THE EFFECTS OF AGING ON TESTICULAR FUNCTION IN THE RABBIT: A BIOCHEMICAL, HORMONAL AND SPERMATOGENIC STUDY

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CHAPTER I

INTRODUCTION

Testicular dysfunction in aging males has been the object of considerable interest. Studies have been stimulated by lowered fertility in economically important domestic animals, decreased sexual capacity in human males and the incidence in human males of prostatic cancer or benign prostatic hypertrophy with advanced age.

Studies on bulls at artificial insemination centers showed that fertility significantly declined with age (Collins, et al., 1962). It has also been amply demonstrated that both the endocrine and germinal elements of the testis exhibit decreased viability and function with advanced age in mammals (Korenchevsky, 1953; Pincus, 1961; Sniffen, 1950). Protein biosynthesis, RNA, and the RNA/DNA ratios were decreased with advanced age in rabbit testicular homogenates (Ewing, 1967). Total lipid, phospholipid, triglycerides and linoleate increased while cholesterol, palmitate, arachidonate and oleate decreased in the testis of the aged bovine (Ahluwalia and Holman, 1965, 1966). In the aging human male, testosterone production, testosterone metabolic clearance (Isurugi, 1967; Young and Kent, 1968; Kirschner and Coffman, 1968; Frick, 1969; Vermeulen, et al., 1972) and free testosterone concentration (Vermeulen, et al., 1972) decreased with advancing age. An increase in the mean plasma luteinizing hormone levels in older human males (Ryan and Fairman, 1968) has been reported.

Numerous theories have been proposed to explain the effect of aging in the intact organism. In order to assess which of these have application to age-related testis dysfunction, experiments are required in which all possible constituents are measured in the same animals.

With these thoughts in mind the present investigation was designed to study the effects of increasing chronological age on testicular metabolic, spermatogenic and endocrine function in individual animals. The major objectives of the study were to determine the effects of aging in rabbits on: 1) testis and pituitary weight; 2) sperm production and storage; 3) biosynthesis of specific chemical constituents in vitro; 4) basal levels of testosterone production and the response to a saturating level of gonadotropin in the perfused testis.

Four groups of male rabbits were maintained under identical conditions. At various ages, seven animals from each group were randomly selected. One testis was removed for in vitro perfusion to evaluate the testosterone production, with and without gonadotropic stimulation. The second testis was removed for determination of several biochemical criteria. These included metabolism of: 1) D-glucose-U-^{14}C into $^{14}CO_2$; 2) L-lysine-U-^{14}C into total protein, residual nucleoprotein, and nuclear acid soluble protein; and 3) sodium acetate-1-^{14}C into total lipid, monoglycerides, diglycerides, triglycerides, non-volatile fatty acids, sterols and sterol esters. The epididymides and perfused testes were homozenized to determine concentrations and production of sperm.

CHAPTER II

REVIEW OF LITERATURE

Introduction

The purpose of this review is to discuss some of the effects of senescence upon mammalian testes. Special emphasis is placed upon those changes which appear to influence the fertility of the organism.

Whenever possible, studies in the rabbit have been cited and compared with results found in other species. Although the number of definitive experiments on the effect of aging on testicular hormonal and metabolic changes is small, this lack has not prevented numerous speculations that decreased testosterone levels, decreased metabolism and cummulative genetic errors are responsible for loss of fertility with advancing age.

Theories on Aging

Numerous theories regarding aging have been reviewed by von Hahn (1966) and Carpenter and Loynd (1968). Basically, these fall into two groups, metabolic and chromosomal. At least two theories involve both chromosomal and metabolic factors. These are the diffusion and integration theories. The diffusion theory suggests that large complex molecules accumulate in the organism more rapidly than they can be removed (Carpenter, 1965). These large molecules could be produced by both normal and abnormal chemical reactions within the body and could explain the accumulation of lipofuscins in human myocardium (Carpenter, D. G. cited

by Carpenter and Loynd, 1968) and the accumulation of cross-linkage in rat tendons (Carpenter and Carpenter, 1966) with aging. Cross-linkage would presumably be caused by these molecules acting directly upon the free functional side groups of the protein molecules creating an irreversible bond which would block the normal function of those molecules (Carpenter and Loynd, 1968). The integration theory as proposed by Carpenter and Loynd (1968) assumes that the major cause of aging is the cross-linkage of all types of molecules, that the cross-linkage rate is roughly proportional to the metabolic rate and is dependent upon the density of chemical free radicals within the system, and that increased stress upon the organism results in biochemical changes which enhance the rate of cross-linkage. The modes by which cross-linkage affects the organism are either non-genetic or genetic. The non-genetic mode is separated further into collagenous and non-collagenous, thus cross-linkage could result in the accumulation of many different types of protein or non-protein waste product molecules. The cross-linkage of genetic material produces mutations whose actions are enhanced by immunologic reactions. The cross-linkage of collagen leads to shrinkage of tissues about the capillaries thereby decreasing blood flow and the supply of oxygen and metabolic precursors. The net result is a reduction in the metabolic efficiency required for survival due to the decreased number of molecules able to perform functions necessary for cellular growth and maintenance. This theory includes most of the other theories and has considerable value in considering the aging process of the organism.

Metabolic theories include: 1) collagen accumulation; 2) waste product accumulation; 3) rate of living based on the metabolic rate of the organism; 4) auto-immunologic response; 5) stress due to normal wear

and tear of living; and 6) cybernetic, involving loss of control over the organism by the nervous system.

The collagen theory is based upon Verzár's original experiments with rat tendons (Verzár, 1956) and has been further refined through later research (Verzár, 1957, 1963; Verzar and Meyer, 1961). Briefly, the theory holds that collagen, a fibrous protein accounting for about 40 per cent of a human's total protein, tends to increase in amount and to cross-link slowly but progressively with age. The cross-linkage causes the collagen fibers to shrink with age and to "choke-off" the surrounding tissue.

The waste product theory of aging proposes that some waste products are not excreted as rapidly as they are formed, therefore they accumulate and slowly poison the organism or otherwise interfere with its operation. Attempts to validate this experimentally were inconclusive (Carrell and Ebeling, 1923). However, it was shown that "waste product" lipofuscins accumulate with age in the myocardium (Strehler, et al., 1959) but there is no evidence to prove that it interferes with the function of the heart muscle (Carpenter and Loynd, 1968) or is involved in testicular dysfunction.

Ruebner (1908) showed a relationship between the life span and the metabolic rate in a wide variety of animals. The greater the metabolic rate, the shorter the life span. However, no consistent relationship was found in an attempt to apply this to man (Pearl, 1928). Therefore, the rate of living theory remains questionable for the organism and would be unlikely to apply to the dysfunction of the testis with aging.

Walford (1962) proposed the autoimmunologic theory of aging. This proposes that mutated cells stimulate immunologic reactions within the

organism's system which degrade and eventually destroy the organism. This concept is supported by increased incidence of immunologic diseases with age but is opposed by the fact that not all elderly people acquire immunologic diseases (Carpenter and Loynd, 1968). Evidence using an immunologic reaction suppressor in rats (Walford, 1966) implies that this theory has some validity but is only a part of the total picture. No data have been obtained to implicate this mechanism in testis dysfunction in aging males.

The stress theory was proposed by Selye (1956, 1960). This theory proposes that stress produces damage to the organism and that rest almost returns the organism to pre-stress condition. Excess stress can terminate the life of an organism and the older the organism the less stress it can withstand before it becomes ill or dies. However, the experimental data do not reveal a close correlation between stress and aging (Carpenter and Loynd, 1968) and there are no data to suggest that it is involved in testicular dysfunction due to aging.

Still (1956, 1958) proposed that the organism be treated as a cybernetic system. He proposed that aging is due to gradual loss of control over the body cells by the nervous system. Both amplification and feedback must be considered with death as a limit to the process. There appears to be some validity to this theory but it is too general in nature to apply to all ramifications of aging (Carpenter and Loynd, 1968). There is no evidence to implicate this mechanism to age related testis dysfunction.

Those theories classified as chromosomal in origin include: 1) cross-linkage of the protein and nucleic acid molecules; 2) loss of genes through chromosomal breakage and deletion through mitosis; 3) alteration

of the information content; and 4) failure of the regulating mechanism at the gene level due to gene repression by the irreversible binding of a repressor molecule to its corresponding structural gene, or to the "operator gene".

In its present form, the cross-linkage theory holds that aging is caused by monotonically increasing cross-linkage of protein and nucleic acid molecules (Bjorksten, 1968) resulting in altered behavior of the molecule. It has also been postulated that free radicals within the organism cause aging through the production of cross-linkages (Harman, 1962). Despite much evidence correlating cross-linkage and aging (Verzár, 1956; Bjorksten, et al., 1962; Alexander and Connell, 1962), and increased longevity when chemicals were administered to mice decreasing the density of free radicals (Harman, 1966), the theory does not explain the chemical and immunologic defects in aged animals. Also, there has been no proof that cross-linkage causes aging (Carpenter and Loynd, 1968).

Loss of genes through chromosomal breakage and deletion through mitosis does not appear to be as important in aging of the organism as in death of specific cells. No quantitative loss of DNA per nucleus with advancing age has been reported. Unequal mitosis can lead to loss of genetic material in one of the daughter cells but such genetic imbalance would probably lead to rapid loss of viability and early cell death (von Hahn, 1966).

Alteration of the information content of DNA can occur via mutations produced by cross-linkage (Smith and O'Leary, 1967) and dissociation (Allison, 1967), as well as, deletions and nucleotide substitutions. These lead to new or nonsense words in the genetic code providing the mechanism for somatic mutation (Curtis, 1964). Since most mutations are

harmful, the organism's DNA becomes less like the original until unable to survive. Curtis (1966b) gathered much evidence supporting this theory. For example, the number of mutations in an organism increase with age. Also, species with higher mutation rates have shorter life spans. Although the amount of somatic mutations in an organism at any given time is inadequate to explain aging and death of the organism (Curtis, 1966a), it may have application to the aging of specific cell types. Therefore, the possible role of alteration of genetic information in the Leydig cells and germinal elements in age-related testis dysfunction cannot be eliminated.

Genetic transcription is dependent upon the presence of a repressor molecule, a co-repressor molecule, inducer molecule and a regulatory gene as postulated by Jacob and Monod (1961). The key to this model of gene regulation is the repressor molecule by which the structural gene is repressed. The inducer molecule leads to inactivation of the repressor and thus to the opening of the gene for transcription. The discovery of the inhibitory activity of histones on RNA and DNA synthesis _in vitro_ (Huang and Bonner, 1962; Littau and Mirsky, 1963; Billen and Hnilica, 1964; Gurley, _et al._, 1964) has stimulated much discussion of their function as regulators of gene activity _in vivo_ (Butler, 1965).

In view of these findings, von Hahn (1966) postulated a theory of aging based on a combination of the Jacob-Monod "operon" model of gene regulation with the facts concerning histone function and the evidence brought forward on the structural and functional state of DNA and nucleoprotein in aging cells. He proposed that the age-related loss in ability of the cell to synthesize RNA and protein are the result of permanent repression of genes through the irreversible binding of the repressor to

it corresponding structural gene, or to the "operator gene", controlling an adjoining sequence of structural genes in an operon. This would result in an accumulation of blocked genes in surviving, non-mitotic cells with advancing age and a progressive loss of available genetic information in the cell. Consequently, this would result in decreased protein synthesis and eventually cell death. In support of this theory, von Hahn (1964/65a) found that the average amount of residual histone in the DNA of the thymus gland of the bovine was greater in the old than young animals. Also, DNA preparations from old animals denatured at higher temperatures than that of young animals containing comparable amounts of histone. It was later determined (von Hahn, 1964/65b) that histones prepared from the thymus of older animals contained more firmly bound DNA than histones from young animals. If testicular dysfunction due to aging is a result of some genetic failure this could be a possible mechanism.

Changes in Breeding Efficiency and Sexual Potency With Aging

Numerous studies involving several bovine breeds have been conducted on animals at artificial insemination centers and on dairy herds at universities. The basic conclusion is that decreased fertility is coincident with increased age (Dawson, 1938; Bowling, et al., 1940; Erb, et al., 1940; Hilder, et al., 1944; Tanabe and Salisbury, 1946; Becker and Dix, 1950; Collins, et al., 1962). An exception to these results was reported by McCollough, et al., (1951). Their results can probably be explained on the basis of early culling of bulls with low fertility.

Franks and Payne (1970) demonstrated that most male C57BL mice were sterile by 24 months of age. Moreover, those males still capable of

breeding showed increased time between litters sired. Sterility appeared to be due to some defect in spermatozoa.

These results seem to be similar to those found in human males. Freeman (1961) noted a sharp decrease in sexual activity in American males at age 65 with 67 percent showing impaired potency by age 70. This corresponds closely to the findings of Finkle, et al. (1959). Both reports pointed out marked exceptions to declining potency upon aging.

Decreased fertility in mammals with advanced age implies that there is some genetic or metabolic change in the germ cells. If this is the case, fetal mortality should be higher for the offspring of older fathers. This was shown to be the case in studies by Sonneborn (1960), using records on humans from the New York City Department of Vital Statistics. There was a consistently high fetal death rate for older fathers. He suggests that dominant lethal genes are concentrated in the testicular tissue in older males. In agreement with this, Bishop (1964) suggests that low fertility in aged bulls used for artificial insemination may be due to embryonic death. Fetal resorption was postulated as a means of eliminating unfit genotypes at a low biological cost. Lethal factors would include those inherited from parents, progressive mutations accumulated in the testes, and those that arise in the stored spermatozoa due to infrequent mating. These results suggest that aging results in dysfunction of mammalian testes. Next we will discuss those gross morphological changes in testes observed as a result of senescence.

Gross Changes With Aging

In keeping with one of the theories of aging, decreased DNA synthesis should be reflected in decreased protein biosynthesis thereby causing a

decrease in the size of the testes of aged animals. In support of this hypothesis, testicular atrophy with subsequent reduction in testicular weight is coincident with aging in male rats (Korenchevsky, et al., 1953). Testicular involution was found to be present by 36 months of age in male rabbits (Ewing, 1967). Hooker (1944) found a reduction in testes weight with increased age in bulls. These studies prove that testis weight declines with advanced age. In keeping with the integrated theory of aging, this testicular atrophy could possibly be related to blood flow changes coincident with aging.

Vascular Patterns and Blood Supply in the Aging Testis

Availability of blood supply is an important factor in maintenance of testicular function. Zhordania and Gotsiridze (1963) claim that surgical omentization revives the endocrinological and spermatogenic function of cryptorchid or aged genitals in the human. Sasano and Ichojo (1969) supported this concept when they showed that seminiferous tubules which were distal to the spermatic arterial blood supply, showed degenerative changes in human males after 40 years of age. Those seminiferous tubules proximal to the spermatic arterial branches were not severely affected until after 70 years of age. Basement membranes of the seminiferous tubules progressively increased in thickness in areas distal to the blood supply up to the sixth decade, after which there was no change. Thickening of the basement membrane and uniform lesions were more numerous after sixty years of age in areas proximal to the blood supply. These results suggest that aging results in changes in blood supply to the testis which may directly or indirectly cause spermatogenic degeneration in the testis.

