MODIFICATION OF GONADAL DIFFERENTIATION IN THE MALE GOLDEN SYRIAN HAMSTER AS A CONSEQUENCE OF CYPROTERONE ACETATE ADMINISTRATION

by

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS (cont.)

PAGE

APPENDIX PAGE

ABSTRACT

Gravid Golden Syrian Hamsters were injected with an antiandrogen cyproterone acetate (a synthetic progestin, SH 714) prior to and during implantation of their embryos. At term, the male pups were examined and compared to controls. Neonatal body and testicular weights were reduced two and three times, respectively, when compared with controls. The testicular histology of the experimental pups was strikingly different from that of the controls. Preferential periodic acid-Schiff's staining for mucoproteins and glycoproteins appeared diminished or absent in the testicular sections when contrasted with the intense magental color seen in the control sections. Mitotic figures were also absent in the testicular sections from the experimental group. Furthermore, the spermatogonial and interstitial types of cells appeared increased in number and hyperchromaticity, but decreased in size when visually compared to controls. The changes in PAS stainability and cellular division were not found in non-reproductive, systemic tissues such as liver and small intestines. The implication of these findings is that embryonic, male gonadal differentation may be under androgen (i.e., hormonal) influence since the differentiation phenomen was hindered by exogenously altering hormonal balances with administration of cyproterone acetate.

INTRODUCTION

Gonadal differentiation is defined for the present study as the progressive development of the primordial reproductive organ to its adult state. The study of gonadal differentiation provides a basis for understanding the normal as well as the abnormal aspects of morphologic sex differentiation. In the past, investigations into embryonic or fetal morphologic sex differentiation [4,38,39,40,84,85] have been concerned with studying the developmental stages of the accessory reproductive organs, their associated glands (e.g., the female Bartholin's glands and the male prostatic gland) and the external genitalia. However, observations [50,51,60,66,88] of endogenous, aberrant gonadal differentiation stimulated the consideration of experimental (i) alteration of gonadal sex [6,7,9,37,44,78] or (ii) postponement or inhibition of gonadal differentiation.[69,78] It is emphasized that the present study was not concerned with alteration of the phenotypic sex of the animal as a whole, but was specifically concerned with the alteration of normal testicular differentiation.

Studies by Neumann, Elger and Kramer[61] demonstrated that administration of a Schering, synthetic progestin, cyproterone acetate (10 mg/Kg body weight/da) to gravid rats from the 13th through the 22nd day of gestation and to the pups for the ensuing 21 days induced the development of vaginae in the male animals.

In addition, such "induced" vaginae were cyclic in response to exogenous estrogen when the pups reached puberty. The administration of cyproterone acetate (also referred to as SH 714) furthermore caused the pups to develop nipples and the potential for mammary gland hypertrophy. The reproductive ducts and seminal vesicles, however, did not differ from the controls; and, rudimentary prostatic glands were present. It is important to note here, that the testes of the neonatal, experimental group appeared histologically identical to those from the control neonates. Treatment of gravid rats with cyproterone acetate from the 7th through the 21st days of gestation only accentuated the forementioned, developmental alterations in the reproductive organs of the male pups.[22] Similar findings have been obtained from studies in the mouse, dog and rabbit[62] and hamster.[53]

At this point it is pertinent to elaborate on the findings of Forsberg and Jacobsohn.[22] Their studies with cyproterone acetate involved the administration of the compound (10mg/Kg body weight/da) to gravid rats from either the 7th, 14th or 15th day of gestation to the end of gestation (i.e., day 22). Their observations indicated that regardless of when their treatments were begun, the final effects seen in the neonates were essentially the same. In all of the male pups, there were definite structural alterations of the accessory reproductive organs and external genitalia. However, the histology of the testes from experimental neonates had a "...normal appearance" and "...was as in the controls." Where alterations were found in the histology of the testes, these occurred in male pups that had been additionally treated with SH 714 for the first 21

postnatal days and examined three months after birth. This finding was not inconsistent with their other data for the following reason. Cyproterone acetate is known to affect the histology of the mature (adult) testis in the rat[52,72] and in man (C.G. Heller and M. Rowley - personal communication). Since rat pups reach puberty 3 weeks post-delivery, Forsberg and Jacobsohn's administration of SH 714 during those three weeks should be expected to affect the testes regardless of prenatal treatments. The common denominator of the treatment schedules followed by Neumann et al.,[61,62,64] and Forsberg et al.,[22,23] was that all of their maternal injections were begun after the implantation of the embryos(day 5 of gestation for the rat) had already occurred. Apparently the presence or absence of the placenta had little bearing upon the outcome of their treatments (the vascularization of the rat placenta is complete about day 12.5).

