THE EFFECTS OF ISOLATION, COHABITATION AND SEGREGATION ON TESTICULAR METABOLISM IN MICE

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1966

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
May, 1971

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ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my major adviser, Dr. Claude Desjardins, for his guidance, encouragement, special interest and personal assistance during the course of this study. I would also extend my thanks to the members of my committee for their suggestions regarding this manuscript. To my colleagues and fellow graduate students I extend my thanks and appreciation for their assistance and thoughtfulness throughout the study.

In addition I would like to thank Sarah Kiesling and Dixie Jennings for typing the manuscript.

Finally, I would like to express my appreciation to my husband, Ed, for his endless encouragement, assistance and sacrifices during the course of this study.

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CHAPTER I

INTRODUCTION

The testes perform two complementary functions, namely the production of gametes (sperm cells) by the seminiferous epithelium and the production of androgens by the interstitial cells of Leydig. These two functions are interrelated and highly susceptible to influences from both the internal and external environment, which in turn can alter the function of the tissue and the behavior of the animal.

Alterations in the internal environment such as failure to provide essential nutrients, hyperglycemia, hypoglycemia and abnormal function of the pituitary, adrenal and thyroid glands have long been recognized as contributing to testis failure. By contrast to the internal environment, the external environment also exerts many influences upon the testes and their functioning, but the effects of these stimuli on testis function have not been well documented. For example, auditory, olfactory, tactile, thermal and photic stimuli have been implicated in altering fertility, sexual maturation, receptivity and sexual behavior.

It has been postulated that changes in sexual behavior brought about by alterations in the social environment may be reflected by changes in the morphology and physiology of the testis and accessory sex organs. This postulate was supported by observing gross and morphological changes in the testes of rodents exposed to widely different social environments which were associated with equally different

behavioral patterns. In view of the fact that previous investigators (24,25,26) noted qualitative differences in the testes and accessory sex glands of rodents reared under isolation, cohabitation and segregation the present study was designed to quantitate the effects of these specific social situations on the spermatogenic and steroidogenic elements of the mouse testis. This was accomplished by determining the concentration of selected biochemical components of the testis, examining glucose metabolism and studying the incorporation of uniformly labeled lysine-U-^{14}C into testis "protein" by teased testis tubules in vitro. The steroidogenic functions of the testis were investigated by determining the incorporation of acetate-1-^{14}C into testosterone-^{14}C and androstenedione-^{14}C.

The results show that the seminal vesicles of mice raised without social contact were smaller than those raised in either segregation or cohabitation. Moreover, the testes of isolated mice tended to incorporate less acetate-^{14}C into testosterone-^{14}C and androstenedione-^{14}C indicating a possible reduction in androgen biosynthesis. The total protein content of the testes of cohabitated mice was higher than that of segregated or isolated mice. The various social groups studied did not influence the amount of glucose oxidized to CO_2 or the amount of lysine-U^{14}C that was incorporated into testis "protein". As expected, the testes and seminal vesicles were smaller at 42 than at 56 days of age regardless of the social group but the adrenal glands of 42 day old males were heavier than those of the 56 day old animals.

CHAPTER II

LITERATURE SURVEY

The testis is subject to many influences from both the internal and external environment in the performance of its dual role as an exocrine and endocrine organ. The reason that testis function can be influenced by both internal and external stimuli lies in the fact that the production of sperm and androgens depends on the hypothalamo-hypophyseal neuroendocrine system which may be regulated by internal (hormonal and nervous) as well as external signals. The types of external stimuli influencing testis function have been associated with either the physical or social environment. For example, physical stimuli such as light, temperature and sound are known to have a profound influence on testis function especially in seasonal breeders. In addition, olfaction, aggression and population density are examples of social stimuli that are capable of influencing testis function. Previous investigations concerned with examining the effects of social stimuli on the testis were limited to observations on testis weight and morphology. Although these criteria can provide qualitative information regarding the effects of such stimuli on the testis it is extremely difficult to indicate the extent and specific effect of these stimuli upon the spermatogenic and steroidogenic elements of the testis without conducting a quantiative investigation. Hence, the present literature survey summarizes relevant information pertaining to the physical and social factors modifying

testis function. In addition, literature associated with quantitating the function of the spermatogenic and steroidogenic elements of the testis was reviewed and related to the approach used to examine testis function in this study.

Effects of Physical Stimuli on Testis Function

Man has recognized for centuries the influence of season on reproduction noting that some animals are continuous breeders while others are seasonal breeders, and yet, there is no season that is not the sexual season for some animals (20). Only in recent years has the role of sensory stimulation and environmental factors on reproduction been studied. The greatest effort has dealt with the effects of light and temperature, but humidity, gravitational forces, magnetic fields, lunar effects and captivity have been studied to some degree in some species (59).

For instance, the effects of light on gonadal activity have been observed in representatives of the boney fishes, amphibians, reptiles, birds and mammals (59). Much early work was done in birds after egg production was increased following lengthend exposures to artificial light (1). Later Rowan showed that recurring periods of light and darkness with the changing season was the primary stimulus increasing gonadal activity in the junco, some finches and the crow (43,44). The response of the testis (increase in size, stimulation of spermatogenesis) to light is variable, sometimes refractory, or followed by regression in different species (59). Nevertheless mammals exhibit a sensitivity to alterations in photoperiod. Bulls and rams experience a reduction in semen quality and fertility during the summer months (20). Moreover

testicular atrophy has been reported in rats, ferrets, and hamsters after long periods of continuous darkness (11).

Seasonal variations in temperature have profound effects upon reproduction in both sexes of many species. The scrotal testes of mammals must be maintained several degrees below body temperature (15), since exposure of the testes to body temperature or to high environmental temperatures causes severe disorganization of the seminiferous tubules, a greater production of abnormal sperm and reduced semen quality in rams (20). By contrast, air conditioning and shearing of rams may improve semen quality and fertility (20).