Spermatogenic Response to Aging

Qualitative histological assessment of spermatogenesis in mammalian testes showed decreased viability and function with increased age (Sniffen, 1950; Korenchevsky, et al., 1953; Pincus, 1961). Segal and Nelson (1959) found over half of the human males examined at ages 60 to 94 years to have testes exhibiting poor to active spermatogenesis, progressive fibrosis, yet functional Leydig cells. Again there were notable exceptions. Sasano and Ichijo (1969) in histological examination of testes of human males aged 14 to 89 years, supported the above conclusions. The mean number of seminiferous tubules containing spermatids was shown to be around 90% during the third and fourth decades, then dropped to 50% in the fifth decade and finally to 10% in the ninth decade. These data prove that spermatogenesis decreased with increased age but do not attempt to correlate spermatogenic decline with testosterone levels or secretion. Likewise, there has been no quantitative estimate of total daily sperm production in aging animals.

Testicular Metabolic Response to Aging

Little is known about the effect of aging on testicular metabolism. In keeping with the hypothesis that aging involves a permanent binding of the repressor molecule to the gene, it would be logical to assume that metabolism is affected in age-related testis dysfunction. Possible sites of influence would be glucose catabolism, oxygen uptake, protein biosynthesis, nucleic acid biosynthesis, lipid patterns and biosynthesis and glycogen storage. Each of these will be examined in the following sections.

Glucose Utilization

In keeping with the gene repression theory of aging it might be expected that aging would result in some defect in testicular glucose catabolism. Numerous *in vivo* studies showed that the amount of glucose supplied to the testis is critical for maintenance of spermatogenesis (Mancine, *et al.*, 1960; Waites and Setchell, 1964; Setchell and Waites, 1964; Setchell, *et al.*, 1965). This is exemplified by experiments in which experimental hypoglycemia caused lesions in the rat testis with no observable effect on other tissues (Mancine, *et al.*, 1960).

In vitro testicular metabolism was demonstrated to be more dependent on exogenous glucose than most tissues (Tepperman, *et al.*, 1949; Davis and Morris, 1963; Ewing and VanDemark, 1963a,b; Davis and Firlit, 1965). Protein biosynthesis associated with the transition of germinal cells in somatic mitosis to the first meiotic prophase was shown to be very sensitive to addition of exogenous glucose (Davis and Firlit, 1965). Testes from senile rabbits appear to utilize glucose in *in vitro* incubation systems, as measured by glucose uptake, as efficiently as younger rabbits. Ewing (1967) found no significant difference in glucose uptake in rabbit testis slices from animals 6 to 36 months of age. However, data from *in vitro* perfusion of rabbit testes was considerably different. Glucose utilization by 36 months old *in vitro* perfused rabbit testes was significantly higher than that of animals 6 months of age. This contradiction could be reconciled if one assumed that the intact perfused organ reflected that some basic shift in intracellular regulation of glucose had occurred during aging. Since aging apparently affects vascular patterns in the testis (Sasano and Ichijo, 1969), it certainly seems logical that

if blood flow through the testis reached a critical point at which oxygen supply was limiting, oxidative phosphorylation would be reduced and glycolysis enhanced. Thus, if decreased glucose catabolism at the cellular level is involved in the aging process, it would appear to be secondary to decreased oxygen supply resulting from decreased arterial function.

Oxygen Uptake

Optimum production of ATP for use in biosynthetic processes is dependent upon an adequate supply of oxygen (Mahler and Cordes, 1966). Since oxygen tension within a tissue reflects, to a large extent, the balance between supply and demand for oxygen in that tissue, it is necessary to examine the relationship between oxygen utilization and testis function. It has been well documented by *in vivo* studies that regulation of oxygen and glucose supply to the testis is critical for maintenance of spermatogenesis (Mancine, *et al.*, 1960; Setchell and Waites, 1964; Waites and Setchell, 1964; Setchell, *et al.*, 1965). This is especially important since direct tissue measurements (Cross and Silver, 1962) and spermatic venous blood measurements (Free and VanDemark, 1968) of O_2 tension indicate the internal environment of the testis may be somewhat hypoxic. This is due to the fact that blood flow through the testis is slow compared to other organs (Setchell and Waites, 1964) and the lower scrotal temperature modifies gas tensions within the tissue (Free and VanDemark, 1968).

Increased age did not have any effect on *in vitro* oxygen uptake by rabbit testis slices (Ewing, 1967). However, *in vitro* studies may not reflect the normal requirements of the tissue but may represent the uncontrolled response of the isolated tissue to high partial pressures of O_2. Studies need to be conducted on the relationship between *in vivo*

oxygen utilization and testis dysfunction in order to determine the effects of aging upon the oxidative phosphorylation necessary for protein biosynthesis, nucleic acid biosynthesis and lipid biosynthesis.

Protein Synthesis

Gene repression by a nuclear molecule is a plausable mechanism by which testis dysfunction in senescence could occur. In keeping with this hypothesis, it is logical to assume that protein biosynthesis is reduced in the testis of aging animals. In support of this, Ewing (1967) found protein content to decrease in rabbit testicular homogenates with advanced age.

Protein biosynthesis *in vitro* is dependent upon several factors which may be involved in testicular dysfunction with advanced age. In experiments with immature rats, Means and Hall (1967) found that a single injection of FSH stimulated testicular protein biosynthesis from ^{14}C lysine, leucine or tyrosine up to 24 days of age but not in older rats. Upon hypophysectomy, FSH stimulated protein biosynthesis beginning at 18 hours after hypophysectomy and continued to do so for at least 25 days (Means and Hall, 1968a). They concluded that FSH stimulated protein biosynthesis in some or all of the cell types of the germinal epithelium regardless of whether or not spermatids were present.

Exogenous glucose did not stimulate protein biosynthesis in the testis before 28 days of age in rats after which it peaked at 40 days of age. Upon hypophysectomy, glucose stimulation of protein biosynthesis decreased gradually until no response was shown at 25 days post-hypophysectomy. Since spermatids appear by day 28 in immature rats, and disappear by day 25 after hypophysectomy in adult rats, Means and Hall

(1968a) concluded that glucose stimulation acted primarily upon spermatids. To determine a possible mechanism, these workers examined the role of glucose on stimulation of ATP production (1968b). In immature rats, neither protein biosynthesis nor ATP concentration of the testis were increased by exogenous glucose whereas in older rats both increased when the tissue was incubated under aerobic conditions. Incubation of tissue under anaerobic conditions prevented both increases of ATP concentration and protein biosynthesis when exogenous glucose was added. Their conclusion was that glucose maintained testicular levels of ATP thereby stimulating testicular protein biosynthesis *in vitro*.

It is concluded that gene repression in aging animals could cause testicular dysfunction by directly reducing protein biosynthesis or by indirectly affecting the mechanisms by which glucose, ATP or FSH influence protein biosynthesis. The influence of age-related testicular dysfunction on nucleic acids will be examined in the following section.

Nucleic Acid Synthesis

Nucleic acid synthesis is generally thought to precede the protein synthesis necessary for germ cell division. Thus it would play a role in the maintenance of full reproductive function. Gene repression at the transcription level would be sufficient to block steroidogenesis and/or spermatogenesis and thus cause testis dysfunction with aging. Recently, a new species of nuclear protein has been found to appear during spermatogenesis whose synthesis could be affected during age-related testis dysfunction. This nuclear protein was found to appear during that part of the spermatogenic cycle when chromatin was condensing in the mouse and bull (Monesi, 1965; Gledhill, *et al.*, 1966). This appears to be an

arginine-rich histone that replaces the lysine-rich histone late in spermatogenesis. This material is not protamine but could belong to a new class of basic proteins recently isolated from sea urchin spermatozoa (Paoletti and Huang, 1969). These basic proteins contain a high amount of lysine, proline and alanine similar to lysine-rich histones but exhibit a high content of arginine and have a lysine/arginine ratio similar to slightly lysine-rich histones.

Since the mechanism of chromatin formation is not clearly understood, the role of this new protein has not been fully determined. Therefore, the role of nucleoproteins in gene repression and their possible relationship to age related testicular dysfunction needs to be examined.

Lipid Patterns and Synthesis

Specific information regarding the role of lipids in the testis is scanty. Lipids are constituents of membranes, mitochondria, microsomes, nuclei and precursors of steroid hormones. They are also involved in cation transport and membrane permeability as well as storage forms of available energy (De Robertis, et al., 1965; Giese, 1968). This includes lipid constituents of the germinal elements and Leydig cells of the testis. Since lipids are important structural components and steroid precursors in the testis, it would be logical to assume that possible gene repression could affect them in age-related testicular dysfunction.

Aging was found to affect testicular lipids in several experiments. Histochemical methods, applied to testicular biopses of human males, have shown that the lipids in Leydig cells gradually increase through 18 years, then decrease gradually by age 80. Sertoli cells showed a continual and gradual increase in lipid with age (Lynch and Scott, 1950). In

conjunction with this, the stainable lipid of Leydig cells in human males increased in amount to age 35 then slowly declined (Engle, 1955). It was assumed that these lipids represented androgens or androgen precursors and mirrored androgen secretion. Support of this is seen in that Vermeulen, et al. (1972) and Kirschner and Coffman (1968) found a distinct decrease in the peripheral plasma concentration of testosterone with advanced age.

Further effects of aging on testicular lipid patterns have been found. Ahluwalia and Holman (1965, 1966) have shown that total lipid in the bovine testis gradually increased from 4 months in utero through 12 years postpartum. Phospholipids and triglycerides generally followed the same trend. Free cholesterol decreased through 12 years of age in the testis of the bovine (Ahluwalia and Holman, 1966). Gradual decrease in testicular cholesterol with aging was also found in rabbits (Ewing, 1967). It appears that cholesterol decreases when the testis has matured enough to produce spermatids and spermatozoa which can catabolize cholesterol (Johnson, 1970). However, further decrease in testicular cholesterol with senescence would imply a shift in lipid metabolism since spermatogenesis is impaired with aging.

Specific fatty acids in the testis also change with aging. Palmitate (16.0), a fatty acid associated with cholesterol decreased steadily through 12 years of age in the bovine testis. Arachidonate (20.4), which can be converted to essential linoleic acid necessary for cholesterol formation, was shown to increase through 12 months then decrease by 12 years of age in testes of the bovine (Ahluwalia and Holman, 1966). These workers also found fatty acids associated with the neutral lipid fraction to change in the testis of the aging bovine. Oleate (18.1) decreased with age and linoleate (18.2) increased with age. Since the three fatty acids

which decrease make up over 60% of the total fatty acid concentration in the normal bull testis (Holman and Hofstetter, 1965), this implies that there is a major shift in lipid metabolism coincident with aging.

Testosterone Production With Aging

The germinal epithelium is dependent upon testosterone and/or its derivatives for maintenance of spermatogenesis (Clermont and Harvey, 1967). Numerous studies showed that spermatogenesis decreased with advanced age in mammalian testes (Sniffen, 1950; Korenchevsky, et al., 1953; Segal and Nelson, 1959; Pincus, 1961). From these reports it would be logical to assume that testosterone production was also affected by aging. This has been shown to be the case in human males using a number of methods. Early work on production of hormones with aging was inconclusive due to lack of specific methods. However, Pincus and coworkers (1955) suggested that androgen production decreased with aging in the human male. Later, Segal and Nelson (1959) examined 181 human males from young adults to age 94. Close correlation was found between urinary 17-ketosteroids decrease, androgen decrease, estrogen increase, and the histology associated with advanced age. Further work established the fact that urinary androgen excretion in human males decreased with increased age (Dorfman, 1948; Brooks, 1964; Ibayashi, et al., 1964; Futterweit, et al., 1964).

With the development of gas-liquid chromatography, competitive protein binding and the double isotope derivative methods, it became feasible to measure androgen concentrations in peripheral plasma in mammals. Testosterone concentration in the peripheral plasma of human males has been found to decrease with aging in a number of studies. Kent and Acone

(1966) found males 40-49 and 80-89 years of age to have significantly lower testosterone concentrations in the peripheral plasma than 20-29 year old males. Peripheral plasma testosterone concentration, as measured by gas-liquid chromatography (Kirschner and Coffman, 1968) and competitive protein binding (Frick, 1969), was lower in human males above 55 years of age than in those 18 to 38 years of age. Further support for decreased plasma testosterone concentration with advanced age was found by Young and Kent (1968) in normal males and by Isurugi (1967) in males with benign prostatic hypertrophy. Recent studies (Vermeulen, et al., 1972) showed that plasma testosterone levels and apparent free testosterone concentration remained within the same range from adolescence until the age of 50 years then decreased rapidly. The metabolic clearance rate of testosterone also decreased in male senescence and therefore produced a significant decrease in the testosterone production rate. These changes correspond well with the fact that the mean plasma luteinizing hormone (LH) was increased in males over 50 years of age (Ryan and Fairman, 1968). In an effort to elucidate the relationship between testosterone and spermatogenic changes with aging the testis must be isolated from the extra-gonadal sites of testosterone production or utilization and pituitary hormone influence since plasma testosterone concentration does not accurately reflect testosterone secretion (Lipsett and Korenman, 1964). Although the testis produces 95% of the testosterone secreted in the normal human male (Gandy and Peterson, 1968; Paulsen, 1968) extra-gonadal sources and other factors must be considered. The adrenal cortex has the potential to produce large amounts of testosterone as shown by abnormal physiological conditions (Lloyd, et al., 1966; Segre, 1967) and in vitro incubation studies (Kase and Kowal, 1962; Ichii, et al., 1963).

Testosterone biosynthesis has also been found in the epididymis and ductus deferens of the rat (Hamilton and Fawcett, 1970), the human prostate, guinea pig kidney and rabbit skeletal muscle (Lipsett and Korenman, 1964). In addition, the liver is capable of converting other steroids to testosterone (Klempien, et al., 1961; Lipsett and Korenman, 1964). From these studies it is evident that a considerable amount of testosterone could be secreted from extra-gonadal sites however in the normal physiological condition these contribute less than 5% in man (Paulsen, 1968). In addition to extra-gonadal sources of testosterone, the rate of metabolic clearance of testosterone must be considered. The rate at which testosterone is metabolized and excreted was found to decrease with increasing age in human males (Kent and Acone, 1966; Vermeulen, et al., 1972). In view of the metabolic changes and possible alteration of testosterone production by the extra-gonadal sites it is evident that the testis must be isolated to measure the debilitating effects of aging on testosterone secretion and the ability of the testis to respond to gonadotropic hormones.

Summary and Conclusions

Research on the effects of aging on reproduction has been largely carried out on laboratory animals including the economically important domestic species. There is no question that aging has a detrimental effect on sexual activity and reproductive ability. Numerous theories have been proposed in an effort to explain the aging process for the organism. The theory proposing the most plausible mechanism for a study of testis dysfunction is that of gene repression by a nuclear molecule.