From these observations of Forsberg et al.,[22,23] one can assume that the direction of the male gonadal differentiation is fixed either before or soon after implantation of the embryo in the uterus. Perhaps it would be more nearly correct to state that the direction of the primordial germ cell potential is fixed at this time. From their post-implantation treatments, Neumann et al.,[62] have suggested that male gonadal differentiation does not depend upon androgenic (i.e., hormonal) influence since their administration of cyproterone acetate did not alter the testicular picture. This point will be again considered in the "DISCUSSION".

Based on these earlier studies with cyproterone acetate, we questioned what would happen to the normal differentiation of the

MATERIALS AND METHODS

General

The classic studies by Graves,[25] Bond,[3] Ward,[82,83] Venable[79] and more recently of Hoffman, Robinson and Magalhaes[35] on the Golden Syrian hamster (Cricetus auratus), have facilitated the basic understanding of this species for experimental embryologic study. The animals themselves are generally of the good temper necessary to sustain the trauma of consecutive injections and they are a clean animal with which to work. Ward's work[83] demonstrated that the female hamster is sexually responsive and comes into heat every fourth night unless the particular strain exhibits a seasonal control over its cycling. Regular cycles facilitate the laboratory breeding of these animals. Also important and convenient for this study is the short period of gestation (about 15.5 days). This characteristic obviously shortens the waiting period for neonatal observations.

Sixteen mature, three month old, Golden Syrian female hamsters (Lakeview Colony, New Jersey) weighing 100-120 grams, were mated with adult males. The probability of pregnancy was determined by microscopic observation of vaginal smears obtained immediately after a copulation interval of 15 minutes. The animals were housed separately in wire mesh cages under conditions of controlled light (6 a.m. to 6 p.m. - lighted) and room temperature conditions.

Four days before the end of their gestation, the animals were transferred to individual plastic boxes containing San.i.cel (Paxton Processing, Portland, Oregon) bedding. Their diet consisted of tap water and Purina Laboratory Chow supplemented twice a week with leafy greens. Retaining the animals in wire mesh cages until four days before delivery apparently did not cause any ill effects as they readily nested in the plastic boxes with commercial bedding.

At 70, 82, 94, 106 and 118 hours post-copulation, the experimental groups of 11 female hamsters were given single, intraperitoneal injections of 10 milligrams per 100 grams body weight of cyproterone acetate. The SH 714 had been dissolved at a concentration of 50 milligrams per milliliter in a 2:25 solution of benzyl benzoate-castor oil. Each hamster in the experimental group thus received a total of 50 milligrams of cyproterone acetate in one milliliter of vehicle. Each of the five control animals received an injected quantity of the vehicle identical to that for the experimental animals. The manner in which this dose level of 50 milligrams was determined is discussed in the "APPENDIX - PRELIMINARY STUDIES - DOSE RESPONSE."

The post-copulation injections in this study coincided with approximately the 41st, 29th, 17th and 5th hours before implantation, and 3 hours, post-implantation. In the hamster, following successful impregnation, implantation occurs approximately 111 hours post-copulation. Obviously, this time is not absolute but can vary within a limited range ($\pm$ 7 hours) depending on the

individual animal.[83] In any case, our treatments were given essentially before and up through implantation.

At the end of gestation (15.5 days) the animals of the control groups delivered their young by natural birth. The cyproterone acetate-treated mothers were anesthetized with ether and their pups obtained by Caesarian section. Some control mothers were also etherized and their offspring obtained by Caesarian section to determine if the anesthetization and/or natural birth had any effect on the results. The body weights were recorded and immediately both testes were removed and weighed while wet. (The term "testes" is used here since these gonads were found in the suprainguinal region of the neonates as opposed to the subrenal location of the neonatal ovaries.) The accessory reproductive organs in the control and experimental groups were too small to be readily dissected and weighed.