Some breeds of beef cattle reach puberty later when exposed to higher temperatures (32.2° C) than control animals raised at 10° C. Exposure of bulls to 40° C had a detrimental effect on spermatogenesis, while swine show no ill effects on reproduction after exposure to high temperature (59). Generally high temperatures have more adverse effects than low temperatures on reproduction (59).

Likewise auditory stimuli effect sexual reproduction; exposure to alarm bells during premating periods decreases the capacity of males to fertilize females and decreases the capacity of females to be fertilized, decreases number of fetuses per litter and leads to an increase in abortions if stimulated within forty-eight hours of the post mating period (63). Moreover, physiological auditory stimuli also have a part in sexual behavior of some species. Cow vocalizations increase semen output and decrease the amount of time necessary for the bull to ejaculate into an artificial vagina; boar sounds increase the incidence of estrus posture in sows; and the male gobiid fish makes grunting sounds to which the gravid female responds (59). The courtship and mating of

many birds would be incomplete without their songs (1).

In addition to temperature, light and sound, there are other factors in the physical environment that can stimulate or inhibit the maturation or development of the gonads. Examples of these are rainfall, nesting sites, lunar influences, gravity and magnetic fields, however the effects of these stimuli were limited to effects on fertility rather than description of morphological or physiological changes in the gonads (59).

Effects of Social Stimuli on Testis Function

Although the physical environment plays an important role in sexual reproduction, animals are not limited to physical stimuli to alter testis function. Social stimuli are primarily perceived by olfactory, visual, auditory or tactile sensations whereas the physical presence of another animal (not necessarily of the opposite sex) may or may not be necessary to elicit the effect.

Individual behavior and endocrine responses vary in a group situation. Mice, rats, dogs and rabbits establish social hierarchies with or without fighting when grouped (6). Subordinant mice show adrenal hypertrophy and gonadal atrophy after being grouped for only 10 days (6). Increases in population density within limits lead to decreases in testicular, seminal vesicle and preputial gland weight in mice (6), probably reflecting smaller seminiferous tubules and partially inhibited spermatogenesis due to decreases in gonadotropin and androgen secretion.

In freely growing populations of mice a significant decline in activity and aggression rate were found as the population reached its upper asymptote. Adrenal weights increased, and testicular and

epididymal weights decreased in these animals (6) indicating that high density can lead to aggressiveness which is reflected in adrenal hypertrophy and gonadal atrophy. Voles, lemmings and muskrats show an inhibition of gonadal maturation and stopping of breeding earlier in the season when the population density is high (6). Aggressive behavior, traditionally a male characteristic, is related to androgen production and sensitization in the young (4). Dominant animals generally are the most aggressive and have larger testes and seminal vesicles indicating a higher androgen level than subordinates (6).

Both males and females are highly susceptible to social contact and the presence or absence of other animals may bring about numerous changes in reproduction. Female mouse sexual maturation is hastened by the presence of a male or male odors (58); olfactory bulb ablation terminates estrus cyclicity in adult mice (61); odors from strange males may block pregnancy after conception (5,41); and rhinencephalic lesions delay puberty in female rats (33). Females after 16 weeks in isolation (no social contact with either sex) were more irritable and hyperactive than controls and showed a decrease in thyroidal, splenic and ovarian weights (60). Offspring from isolated mothers showed retarded gonadal development (45).

Male rats housed for several months in rooms containing no females and caged either singly or in groups of four exhibited testicular atrophy and subsequent degeneration of secondary sex organs and a consequent loss of libido. Reversal of testicular atrophy was achieved after contact with females or female odors (50) indicating the importance of social stimuli in the male for the maintenance of testicular integrity.

Male guinea pigs reared socially and in isolation were castrated after the sexual performance of the social group was determined to be significantly higher than the sexual behavior of the isolated group. Ten weeks post-castration testosterone propionate injections were begun and each group returned to near the level of behavior characteristic of the pre-castration period, but the isolation group still remained lower in sexual performance than the socially reared group, suggesting that previous social experience may have great influence on the response of castrate rats to testosterone propionate (57).

By subdividing the function of the male reproductive system into its secretory and erection components and subjecting the animals to varying levels of social contact, Thomas and Neiman demonstrated that the secretory system is subject to androgen levels whereas exercise or some degree of tactile stimulation is important for maintenance of erection in rats (53). They suggest that some aspect of intromission stimulates the production of sufficient androgen to maintain the secondary reproductive organs, while the erection organs require practice in copulation or exercise to maintain them.

According to Beach male rats reared in isolation responded to sexual opportunities faster than males reared either with females or other males (2) whereas, male rats raised in cohabitation with sexually receptive females had larger accessory reproductive organs and kidneys post puberty than male rats raised with ovariectomized females or in all male groups (19). This would indicate that the presence of a receptive female is necessary for proper maintenance of testicular androgen secretion which conflicts with Beach's data cited earlier. Further experimentation has substantiated the latter idea for social conditions

and female odors play minor roles in maintaining male sexual behavior relative to the physical presence of a female which allows for heterosexual mating, the major factor in preventing atrophy of the male reproductive system (24).

Also in contrast to Beach's earlier work, in which isolated male rats were more sexually aggressive than socially reared rats, Folman and Drori found that male rats isolated from weaning exhibited disorientation and inability to achieve intromission in two to three copulation tests with receptive females (25). Gerall found that as the period of isolation prior to the first mating test increased, the ability to achieve successful mounts decreased, whereas light and dark, and lack of practice were not significant in reducing successful mounts (30).