This review amply demonstrates that there is testicular atrophy

while fertility, blood supply, and spermatogenesis decrease in the testis coincident with advancing age. Likewise, protein synthesis, RNA, and RNA/DNA decrease in the testis with aging. Total lipid, phospholipid, triglyceride and linoleate increased while cholesterol, palmitate, arachidonate and oleate decreased in testes of senile animals. Testosterone production, testosterone metabolic clearance, and free plasma testosterone decreased with advancing age. These reports indicate a number of areas in which gene repression could be involved in testicular dysfunction with aging.

CHAPTER III

MATERIALS AND METHODS

Materials

Chemicals

Special chemicals and radioisotopes are listed as discussed in this chapter. All chemicals used were either reagent, analytical or spectrograde except those used for gas-liquid chromatography which were redistilled nanograde.

Animals

Sexually mature New Zealand white male rabbits, obtained from the Redwood Game Farms, Salt Lake City, Utah, were used throughout the experiments. The animals were obtained at four months of age in separate groups and at intervals allowing all experiments to be conducted in a single six month period. All animals were housed in separate stainless steel cages in air conditioned rooms with a 14:10 (L:D) photoperiod. Water was provided ad libitum and four ounces of Purina rabbit chow were available each day. All animals were treated for minor ailments by the staff veterinarian and kept sexually inactive throughout the aging period.

Methods

Collection of Organs

Two animals from the same age group were anesthetized with sodium pentobarbital. Testes to be used for *in vitro* perfusion were randomly selected and removed from both animals as previously described by VanDemark and Ewing (1963). The remaining testes and epididymides were removed and placed in ice cold 0.154M NaCl. The animals were then decapitated and the pituitary gland was removed for weight determination.

Testicular Perfusion

The perfusion technique was that described by VanDemark and Ewing (1963) with the modifications of Ewing and Eik-Nes (1966). The perfusion media was that described by Johnson and Ewing (1971).

After an initial 1/2 hour warming period, the testes were perfused for 2 hours with media containing no exogenous gonadotropic hormone (GTH). Ovine NIH-FSH-S7 (15 mμg/ml media) and ovine NIH-ICSH-S16 (30 mμg/ml media) were added after the second hour of perfusion. Recent studies show that these levels of GTH are saturating (Johnson and Ewing, 1971). Testes were perfused for four hours after addition of GTH. The venous effluent was collected at hourly intervals and assayed for testosterone. In addition, blood flow through the testis was determined at hourly intervals.

Following *in vitro* perfusion, each testis was divided into two portions and one half fixed in bouins solution for histological examination. The other half of the perfused testis was homogenized for hemacytometric quantitation of spermatids and spermatozoa.

Testosterone Determination

Testosterone present in the venous effluent of *in vitro* perfused rabbit testes was extracted, isolated and quantitated according to Brownie, *et al.* (1964) as modified by Kirschner and Coffman (1968). Authentic testosterone heptafluorobutyrate was compared to that isolated from the testicular venous effluent with a prototype of the LKB-9000 gas chromatograph-mass spectrometer (Ryhage, 1967; Waller, 1967). The mass spectra of material quantitated via GLC was similar to authentic testosterone heptafluorobutyrate. Radioactivity measurements of testosterone-1, 2-^{3}H was used to assess recovery through the testosterone assay method. This was accomplished by counting in a toluene scintillation fluid (Desjardins and Ewing, 1971) in a three channel Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3365, equipped with a ^{137}Cs Automatic External Standard device.

Incubation Tissue Preparation

One testis per rabbit was removed as described previously, placed in 0.154M NaCl on ice, weighed, the tunica removed and immersed in Krebs-Ringers bicarbonate buffer at pH 7.4 which had been gassed with 95% oxygen-5% carbon dioxide for 15 minutes. Testicular slices were prepared using a chilled Stadie-Riggs hand microtome. Glucose was added to all incubation flasks and weighed portions of testis slices were incubated on an Eberbach metabolic shaker at 37.5^{o}C for 2 hours under 95% oxygen-5% carbon dioxide.

Metabolism of Radioisotopic Precursors Into Biochemical Constituents

D-Glucose-U-^{14}C Catabolism Into $^{14}CO_2$. One hundred milligrams of testis slices were weighed and placed in the outer vessel of Warburg flasks on ice. To this were added 3.8 ml of oxygenated Krebs-Ringers bicarbonate buffer (pH 7.4), 0.1 ml of D-glucose (10.0 millimolar), and 0.1 ml of D-glucose-U-^{14}C (0.5 microcuries/3 ml media, obtained from New England Nuclear, Boston, Mass. in 90% ethanol, specific activity 14.7 mCi/mM). Each flask was aerated under 95% O_2-5% CO_2 for 15 seconds, capped with a serum stopper and incubated as described above. The reaction was stopped by placing each flask on ice and adding 0.25 ml of 5N H_2SO_4 to the incubation media with a glass tuberculin syringe equipped with a 20 gauge needle. Immediately afterward, 0.25 ml of hydroxide of hyamine was added to the center well of each flask as a $^{14}CO_2$ trapping agent (Rapkin, 1961). The flasks were returned to the shaker and gently agitated for two hours at room temperature to trap the $^{14}CO_2$. Contents of the center well were quantitatively transferred in 1.0 ml of methanol to a scintillation vial with a Pasteur pipette. Fifteen milliliters of Bray's solution (Bray, 1960) was added to each vial as a scintillation cocktail and the radioactivity was determined in a Packard Tri-Carb Scintillation Spectrometer.

A zero control flask with D-glucose-U-^{14}C was carried through all procedures for correction of the experimental values. This consisted of a flask containing approximately 100 mg of sliced testis, 3.8 ml of oxygenated Krebs-Ringers bicarbonate buffer (pH 7.4), 0.1 ml of D-glucose (10 millimolar), and 0.1 ml of D-glucose-U-^{14}C (0.5 microcuries/flask). Immediately upon addition of the radioisotope, 0.25 ml of 5 N H_2SO_4 was

added to the flask to stop the reaction. This flask was incubated, hydroxide of hyamine added, then carried through the $^{14}CO_2$ trapping procedure, transferred and counted as described for all other flasks. Any radioactive counts found in the zero control flask were subtracted from the experimental values. These values represented about two percent of the total radioactivity in the experimental tissue.

Sodium Acetate-1-^{14}C Incorporation Into Testicular Lipids. Total lipid and lipid classes were isolated from approximately 200 mg of testis slices according to the methods described by Mangold (1961). Two hundred milligrams of testis slices were incubated in a 25 ml Erlenmeyer flask with 2.7 ml of oxygenated Krebs-Ringers bicarbonate (pH 7.4), 25 microcuries/flask of sodium acetate-1-^{14}C (obtained from New England Nuclear, Boston, Mass. in 90% ethanol, specific activity 60.8 mCi/mM), 2.5 millimolar sodium acetate, and 10 millimolar glucose to make a final volume of three milliliters. All flasks were aerated for 15 seconds under 95% O_2-5% CO_2 and sealed with a serum stopper. Following incubation at 37.5°C for 2 hours, the contents of each flask were homogenized and placed in a 50 ml beaker with 35 ml chloroform:methanol (2:1, V:V), along with the washings obtained with 2 ml of 0.85% NaCl. Contents of the beaker were stirred 4-5 times over a period of 8 hours after which they were filtered through No. 1 Whatman filter paper. The residue was washed with chloroform:methanol (2:1, V:V). The beaker containing this filtrate was placed in a beaker 6-8 times larger and tap water was added to approximately 1 cm of the top of the beaker containing the filtrate. After this had cleared, tap water was added to the larger beaker until the smaller beaker containing the filtrate was flooded and well covered. Both beakers were allowed to stand 7-8 hours in an open area to enable convection

currents to carry the methanol into the aqueous phase for evaporation. As much water as possible was removed with a suction device. The lipid containing beaker was covered and placed in the freezer for 1-2 hours. Ice was removed with forceps and rinsed with ice cold chloroform. The lipid extract was filtered into large conical centrifuge tubes through funnels containing glass wool covered with 2-3 grams of anhydrous sodium sulfate. The chloroform-lipid extract was evaporated to dryness under nitrogen and the lipids were resuspended in 2.0 ml of chloroform:methanol (2:1, V:V). An aliquot (0.2 ml) was removed, placed in scintillation vials with a toluene scintillation cocktail (Desjardins and Ewing, 1971) for determination of incorporation of sodium acetate-1-^{14}C into total lipid.

Zero control samples were carried through all procedures to determine the effectiveness of the extraction technique to remove unincorporated radioisotope. These flasks contained amounts of tissue, Krebs-Ringers bicarbonate buffer, sodium acetate, glucose and sodium acetate-1-^{14}C equivalent to the experimental flasks. However, these flasks were not incubated and the contents were immediately homogenized and placed in chloroform:methanol to stop the reaction. After the extraction described above for the experimental flasks, the radioactivity was assessed and subtracted from the experimental values. These values represented less than one percent of the total radioactivity in the experimental tissue.

Lipid classes were separated from aliquots of the lipid extract via Silica gel thin-layer chromatography (TLC) using two solvent systems. The TLC plates contained aliquots of the experimental lipid suspension and a standard mixture consisting of 1 mg/ml each of cholesterol, cholesteryl acetate, 1,3-diolein, oleic acid, bovine sphingomyelin,

tripalmitin (Applied Science Lab, Inc., P.O. Box 440, State College, Pennsylvania) and monopalmitin (The Hormel Institute, Austin, Minnesota). TLC plates were developed in petroleum ether:ethyl ether:glacial acetic acid (90:10:1, V:V:V) followed by air drying and development in ethyl ether:petroleum ether:glacial acetic acid (70:30:1) to a height of 7.5 cm from the origin. Standard lipid classes were visualized by 5 minutes exposure in an iodine vapor tank and/or 30 second spray with 0.2% solution of 2', 7'-dichlorofluorescein (Eastman Kodak Chemicals, No. 373, Rochester, New York) in isopropyl alcohol to permit visibility under ultraviolet light. Experimental lanes on the plate were covered with a sheet of Saran Wrap prior to spraying. Unsprayed experimental spots were eluted with a glass wool packed Pasteur pipette under vacuum. Silica gel eluted from areas corresponding in chromatographic mobility to the standard was washed with chloroform:methanol (2:1, V:V) into a scintillation vial which was then evaporated to dryness. Fifteen milliliters of toluene scintillation cocktail (Desjardins and Ewing, 1971) were added to each vial and incorporation of sodium acetate-1-^{14}C into lipid classes was assessed.

L-Lysine-U-^{14}C Incorporation Into Testicular Total Protein. Total protein was isolated according to methods described by Ewing and coworkers (1969). Approximately 100 mg of testis tissue slices were incubated as described previously in flasks containing Krebs-Ringers bicarbonate buffer at pH 7.4, D-glucose (10 millimolar), L-lysine hydrochloride (0.10 millimolar) and L-lysine-U-^{14}C (0.25 microcuries/3 ml media obtained from New England Nuclear, Boston, Mass. in 0.01N HCl solution, specific activity 257-269 mCi/mM). Each flask was aerated for 15 seconds under 95% O_2-5% CO_2 and capped with a serum stopper. After 2 hours of incubation at

37.5°C under 95% O_2-5% CO_2, the reaction was stopped by adding 4.0 ml of 20% ice cold trichloroacetic acid (TCA) containing 0.1% of lysine hydrochloride to the incubation flask. The tissue was then homogenized in a glass homogenizer fitted with a teflon pestle and centrifuged. The precipitate was washed two times with 2.5 ml of 10% TCA-0.1% lysine hydrochloride and heated in a water bath at 90°C for 15 minutes in 2.5 ml of 10% TCA-0.1% lysine hydrochloride. The TCA precipitate was further washed one time each in 5 ml of 10% TCA-0.1% lysine hydrochloride and 5 ml of ethanol:ethyl ether (3:1, V:V). The final pellet was dissolved in 0.5 ml of 97% (W/V) formic acid allowing measurement of radioactivity of the TCA precipitable protein in Bray's solution (Bray, 1960) in a Packard Tri-Carb Scintillation Spectrometer.

Zero control samples were isolated along with the experimental group. These consisted of flasks containing amounts of testis tissue, Krebs-Ringers bicarbonate buffer at pH 7.4, D-glucose, L-lysine hydrochloride and L-lysine-U-^{14}C equivalent to the experimental flasks. Immediately after addition of the radioisotope to the zero control flask, the reaction was stopped by adding 4.0 ml of 20% ice cold TCA-0.1% of lysine hydrochloride. The contents of the flask were not incubated but immediately homogenized, centrifuged, and taken through the isolation procedure outlined for the experimental flasks above. The final pellet was dissolved and the radioactivity assessed as described for the experimental samples. Any radioactivity present in the zero control vial was subtracted from all experimental values. These values represented less than one percent of the total radioactivity in the experimental tissue.

L-Lysine-U-^{14}C Incorporation Into Testicular Nuclear Fractions. Residual nucleoprotein (RNP) and nuclear acid soluble protein (NASP) were

isolated from 1.0 g ± 20% of testis slices. Often extra tissue from randomly selected, unaged, healthy rabbit testes was added to the extraction tubes to give a total mass of 2.0g after the incubation with radioisotope was stopped. This extra tissue was added to insure sufficient mass of tissue to carry out the physical manipulations required to isolate RNP and NASP ^{14}C containing fractions. Tissue from the experimental animals was incubated in Krebs-Ringers bicarbonate buffer at pH 7.4 with 5.0 millimolar D-glucose, 0.12 millimolar lysine hydrochloride, and 0.1 microcurie/ml of L-lysine-U-^{14}C (obtained from New England Nuclear, Boston, Massachusetts in 0.01N HCl solution, specific activity 257-269 mCi/mM). Each flask contained approximately 500 mg of experimental testis slices and a final volume of 10 milliliters. Prior to incubation, all flasks were gassed for 15 seconds with 95% O_2-5% CO_2 and capped with rubber stoppers. After incubation at 37.5°C for 2 hours the reaction was stopped by placing the flasks on ice and adding lysine hydrochloride to a final concentration of 0.1 percent. Two flasks containing testis tissue from a single experimental animal were pooled and unincubated non-experimental testis tissue was added if necessary, to bring the final mass of tissue to approximately 2.0 grams in the extraction tube. The pooled materials were centrifuged at 1500 xg for 10 minutes at 4°C and washed two times in 20 volumes of 0.1% NaCl-0.1% lysine hydrochloride. Nuclear material was then isolated by a modification of the methods of Chauveau, et al. (1956) as described by Ewing and coworkers (1969). This involved resuspension of the washed tissue pellet in 10 ml of a 0.25M sucrose, 5mM mercaptoethanol, 3mM $MgCl_2$ solution and homogenization for 15 strokes in a glass tube fitted with teflon pestle, suspended in an ice bath. The homogenate was filtered through two layers of cheese cloth and

centrifuged at 1500 xg at 4°C. The pellet was then resuspended in 10 ml of 2.0 M sucrose, 5mM mercaptoethanol, 3mM $MgCl_2$ and 12.5 ml of the same solution was layered below the tissue suspension. The interface between the two phases was mixed gently and the tube was centrifuged for 2 hours at 27,000 xg at 4°C. The nuclear pellet was washed in 20 ml of 0.9% NaCl-0.1% lysine hydrochloride and centrifuged at 1500 xg for ten minutes.