In addition to the testicular tissue from the neonates of the sixteen females mentioned above, sections of liver and small intestines were obtained from control and experimental neonates of other females. These sections were stained with hematoxylin and eosin.

Following the observations made on the gonadal tissues (see "RESULTS - Light Microscopy"), a subsequent effort was made to obtain gonadal tissues from 12, 13, 14 and 15 day-old fetuses of mothers similarly treated with cyproterone acetate. The purpose of this was to examine any sequential effects of cyproterone acetate administered prior to and up through implantation. Histologic preparation of these tissues was identical to that described below for the neonatal gonads.

Light Microscopy

The excised, right testes from the control and experimental pups were placed directly into Helly's fixative (Zenker's formol solution). Subsequent dehydrations in ethanol, clearing in cedarwood oil and embedding in 6% Piccolyte S0115 Paraplast were carried out in the manner suggested by Rowley and Heller.[70] Sections of the testicular tissue were cut at a 5 micron thickness on an "AO Spencer" microtome. The mounting of the control and experimental tissue sections onto histologic slides and the subsequent, simultaneous staining procedure were also carried out in the manner suggested by Rowley and Heller[70] for the periodic acid-Schiff's reaction. The only modification of their procedure employed a decrease in the length of time suggested for the Harris' hematoxylin counter-staining from 1 minute to three seconds. Stained sections were viewed and photographed with a Zeiss photomicroscope.

Quantitative data were also obtained with respect to comparing the number of nuclei per microscopic field in control and experimental tissues. A microprojector was used to magnify the microscopic fields with a high-dry objective lens for counting nuclei. The actual image projected onto a white screen was a circular field. Because of the tedious nature of the counting process, only one-quarter of any circular field was used for quantitative purposes. As the circular microscopic field image remained constant in area, it was also possible to keep constant the quarter portion size of any projected image on the screen by means of a cardboard frame attached to the microprojector lens. Several

kodachrome slides of the microscopic fields were also employed for similar quantitation. However, because of the technical difficulty in taking kodachrome slides of large microscopic fields, the actual areas counted by this latter method were limited.

DNA determination

The left testes from the control and experimental pups were removed and excess connective tissue and fat were carefully removed from around the organ. The testes were then individually weighed on pre-weighed powder paper and placed into 0.7 milliliters of distilled ice water in glass centrifuge tubes. A crude homogenate was obtained by freezing and thawing the solutions of tissue and water 6 times in a dry ice-acetone bath to promote cellular autolysis. Nucleic acid extraction was carried out with 0.6 milliliters of the homogenate using the method described by Schneider.[73] (See "APPENDIX")

The DNA determination was carried out on the nucleic acid extraction preparation according to the method described by Burton.[8] (See "APPENDIX") DNA standards were prepared each time and their optical densities read along with those of the extracts on a Beckman DB-G spectrophotometer.

RESULTS

The body and left testicular weights of the control and experimental neonates are shown in Table 1 ("APPENDIX") along with their p-levels of significance. Both the body and left testicular weights of the experimental pups were significantly less than those of the control pups.

In the uterine horns of every cyproterone acetate-treated mother, there were indications that implantation, some development and subsequent resorption of a number of fetuses had occurred. These indications were the gross findings of amorphic entities attached to the lumen walls. Moreover, these entities were evenly and linearly spaced relative to the amniotic sacs present with completely formed hamster fetuses. The total numbers of pups for each of the cyproterone acetate-treated mothers were less than those for the controls, i.e., control pups numbered from nine to fifteen in a litter per mother compared to experimental pups who numbered from one to four per mother. It is a reasonable consideration that the difference in litter size between the two groups was a consequence of the cyproterone acetate treatment.