From these observations it appears obvious that social contact both pre- and post-puberty is necessary to elicit the normal patterns of sexual behavior in male and female animals. Furthermore, isolation of animals severely handicaps their sexual performance and may influence their offspring adversely. All of these observations have dealt primarily with changes in behavior brought about by variations in social contact. Little experimental effort has been made to see how these changes in behavior are mediated and to see if the morphology and physiology of the reproductive organs is changed prior to, concommitant with or after a change in behavior.

Prepubertal habitation conditions of male mice have been found to alter the morphology of the reproductive system under several circumstances. Males raised in cohabitation with adult females exhibited larger testes and epididymides, larger seminiferous tubules and were significantly more sexually mature, than males raised in isolation or

with all males. These differences were not noticed if the males were raised with ovariectomized females. Seminal vesicles, prepuital glands and kidney weights (all androgen dependent organs) showed no significant differences between groups, "suggesting that pubertal acceleration is mediated primarily by the action of follicle stimulating hormone (FSH) rather than by interstitial cell hormone (ICSH) and its consequent androgen production" (26). These observations agree with those made by Vandenberg on the earlier attainment of first estrus by female mice exposed to males as opposed to non-exposed females (58). These effects appear to be due to gonadotropin stimulation in the young rather than to mating stimulation probably mediated by the gonadal hormones as discussed previously in adult rats.

Relationship Between External Signals and Internal Environment

From the foregoing discussion it is obvious that the external environment exerts many influences on reproduction and sexual behavior. It should be equally apparent that some mechanism operable within the organism must translate these external signals from the environment into internal signals to which the gonads are tuned. The interrelationships between the external and internal signal form the basis for the concept of a neuro-endocrine reflex, an explanation for the mechanism by which the external signals mediated via neurons trigger the release of chemical messengers which act on the gonads.

Early in this century observations were made relating the brain to control of reproductive function in dogs, but it wasn't until 1927 that Smith demonstrated that testicular atrophy invariably follows hypophysectomy (11). Replacement therapy with pituitary extracts and the fact

that testicular nerves are primarily vasomotor (31) implicated a hormonal substance rather than the nervous system in control of testicular function (62). The gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), have now been isolated in pure form and shown to prevent testicular atrophy following hypophysectomy (59). The pituitary and hypothalamus are intimately associated via the hypophyseal portal vessels (13,59) and this connection proved to be one of the missing links in the translation of external to internal signals.

Chemotransmitters (releasing factors) have been isolated from the hypothalamus which upon injection or infusion into the pituitary cause a release of FSH or LH (59). Hypothalamic lesions in the region of the arcuate nucleus and median eminence led to the absence of spermatogenesis, Leydig cell involution, and atrophy of secondary sex organs (11), further implicating the hypothalamus as the central connection in the neuroendocrine control of testicular function. These lesions did not interfere with other endocrine functions, and appeared to act by depleting pituitary stores of LH and FSH (11). Evidence indicates that the gonadotropins and androgens interact to control blood plasma levels of each other via a "negative feedback mechanism" (13,23,46). Various hypothalamic areas have proved sensitive to steroid hormone implantation leading to alterations in the testes, hormone levels and accessory gland weight (12,14).

Extra-hypothalamic pathways which affect male reproduction in some species include the limbic system, pineal gland and neocortex (11,59). Thus, the hypothalamus can be visualized as a giant "switchboard" receiving messages from every part of the body via chemical transmitters and nervous impulses and from the external environment via the special

senses and thalamus. "These messages are received, 'decoded', and 'decisions' quickly made, which are sent in the form of instructions over one or another outflow system." (51). Thus the hypothalamo-hypophyseal-neuroendocrine reflex provides the translation from external to internal signals.

Gonadotropins and Testis Function

Promotion of testicular growth by gonadotropins has already been mentioned. Now let us consider gonadotropin control of testicular function. Both testosterone and Δ^4-androstenedione have been isolated in spermatic blood, and administration of FSH, LH and human chorionic gonadotropin (HCG) to anesthetized dogs results in increased secretion of testosterone without increasing blood flow in the spermatic vein (22). Upon infusing dogs with acetate-1-^{14}C and administering HCG, LH or pregnant mare serum (PMS) the specific activity of testosterone-^{14}C increased. This indicated de novo synthesis of testosterone, rather than release of stored hormone (21). HCG increased urinary estrogens, free and conjugated urinary testosterone, and plasma testosterone. The effects of LH, FSH, and PMS on testosterone secretion in man are inconclusive (21).

In addition to the in vivo work on testosterone secretion, much data is now available on the in vitro effects of gonadotropins on androgen production. Testes slices from several species respond to PMS, HCG, LH, and FSH containing LH with increased rate of production of testosterone-^{14}C from acetate-1-^{14}C. It is thought that the primary effect of gonadotropins is on the interstitial cells of Leydig, but FSH is known to also have a spermatogenic effect (21).

After studying the effects of testosterone propionate (TP), LH and FSH on spermatogenesis in the hypophysectomized and normal rat, Clermont and Harvey concluded that neither hormone altered the rate of development of germ cells in the tubules. No treatment maintained the levels of spermatogenesis observed in normal rats and no marked differences were seen in the cell counts between groups. In hypophysectomized TP treated rats there was an initial drop in spermatogonial stem cells which plateaued after one cycle of the seminiferous epithelium (7). They suggested that rather than acting directly on the germ cells, the hormones act directly on the milieu in which the cells develop and hence a modification in the milieu would be reflected in the whole germ cell population. Later work has suggested that the gonadotropins are not necessary for the initiation of spermatogenesis up to pachytene spermatocytes, that testosterone may be necessary for type A spermatogonial formation and is necessary for the reduction division of primary spermatocytes. The completion of spermiogenesis requires FSH (7,48). Gonadotropins clearly exert a control over testicular function, but how they act is still a subject of much controversy.