Chromatin material was isolated from the nuclei via the following steps. The isotonic saline washed nuclear pellet was suspended in 20 volumes of 0.05 M Tris buffer (pH 8.0 at 4°C) and centrifuged at 10,000 xg for 15 minutes according to Bonner, et al. (1968). This pellet was centrifuged, successively, in 20 volumes of 0.005 M and 0.001 M Tris buffer (pH 8.0) at 1500 xg for 15 minutes at 4°C. This was followed by resuspension in 0.001 M Tris buffer (pH 8.0) with centrifugation at 5000 xg for 30 minutes according to Paoletti and Huang (1969). NASP fraction was extracted from the chromatin by shaking the chromatin pellet overnight at 4°C in 3.0 ml of 0.25N HCl and collected by centrifugation at 12,000 xg for 20 minutes at 4°C. The supernatant was saved and the chromatin pellet was washed again in 2.0 ml of 0.25N HCl. The pellet contained the RNP and DNA. Both HCl extracts were combined, 1 mg bovine serum albumen and 4 mg calf thymus histone were added and the NASP fraction precipitated by adding ice cold TCA to a final concentration of 20%. This was followed by centrifugation at 12,000 xg for 20 minutes at 4°C. The NASP and RNP pellets were carried through the isolation procedure described for total protein and incorporation of L-lysine-U-^{14}C into RNP and NASP fractions, suspended in 15 ml of Bray's solution (Bray, 1960), were assessed in a Packard Tri-Carb Scintillation Spectrometer.

Zero time control samples were carried through the method. Each of

two control flasks consisted of 1.0 g of testis slices, 9.4 ml of Krebs-Ringers bicarbonate buffer at pH 7.4, 5 millimolar D-glucose, 0.12 millimolar lysine hydrochloride, and 0.1 microcurie/ml of L-lysine-U-^{14}C. Immediately upon addition of the radioisotope, the two flasks were pooled and carried through the complete extraction procedure for RNP and NASP described above without incubation. After isolation of the RNP and NASP fractions from the control flask, radioactivity was assessed as described and subtracted from the experimental values. The zero control values for RNP and NASP represented about two percent of the total radioactivity in the experimental tissue.

Epididymal and Testicular Sperm Counts

One epididymis per animal was removed as described previously, trimmed and separated into two parts consisting of head-body and tail. Each part was weighed and minced in a watch glass containing 3.0 ml of saline. A Waring blender, with a micro attachment, was used to homogenize the tissue to which varying amounts of SMT solution were added (Amann and Lambiase, 1969). All epididymal tissue was homogenized for three minutes. Final volumes of homogenates and washings consisted of 150 ml for the tail and 30 ml for the head-body of the epididymis. A 10 ml aliquot of each was taken and stored for 1-24 hours at 5^{o}C. Spermatozoa concentrations were estimated in duplicate by two observers using phase contrast illumination of the spermatozoa in American Optical Spencer Bright-Line hemacytometers.

Testicular spermatozoa concentrations were determined on one-half of the perfused testes using the above method with the exception that homogenization was one and one-half minutes and the final volume of homogenate

and washings was 50 milliliters.

Total daily sperm production was estimated from testicular sperm reserves by using 3.43 days as a time divisor (Amann and Lambiase, 1969).

Pituitary Gland Weight

Each pituitary gland was removed, as described in an earlier section, and placed in a beaker on ice. After blotting and trimming, the whole gland was weighed. The anterior and posterior lobes were dissected and weighed separately.

Statistical Design and Analysis

The experiment was arranged in a completely randomized design within each age group. Analyses of variance were calculated for each criterion by the methods of Snedecor and Cochran (1967) and Duncan's New Multiple Range Test (Steele and Torrie, 1960) was used to determine the significance of individual means when significant treatment effects were observed. All statistical analyses are summarized in the Appendix.

CHAPTER IV

RESULTS

The effects of aging on spermatogenesis, steroidogenesis and the biosynthetic capacity of the testis in male rabbits 6, 12, 24, and 36 months of age were studied.

The specific objectives of the study were to determine the effects of aging in rabbits on: 1) body, testis, and pituitary gland weights; 2) testicular and epididymal spermatid and spermatozoa concentrations; 3) total daily sperm production of the testis; 4) metabolism of radioisotopic precursors into testicular $^{14}CO_2$, total protein, residual nucleoprotein (RNP), nuclear acid soluble protein (NASP), lipid and lipid classes; and 5) the testosterone secretion rate in perfused testes.

Characterization of *in Vitro* Incubation Systems for Metabolism of Radioisotopic Precursors Into Testicular $^{14}CO_2$, Total Protein, Residual Nucleoprotein (RNP), Nuclear Acid Soluble Protein (NASP), and Lipids in Rabbits

Preliminary experiments were carried out in rabbit testis slices to characterize the incubation systems in which metabolism of D-glucose-U-^{14}C into $^{14}CO_2$, sodium acetate-1-^{14}C into lipids, and L-lysine-U-^{14}C

into total protein, RNP and NASP. Experiments were conducted to determine the concentration of each radioisotopic precursor necessary to saturate the exogenous tissue pools and thus to obtain an accurate estimate of the radioisotopic metabolism due to treatment. These included the concentration necessary for metabolism of: 1) D-glucose-U-^{14}C into $^{14}CO_2$ in the presence of exogenous glucose; 2) sodium acetate-1-^{14}C into total lipid in the presence and absence of exogenous glucose; 3) L-lysine-U-^{14}C into RNP and NASP with exogenous glucose; and 4) L-lysine-U-^{14}C into total protein in the presence and absence of exogenous glucose. After the optimum concentration for metabolism of each isotopic precursor into its biochemical constituent was determined, studies were performed to determine the incubation time length necessary to obtain maximum metabolism of each radioisotope. Each experiment will be described in detail.

Determination of Concentration Necessary to Obtain Optimum Metabolism of Precursors Into Rabbit Testicular $^{14}CO_2$, Total Protein, Total Lipid, RNP and NASP

Catabolism of D-Glucose-U-^{14}C Into Testicular $^{14}CO_2$. Experiments were conducted to determine the concentration of glucose necessary to obtain optimum catabolism under physiological conditions into testicular $^{14}CO_2$. This incubation system consisted of 200mg $\pm$ 10% of rabbit testis slices, sufficient aerated Krebs-Ringers bicarbonate buffer at pH 7.4 to give a final volume of 3 milliliters, and increasing concentrations of glucose. The glucose solution added was a mixture of D-glucose and D-glucose-U-^{14}C (specific activity 37 dpm/pM). The initial flask was a 1.25 millimolar concentration of the above mixture and volumes were

doubled in subsequent flasks until a final 20 millimolar concentration was reached (Table I). In all flasks the specific activity was held constant. Incubation was carried out under 95% O_2-5% CO_2 at 37.5°C on an Eberbach metabolic shaker. Trapping of $^{14}CO_2$ and assessment of radioactivity were carried out as described previously. Catabolism of glucose into testicular $^{14}CO_2$ increased with increasing concentrations of glucose and was found to be saturating at 5 millimolar. No further catabolism of D-glucose-U-^{14}C into $^{14}CO_2$ was found with increased glucose concentrations (Table I).

TABLE I

THE EFFECT OF FINAL D-GLUCOSE CONCENTRATION ON THE CATABOLISM OF D-GLUCOSE-U-^{14}C INTO RABBIT TESTICULAR $^{14}CO_2$ IN VITRO[a]

Replicate	Glucose Concentration (millimolar)				
	1.25	2.5	5.0	10.0	20.0
1	2,664[b]	4,058	5,273	5,626	5,545
2	3,587	3,109	4,660	4,534	4,784
Mean	3,126	3,583	4,966	5,080	5,165

[a]Incubation was performed at 37.5°C for 2 hours in Krebs-Ringers bicarbonate buffer at pH 7.4. The specific activity of D-glucose-U-^{14}C was 37.0 dpm/pM.

[b]Results are expressed in cpm/100 mg wet weight of tissue equivalent/2 hours.

Incorporation of L-Lysine-U-^{14}C Into Testicular Total Protein. Experiments were conducted to determine the concentration of lysine necessary to obtain optimum incorporation into testicular total protein. The basic incubation system consisted of 100mg ± 10% of rabbit testis slices and sufficient aerated Krebs-Ringers bicarbonate buffer at pH 7.4 to give a final volume of 3 ml after other constituents were added. One series was conducted with exogenous glucose (10 millimolar). Lysine (specific activity of 1850 dpm/pM) was increased in 0.016 millimolar amounts to a final 0.10 millimolar concentration (Table II). All incubations were carried out under 95% O_2-5% CO_2 at 37.5°C in an Eberbach metabolic shaker for 2 hours. Total protein was isolated and radioactivity assessed by methods previously described. Incorporation of L-lysine-U-^{14}C into total protein increased with increasing lysine concentrations through 0.05 millimolar in both the presence and absence of exogenous glucose. Increased lysine concentrations in excess of 0.05 millimolar did not increase L-lysine-U-^{14}C incorporation in either system. Incorporation of lysine into total protein was determined to be optimal in the presence of exogenous glucose (Table II).

TABLE II

THE EFFECT OF FINAL L-LYSINE CONCENTRATION AND PRESENCE OR ABSENCE OF GLUCOSE ON THE IN VITRO INCORPORATION OF L-LYSINE-U-^{14}C INTO TESTICULAR TOTAL PROTEIN[a]

Experimental Conditions	Lysine Concentration (millimolar)					
	0.016	0.033	0.050	0.066	0.083	0.100
Minus Exogenous Glucose	5,793[b]	8,977	10,479	12,228	13,509	10,869
Plus Exogenous Glucose[c]	7,617	12,217	16,739	18,042	16,265	20,075

[a]Incubations were performed at 37.5°C for 2 hours in Krebs-Ringers bicarbonate buffer pH 7.4. The specific activity of L-lysine-U-^{14}C was 1850 dpm/pM.

[b]Results are expressed as cpm/100 mg wet weight of tissue equivalent/2 hours.

[c]The exogenous glucose concentration was 10 millimolar.

Incorporation of L-Lysine-U-^{14}C Into Testicular RNP and NASP. Experiments were conducted to determine the concentration of lysine necessary to obtain optimum incorporation into testicular RNP and NASP. This system consisted of four 125 ml Erlenmeyer flasks with each flask containing 500 mg $\pm$ 10% of rabbit testis slices, glucose (5 millimolar), and sufficient aerated Krebs-Ringers bicarbonate buffer (pH 7.4) to give a final volume of 10 ml after addition of lysine. Increasing volumes of lysine were added to give concentrations from 0.01 to 0.2 millimolar (Table III). In all flasks, the specific activity was 1850 dpm/pM. After incubation of all flasks at 37.5°C for 2 hours under 95% O_2-5% CO_2 in an Eberbach metabolic shaker, the contents of the four corresponding flasks were pooled. RNP and NASP were isolated and radioactivity was

assessed according to methods described previously. Incorporation of lysine into RNP and NASP increased with increased lysine up to 0.12 millimolar concentration then plateaued (Table III). It was concluded that 0.12 millimolar concentration would be a saturating level for use in subsequent experiments.

TABLE III

THE EFFECT OF FINAL L-LYSINE CONCENTRATION ON THE IN VITRO INCORPORATION OF L-LYSINE-U-^{14}C INTO SEVERAL TESTICULAR PROTEIN FRACTIONS[a]

Testicular Protein Fraction	Lysine Concentration (millimolar)				
	0.01	0.03	0.06	0.12	0.20
Residual Nucleoprotein	28[b]	92	116	199	174
Nuclear Acid Soluble Protein	42	69	102	236	248

[a]Incubations were performed at 37.5°C for 2 hours in Krebs-Ringers bicarbonate buffer pH 7.4. The final glucose concentration was 5 millimolar. The specific activity of L-lysine-U-^{14}C was 1850 dpm/pM.

[b]Results are expressed as cpm/100 mg wet weight of tissue equivalent/2 hours.

Incorporation of Sodium Acetate-1-^{14}C Into Testicular Total Lipid. Experiments were conducted to determine the concentration of sodium acetate necessary to obtain optimum incorporation of sodium acetate-1-^{14}C into testicular total lipid. The basic system consisted of 250 mg ± 10% of rabbit testis slices and sufficient aerated Krebs-Ringers bicarbonate buffer at pH 7.4 to give a final volume of 3 ml after other constituents

were added. One system contained 10 millimolar exogenous glucose and the other contained no glucose. Both systems contained acetate-1-^{14}C with a specific activity of 7397 dpm/pM. Acetate was increased from 0.5 to 3.0 millimolar concentration (Table IV). Incubation was carried out under 95% O_2-5% CO_2 at 37.5°C for 2 hours in an Eberbach metabolic shaker. Extraction and assessment of radioactivity of the total lipid fraction was accomplished by the methods previously described. Incorporation of acetate-1-^{14}C into total lipid, with and without exogenous glucose, increased with increasing acetate concentration. Optimum incorporation occurred at 2.0 millimolar concentration.

TABLE IV

THE EFFECT OF FINAL SODIUM ACETATE CONCENTRATION AND PRESENCE OR ABSENCE OF GLUCOSE ON THE IN VITRO INCORPORATION OF SODIUM ACETATE-1-^{14}C INTO TESTICULAR TOTAL LIPID FRACTION[a]

Experimental Conditions	Sodium Acetate Concentration (millimolar)				
	0.5	1.0	2.0	2.5	3.0
Minus Exogenous Glucose	12,044[b]	7,883	19,401	14,014	9,574
Plus Exogenous Glucose[c]	61,606	107,084	128,805	105,708	96,405

[a]Incubation was performed at 37.5°C in Krebs-Ringers bicarbonate buffer at pH 7.4. The specific activity of sodium acetate-1-^{14}C was 7397 dpm/pM.

[b]Results are expressed in cpm/100 mg wet weight of tissue equivalent/2 hours.

[c]Exogenous glucose concentration was 10 millimolar.

In conclusion, it was determined that metabolism of radioisotopic precursors into $^{14}CO_2$, total protein, RNP, NASP, and total lipid proceeded optimally in the presence of exogenous glucose. Levels of glucose above 0.5 millimolar (specific activity of 37 dpm/pM) were found to be saturating for catabolism of D-glucose-U-^{14}C into testicular $^{14}CO_2$. Incorporation of L-lysine-U-^{14}C (specific activity of 1850 dpm/pM) into testicular total protein was found to plateau at 0.05 millimolar concentration. The saturating level of L-lysine-U-^{14}C (specific activity of 1850 dpm/pM) for incorporation into testicular RNP and NASP was found to be 0.12 millimolar. Two millimolar sodium acetate-1-^{14}C (specific activity of 7397 dpm/pM) was determined to be optimal for incorporation of acetate into testicular total lipid.

