min at 4°C
  - decant

**Leydig Cells**

- dispersed Leydig cells 2 x 10<sup>5</sup> to 1 x 10<sup>6</sup> L.C./1ml Med. 199
- incubate ½ hr at 37°C or 18 hr at 4°C
- add 1.0ml ice cold TEMb
- centrifuge 800 xg 10 min at 4°C
- wash cells 3 x with TEMb
- add 1ml ethanol to cells in each tube
- extract 10 min at 37°C
- decant ethanol into counting vial
- count
Chronic hCG Stimulation

**Inject 5 IU hCG**

- **Day 1 - Day 10**
  - Collect blood sample 1 hr post hCG on D1, D2, D4, D7 (+ basal)
  - Collect TV blood
  - Castrate
  - TP blood
  - One testis

**Extract**

- Separate E2, T, P, 20αOHP by column chromatography
- Quantify T, E2, P, 20αOHP by RIA
- Incubate 2 hr at 34 C under 95% O2, 5% CO2 with and without hCG

**Testis tissue**

- 250 mg/5ml KRB

**Extract**

- Quantify T by RIA
FIGURE 6

Aromatization and Metabolism of T

testis

250 mg tissue/5ml KRB

↓

preincubation - 30 min at 34°C under 95% O2 / 5% CO2

↓

strain out tissue

↓

resuspend tissue and incubate in 5ml KRB with 0.3 n moles 7-^3HT +/- hCG 2 hr at 34°C

↓

strain out tissue

↓

extract supernatant

↓

TLC separate E1, E2, E3, A4D, T 7ΩOHT and 7^-OH Α4D

↓

elute, dry down

↓

count

dispersed L.C. 5 x 10^5 L.C./0.5 ml Med. 199

↓

preincubation - 30 min at 34°C under 95% O2 / 5% CO2

↓

spin 800 xg 10 min at 4°C

↓

resuspend cells in 0.5 ml Medium 199 +/- hCG and incubate +7^-Ε-T 2 hr at 34°C

↓

spin 800 xg 10 min at 4°C

↓

extract supernatant 2x with 10x vol. ether
FIGURE 7

T Disappearance and MCR

Castrate → 2 wks (or more) later - inject 100 μg T via cardiac puncture

draw blood samples 5-180 min post castration/injection

draw hematocrit and replace blood with saline at each sampling

extract serum 2x with 10x vol of solvent

quantify T by RIA
figure 8
RIA STANDARD CURVE
figure 9
ELUTION PROFILE

ELUTION FRACTION (ML.)

<table>
<thead>
<tr>
<th>CPM x 10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>1</td>
</tr>
</tbody>
</table>

P, 20 α P, T, E2

2nd SYSTEM
Figure 10: Testosterone
Serum samples from D1-D10 of chronic hCG stimulation and basal sample were extracted, column separated and quantified by RIA for Testosterone. Results were calculated as ng/ml of serum.
Testosterone
figure 10

DAY

NG/ML SERUM

- YOUNG
- OLD

1 2 3 4 5 6 7 8 9 10
Figure 11: Estradiol
Serum samples from D1-D10 of chronic hCG stimulation and basal sample were extracted, column separated and quantified by RIA for Estradiol. Results were calculated as pg/ml of serum.
Figure 11

 Estradiol

 PG/ML SERUM

 DAY

 1 2 3 4 5 6 7 8 9 10

 O Y

 ** **
Figure 12: Progesterone
Serum samples from D1-D10 of chronic hCG stimulation and basal sample were extracted, column separated and quantified by RIA for Progesterone. Results were calculated as ng/ml of serum.
PROGESTERONE

Figure 12

NG/ML SERUM

DAY
Figure 13: 20α Hydroxyprogesterone
Serum samples from D1-D10 of chronic hCG stimulation and basal sample were extracted, column separated and quantified by RIA for 20 Hydroxyprogesterone. Results were calculated as ng/ml of serum.
20α HYDROXYPROGESTERONE

Figure 13

NG/ML SERUM

DAY

1 2 3 4 5 6 7 8 9 10

Y O
Figure 14: Testis tissues from rats that were chronically stimulated \textit{in vivo} were incubated with 0-100 mIU hCG. The incubates were extracted and quantified for testosterone by RIA. The results of this assay were compared with non-chronically stimulated tissue assayed previously in this laboratory.
IN VITRO STIMULATION OF IN VIVO CHRONICALLY STIMULATED TESTES