Testicular Metabolism

The testis is composed of the seminiferous epithelium, which carries on the exocrine function of the testis, and interstitial tissue composed of the Leydig cells whose function is endocrine and Sertoli cells whose function is supportive. The seminiferous epithelium represents approximately 90% of the normal adult testes, and therefore metabolic data from normal testes largely reflects tubular metabolism (42). Conditions that result in degeneration of germinal epithelium

(i.e. longterm cryptorchid rats, x-ray treated rats, furacin fed rats, or hypoglycemic rabbits) reflect primarily the metabolism of the interstitial tissue (29).

Spermatogenesis commences at puberty and is a continuous process within the seminiferous epithelium (34). Spermatogenesis has been studied in great detail in several species (7,8,9,39,40), but for the present review only four classes of cells will be considered. The most immature cells are spermatogonia, then the spermatocytes, spermatids and finally mature sperm (31). Spermatogenesis in the mouse is divided into four cycles the duration of which is 34.5 days (39). In this experiment the seminiferous tubules of the mice treated for 35 days would be expected to have completed the four cycles once during the course of treatment.

The aspects of testicular metabolism of interest in this study were the metabolism of glucose to carbon dioxide, amino acid incorporation into protein and steroidogenesis (specifically the synthesis of testosterone-^{14}C and Δ^4-androstenedione-^{14}C from acetate-1-^{14}C.

By virtue of its position outside of the body cavity the scrotal testis is subjected to temperatures much lower than normal body temperature. The optimum temperature for lysine incorporation into testicular protein is 32^{o} C in the mouse and rat (15) indicating that the testicular protein synthesizing systems are more heat labile than similar systems in the liver, kidney and spleen which show maximum incorporation at 38^{o} C. There is data to indicate that glucose can protect against the inhibition exerted by high temperatures or protein labeling (15). A higher incidence of testicular protein labeling was found in the cryptorchid testis than the scrotal testis, indicating that the more

immature cell types (spermatogonia and primary spermatocytes) are responsible for protein biosynthesis (18).

Radioautographic studies indicate that in the absence of glucose the resting spermatocytes are the most heavily labeled cells in the seminiferous epithelium, while in the presence of glucose the pachytene spermatocytes and spermatids show the greatest degree of labeling (14, 16,18). This has lead to the hypothesis that temperatures exceeding 32^{o} C may lead to rapid utilization of glucose by the maturing spermatids, which quickly depletes the testicular glucose supply. The reduced glucose level then cannot protect certain temperature sensitive enzymes associated with protein synthesis against the elevated temperature. The result is irreversible damage to the testicular protein synthesizing systems of the rat testis (15). In rat testes the primary sites of glucose oxidation are the more advanced germinal cells (spermatocytes and spermatids (27)).

A high degree of dependence of the testes upon glucose was demonstrated by studying the incorporation of labeled lysine into testicular protein. Glucose increased the incorporation of lysine-^{14}C into labeled testicular protein by over 600% while the head of the epididymis showed a 150% increase and all other tissues tested showed less than a 50% increase in ^{14}C labeling (17). It appears then that there is a unique and characteristic stimulation of testicular protein biosynthesis by glucose and for this reason glucose is used in the incubation media. In addition to its affects on protein synthesis and tissue integrity (28), glucose has been implicated in contributing to the maximum oxygen consumption of testes (52) and increased ATP synthesis in the testes <u>in vitro</u> (37). The effect of glucose on the biosynthesis of testis proteins has

been shown to be due in part to increased amino acid transport but this is not sufficient to account for the entire effect (38). Other mechanisms which may be mediated by glucose acting via ATP are amino acid activation, RNA synthesis, or formation of peptide bonds all of which must preclude protein biosynthesis (38).

The elucidation of cholesterol synthesis from acetate and the subsequent synthesis of steroid hormones from cholesterol has made it possible to study the entire biosynthetic pathway of steroid hormones and the effects of the presence or absence of specific precursors on the synthetic process (36,54,59). Steroidogenesis in the testes is confined primarily to the interstitial cells of Leydig, but it has been difficult to show that the constituents of the seminiferous tubules play no role in steroid biosynthesis (34). Hall, Irby and deKrester have shown that seminiferous tubules stripped of interstitial cells failed to incorporate acetate-1-^{14}C into testosterone-^{14}C while the remaining cells did synthesize testosterone-^{14}C from labeled acetate (32). Both interstitial cells and seminiferous tubules converted progesterone-4-^{14}C to testosterone-^{14}C and androstenedione-^{14}C, but only the interstitial cells were able to use cholesterol-7-α-^{3}H as a substrate for testosterone-^{3}H or androstenedione-^{3}H biosynthesis (32). These observations indicate that only the interstitial cells have the enzymes necessary to synthesize androgens from cholesterol, and that the major biosynthetic mechanisms responsible for steroidogenesis reside in the interstitial elements of the testis.

It is suggested that the secretion of androgen by the seminiferous tubule exerts a local effect on the testis rather than being secreted into the general circulation. Glucose-U-^{14}C can act as a carbon source

for testicular steroids in addition to its role in the generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH), an obligatory cofactor for steroid synthesis, via the hexose monophosphate shunt (10).

The differentiation of the steroidogenic process in the testis is not a unidirectional process, but rather, a complicated pattern of stimulation and repression of enzyme synthesis or activity involved with androgen metabolism (49). Hypophysectomy, administration of gonadotropins, and the maturity of the animal all seem to alter the use of available substrates for steroid biosynthesis (49). The role of gonadotropins in steroid biosynthesis and the effects of steroids on the central nervous system have been alluded to above.