The Effect of Incubation Time in Vitro on the Metabolism of Radioisotopic Precursors Into Testicular Constituents

Time course studies were necessary to determine that metabolism of each radioisotopic precursor was constant with time and to determine the length of time required to obtain metabolism of adequate amounts of radioisotopic precursors into testicular constituents necessary to obtain measurable radioactivity in each biochemical fraction. The effect of time on the metabolism of D-glucose-U-^{14}C into $^{14}CO_2$, sodium acetate-1-^{14}C into total lipid, and L-lysine-U-^{14}C into total protein, RNP and NASP are summarized in Table V. Incorporation of radioisotopic precursors into total protein and total lipid were carried out in the presence and absence of exogenous glucose (10 millimolar) over time periods of 30 to 120 minutes. Incorporation of L-lysine-U-^{14}C into RNP and NASP, and catabolism of D-glucose-U-^{14}C into $^{14}CO_2$, were carried

out over time periods of 30 to 120 minutes only in the presence of exogenous glucose (5.0 and 10 millimolar, respectively).

TABLE V

CHANGES IN THE METABOLISM OF D-GLUCOSE-U-^{14}C INTO $^{14}CO_2$, SODIUM ACETATE-1-^{14}C INTO TOTAL LIPID AND L-LYSINE-U-^{14}C INTO TOTAL PROTEIN, NUCLEAR ACID SOLUBLE PROTEIN AND RESIDUAL NUCLEAR PROTEIN WITH CHANGE IN INCUBATION TIME IN ADULT RABBIT TESTICULAR TISSUE IN THE PRESENCE AND ABSENCE OF GLUCOSE[a]

Testicular Fraction	Minus Exogenous Glucose			Plus Exogenous Glucose		
	Incubation Length (Minutes)			Incubation Length (Minutes)		
	30	60	120	30	60	120
$^{14}CO_2$[b]	-----	-----	-----	877	2,889	5,075
Total Protein[b]	2,391	4,019	9,251	1,198	7,730	15,512
Residual Nuclear Protein	-----	-----	-----	70	123	244
Nuclear Acid Soluble Protein	-----	-----	-----	74	130	313
Total Lipid	5,881	5,238	12,636	5,312[b]	12,124[b]	44,778[b]

[a]Incubations were performed at 37.5°C for 30-120 minutes in Krebs-Ringers bicarbonate buffer at pH 7.4. The specific activity of D-glucose-U-^{14}C was 37 dpm/pM, L-lysine-U-^{14}C was 1850 dpm/pM, and sodium acetate-1-^{14}C was 7,397 dpm/pM. Results are expressed in cpm/100 mg wet weight of tissue equivalent/2 hours. The exogenous glucose concentration was 10 millimolar for $^{14}CO_2$, total protein and total lipid and 4.0 millimolar for RNP and NASP.

[b]Average of 2 incubations.

The Effect of Time on the Catabolism of D-Glucose-U-^{14}C Into Testicular $^{14}CO_2$. The incubation system for catabolism of D-glucose-U-^{14}C into $^{14}CO_2$ consisted of 200 mg ± 10% of rabbit testis slices, 10 millimolar D-glucose-U-^{14}C (specific activity of 37 dpm/pM), and aerated Krebs-Ringers bicarbonate buffer (pH 7.4) to give a final volume of 3.0 milliliters. Each flask was incubated at 37.5°C under 95% O_2-5% CO_2 on an Eberbach metabolic shaker for 30, 60, or 120 minutes. The $^{14}CO_2$ was trapped, quantitatively transferred and the radioactivity assessed as described previously. Catabolism of D-glucose-U-^{14}C into $^{14}CO_2$ with time, in the presence of exogenous glucose, was found to increase linearly with maximum catabolism at 2 hours (Table V).

The Effect of Time on the Incorporation of Sodium Acetate-1-^{14}C Into Testicular Total Lipid. The incubation system for incorporation of sodium acetate-1-^{14}C into total lipid consisted of 250 mg ± 10% of rabbit testis slices, 2.5 millimolar sodium acetate-1-^{14}C (specific activity of 7,397 dpm/pM), and sufficient aerated Krebs-Ringers bicarbonate buffer (pH 7.4) to give a final volume of 3.0 milliliters. Ten millimolar D-glucose was added to flasks in experiments involving the presence of exogenous glucose. Each flask was incubated at 37.5°C under 95% O_2-5% CO_2 on an Eberbach metabolic shaker for 30, 60, and 120 minutes. Total lipid was extracted and radioactivity assessed as described previously. Incorporation of radioactive acetate into total lipid was found to increase slightly with time in the absence of exogenous glucose. In the presence of exogenous glucose, incorporation of acetate into total lipid was 3.5 times higher after 120 minutes than when glucose was absent. The optimum length of time for incorporation of radioactive acetate into total lipid was determined to be 2 hours (Table V).

The Effect of Time on the Incorporation of L-Lysine-U-^{14}C Into Testicular Total Protein. The incubation system for incorporation of L-lysine-U-^{14}C into total protein consisted of 100 mg ± 10% of rabbit testis slices, 0.083 millimolar L-lysine-U-^{14}C (specific activity of 1850 dpm/pM) and sufficient aerated Krebs-Ringers bicarbonate buffer (pH 7.4) to give a final volume of 3.0 milliliters. Ten millimolar glucose was added in exper-ments involving exogenous glucose. Each flask was incubated at 37.5°C under 95% O_2-5% CO_2 in an Eberbach metabolic shaker for 30, 60 or 120 minutes. Total protein was isolated and radioactivity assessed as described previously. Optimum incorporation occurred after 120 minutes of incubation with exogenous glucose. Incorporation of lysine into total protein increased with time both with and without exogenous glucose. However, the incorporation of L-lysine-U-^{14}C into total protein in the presence of exogenous glucose was approximately two times greater than in the absence of glucose (Table V).

The Effect of Time on the Incorporation of L-Lysine-U-^{14}C Into Testicular RNP and NASP. The incubation system for incorporation of L-lysine-U-^{14}C into testicular RNP and NASP consisted of four 125 ml Erlenmeyer flasks. Each flask contained 500 mg ± 10% of rabbit testis slices, L-lysine-U-^{14}C (0.1 millimolar with a specific activity of 1850 dpm/pM), glucose (5.0 millimolar), and sufficient aerated Krebs-Ringers bicarbonate buffer (pH 7.4) to give a final volume of 10 milliliters. No experiments were conducted without exogenous glucose. All flasks were incubated at 37.5°C under 95% O_2-5% CO_2 in an Eberbach metabolic shaker for 30, 60 and 120 minutes. At the termination of the incubation, the contents of all corresponding flasks were pooled, RNP and NASP were isolated, and the radioactivity assessed as previously described.

Incorporation of lysine into RNP and NASP were both linear with time with the maximum incorporation occuring after 120 minutes (Table V).

It was concluded from the time course studies that metabolism of D-glucose-U-^{14}C into $^{14}CO_2$, sodium acetate-1-^{14}C into total lipid and L-lysine-U-^{14}C into total protein, RNP and NASP in rabbit testis slices was linear in the presence of glucose through a two hour incubation period.

The Effect of Aging on Body Weight, Testis Weight, and Pituitary Gland Weight in Male Rabbits

Results in Table VI show the effects of aging on body weight, testis weight and pituitary gland weight in male rabbits. Body weight increased through 24 months and declined thereafter.

Body weight of the 3 year old animals was significantly higher ($P < 0.05$) than that of the 6 month old group. The 6-month old animals were significantly ($P < 0.05$) lower than the 12 and 24-month old groups and the 12-month old rabbits were significantly lower ($P < 0.05$) than the 24-month old animals. The 36-month old animals were not significantly ($P > 0.25$) lower than the 12 or 24-month old animals.

Total paired testes weight in rabbits increased with age through 24 months and decreased by 36 months of age. The 24-month old rabbits had testes significantly larger ($P < 0.05$) than the 6-month old rabbits but the testes of 36-month old animals were not significantly larger than 6-month old rabbits.

Total pituitary gland weight in rabbits increased gradually through 24 months, then decreased dramatically by 36 months of age. Total

pituitary gland weight in 36-month old animals was significantly lower ($P < 0.01$) than that of 6, 12 and 24-month old animals. No differences ($P > 0.25$) were found in total pituitary gland weights in animals between the ages of 6 and 24 months.

TABLE VI

THE EFFECT OF AGING ON THE MEAN BODY WEIGHT, TESTIS WEIGHT, AND PITUITARY GLAND WEIGHT IN RABBITS

	Age in Months			
	6	12	24	36
Body Weight (Kg)*	3.52 ± 0.05[a]	3.93 ± 0.14	4.31 ± 0.14	4.05 ± 0.13
Total Paired Testes Weight (g)*	4.12 ± 0.10	4.90 ± 0.11	5.57 ± 0.10	4.51 ± 0.70
Testes Weight, g/Body Weight, kg	1.17 ± 0.08	1.26 ± 0.11	1.30 ± 0.08	1.13 ± 0.15
Pituitary Gland Weight (mg)**	28.0 ± 1.5	28.2 ± 0.9	28.9 ± 1.1	23.1 ± 1.2
Anterior Pituitary Gland Weight (mg)	19.6 ± 1.4	19.0 ± 0.9	22.5 ± 1.0	19.5 ± 1.4
Posterior Pituitary Gland Weight (mg)**	9.0 ± 0.5	9.2 ± 0.7	6.4 ± 0.4	4.2 ± 0.1

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

*Analysis of Variance shows significant differences at the 0.05 level.

**Analysis of Variance shows significant differences at the 0.01 level.

Anterior pituitary gland weight appeared to increase through 24 months then decrease by 36 months of age in male rabbits. However, no significant ($P > 0.25$) differences were found between any of the age groups. Posterior pituitary gland weight increased slightly through 12 months then decreased 50 percent by 36 months. The 6 and 12-month old animals had significantly higher ($P < 0.01$) posterior pituitary gland weights than the 24 or 36-month old rabbits. In addition, the mean posterior pituitary gland weight of the 36-month old animals was significantly lower ($P < 0.01$) than that of the 24-month old animals.

In summary, body weight and total paired testes weight increased with age through 24 months then decreased in older animals. Total pituitary gland weight increased gradually through 24 months then significantly decreased by 36 months of age; anterior pituitary gland weight did not significantly ($P > 0.25$) change with age but posterior pituitary gland weight decreased after 12 months of age in male rabbits. These results coupled with the fact that 30 percent of the 36 month old group had died prior to the experiment suggest that the rabbits were suffering the ravages of aging by 36 months.

The Effect of Aging on Total Daily Sperm Production, Testicular and Epididymal Spermatozoan and Spermatid Numbers in Male Rabbits

Data are shown in Table VII for the effects of aging on the number of spermatids and spermatozoa present in the testis and epididymides. Total spermatids and spermatozoa per testes pair increased through 24 months, then decreased dramatically by 36 months in rabbits. Six and 36

month old rabbits were significantly lower ($P < 0.05$) than the 24-month old rabbits. The 36-month old animals were not significantly ($P > 0.25$) lower than the 6 or 12-month old groups. The total concentration of spermatids and spermatozoa per testes pair is directly dependent upon their concentration per gram of testis tissue and the total daily sperm production. Therefore, these were determined.

TABLE VII

THE EFFECT OF AGING ON THE MEAN NUMBER OF SPERM ($X\ 10^6$) RECOVERED FROM RABBIT TESTES AND EPIDIDYMIDES

Number Spermatids and Spermatozoa	Age in Months			
	6	12	24	36
Total/Paired Testes*	355 ± 11[a]	582 ± 76	743 ± 77	397 ± 41
Total/Gram of Testis**	86 ± 6	114 ± 15	134 ± 13	88 ± 8
Total/Head and Body of the Epididymis**	61 ± 20	134 ± 21	155 ± 20	48 ± 11
Total/Tail of the Epididymis*	227 ± 28	696 ± 42	527 ± 63	268 ± 111
Total Daily Sperm Production/Rabbit*	103 ± 10	170 ± 22	217 ± 20	116 ± 18

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

*Significant at the 0.05 level.

**Significant at the 0.01 level.

Spermatids and spermatozoa per gram of testis increased through 24 months and declined by 36 months of age in rabbits (Table VII). The 6, 12 and 36-month old animals were all significantly lower ($P < 0.01$) than the 24-month old group. Six and 36-month old animals were significantly lower ($P < 0.01$) than the 12-month old group but were not significantly ($P > 0.25$) different from each other.

Total daily sperm production (TDSP) in male rabbits increased linearly through 24 months then decreased dramatically by 36 months of age (Table VII). Again, the TDSP in 6 and 36-month old animals were not significantly ($P > 0.25$) different from each other but both were significantly lower ($P < 0.05$) than the 24-month old rabbits.

Total numbers of spermatids and spermatozoa in the head-body of the epididymis of rabbits increased through 24 months then decreased by 36 months (Table VII). Six and 36-month old animals were significantly lower ($P < 0.01$) than the 12 and 24-month old groups but were not significantly ($P > 0.25$) different from each other.

Total numbers of spermatids and spermatozoa in the tail of the epididymis of rabbits increased through 12 months then decreased linearly by 36 months of age (Table VII). Six and 36-month old animals were significantly lower ($P < 0.05$) than 12-month old groups but not significantly ($P > 0.25$) different from each other. There was no significant ($P > 0.25$) difference between the 36 and 24-month old rabbits.

In summary, total spermatids and spermatozoa per testes pair and per gram of testis tissue increased through 24 months then decreased by 36 months of age. Total daily sperm production and spermatids-spermatozoa in the head-body of the epididymis increased through 24 months then decreased by 36 months of age. Spermatids and spermatozoa in the tail of

the epididymis increased through 12 months then decreased by 36 months of age in rabbits. Since the production and excretion of spermatozoa depend on glucose catabolism for energy to biosynthesize protein and lipids it seemed appropriate to measure the incorporation of radioactive precursors into specific testicular constituents.

The Effect of Aging on Metabolism of Radioisotopic Precursors Into Testicular Constituents in Rabbits

In Vitro Catabolism of D-Glucose-U-^{14}C Into Testicular $^{14}CO_2$

Production of CO_2 is a direct measure of intracellular oxidative metabolism necessary for efficient energy production. Glucose is the primary substrate for the oxidative metabolic process in testicular tissue (Dickens and Greville, 1933; Ewing and VanDemark, 1963a,b; Means and Hall, 1968b). Therefore an assessment of the catabolism of D-glucose-U-^{14}C into testicular $^{14}CO_2$ is one method of determining the effect of aging on oxidative metabolism. This is especially important in light of the evidence that glucose availability is critical for the maintenance of spermatogenesis (Mancine, et al., 1960; Waites and Setchell, 1964; Setchell and Waites, 1964; Setchell, et al., 1965).

Catabolism of D-glucose-U-^{14}C into $^{14}CO_2$, in the presence of a saturating concentration of exogenous glucose in testis slices of aging rabbits is summarized in Table VIII. All age groups produced approximately equal amounts of $^{14}CO_2$ and no significant ($P > 0.25$) differences were found. These results indicate that rabbit testis tissue from senile animals is capable of normal levels of oxidative metabolism, provided

sufficient glucose and oxygen are available.