Figure 14
Figure 15a: Saturation curve of a cytosol estrogen receptor preparation showing non-specific, specific, and total binding.
Figure 15a

- Total binding
- Non-specific binding
- Specific binding

Y-axis: $3H_2E_2$ bound in fm
X-axis: $3H_2E_2$ free in pm
Figure 15b: Scatchard analysis of data from 15a showing $K_a$ and concentration of receptors.
Figure 15b

\[ n = 3.05 \text{ fm/mg protein} \]

\[ K_a = 2.54 \times 10^{10} \text{ m}^{-1} \]
ethyl ether : chloroform (4:1)

chloroform : ethanol (95:5)

chloroform : ethanol (92:8)

E₁ + T

E₂

E₄

E₅

chloroform ; ethanol (95:5)

chloroform + E₂

E₄ + E₅

extracted tissue or cell sample

TLC Chromatography Developing Systems

figure 16
Figure 17: Experiment A
Disappearance of testosterone from circulation of 4m rats (n=7) and 18m rats (n=9) after castration. Line (_ _ _) shows time at which 1/2 the basal amount remained in circulation. Results are expressed as ng/ml serum. Testosterone levels were determined by T-RIA of extracted serum samples.
Figure 17

EXP A

- 4 m N=7
- 18 m N=9

T-REMAINING NG/ML SERUM

TIME AFTER CASTRATION (hr.)
Figure 18: Experiment B
Comparision of disappearance of testosterone from circulation of 4m vs 18m rats after castration. Line (___) shows time at which 1/2 the basal amount remained in circulation. Results are expressed as ng/ml serum. Testosterone levels were determined by T-RIA of extracted serum samples.
Figure 18

EXP B

4 m – N=9
18 m – N=4

T-remaining ng/ml serum

Time after castration (min)
Figure 19: Experiment C

Disappearance of 100 ug injected testosterone in 4m vs 18m castrated male rats. Line (---) shows time at which 1/2 the basal amount remained in circulation. The rats were castrated at least 2 weeks prior to the injection. The testosterone was administered via cardiac puncture. Testosterone levels were determined by T-RIA of extracted serum samples.
T - REMAINING NG/ML SERUM

TIME AFTER INJECTION (min)

EXP C

4

N=6

N=8

figure 19
Figure 20 MCR line equations

EXP C

\[ y = A e^{-\alpha t} + B e^{-\beta t} \]

\[ y_1 = B e^{-\beta t} \]

\[ y_2 = A e^{-\alpha t} \]

FRACTION OF INJECTED DOSE/L. BLOOD

MIN. AFTER INJECTION
Figure 21a: Experiment B
Typical metabolic clearance rate (MCR) of testosterone in 4m rat after castration.
Results are expressed as fraction of basal value of testosterone/liter of whole blood. MCR is calculated as 1/d and as 1/kg/d. The clearance follows a typical 2 component or 2 pool clearance pattern and t 1/2 is given for both early and late times.
Figure 21a

\[ T \ MCR \]

4m

\[ MCR = 13.4 \ l/d \]
\[ = 34.0 \ l/kg/d \]
\[ t_{1/2}^a = 9.7 \text{ min} \]
\[ t_{1/2}^\beta = 151.7 \text{ min} \]

MIN. AFTER CASTRATION
Figure 21b: Experiment B
Typical metabolic clearance rate (MCR) of testosterone in 18m rat after castration. Results are expressed as fraction of basal value of testosterone/ liter of whole blood. MCR was not calculated because of fluctuating clearance pattern.
FIGURE 21b

18 m  n = 7

MIN. AFTER CASTRATION
Figure 22a: Experiment C
Metabolic clearance of rate (MCR) testosterone in 4m rats after castration following cardiac injection of 100 ug testosterone. Results are expressed as fraction of basal value of testosterone/ liter of whole blood. MCR is calculated as l/d and as l/kg/d. The clearance follows a typical 2 component or 2 pool clearance pattern and t 1/2 and t 1/2 are shown.
Figure 22a