The multiplicity of external factors impinging upon the organism which can alter his reproductive behavior have been discussed and some explanation offered for their translation into an internal signal to which the testis and in turn the organism can respond. It was expected that factors which alter sexual behavior would in turn cause some alteration in testicular function and accessory gland morphology.

CHAPTER III

MATERIALS AND METHODS

Housing of Animals

Albino, male, 21-day old (weanling) ICR-Swiss strain mice were purchased from Canadian Breeding Laboratory, St. Constance, Quebec and used in all experiments. Immediately upon arrival, the animals were divided into three experimental groups: Group I - Isolation: one male per cage; Group II - Cohabitation: two males and one adult estrus female mouse (ICR-Swiss strain injected twice weekly with 100 µg estradiol benzoate in 0.1 ml cottonseed oil) per cage; Group III - Segregation: three males per cage.

Groups I and III were housed separate from Group II in a room in which no female mice were housed and both rooms were maintained on a photoperiod of 14 hours light and 10 hours dark at approximately 24° C. The isolation cages were compartmentalized so that all animals in each group had similar amounts of floor space (approximately 24 in^2/animal). Mice were provided with Purina Lab Chow and water ad libitum during the course of the experiment. The animals were maintained under these housing conditions for a period of either 3 or 5 weeks or until they were 42 or 56 days of age.

Preparation and Incubation of Testes

After 3 or 5 weeks in one of the three housing conditions, animals were sacrificed by cervical dislocation, the testes quickly removed and placed in ice cold 0.154 M KCl. The seminal vesicles, prostate and adrenals were subsequently removed and weighed. After removing the tunics from the testes, the seminiferous tubules were teased according to Umbriet et al. (55) and the testes from several mice (i.e. isolation 3 weeks) were pooled within treatments to obtain sufficient tissue for metabolic studies. The teased tubules (approximately 200 mg) were placed in incubation flasks with 3.0 ml of Krebs-Ringer-Bicarbonate, pH 7.4, (KRB) (55) containing 8 mM glucose (determined in preliminary studies to be a saturation level) and the appropriate isotope: D-glucose-1-^{14}C, 0.5 µc/flask; D-glucose-6-^{14}C, 0.5 µc/flask; L-lysine-U-^{14}C, 0.5 µc/flask; or sodium acetate-1-^{14}C, 25 µc/flask.

The glucose incubation flasks were gassed with 95% O_2-5% CO_2 and incubated in a closed system for 2 hours at 32° C in a shaking water bath. To stop the reaction 0.25 ml of 10 N H_2SO_4 was injected into the incubation vessel and 0.25 ml of Hydroxide of Hyamine (Packard Instr. Co., Downers Grove, Ill.) was injected into the center well to capture the evolved $^{14}CO_2$. The flasks were then shaken for an additional 2 h hours at room temperature. The trapped CO_2 was removed using 1 ml of methanol and transferred to a scintillation vial to which 10 ml of Bray's solution (3) was added.

The lysine and acetate incubations were gassed with 95% O_2-5%$_2CO_2$ and incubated in an open system at 32° C in a shaker bath. After 1 hour the lysine reaction was stopped by adding 0.3 ml 5 N perchloric acid (PCA) to the reaction flask and homogenizing the reaction mixture in

2 ml 0.5 N PCA. The acetate reaction was stopped after the flasks were incubated for 2 hours by homogenizing the entire flask contents in 5 ml 0.154 M KCl and storing the homogenates at -20° C.

Approximately 50 mg of teased tubules, from each treatment group, was placed in 2 ml 95% ethanol and stored at room temperature for analysis of nucleic acid content according to the method of Schmidt and Thannhauser (47). In addition, 50 mg of teased testis tubules were homogenized in 5 ml 0.154 M KCl and stored at -20° C prior to analyzing for total protein content according to the Lowry procedure (35).

Isolation of Acid Precipitable Material From Lysine Incubation

The PCA precipitable material from the lysine incubation was separated in a Sorvall superspeed refrigerated centrifuge model RC2-B at 19,5000 rpm for 20 minutes. The precipitate was resuspended in 1.5 N trichloroacetic acid (TCA) and placed in a 90° C water bath for 15 minutes, then recentrifuged. The precipitate was successively washed using 95% ethanol, chloroform:methanol (2:1), benzene, and ethyl ether. Finally, the precipitate was resuspended in 0.5 N NaOH and hydrolyzed by heating at 90° C for 2 hours. An aliquot (0.2 ml) of the NaOH hydrolysate was placed in a scintillation vial with 10 ml of Bray's (3) solution for scintillation counting.

Steroid Extraction and Separation Using Thin Layer Chromatography (TLC)

The homogenates which were incubated with acetate-^{14}C were thawed and placed in an extraction tube containing non-radioactive testosterone and Δ^4-androstenedione. The tube contents were extracted three times with 2.5 volumes of ice cold dichloromethane. The dichloromethane

extract was evaporated under nitrogen, reconstituted in 15 ml of diethyl ether, washed once with 0.1 N NaOH and twice with water. The extract was then concentrated, dried and dissolved in 3 drops of benzene. This solution was spotted on a thin layer plate and chromatographed in a solvent system containing chloroform:toluene:methanol:water (60:120:20:1). Thin layer plates were visualized under an ultra-violet lamp and material that had the same chromatographic mobility as authentic testosterone and Δ^4-androstenedione was scrapped off the plate and eluted from the silica gel with benzene. The benzene extracts were evaporated under nitrogen, concentrated, dried, dissolved in 3 drops of benzene, spotted as before and then run in a benzene:ethyl acetate (65:35) system. The testosterone and Δ^4-androstenedione spots corresponding to authentic standards were eluted into counting vials with 5 ml ethyl acetate which was evaporated under nitrogen before adding 10 ml of toluene scintillation cocktail (15.3 g PPO (2, 5 diphenyl oxazole) 0.153 g POPOP (1,4-bis-2-(5 phenyl oxazole)-benzene) dissolved in 3.79 ℓ of toluene).