TABLE VIII

THE EFFECT OF AGING ON IN VITRO CATABOLISM OF D-GLUCOSE-^{14}C INTO RABBIT TESTICULAR $^{14}CO_2$ IN THE PRESENCE OF GLUCOSE[a]

	Age in Months			
	6	12	24	36
Mean	7,823	7,085	8,365	7,492
Standard Error	± 1,224	± 1,446	± 1,994	± 1,090

[a]Each value is expressed in cpm/100 mg wet weight of tissue equivalent/2 hours incubation. The exogenous glucose concentration was 10 millimolar. The specific activity of glucose was 37 dpm/pM.

In Vitro Incorporation of L-Lysine-U-^{14}C Into Testicular Protein Fractions

Incorporation of L-lysine-U-^{14}C into total protein in rabbit testis slices increased through 12 months then decreased slowly by 36 months of age (Table IX). No significant ($P > 0.25$) difference between age groups was shown due to variation between animals. These results suggest that there is some relationship between protein synthesis and spermatogenesis in the testis of aging rabbits.

Incorporation of L-lysine-U-^{14}C into testicular residual nucleoprotein (RNP) in the presence of exogenous glucose and saturating levels of lysine hydrochloride, increased almost linearly through 24 months of age, then decreased by 36 months (Table IX). The 24 month old group was

significantly higher ($P < 0.01$) than the 6-month old group. These data imply that there is a direct relationship between RNP biosynthesis and spermatogenesis in aging rabbit testes.

TABLE IX

THE EFFECT OF AGING ON THE IN VITRO INCORPORATION OF L-LYSINE-U-^{14}C INTO VARIOUS RABBIT TESTICULAR COMPONENTS IN THE PRESENCE OF GLUCOSE[a]

Testicular Protein Fraction	Age in Months			
	6	12	24	36
Total Protein[b]	12,971 ±1,473	20,075 ±2,841	17,667 ±1,240	16,806 ±2,022
Residual Nucleoprotein[c]**	302 ± 82	476 ± 34	648 ± 76	507 ± 101
Nuclear Acid Soluble Protein[c]	273 ± 79	313 ± 44	431 ± 52	314 ± 46

[a]Each value represents the mean-zero control ± standard error expressed in cpm/100 mg wet weight of tissue equivalent/2 hours. The specific activity of lysine in all experiments was 1850 dpm/pM.

[b]Exogenous glucose concentration was 10 millimolar.

[c]Exogenous glucose concentration was 5 millimolar.

**Significant differences at the 0.01 level.

Testicular incorporation of L-lysine-U-^{14}C into nuclear acid soluble protein (NASP), in the presence of exogenous glucose and saturating levels of lysine hydrochloride, also increased through 24 months then decreased by 36 months of age in rabbits. These differences were not significant

($P > 0.25$) due to experimental variation. However, the trend in NASP synthesis was similar to that of RNP, total protein, and spermatogenesis. Therefore, these data suggest close relationship between protein biosynthesis *in vitro* and spermatogenesis in aging rabbits.

In summary, total protein RNP and NASP biosynthesis *in vitro* in rabbit testicular tissue increased through 24 months, then declined by 36 months of age. These data would be in keeping with an effect of aging on the biosynthesis of a specific acidic nuclear protein in the rabbit testes.

In Vitro Incorporation of Sodium Acetate-1-^{14}C Into Testicular Lipids

Lipids are constituents of membranes, mitochondria, microsomes, nuclei and precursors of steroid hormones. They are also involved in cation transport and membrane permeability as well as storage forms of available energy (De Robertis, *et al.*, 1965; Giese, 1968). Maintenance of testis size and spermatogenic function appears to be dependent on dietary essential fatty acids (Panos and Finerty, 1954). Lipid changes in the testis coincident with aging have not been extensively studied. Histochemical methods, applied to testicular biopses of human males, have shown that the lipids in Leydig cells gradually increase through 18 years, then decrease by age 80. Sertoli cells showed a continual and gradual increase in lipid with age (Lynch and Scott, 1950). Since lipids are important constituents of the germinal epithelium and Leydig cells, and show general change with age, a study of the incorporation of acetate into specific lipid classes with aging was considered essential in this study.

Data are shown in Table X for the *in vitro* incorporation of sodium

acetate-1-^{14}C into testicular total lipid and various lipid classes with age in the male rabbit. Due to loss of samples from technical error, results of the 6-month old group are not included in this comparison. Testes from 24 and 36-month old animals incorporated more acetate-1-^{14}C ($P < 0.01$) into triglycerides and sterol esters which co-chromatograph with triolein and cholesteryl acetate, respectively, than did testes from 12-month old animals. This incorporation into triglycerides and sterol esters steadily increased through 36 months of age.

Reference to Table X shows variation with age in the incorporation of acetate-1-^{14}C into: 1) total lipid; 2) polar lipids which co-chromatograph with sphingomyelin; 3) monoglycerides which co-chromatograph with monopalmatin; 4) diglycerides which co-chromatograph with diolein; 5) non-volatile fatty acids which co-chromatograph with oleic acid; and 6) sterols which co-chromatograph with cholesterol. However, the magnitude and direction of variation with age in incorporation of acetate into these lipid classes were not statistically significant ($P > 0.25$).

TABLE X

THE EFFECT OF AGING ON THE IN VITRO INCORPORATION OF SODIUM ACETATE-1-^{14}C INTO RABBIT TESTICULAR TOTAL LIPIDS AND LIPID FRACTIONS IN THE PRESENCE OF GLUCOSE[a]

Testicular Lipid	Age in Months		
	12	24	36
Total Lipid	105,708 ±13,495	108,532 ±20,202	95,307 ±13,526
Polar Lipid	25,904 ± 4,539	30,811 ± 8,595	15,192 ± 4,297
Monoglycerides	1,466 ± 264	1,784 ± 288	2,209 ± 497
Diglycerides	5,983 ± 741	5,833 ± 997	7,061 ± 1,921
Triglycerides**	6,859 ± 108	27,356 ± 5,387	38,362 ± 7,487
Non-Volatile Fatty Acids	12,619 ± 3,171	7,001 ± 3,190	2,652 ± 460
Sterols	6,069 ± 1,782	6,353 ± 1,315	5,018 ± 1,561
Sterol Esters**	945 ± 14	1,556 ± 280	1,861 ± 397

[a]Each value represents the mean-zero control ± standard error expressed in cpm/100 mg wet weight of tissue equivalent/2 hours. The specific activity of sodium acetate in all experiments was 7397 dpm/pM. The exogenous glucose concentration was 10 millimolar.

**Significant differences at the 0.01 level.

The Effect of Aging on Testosterone Secretion Rate in the *in Vitro* Perfused Rabbit Testis

Previous experiments by Isurugi (1967) and Vermeulen, *et al.* (1971) failed to determine whether decreased testosterone concentration in peripheral blood of senile men was due to decreased gonadotropic hormone (GTH) secretion, decreased testicular androgen production in response to GTH, or to increased testosterone metabolism and excretion. It is impossible to determine which mechanism is acting in intact males by monitoring peripheral testosterone levels due to the production of adrenal androgens, peripheral conversion of precursors to androgens, interaction between the anterior pituitary gland and the testis, and the possible alteration of androgen metabolism and excretion with age. Isolation of the testes in an *in vitro* perfusion apparatus allowed the use of an artificial medium essentially devoid of GTH. Thus, basal levels of testosterone secretion could be measured in the absence of GTH, and the testis could be challenged with sufficient quantities of exogenous GTH to stimulate maximum testosterone secretion.

The effect of age on testosterone secretion of perfused rabbit testes is shown in Figure 1. Testosterone secretion during the first two hours was similar for all groups. This period represented the basal level of secretion since no exogenous GTH was present in the perfusion medium. All testes responded with increased testosterone secretion to a saturating level of GTH added at the end of the second hour of perfusion. Six-month old rabbit testes appeared to secrete more testosterone in response to GTH than any other group. When data from the fifth and sixth hours of *in vitro* perfusion were averaged, testosterone secretion was 0.83, 0.73,

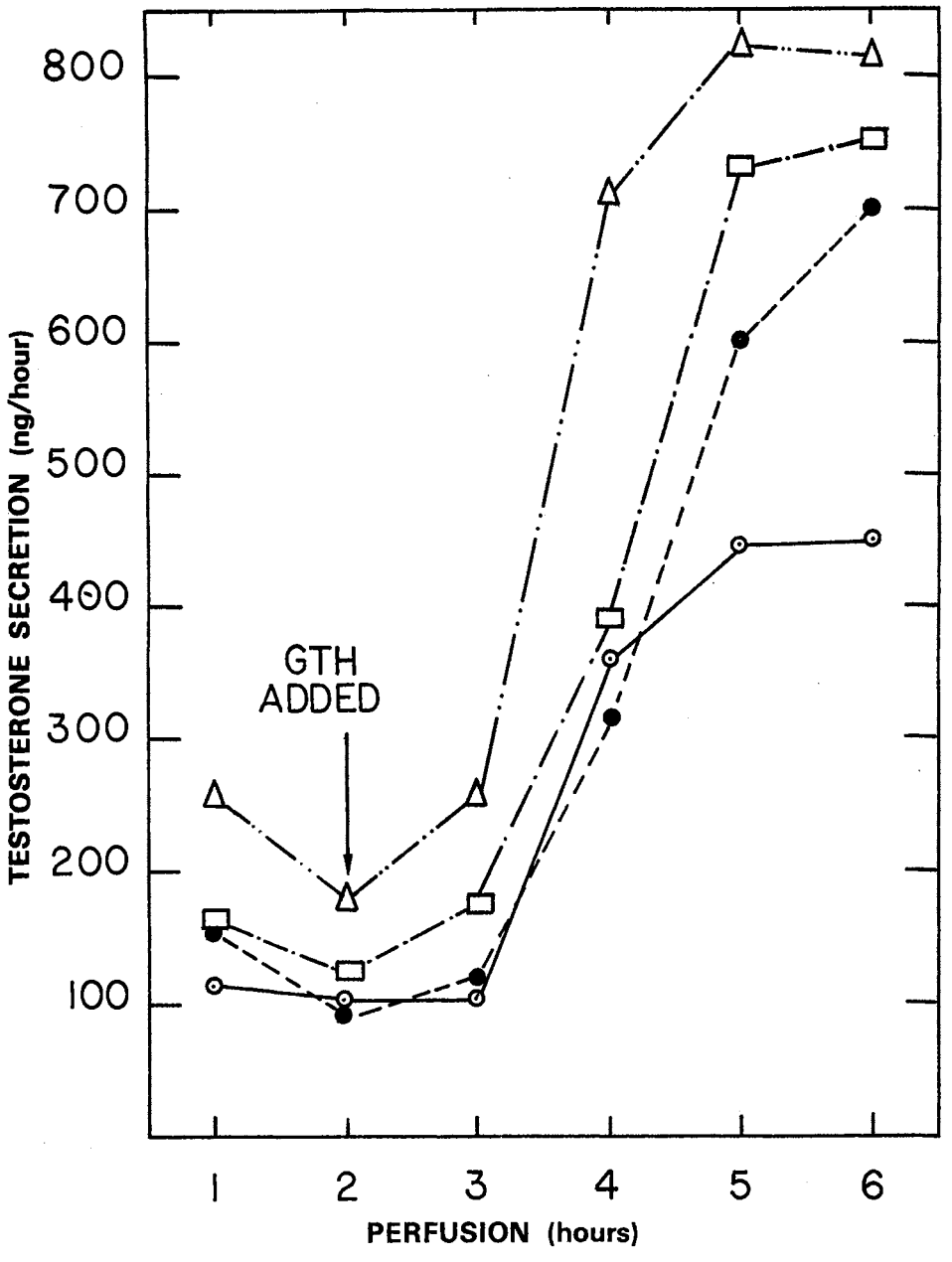

Figure 1. Testosterone Secretion by Perfused Rabbit Testes. Δ—··—Δ = 6 month old rabbits (n = 9); □— — —□ = 12 month old rabbits (n = 7); ●— — —● = 24 month old rabbits (n = 9); and ⊙———⊙ = 36 month old rabbits (n = 7)

0.65, and 0.45 μg/hr in testes from 6, 12, 24 and 36-month old rabbits, respectively. Comparison of individual treatment means showed no significant ($P > 0.25$) difference between the rate of testosterone secretion in testes from 6, 12 and 24-month old rabbits, but that there was a significant difference ($P < 0.05$) between the testes of 6 and 36-month old rabbits. Therefore, perfused testes from 36-month old rabbits failed to secrete as much testosterone as testes from 6-month old rabbits when maximally stimulated with gonadotropic hormones.

CHAPTER V

DISCUSSION

The testis may be a good model for aging since its dysfunction occurs before the debilitating effects of senility are apparent in many other tissues.

Previous investigations suggested that androgen production declines in man (Isurugi, 1967; Vermeulen, et al., 1971; Vermeulen, et al., 1972), that testes atrophy in rats and rabbits (Korenchevsky, et al., 1953; Ewing, 1967), and that fertility declines in the bovine (Collins, et al., 1962) with advanced age. Careful study of the effects of aging on testicular endocrine and germinal elements has not been carried out in the same animal. The present study was initiated to examine in each male rabbit, the effect of aging on: 1) testis size and sperm production; 2) the relationship between glucose catabolism, biosynthesis of specific lipid, protein and nucleoprotein fractions and spermatogenesis; 3) the capacity of the Leydig cell to secrete testosterone; and 4) the relationship between testosterone secretion and spermatogenesis. The ages selected were 6, 12, 24 and 36 months. Only those animals in apparent good health were used. This is seen in that body weight increased then plateaued between 24 and 36 months of age. Therefore, any changes in testis function must be due to the aging process and not secondary to some debilitating illness.

The Effect of Aging Upon Testis Size and Sperm Production

What is the effect of aging upon testis size and sperm production? Total paired testis weight increased through 24 months and was decreased by 36 months. This is in agreement with data presented by Ewing (1967). This decrease in testicular size with senescence implies that there is a decrease in spermatogenesis.

That spermatogenesis is reduced with aging is seen in the fact that spermatids and spermatozoa, expressed both per gram of testis weight and per testis pair, and total daily sperm production increased linearly through 24 months and decreased sharply by 36 months of age in male rabbits. These changes were accompanied by similar changes in spermatozoan numbers found in the head-body and tail of the epididymides. All of these data support the concept that spermatogenesis decreased between 24 and 36 months of age in rabbits. However, these results provide no information concerning the mechanisms mediating this age dependent change in sperm production.