Light Microscopy - control sections (Figures 1,2,5,6,7)

The prominent feature seen is the moderate intensity of the periodic acid-Schiff's (PAS) magenta stain in the basement membranes of the seminiferous cords and in the interstitial ground

substance. This phenomenon is as would be expected since some of the structural entities making up these tissues are mucoproteins and glycoproteins sensitive to the PAS reaction. The relative differences between the intensities of staining throughout the sections reflect the variable amounts of connective tissues present. Also evident in the interstitial fields are outlined gaps that could be attributable to the extraction of lipids during the ethanol dehydrations required for clearing and staining the tissue sections. Lipid staining itself was not done. Many ovoid, mesenchymal cells (fibroblasts?) are present in the interstitial areas along with occasional, fetal Leydig cells. The distinguishing feature of the latter is their prominent, eccentric round nucleus with a prominent, dense nucleolus. These cells are considered to be of the fetal type because (i) the cytoplasm appear sparse or absent as would be expected if cytoplasmic lipids were present in the fetal Leydig cells;[65] and, (ii) the adult Leydig cells do not normally appear until puberty is reached (when the pup is three weeks old). Occasional mitotic figures are also found in the interstitial areas. Whether they represent dividing mesenchymal cells or Leydig cells cannot be discerned at this time.

The basement membrane (the membrana propria) surrounding each seminiferous cord contains long, flat, dark nuclei. This membrane is also generally considered to have constituents of reticular and elastic fibers. The internal architecture of the cords is well organized. Along the inner margin of the membrane an occasional Sertoli cell is encountered. The Sertoli cell is distinguished by its large, dark nucleolus, dustlike chromatin and a few chromophilic

clumps along the nuclear membrane. The nuclear membrane also exhibits one or more infoldings and the cytoplasmic processes are in contact with adjacent spermatogonia.

Within the seminiferous cords are the light or dark nuclei of three of the presently known types of spermatogonia.[71] According to the earlier classifications of Leblond and Clermont,[46] Clermont and Perey,[10] Perey, Clermont and Leblond[68] and Clermont and Huckins,[11] these three are type A, type In (intermediate) and type B. The former two line the inner aspect of the basement membrane and the latter type constitutes the core of the seminiferous cords. Type A and type In are similar in that they both have ovoid nuclei and one or more hyperchromatic nucleoli. Their cytoplasm is difficult to distinguish; but where seen, is weakly positive to the PAS staining. These two spermatogonial types differ from each other in the densities of their chromatin granulations. The In type has a nuclear membrane, well delineated by the adherence of deeply stained clumps of chromatin. The nuclear membrane of type A is not as well delineated. Therefore,the In type nuclei appears darker than those of type A.

Type B spermatogonia are easily distinguished from the other types by their large, spherical nuclei. Here, the chromatin granulation is so fine that each nucleus is homogenously dark. Delineation of their nuclear membranes by clumps of chromatin is not as evident as that of type In. Nucleoli are dark and peripheral or eccentric. The cytoplasm is weakly PAS-positive and appears vacuolated. The cell borders are evident.

Various stages of mitosis are present within different seminiferous cords. These represent the normal, mitotic replication of the spermatogonia. Spermatocytes are not evidenced.

Light Microscopy - cyproterone acetate sections (Figures 3,4,8,9)

The histology of the experimental sections is quite different from that of the controls. The PAS reaction is seen as a very pale pink (i.e., weakly positive). As these sections were stained simultaneously with controls, this color difference, contrasted with controls cannot be considered an artifact of histologic technique. The large, clear, interstitial gaps are again present. Mesenchymal cells (fibroblasts?) are prominent in the interstitial fields and some have very hyperchromatic nuclei. An increase in the number of Leydig cells is seen. These Leydig cells exhibit an acidophilic cytoplasm. Presumedly, ethanol dehydration during the histologic process did not effect their cytoplasm as it did those of the control Leydig cells.[65]

The basement membranes of the seminiferous cords appear intact. However, the germinal epithelial architecture is poorly organized. Gaps are evident between single spermatogonia and between groups of spermatogonia. The marginal, spermatogonial cell types are difficult to differentiate from each other because of their hyperchromicity. It is noteworthy that the central spermatogonia appear smaller than their counterparts in the controls. Spermatogonium type In is seen as being more prevalent than type A. Type B, as described above for the controls, cannot be specifically

demonstrated. Either it is absent from the cords or too closely resembles type In. The Sertoli cells can be distinguished from the spermatogonial types by their infolded nuclear membranes. No mitotic stages are evidenced throughout the microscopic fields.