Determination of Radioactivity

Radioactivity of all samples was determined using a Packard Tri-Carb Liquid Scintillation Counter Model 574. After adding 10 ml of scintillation fluid to each sample, counts were accumulated for 10 minutes (50% efficiency) twice against an internal standard and expressed as dpm per 100 mg of tissue.

Statistics

The data were subjected to analysis of variance by assuming a fixed model, two factor analysis, and if found to be significant Duncan's Multiple-Range Test was used to examine the difference between treatment means.

Chemicals

D-glucose-1-^{14}C (14.1 mCi/m mole, lot 292-178); D-glucose-6-^{14}C (14.7 mCi/m mole, lot 379-238); uniformly labeled-l-lysine-^{14}C (270 mCi/m mole, lot 459-233); and sodium acetate-1-^{14}C (56.9 mCi/m mole, lot 318-261) were purchased from New England Nuclear Corporation. All chemicals used in these experiments were analytical reagent grade and purchased from either Baker, Phillipsburg, N. J., Mallinkrodt, St. Louis, Mo., or Packard Inst. Co., Downers Grove, Ill. The solvents used in the extractions, TLC and scintillation counting were either Spectrar or Nanograde quality.

CHAPTER IV

RESULTS

The objectives of this study were: (1) to confirm previous studies on the effects of housing conditions of mice on testicular and accessory sex gland morphology and (2) to determine if changes in morphology are reflected in metabolic alterations in the testis.

The average body and organ weights for mice exposed to isolation, cohabitation and segregation for either 3 or 5 weeks after weaning are summarized in Table I. Body weights of mice treated for 5 weeks were significantly larger ($P < 0.05$) than those in similar social groups treated for 3 weeks. Testes and seminal vesicle weights of isolated and cohabitated mice treated for 5 weeks were larger than those of their counterparts after 3 weeks in similar housing conditions. The prostate glands of mice cohabitated for 5 weeks were slightly larger than those of mice cohabitated for 3 weeks, whereas mice exposed to isolation and segregation for 5 weeks had smaller prostates than their counterparts in the 3 week group. The adrenal glands of mice isolated or segregated for 5 weeks were significantly smaller ($P < 0.05$) than those in similar groups treated for 3 weeks. By contrast, there was no significant difference ($P > 0.25$) in adrenal gland weight following cohabitation for either 3 or 5 weeks. The adrenals of mice cohabitated for 5 weeks were larger ($P < 0.05$) than the adrenals of segregated or isolated mice treated for 5 weeks.

TABLE I

BODY AND ORGAN WEIGHTS OF MALE MICE REARED IN ISOLATION, COHABITATION OR SEGREGATION FOR EITHER 3 OR 5 WEEKS AFTER WEANING[1]

Treatment		Body Weight	Testes	Seminal Vesicles	Prostate	Adrenals
Duration	Social Group	g	mg%	mg%	mg%	mg%
3	Isolation	25.7** ±0.9	549.9** ±59.9	187.1*,** ±17.3	251.2 ±17.1	32.6** ±4.4
	Cohabitation	25.5** ±0.7	525.6** ±30.9	165.7*,** ±20.9	186.5 ±18.4	27.5** ±1.7
	Segregation	25.8** ±0.8	598.0** ±50.7	215.7*,** ±18.2	241.4 ±13.4	30.1** ±1.7
5	Isolation	30.6** ±0.9	685.0*,** ±17.5	224.7*,** ±17.3	238.6 ±15.1	21.9** ±1.9
	Cohabitation	31.0** ±0.7	722.5** ±20.7	242.0*,** ±17.7	254.5 ±15.3	26.0** ±1.5
	Segregation	30.5** ±0.7	679.7** ±22.8	282.7*,** ±26.8	238.3 ±13.3	21.7 ±2.9

[1]Each value represents the mean ± standard error for 18 mice.

*($P < 0.05$) for social group.

**($P < 0.01$) for duration of treatment.

The DNA and RNA content of testes showed no significant differences ($P > 0.25$) between social groups or duration of treatment (Table II). The concentration of total protein of mice cohabitated for 5 weeks was greater ($P < 0.05$) than that observed in mice segregated for 5 weeks or of mice housed in any social group for 3 weeks. Mice cohabitated for 3 weeks tended to show higher testicular protein levels than mice isolated or segregated for 3 weeks. Each social group had a higher protein content in the testes after 5 weeks of treatment than was observed after 3 weeks, although the differences were significant ($P < 0.05$) only in the cohabitated and isolated groups.

Metabolic data on the in vitro oxidation of glucose-1-^{14}C and glucose-6-^{14}C is summarized in Table III. The testes of mice exposed to isolation, cohabitation and segregation oxidized similar amounts of glucose-1-^{14}C and glucose-6-^{14}C to $^{14}CO_2$. In addition, there was no significant difference in glucose utilization between mice treated for either 3 or 5 weeks.

The data in Table IV show the results of the experiments on the incorporation of L-lysine-U-^{14}C into testicular protein. Cohabitated males had the greatest amount of testicular protein biosynthesis, but these differences were not significant ($P \cong 0.07$). There was relatively little change in the amount of lysine-U-^{14}C incorporated into testis protein after treatment for 3 and 5 weeks.