The Effect of Aging on Glucose Catabolism, Protein Biosynthesis and Lipid Biosynthesis in Rabbit Testes

Can reduced spermatogenesis be related to decreased glucose catabolism, biosynthesis of testicular protein fractions or to biosynthesis of specific lipid fractions? Aging had no apparent effect upon <u>in vitro</u> catabolism of D-glucose-U-^{14}C to testicular $^{14}CO_2$. These results in the <u>in vitro</u> system show that the glycolytic, citric acid cycle and

oxidative phosphorylating enzymes necessary for ATP production are present and that their activity is not impaired. This is shown by the production of $^{14}CO_2$ in tissue from aging animals in response to saturating levels of glucose and O_2. However, the in vitro response may not be a true reflection of the in vivo conditions. Glucose and O_2 may not be present in adequate amounts in the aging testis if blood flow is not sufficient. In support of this, Sasano and Ichijo (1969) found that seminiferous tubules showed degenerative changes and increased thickness of the basement membrane in areas distal to the spermatic artery in human males above 40 years of age. Thus, from these results we have shown that decreased spermatogenesis in aging is not directly caused by decreased glucose catabolism. If there is a relationship between lower glucose catabolism and decreased spermatogenesis, it is probably secondary to decreased blood supply.

Spermatogenic changes should be accompanied by similar changes in cytoplasmic and nuclear protein synthesis. We have now shown this to be the case. In vitro incorporation of L-lysine-U-^{14}C into testicular total protein increased through 12 months then decreased gradually with aging. Incorporation of radioactive lysine into residual nucleoprotein (RNP) increased significantly through 24 months then decreased by 36 months of age. The same pattern of incorporation was found for nuclear acid soluble protein (NASP). These data suggest that the biosynthesis of a specific nucleoprotein moiety is decreased with advancing age and that the decreased total protein synthesis may be a secondary effect. The fact that nucleoprotein synthesis follows the same age associated pattern as in vitro testosterone secretion and daily sperm production suggests a cause effect relationship. By extension of this logic these data argue for a

dependence upon testosterone for the production of these nuclear proteins and the involvement of RNP and NASP in the spermatogenic decline with senescence. The data of Ewing, et al. (1969) lend support to this hypothesis in that the incorporation of amino acids into RNP and NASP in rat testes is associated with the appearance of spermatocytes, spermatids and spermatozoa.

Lipid biosynthesis data for 6-month old male rabbits were lost due to technical error. Therefore, data from 12, 24, and 36-month old rabbits were available for analysis. Incorporation of acetate-1-^{14}C into total lipid, polar lipids, monoglycerides, diglycerides, non-volatile fatty acids, and sterols did not change between 12 and 36 months of age. In contrast, the incorporation of acetate into triglycerides and sterol esters was significantly higher in the testes of 24 and 36-month old rabbits than in that of 12-month old animals. This corresponds to work by Ahluwalia and Holman (1965, 1966) in which triglycerides were increased in the testis of the aging bovine. Comparison of the change in sterol ester is difficult since the only values reported are for younger animals. In rats cholesterol ester apparently decreases in the testis between 28 and 200 days of age (Davis, et al., 1966; Johnson, 1967, cited by Johnson, 1970). Hafiez and coworkers (1972) found cholesterol ester in the testis of hypophysectomized rats to decrease when LH or a combination of LH and prolactin were administered. These changes were accompanied by a significant increase in androgen production. These data imply that an increase in cholesterol ester might reflect a decrease in steroid secretion and thus a decrease in spermatogenesis coincident with aging.

In experiments designed to examine the action of hormones on lipid biosynthesis, Butler, et al. (1967) administered testosterone (3 mg/day)

to rats for 15 days with FSH added during the last 10 days. Testicular triglycerides and cholesterol ester were shown to increase. It was suggested that FSH stimulated esterfication of the cholesterol. Triglyceride and sterol ester increase coincident with aging implies that lipid utilization decreases. Triglycerides would be considered as storage forms of fatty acids and cholesteryl ester would be a storage form of cholesterol. Increased concentrations of cholesteryl ester would imply that spermatids and spermatozoa are not catabolizing cholesterol rapidly enough to shift the cholesterol/esterified cholesterol ratio to a predominance of cholesterol (Johnson, 1970).

The Effect of Aging Upon the Capacity of the Leydig Cell to Secrete Testosterone in Perfused Rabbit Testes

Spermatogenesis (Clermont and Harvey, 1967) and nitrogen retention necessary for protein synthesis are dependent upon testosterone secretion by the testis in males. The decline in total daily sperm production coincident with aging implies that testosterone secretion was decreased. This was shown to be the case.

The testosterone secretion rate from the <u>in vitro</u> perfused testis was lower, but not significantly lower, in the 36-month old rabbits than in the 6 month old animals prior to stimulation with GTH. Upon addition of GTH, testes from younger animals secreted more testosterone than testes from the aged group. This is shown by the fact that testosterone secretion in the 36-month old group was significantly lower than in the 6-month old animals after 5-6 hours of <u>in vitro</u> perfusion. The results are in agreement with data found in man where lower plasma testosterone levels

(Kirschner and Coffman, 1968) and lower apparent free testosterone were found with senescence (Vermeulen, et al., 1972). In the intact organism, the lower free testosterone concentration with increased age is partially due to the increased binding capacity of plasma testosterone to a globulin molecule. Changes in the binding capacity appear to be secondary to testosterone concentration but could be related to a shift in the estradiol/testosterone ratio. It was shown that the metabolic clearance of testosterone declined, that 5α reductase activity in the liver increased and suggested that there were decreased androgen receptors in target tissues with advanced age (Vermeulen, et al., 1972). Although these factors are involved in testosterone decrease in the intact organism, the present data show that the testes of aged animals do not secrete as much testosterone in response to GTH as younger animals. Thus, lower circulating testosterone levels are directly related to decreased secretion from the testes.

The difference in rate of testosterone secretion between age groups is coincident with the decrease in total daily sperm production. The failure of 36 month old rabbit testes to secrete as much testosterone as the testes of 6 month old animals is not easily explained. It is impossible to say whether this difference was due to decreased number of Leydig cells, altered populations of Leydig cell types, refractoriness of Leydig cells to exogenous gonadotropic hormones, decreased activity of enzymes needed for testosterone biosynthesis, gene repression of testosterone biosynthetic enzymes by specific nucleoproteins, or to an increased requirement for metabolic precursors. Whether the decrease in sperm production is due to decreased testosterone production in aged rabbits awaits experiments to determine the minimum 24 hour in vivo testosterone

production required to maintain spermatogenesis and the 24 hour *in vivo* testosterone production in aged rabbits. These experiments are now feasible since a technique has been recently described for estimation of *in vivo* testosterone production rates in unanesthetized, restrained rabbits (Goodwin, 1972).

CHAPTER VI

SUMMARY AND CONCLUSIONS

The effects of senescence upon the biochemical, steroidogenic, and spermatogenic elements of the mammalian testes have not been extensively examined. It has been inferred, from experiments in man (Vermeulen, et al., 1971; Isurugi, 1967) that androgen production declines, from rats and rabbits (Korenchevsky, et al., 1953; Ewing, 1967), that testes atrophy, and from the bovine (Collins, et al., 1962) that fertility declines with advancing age. It is also unclear whether a decline in testosterone production in man is coincidental with, or precedes, the decline in libido and fertility with advancing age. Therefore, this study undertook to examine the effects of age on in vitro testosterone secretion, spermatozoa production and biosynthesis of specific testicular constituents in the testes of rabbits from 6 months through 36 months of age.

These experiments demonstrated that body weight increased through 24 months then decreased by 36 months. Total spermatids and spermatozoa per testes pair increased through 24 months then decreased by 36 months. Likewise, spermatids and spermatozoa per gram of testis tissue, along with total daily sperm production, increased through 24 months then decreased by 36 months. Concentration of spermatozoa and spermatids in the head-body of the epididymus also increased through 24 months and decreased by 36 months. However, concentration of spermatozoa and spermatids in the tail of the epididymus increased only through 12 months then decreased

with aging.

The experiments with radioisotopic precursors demonstrated that there were no significant differences in testicular metabolism of glucose into $^{14}CO_2$, or acetate-1-^{14}C into total lipid, diglycerides and sterols. Incorporation of the radioisotope, lysine, into total protein increased through 12 months then decreased gradually with age. Lysine incorporation into RNP and NASP increased through 24 months then decreased by 36 months. Sodium acetate-1-^{14}C incorporation into triglycerides and sterol esters increased with age while incorporation into non-volatile fatty acids decreased with age. Acetate incorporation into monoglycerides increased slightly with age whereas the incorporation of acetate into phospholipids increased slightly to 24 months, then decreased by 36 months.

Experiments with *in vitro* perfused rabbit testes demonstrated that the testosterone secretion rate of 36 month old animals was less than that from younger animals.

In conclusion, the results from these experiments prove that aging has a definite effect on testicular function in rabbits. The increase in sperm production through 24 months of age indicates that testosterone secretion is adequate to maintain spermatogenesis. Decreased daily sperm production, coincident with reduced testosterone secretion, by the *in vitro* perfused testis between 24 and 36 months is suggestive of a cause effect relationship.

Whether or not impaired testosterone production causes decreased sperm production in aged rabbits awaits experiments demonstrating the minimum 24 hour production *in vivo* required to maintain spermatogenesis in rabbits and the 24 hour testosterone production *in vivo* in aged

rabbits. These experiments are now feasible since a method has been described by Goodwin (1972) for estimation of *in vivo* testosterone in unanesthetized, restrained rabbits.

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APPENDIX

TABLE XI

ANALYSIS OF VARIANCE OF BODY WEIGHTS OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	575.200	575.200	---
Treatment	3	3.270	1.090	7.985***
Error	33	4.505	0.137	---
Total	36	7.775	---	---

***(P < 0.005).

TABLE XII

DUNCAN'S NEW MULTIPLE-RANGE TEST[1] APPLIED TO THE MEAN BODY WEIGHT OF AGING RABBITS

Treatment (months)	6	12	36	24
Mean	3.517	3.926	4.0514	4.3101
Value of p (d.f. = 33)	2	5% 3	4	
SSR	2.881	3.031	3.114	
LSR ($s_{\bar{x}}$ = 0.117)	0.3371	0.3546	0.3643	

[1]Steel and Torrie (1960).

TABLE XIII

ANALYSIS OF VARIANCE OF TOTAL PAIRED TESTES WEIGHT OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	850.5	850.50	---
Treatment	3	11.0	3.67	3.001*
Error	33	40.1	1.22	---
Total	36	51.1	---	---

*($P < 0.05$)

TABLE XIV

DUNCAN'S NEW MULTIPLE-RANGE TEST[1] APPLIED TO THE MEAN OF THE TOTAL PAIRED TESTES WEIGHT OF AGING RABBITS

Treatment (months)	6	36	12	24
Mean	4.12	4.51	4.90	5.57
Value of p (d.f. = 33)	2	5% 3	4	
SSR	2.881	3.031	3.114	
LSR	1.006	1.058	1.087	
($s_{\bar{x}}$ = 0.3492)				

[1]Steel and Torrie (1960).

TABLE XV

ANALYSIS OF VARIANCE OF TESTES WEIGHT, G/BODY WEIGHT, KG OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	55.370	55.3700	---
Treatment	3	0.130	0.0433	0.4418
Error	33	3.233	0.0980	---
Total	36	3.363	---	---

TABLE XVI

ANALYSIS OF VARIANCE OF TOTAL PITUITARY GLAND WEIGHT OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	27,734	27,734	---
Treatment	3	159	53	3.533*
Error	33	504	15	---
Total	36	663	---	---

*($P < 0.05$).

TABLE XVII

DUNCAN'S NEW MULTIPLE-RANGE TEST[1] APPLIED TO THE MEAN OF THE TOTAL PITUITARY GLAND WEIGHT OF AGING RABBITS

Treatment (months)	36	6	12	24
Mean (mg)	23.1	28.0	28.2	28.9
Value of p (d.f. = 33)	2	1% 3	4	
SSR	3.869	4.039	4.142	
LSR	4.738	4.947	5.073	
($s_{\bar{x}}$ = 1.2247)				

[1]Steel and Torrie (1960).

TABLE XVIII

ANALYSIS OF VARIANCE OF ANTERIOR PITUITARY GLAND WEIGHT OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	15,116	15,116.00	---
Treatment	3	78	2.60	0.20
Error	33	430	13.00	---
Total	36	508	---	---

TABLE XIX

ANALYSIS OF VARIANCE OF POSTERIOR PITUITARY GLAND WEIGHT OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	2,059	2,059.00	---
Treatment	3	135	45.00	16.85***
Error	33	88	2.67	---
Total	36	223	---	---

***($P < 0.005$).

TABLE XX

DUNCAN'S NEW MULTIPLE-RANGE TEST[1] APPLIED TO THE MEAN OF THE POSTERIOR PITUITARY GLAND WEIGHT OF AGING RABBITS

Treatment (months)	36	24	6	12
Mean (mg)	4.2	6.4	9.0	9.2
Value of p (d.f. = 33)	2	1% 3	4	
SSR	3.869	4.039	4.142	
LSR	1.999	2.087	2.140	
($s_{\bar{x}} = 0.5167$)				

[1]Steel and Torrie (1960).

TABLE XXI

ANALYSIS OF VARIANCE OF TOTAL SPERM IN PAIRED TESTES OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	9,741,357	9,741,357	---
Treatment	3	589,062	196,354	4.54**
Error	33	1,426,578	43,229	---
Total	36	2,015,640	---	---

** ($P < 0.01$).

TABLE XXII

DUNCAN'S NEW MULTIPLE-RANGE TEST[1] APPLIED TO THE MEAN OF THE TOTAL SPERM IN PAIRED TESTES OF AGING RABBITS

Treatment (months)	6	36	12	24
Mean x 10^6	354.9	396.5	581.8	742.8
Value of p (d.f. = 33)	2	5% 3	4	
SSR	2.881	3.031	3.114	
LSR ($s_{\bar{x}}$ = 65.75)	189.4	199.3	204.7	

[1] Steel and Torrie (1960).

TABLE XXIII

ANALYSIS OF VARIANCE OF SPERM PER GRAM OF WET TESTES WEIGHT OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	402,295	402,295	---
Treatment	3	10,020	334	4.53**
Error	33	39,209	118	---
Total	36	49,224	---	---

** ($P < 0.01$).

TABLE XXIV

DUNCAN'S NEW MULTIPLE-RANGE TEST[1] APPLIED TO THE MEAN OF SPERM PER GRAM OF WET TESTIS WEIGHT OF AGING RABBITS

Treatment (months)	6	36	12	24
Mean x 10^6	86.3	87.6	114.1	134.0
Value of p (d.f. = 33)	2	1% 3	4	
SSR	3.869	4.039	4.142	
LSR	13.29	13.87	14.23	
($s_{\bar{x}}$ = 3.435)				

[1]Steel and Torrie (1960).

TABLE XXV

ANALYSIS OF VARIANCE OF THE TOTAL DAILY SPERM PRODUCTION PER RABBIT IN AGING ANIMALS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	880,413	880,413	---
Treatment	3	77,334	25,778	7.9***
Error	33	106,864	3,238	---
Total	36	184,198	---	---

*** ($P < 0.005$)

TABLE XXVI

DUNCAN'S NEW MULTIPLE-RANGE TEST[1] APPLIED TO THE MEAN OF THE TOTAL DAILY SPERM PRODUCTION PER RABBIT IN AGING ANIMALS

Treatment (months)	6	36	12	24
Mean x 10^6	103.4	115.6	169.6	216.7
Value of p (d.f. = 33)	2	5% 3	4	
SSR	2.881	3.031	3.114	
LSR ($s_{\bar{x}}$ = 18)	51.86	54.66	56.05	

[1] Steel and Torrie (1960).