T MCR

\[ 4m \]
\[ \text{MC R} = 44.7 \, l/d \]
\[ = 94.0 \, l/kg/d \]

\[ \frac{1}{\lambda_\alpha} = 4.7 \, \text{min} \]
\[ \frac{1}{\lambda_\beta} = 35.9 \, \text{min} \]
Figure 22b: Experiment C
Metabolic clearance rate (MCR) of testosterone in 18m rats after castration following cardiac injection of 100 ug testosterone. Results are expressed as fraction of basal value of testosterone/liter of whole blood. MCR is calculated as 1/d and as 1/kg/d. The clearance follows a typical 2 component or 2 pool clearance pattern and t 1/2 and t 1/2 are shown.
Figure 22b

\[ T \text{ MCR} \]

18m

\[ \text{MCR} = 57.1 \text{ l/d} \]
\[ = 87.7 \text{ l/kg/d} \]
\[ t/2_a = 10.2 \text{ min} \]
\[ t/2_{\beta} = 83.8 \text{ min} \]

MIN. AFTER INJECTION
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Chan, S.W.C., and McFarlin, S. (1981). Decreases in testosterone and
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The effect of inhibition of aromatization on secretion of gonadotrophins and testosterone in male Rhesus Monkeys, Endocrine Soc. Prog. and Abstracts, 64 Annual Meeting: 199.


Hart, R.W., and Setlow, R.B. (1974). Correlation between deoxyribonucleic acid excision repair and life span in a number of


tissue, and genotype, Lab Invest. **23**: 86-97.


Reiss, V., and Rothstein, M. (1975). Age related changes in isocitric lyase from the free living nematode,
Turbatrix aceti, J. Biol. Chem. 250: 826-830.


APPENDIX

**KHB - Krebs Hensleit Buffer pH 7.5**

Use: Tissue Incubations

- 20.0 ml 4.50% NaCl
- 0.8 ml 5.75% KCl
- 0.6 ml 6.10% CaCl
- 0.2 ml 10.55% KH₂PO₄ (anhydrous)
- 0.2 ml 19.10% MgSO₄ 7H₂O
- 5.25 ml 5.20% NaHCO₃ (make fresh each time)

Add:

- 102.95 ml distilled H₂O
- 130 ml total volume. Adjust pH if necessary with 1 N NaOH or 1 N HCl

For testicular incubations add 0.2% glucose.
Bubble with 95% - 5% O₂-CO₂ for 10 minutes before using.

**Color Developing Spray**

Use: Thin Layer Chromatography

- 95 ml glacial acetic acid
- 3 ml concentrated sulfuric acid
- 2 ml anise aldehyde

Spray onto sample standards using pressurized sprayer, develop for 10-30 min at 80°-110°C or until color is well developed. Can be stored at room temperature for several months. Discard when solution turns dark reddish brown. Very corrosive - use in hood.

**Tris - EDTA - Molybdate Buffer pH 7.4**

Use: Estrogen Receptor Experiments

- 0.01 M Tris
- 0.0015 M EDTA 4Na
- 10.00 M Molybdate

Adjust pH at 5°C.
PBS with 0.1% gelatin pH 7.4

Use: Steroid Radioimmunoassay

0.8 % Na HPO anhydrous
0.54% NaH$_2$PO$_4$•H$_2$O
0.1 % Na azide
0.9 % NaCl
0.1 % gelatin

Heat slightly and stir to dissolve.

**hydroxysteroid Dehydrogenase Stain** (modified from Weibe)

Use: Staining Dispersed Leydig Cells

3.0 mM NADH
0.5 mM NBT (nitrobluetetrazolium)
0.4 mM DHA (dehydroisoandrosterone)

Dissolve each separately in 2:8 propylene glycol/saline.
Store at 4°C

To stain cells: Dilute dispersed Leydig cells to approximate counting concentration. Use 1 part diluted cells: 1 part NBT: 1 part DHA. Incubate at 34°C for 10-15 min. Count stained cells in hemacytometer.

**Scintillation Counting Fluors:**

for aqueous samples:

8 g Omnifluor
1 l Triton X-100
2 l toluene

Stir until dissolved.
Store in the dark.

for non-aqueous samples:

4.0 g PPO
0.1 g POPOP
4.0 l toluene

Stir until dissolved.
Store in the dark.