The results for the metabolic transformations of acetate-1-^{14}C into labeled androgens are summarized in Table V. The segregated males showed the greatest incorporation of labeled acetate-1-^{14}C into testosterone in both the 3 and 5 week treatment groups. In contrast, incorporation of acetate-1-^{14}C into Δ^4-androstenedione was greater in the

TABLE II

NUCLEIC ACID AND TOTAL PROTEIN CONTENT OF TESTES OF MICE REARED IN ISOLATION, COHABITATION OR SEGREGATION FOR EITHER 3 OR 5 WEEKS AFTER WEANING[1]

Treatment		Total Protein	Nucleic Acid	
Time	Social Group	µg/mg tissue	DNA	RNA
			µg/mg tissue	
	Isolation	50.33* ±4.99	6.33 ±0.22	1.22 ±0.07
3	Cohabitation	53.81* ±6.08	6.87 ±0.53	1.27 ±0.11
	Segregation	50.41* ±3.05	6.43 ±0.52	1.28 ±0.10
	Isolation	58.49* ±3.70	6.29 ±0.27	1.22 ±0.03
5	Cohabitation	61.02* ±5.47	6.55 ±0.29	1.31 ±0.09
	Segregation	53.02* ±6.17	6.28 ±0.24	1.31 ±0.07

[1]Each value represents the mean ± standard error for six determinations.

*($P < 0.01$) for social group and duration of treatment.

TABLE III

EVOLUTION OF $^{14}CO_2$ FROM EITHER GLUCOSE-1-^{14}C OR GLUCOSE-6-^{14}C FROM TESTES OF MICE REARED IN ISOLATION, COHABITATION OR SEGREGATION FOR EITHER 3 OR 5 WEEKS AFTER WEANING (DPM/100 MG TISSUE)[1]

Treatment		$^{14}CO_2$ from G-1-^{14}C	$^{14}CO_2$ from G-6-^{14}C
Duration	Social Group		
	Isolation	29,074 ±6,445	26,708 ±3,677
3	Cohabitation	31,235 ±4,557	22,784 ±2,448
	Segregation	34,913 ±4,342	25,610 ±2,935
	Isolation	35,848 ±4,655	26,019 ±3,148
5	Cohabitation	24,897 ±5,867	28,743 ±4,044
	Segregation	32,107 ±3,819	27,574 ±3,654

[1]Each value represents the mean ± standard error of six determinations.

TABLE IV

INCORPORATION OF L-LYSINE-U-^{14}C INTO TESTICULAR PROTEIN OF MICE REARED IN ISOLATION, COHABITATION OR SEGREGATION FOR EITHER 3 OR 5 WEEKS AFTER WEANING[1]

Treatment		Testicular Protein
Duration	Social Group	
	Isolation	1259 ±228
3	Cohabitation	1421 ±204
	Segregation	1232 ±186
	Isolation	1147 ±149
5	Cohabitation	1484 ±249
	Segregation	1170 ±157

[1]Each value represents the mean ± standard error for six determinations.

TABLE V

INCORPORATION OF ACETATE-1-^{14}C INTO TESTOSTERONE-^{14}C AND Δ^4-ANDROSTENEDIONE BY TESTES OF MICE REARED IN ISOLATION, COHABITATION OR SEGREGATION FOR EITHER 3 OR 5 WEEKS AFTER WEANING[1]

Treatment		Testosterone	Δ^4-androstenedione
Duration	Social Group		
3	Isolation	27.5 ± 4.8	320 ±105
	Cohabitation	30.0 ± 5.3	517 ±216
	Segregation	78.3 ±33.2	445 ±155
5	Isolation	69.0 ±23.7	444 ±121
	Cohabitation	65.2 ±22.3	350 ± 41
	Segregation	73.0 ±33.5	543 ±137

[1]Each value represents the mean ± standard error for six determinations.

isolated and segregated males treated for 5 weeks, but less in the cohabitated males, than in their counterparts in the 3 week group. Although these differences were not significant, a greater amount of acetate-1-^{14}C was incorporated into Δ^4-androstenedione-^{14}C than into testosterone-^{14}C regardless of the treatment imposed.

CHAPTER V

DISCUSSION

The results of this study indicate that deprivation of social contact causes changes in the accessory sex organs and in some aspects of testicular metabolism in mice. However in the present experiment some of these changes are the result of the age of the animal as well as the social group in which it was treated.

The increase in body weight noted between the 3 and 5 week treatment groups may be attributed to the differences in age of the animals, since the 3 week group had not passed through puberty whereas the 5 week group may be regarded as post-pubertal (26). The testes weights of cohabitating males were smaller than either the isolated or segregated males after 3 weeks in their particular social group, whereas after 5 weeks in their respective groups the cohabitating males had larger testes than either of the other groups. This data substantiates similar results reported by Fox (26) and suggests that the prepubertal mouse is less susceptible to influences from females with regard to sexual maturation than is the post-pubertal animal. Moreover, these results suggest that cohabitation accelerates testis growth. In the adult animals, the slightly smaller testes of the segregated males could reflect an adrenal-gonadal response to stress imposed by the social group, heirarchy formation and fighting. Christian has reported that male mice establish heirarchies when grouped with one animal

dominant and others subordinate (6). These animals usually exhibit adrenal hypertrophy and gonadal atrophy in response to this stressful situation (6). However, in this experiment, adrenal weight did not increase with increased length of treatment so there is no evidence to support the idea of adrenal hypertrophy. Increases in adrenal weight are not always indicative of hypertrophy and must be coupled with increases in cortical width to suggest hypertrophy conclusively (26).

The changes in prostate weight reflect a pattern similar to that of the testes in which the smallest prostates were found in males cohabitated for 3 weeks and the largest prostates noted in males cohabitated for 5 weeks. This data supports the concept that the presence of a female accelerates reproductive development in post-pubertal male mice. Furthermore, this concept was substantiated by the fact that little difference was observed in prostate weight among the isolated or segregated males.