TABLE XXVII

ANALYSIS OF VARIANCE OF TOTAL SPERM IN THE HEAD AND BODY OF THE EPIDIDYMIS OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	396,249	396,249	---
Treatment	3	75,662	25,220	8.67***
Error	33	95,910	2,906	---
Total	36	171,527	---	---

*** ($P < 0.005$).

TABLE XXVIII

DUNCAN'S NEW MULTIPLE-RANGE TEST[1] APPLIED TO THE MEAN OF THE TOTAL SPERM IN THE HEAD AND BODY OF THE EPIDIDYMIS OF AGING RABBITS

Treatment (months)	36	6	12	24
Mean x 10^6	48.0	61.2	133.7	154.6
Value of p (d.f. = 33)	2	1% 3	4	
SSR	3.869	4.039	4.142	
LSR ($s_{\bar{x}}$ = 17.0469)	66.00	68.90	70,61	

[1]Steel and Torrie (1960).

TABLE XXIX

ANALYSIS OF VARIANCE OF TOTAL SPERM IN THE TAIL OF THE EPIDIDYMIS OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	7,971,520	7,971,520	---
Treatment	3	1,188,315	396,105	4.72**
Error	33	2,764,764	83,780	---
Total	36	3,953,079	---	---

**($P < 0.01$).

TABLE XXX

DUNCAN'S NEW MULTIPLE-RANGE TEST[1] APPLIED TO THE MEAN OF THE TOTAL SPERM IN THE TAIL OF THE EPIDIDYMIS OF AGING RABBITS

Treatment (months)	6	36	24	12
Mean x 10^6	226.5	268.0	526.9	696.0
Value of p (d.f. = 33)	2	5% 3	4	
SSR	2.881	3.031	3.114	
LSR	263.7	277.4	285.0	
($s_{\bar{x}}$ = 91.5314)				

[1]Steel and Torrie (1960).

TABLE XXXI

ANALYSIS OF VARIANCE OF CATABOLISM OF D-GLUCOSE-U-^{14}C INTO TESTICULAR $^{14}CO_2$ OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	2,198,013,289	2,198,013,289	---
Treatment	3	8,633,962	2,877,987	0.13
Error	33	730,825,339	22,146,222	---
Total	36	739,459,301	---	---

TABLE XXXII

ANALYSIS OF VARIANCE OF INCORPORATION OF L-LYSINE-U-^{14}C INTO TESTICULAR TOTAL PROTEIN OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	9,845,801,338	9,845,801,338	---
Treatment	3	249,908,767	83,302,922	2.44
Error	31	1,059,357,455	34,172,821	---
Total	34	1,309,266,222	---	---

TABLE XXXIII

ANALYSIS OF VARIANCE OF INCORPORATION OF L-LYSINE-U-^{14}C INTO TESTICULAR RESIDUAL NUCLEOPROTEIN OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	8,568,055	8,568,055	---
Treatment	3	602,855	200,952	4.04*
Error	33	1,642,219	49,764	---
Total	36	2,245,074	---	---

*($P < 0.05$)

TABLE XXXIV

DUNCAN'S NEW MULTIPLE-RANGE TEST[1] APPLIED TO THE MEAN INCORPORATION OF L-LYSINE-U-^{14}C INTO TESTICULAR RESIDUAL NUCLEOPROTEIN OF AGING RABBITS

Treatment (months)	6	12	36	24
Mean	302	476	507	648
Value of p (d.f. = 33)	2	1% 3	4	
SSR	3.869	4.039	4.142	
LSR ($s_{\bar{x}}$ = 70.55)	273.0	285.0	292.2	

[1]Steel and Torrie (1960).

TABLE XXXV

ANALYSIS OF VARIANCE OF INCORPORATION OF L-LYSINE-U-^{14}C INTO TESTICULAR NUCLEAR ACID SOLUBLE PROTEIN OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	4,044,791	4,044,791	---
Treatment	3	137,590	45,863	1.423
Error	32	1,031,151	32,223	---
Total	35	1,168,741	---	---

TABLE XXXVI

ANALYSIS OF VARIANCE OF INCORPORATION OF SODIUM ACETATE-1-^{14}C INTO TESTICULAR TOTAL LIPID OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	296,531,809,689	296,531,809,689	---
Treatment	2	455,753,536	227,876,768	0.088
Error	24	62,487,929,516	2,603,663,730	---
Total	26	62,943,683,052	---	---

TABLE XXXVII

ANALYSIS OF VARIANCE OF INCORPORATION OF SODIUM ACETATE-1-^{14}C INTO TESTICULAR POLAR LIPIDS OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	16,799,784,001	16,799,784,001	---
Treatment	2	1,019,246,923	509,623,462	1.318
Error	24	9,278,494,046	386,603,919	---
Total	26	10,297,740,789	---	---

TABLE XXXVIII

ANALYSIS OF VARIANCE OF INCORPORATION OF SODIUM ACETATE-1-^{14}C INTO TESTICULAR MONOGLYCERIDES OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	85,194,734	85,194,734	---
Treatment	2	2,278,020	1,139,010	1.132
Error	24	24,156,494	1,006,521	---
Total	26	26,434,514	---	---

TABLE XXXIX

ANALYSIS OF VARIANCE OF INCORPORATION OF SODIUM ACETATE-1-^{14}C INTO TESTICULAR DIGLYCERIDES OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	1,040,162,854	1,040,162,854	---
Treatment	2	7,014,310	3,507,155	0.327
Error	24	257,522,580	10,730,108	---
Total	26	264,536,890	---	---

TABLE XL

ANALYSIS OF VARIANCE OF INCORPORATION OF SODIUM ACETATE-1-^{14}C INTO TESTICULAR TRIGLYCERIDES OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	13,812,179,298	13,812,179,298	---
Treatment	2	3,972,850,903	1,986,425,452	9.584***
Error	24	4,974,367,791	207,265,325	---
Total	26	8,947,218,694	---	---

*** ($P < 0.005$).

TABLE XLI

DUNCAN'S NEW MULTIPLE-RANGE TEST[1] APPLIED TO THE MEAN OF INCORPORATION OF SODIUM ACETATE-1-^{14}C INTO TESTICULAR TRIGLYCERIDES OF AGING RABBITS

Treatment (months)	12	24	36
Mean	6,859	27,356	38,362
Value of p (d.f. = 24)		1%	
	2	3	
SSR	3.96	4,14	
LSR	18,029	18,848	
($s_{\bar{x}}$ = 4552.64)			

[1]Steel and Torrie (1960).

TABLE XLII

ANALYSIS OF VARIANCE OF INCORPORATION OF SODIUM ACETATE-1-^{14}C INTO TESTICULAR NON-VOLATILE FATTY ACIDS OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	1,708,248,765	1,708,248,765	---
Treatment	2	423,412,632	211,706,316	2.78
Error	24	1,829,674,847	76,236,452	---
Total	26	2,253,087,479	---	---

TABLE XLIII

ANALYSIS OF VARIANCE OF INCORPORATION OF SODIUM ACETATE-1-^{14}C INTO TESTICULAR STEROLS OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	940,342,059	940,342,059	---
Treatment	2	7,787,490	3,893,745	0.172
Error	24	544,055,964	22,668,999	---
Total	26	551,843,454	---	---

TABLE XLIV

ANALYSIS OF VARIANCE OF INCORPORATION OF SODIUM ACETATE-1-^{14}C INTO TESTICULAR STEROL ESTERS OF AGING RABBITS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Mean	1	53,577,228	53,577,228	---
Treatment	2	3,800,127	1,900,064	9.56**
Error	24	4,770,228	198,760	---
Total	26	8,570,355	---	---

** ($P < 0.01$).

TABLE XLV

DUNCAN'S NEW MULTIPLE-RANGE TEST[1] APPLIED TO THE MEAN INCORPORATION OF SODIUM ACETATE-1-^{14}C INTO TESTICULAR STEROL ESTERS OF AGING RABBITS

Treatment (months)	12	24	36
Mean	945	1556	1861
Value of p (d.f. = 24)	2	1% 3	
SSR	3.96	4.14	
LSR	558.3	583.7	
($s_{\bar{x}}$ = 140.99)			

[1]Steel and Torrie (1960).

TABLE XLVI

ANALYSIS OF VARIANCE OF TESTOSTERONE SECRETION RATE OF THE TESTIS OF SIX AND THIRTY-SIX MONTH OLD RABBITS USING THE MEAN OF THE FIFTH AND SIXTH HOURS OF IN VITRO PERFUSION WITH A SATURATING LEVEL OF FSH AND ICSH ADDED AT THE END OF THE SECOND HOUR OF PERFUSION

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Treatment	1	0.559	0.559	5.22*
Error	13	1.389	0.107	---
Total	14	1.948	---	---

*($P < 0.05$).

TABLE XLVII

THE LEVEL AND DIRECTION OF SIGNIFICANT DIFFERENCES OF SIX-MONTH OLD RABBITS FROM OTHER AGE GROUPS[a]

	Age in Months		
	12	24	36
WEIGHTS			
Body Weight	<[b]	<	<
Total Paired Testes Weight	=[c]	<	=
Pituitary Gland Weight	=	=	>>[d]
Posterior Pituitary Gland Weight	=	>>	>>
SPERM COUNTS			
Total Sperm/Paired Testes	<	<	=
Total Sperm/g Testis	<<[e]	<<	=
Sperm in Epididymis:			
Head and Body	<<	<<	=
Tail	<	<	=
Total Daily Sperm Production	<	<	=
IN VITRO TESTICULAR INCORPORATION			
L-lysine-U-^{14}C into RNP	=	<<	=
IN VITRO TESTICULAR PERFUSION[f]			
Testosterone Secretion Rate:			
Fifth and Sixth Hours	=	=	>[g]

[a]Each column shows the presence or absence and magnitude of significant difference of the age group listed from the 6-month old group.

[b]Six months is significantly less at $P < 0.05$ level.

[c]Six months is not significantly different.

[d]Six months is significantly greater at $P < 0.01$ level.

[e]Six months is significantly less at $P < 0.01$ level.

[f]Saturating level of ovine NIH-FSH-S7 (15 mμg/ml media) and ovine NIH-ICSH-S16 (30 mμg/ml media) added at the end of the second hour of perfusion.

[g]Six months is significantly greater at $P < 0.05$ level.

TABLE XLVIII

THE LEVEL AND DIRECTION OF SIGNIFICANT DIFFERENCES OF TWELVE-MONTH OLD RABBITS FROM OTHER AGE GROUPS[a]

	Age in Months		
	6	24	36
WEIGHTS			
Body Weight	>[b]	<[c]	=[d]
Pituitary Gland Weight	=	=	>>[e]
Posterior Pituitary Gland Weight	=	>>	>>
SPERM COUNTS			
Total Sperm/Paired Testes	>	=	=
Total Sperm/g Testis	>>	<<[f]	>>
Sperm in Epididymis:			
Head and Body	>>	=	>>
Tail	>	=	>
Total Daily Sperm Production	>	=	=
IN VITRO TESTICULAR INCORPORATION			
Sodium Acetate-1-^{14}C into:			
Triglycerides	-[g]	<<	<<
Sterol Esters	-	<<	<<

[a]Each column shows the presence, or absence, and magnitude of significant difference of the age group listed from the 12-month old group.

[b]Twelve-month old group is significantly greater at the $P < 0.05$ level.

[c]Twelve-month old group is significantly less at the $P < 0.05$ level.

[d]Twelve-month old group is not significantly different.

[e]Twelve-month old group is significantly greater at the $P < 0.01$ level.

[f]Twelve-month old group is significantly less at the $P < 0.01$ level.

[g]Data not included.

TABLE XLIX

THE LEVEL AND DIRECTION OF SIGNIFICANT DIFFERENCES OF TWENTY-FOUR-MONTH OLD RABBITS FROM OTHER AGE GROUPS[a]

	Age in Months		
	6	12	36
WEIGHTS			
Body Weight	>[b]	>	=[c]
Total Paired Testes Weight	>	=	=
Pituitary Gland Weight	=	=	>>[d]
Posterior Pituitary Gland Weight	<<[e]	<<	>>
SPERM COUNTS			
Total Sperm/Paired Testes	>	=	>
Total Sperm/g Testis	>>	>>	>>
Sperm in Epididymis:			
Head and Body	>>	=	>>
Tail	>	=	=
Total Daily Sperm Production	>	=	>
IN VITRO TESTICULAR INCORPORATION			
L-lysine-U-^{14}C into RNP	>>	=	=
Sodium Acetate-1-^{14}C into:			
Triglycerides	-[f]	>>	=
Sterol Esters	-	>>	=

[a]Each column shows the presence, or absence, and magnitude of significant difference of the age group listed from the 24-month old group.

[b]Twenty-four-month old group is significantly greater at $P < 0.05$ level.

[c]Twenty-four-month old group is not significantly different.

[d]Twenty-four-month old group is significantly greater at $P < 0.01$ level.

[e]Twenty-four-month old group is significantly less at $P < 0.01$ level.

[f]Data not included.

TABLE L

THE LEVEL AND DIRECTION OF SIGNIFICANT DIFFERENCES OF THIRTY-SIX-MONTH OLD RABBITS FROM OTHER AGE GROUPS[a]

	Age in Months		
	6	12	24
WEIGHTS			
Body Weight	>[b]	=[c]	=
Pituitary Gland Weight	<<[d]	<<	<<
Posterior Pituitary Gland Weight	<<	<<	<<
SPERM COUNTS			
Total Sperm/Paired Testes	=	=	<[e]
Total Sperm/g Testis	=	<<	<<
Sperm in Epididymis:			
Head and Body	=	<<	<<
Tail	=	<	=
Total Daily Sperm Production	=	=	<
IN VITRO TESTICULAR INCORPORATION			
Sodium Acetate-1-^{14}C into:			
Triglycerides	-[f]	>>[g]	=
Sterol Esters	-	>>	=
IN VITRO TESTICULAR PERFUSION[h]			
Testosterone Secretion Rate			
Fifth and Sixth Hours	<	=	=

[a]Each column shows the presence, or absence, and magnitude of significant difference of the age group listed from the 36-month old group.

[b]Thirty-six-month old group is significantly greater at $P < 0.05$ level.

[c]Thirty-six-month old group is not significantly different.

[d]Thirty-six-month old group is significantly less at $P < 0.01$ level.

[e]Thirty-six-month old group is significantly less at $P < 0.05$ level.

[f]Data not included.

[g]Thirty-six month old group is significantly greater at $P < 0.01$ level.

[h]Saturating level of ovine NIH-FSH-S7 (15 mμg/ml media) and ovine NIH-ICSH-S16 (30 mμg/ml media) added at the end of the second hour of perfusion.

VITA

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