Light Microscopy - quantitative

Table 2 ("APPENDIX") compares the data obtained by counting the nuclei in control and experimental tissues as described in the "MATERIALS AND METHODS" section. P-levels were not calculated since the control and experimental values were of obvious difference and significance. The ratio between the two groups was 1 to 2.47, control to experimental. The ratio between the two groups obtained from counts made from the kodachrome slides of smaller microscopic fields was 1 to 1.84, control to experimental. The lower ratio obtained in the kodachrome counts may be attributed to the smaller sample number available for counting from the smaller fields on the kodachrome slides.

Light Microscopy - nonreproductive, systemic tissues (Figures 10 & 11)

When our observations of the gonadal tissues indicated that the lack of mitotic figures and the loss of periodic acid-Schiff's staining sensitivity were prominent histologic features of the experimental testes, it became necessary to determine whether these phenomena were limited to the testes or also found in non-reproductive, systemic tissues. Following a microscopic examination of liver, small intestines and other tissues of hematoxylin and eosin stained, serial sections of 12 through 14 day-old fetuses, we determined that mitotic figures were present in both the control

and experimental tissues. The intensities of PAS stained sections were comparable between the two groups. These observations seem to imply that the mitotic inhibition by cyproterone acetate was limited to the testicular tissue.

Light Microscopy - 12 to 15 day old fetal gonadal tissues (Figures 12 through 19)

12-day old fetal gonad - control (Figure 12)

The histologic picture seen is that of the indifferent or undifferentiated gonad. In a transverse section of the fetus, the gonads are positioned ventromedial to the fetal kidney and covered by a layer of coelomic epithelium, the "germinal" epithelium. The internal architecture of the gonad consists of a loosely arranged epithelial mass. The nuclei seen in the epithelial mass are those of primordial germ cells and mesenchymal or fibroblast cells. The primordial germ cells are distinguished by their large, round nuclei and prominent centric or eccentric nucleoli. Some cells also have a clear perinuclear space, probably representative of cytoplasmic organelles. Periodic acid-Schiff positive strands extend among groups of cells of the epithelial mass. These strands probably represent the earliest stages of intercellular material separating primitive sex cords which extend from the surface epithelium and become associated with the primordial germ cells. Mitotic figures are particularly evident in the germinal epithelium.

12-day old fetal gonad - experimental (Figure 13)

The histologic picture of the indifferent gonad is similar to that of the 12-day control described above. However, the periodic acid-Schiff positive strands interspersed among the cells of the epithelial mass are not as evident; and, mitotic figures are absent.

13-day old fetal gonad - control (Figure 14)

The gonad continues to exhibit the histologic appearance of the undifferentiated state. The picture is not much changed from that of the 12-day gonad except that there is an increase seen in the presence of the mesenchymal or fibroblast components. Their nuclei are primarily seen in the branching and anastomosing strands between the primitive sex cords previously described. Mitotic figures are present.

13-day old fetal gonad - experimental (Figure 15)

The absence of mitotic figures and diminution of the periodic acid-Schiff staining distinguish this gonad from the control. The presence of the elongated nuclei of the mesenchymal or fibroblast cells, interspersed between the cells of the epithelial mass, is evident.

14-day old fetal testis - control (Figure 16)

Grouping of primordial germ cells and of spermatogonia type A, transformed from primordial germ cells, has occurred and is

easily demonstrated by the periodic acid-Schiff staining of connective tissue strands surrounding these groups. After the indifferent gonad has begun to differentiate towards the male direction, the strands, previously described for the 13-day old gonad, contribute to the formation of the tunica propria surrounding each seminiferous cord. In a transverse section of the testis, the seminiferous cords are seen as not having yet developed tortuosity because their cross-sections are round or oval in shape. Such tortuosity would represent an obvious consequence of longitudinal growth of the cords within the space limited by the capsule of the testis. Within the seminiferous cords are the nuclei of the Sertoli cells and of what are probably spermatogonia of type A. The Sertoli cell nuclei are seen predominantly in the "parallel" position.[46] Leydig cells are not evident in the interstitial area.