Seminal vesicles are generally more sensitive than the prostate to androgen stimulation or deprivation (54). This is reflected by the very small differences observed between prostate weights among social groups especially in the adult animal as compared to the larger differences in seminal vesicle weight among social groups. The influence of the female on the adult animal was observed as evidenced by the greater ($P < 0.05$) increase in seminal vesicle weight in the mice cohabitated for 5 weeks versus 3 weeks, compared to the smaller weight gains evidenced by isolated males. Deprivation of all social contact from weaning to adulthood appears to contribute to seminal vesicle atrophy. Since androgens stimulate accessory sex organs it is reasonable to suggest that the smaller seminal vesicles of adult isolated mice may be

associated with a reduction in androgen synthesis. This assumption appears justified since animals lacking social contact for 3 weeks showed less labeled acetate incorporation into testosterone and Δ^4-androstenedione than either of the groups experiencing social contact. Androstenedione, an immediate precursor of testosterone, showed a higher degree of labeling from labeled acetate than did testosterone, indicating a reduction in the activity of the enzyme which converts Δ^4-androstenedione into testosterone since these animals had not passed through puberty. The drop in acetate incorporation into both testosterone and Δ^4-androstenedione in the 5 week cohabitating males is not understood at this time.

The relatively constant DNA levels in all social groups for both durations of treatment indicate that at these stages of development the testes were operating on a plateau that appears to be independent of social stimuli and age of the animals. The RNA content of the testes was also relatively constant among treatment groups. Significant differences ($P < 0.05$) were noted in the total protein content of cohabitated and isolated mice treated for 3 weeks compared to their counterparts in the 5 week groups. The cohabitated males treated for 5 weeks showed higher ($P < 0.05$) testes protein levels than segregated males. Although the differences were not significant at 3 weeks, the cohabitated males did show higher protein levels than either isolated or segregated males. This would indicate that the physical presence of a female may accelerate protein biosynthesis in some undetermined manner. It was hoped that a change in RNA content could be shown and correlated with the change in protein content to further substantiate the idea of increased protein synthesis with heterosexual social contact. Although

this did not occur it is possible that the method used to determine RNA failed to distinguish between the many types of RNA present in the testis and no attempt was made to fractionate the testicular tissue into subcellular components to facilitate quantitation of various types of RNA.

Increased protein biosynthesis is also indicated by the incorporation of labeled lysine into mouse testicular protein. Cohabitating males showed greater amounts of testicular protein from the labeled precursor than either the isolated or segregated males in both age groups. Little variation was noted in the degree of incorporation of lysine-^{14}C for a given social group regardless of the duration of treatment. These data also suggest that the presence of the female accelerates protein biosynthesis.

The testis has a unique dependence on glucose as an energy source for several metabolic processes that is not found among several other organs (15). Thus, it would seem that one mechanism by which testicular metabolism could be altered is in the oxidation of glucose to carbon dioxide to supply energy for metabolic processes. Testes tubules incubated with glucose-1-^{14}C or glucose-6-^{14}C from the different groups showed no consistent changes for either isotope in any of the social or age groups tested. The shift in glucose-6-^{14}C oxidation by the cohabitating males from the lowest after 3 weeks to the greatest amount of $^{14}CO_2$ evolved after 5 weeks may correspond to an increase in steroid biosynthesis in the adult animal, because glucose-6-^{14}C is utilized by the hexose monophosphate shunt which produces NADPH, an obligatory cofactor in steroid biosynthesis (10).

Further studies on larger groups of animals with more replicates

would reduce the variability observed in some of the data reported here and would be useful in elucidating further the effects of social contact imposed by housing conditions on testicular metabolism. Experiments in which animals were housed under the three social conditions for several different periods of time would suggest more conclusively whether the effects of social contact are age dependent as was suggested by the data reported from this study. The animals in the 3 week treatment group were just reaching the age of puberty and since numerous physiological changes are known to occur at this time, perhaps the effects due to social stimulation were masked. Since weanling mice were used in all experiments, it is possible that the social contact with litter mates and the mother from birth to 3 weeks of age was a factor in the response the mice made to subsequent social grouping post weaning, for other workers have suggested that previous experience is an important factor in response to hormone treatment and to successful mating (25,30,56,57).

CHAPTER VI

SUMMARY AND CONCLUSIONS

Both the endocrine and exocrine functions of the testes are subject to influences from the internal and external environment. One of these external factors, social contact or its deprivation, has been shown to alter the morphology of the testes and to cause alterations in the accessory sex organs, and these changes are reflected in behavioral modifications. These experiments were designed to show that the changes in behavior and reproductive organ morphology by varying levels of social contact are preceeded by changes in testicular metabolism and function.

Male mice were reared in isolation, cohabitation or segregation for either 3 or 5 weeks post weaning after which the following criteria were used to assess the effects of social contact on testicular metabolism: total protein and nucleic acid content, glucose-1 and glucose-6 oxidation to carbon dioxide and protein and androgen biosynthesis using labeled precursors.

The results indicate that the presence of a female is important in maintaining accessory sex gland integrity and some aspects of metabolism in the testes. Total protein content, lysine incorporation into protein and acetate incorporation into androgen were generally greater in cohabitating males treated for 5 weeks. Seminal vesicles atrophied when the animals were deprived of social contact. Nucleic acid content of

testes and glucose oxidation to carbon dioxide was not affected by social grouping.

Further experiments on larger numbers of animals treated for several time periods would be useful in determining if the social environment of an animal has its greatest effects pre-puberty, at puberty or post-puberty. This data could be of great importance in the breeding and rearing of animals for scientific research, especially in the area of reproductive physiology.

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