14-day old fetal testis - experimental (Figure 17)

The early configuration of the seminiferous cords is not as well demonstrated in the sections of these testes because of a diminution in the periodic acid-Schiff staining of connective tissue strands and the early tunica propria. Also apparent is the hyperchromaticity of the nuclei both within and outside of the seminiferous cords.

15-day old fetal testis - control (Figure 18)

The histologic sections demonstrate an increase in the interstitial spaces between the seminiferous cords. Evident within these spaces are the elongated nuclei of the fibroblast cells and

adipose cellular outlines. A slight tortuosity in some of the sections of seminiferous cords is present. Within the cords are the nuclei of the Sertoli cells and spermatogonia types A, In and B. Mitotic figures and periodic acid-Schiff staining are present.

15-day old fetal testis - experimental (Figure 19)

The histologic picture of this testis differs from the 15-day control sections by its lack of mitotic figures, diminished to absent periodic aicd-Schiff staining and the hyperchromaticity of nuclei within and outside of the seminiferous cords. Slight tortuosity of the seminiferous cords and the increase in interstitial spaces are present as in the controls.

DNA Determination - testicular tissue level (Table 1)

Analytic procedures were carried out on the control and experimental testicular tissues in order to determine whether or not a gross correlation could be demonstrated between the presence (controls) or absence (experimental) of the mitotic figures in the histologic sections and the total DNA content per control and per experimental testes. The mean total DNA content per experimental testis (14.5 ug $\pm$ 5.5) was not significantly different from that of the controls (14.9 ug $\pm$ 4.0).

DISCUSSION

Cyproterone acetate (SH 714) has been characterized as an antiandrogen as well as a progestin because of its ability to efficiently antagonize the effects of exogenous androgens upon the reproductive organs of the hypophysectomized male rat.[64] Neumann and Kramer[63] postulated that the mechanism behind SH 714's antiandrogenic effect involved a competitive antagonism by SH 714 for androgenic receptors in the end organs (i.e., structures sensitive to and influenced by plasma levels of androgens).

Evidence for this mechanism was demonstrated by Bloch and Davidson's study[2] in which the implantation of cyproterone pellets within the basal hypothalamus of intact, adult male rats elicited a hypertrophy of the seminal vesicles, prostates and testes. Previous studies by Davidson and Sawyer[12] had indicated that the median nucleus of the basal hypothalamus was the "...site of inhibitory action of testosterone on gonadotropic secretion." That is to say, this site in the basal hypothalamus was sensitive to the plasma levels of testosterone. Such sensitivity, in turn, would dictate the secretion level of gonadotropin-releasing factors in quantities <u>inverse</u> to the plasma levels of testosterone. Therefore, the hypertrophy reported by Bloch and Davidson[2] attributed to an increase in gonadotropin-releasing factors from the basal hypothalamus consequent to a blockade by cyproterone acetate of the hypothalamic sites sensitive to plasma levels of androgens.

Recent studies with ^{3}H-testosterone and its prostatic metabolite, ^{3}H-dihydrotestosterone, by Stern and Eisenfeld[75] have lent additional clarification to the mechanism Neumann and Kramer[63] postulated for cyproterone acetate. In their studies, adult male rats, castrated 3 weeks previously, were injected intravenously with 0.1 ug of ^{3}H-testosterone in aqueous solution per 100 grams of body weight. Prior to receiving the radioactive testosterone, the experimental males intravenously received the cyproterone (1 mg/100 gms body weight); control males received the ethanol vehicle alone. From the subsequent chromatographic and scintillation counter data, Stern and Eisenfeld determined there was a significant 20% and 15% decrease from control values in the concentrations of the ^{3}H-testosterone and ^{3}H-dihydrotestosterone levels, respectively, in the seminal vesicles from the experimental group. No significant difference was found between the ratios of labeled testosterone to labeled dihydrotestosterone from the control and experimental groups. Because of this unchanged ratio, Stern and Eisenfeld suggested the cyproterone did not decrease the labeled concentrations in the seminal vesicles by selective, direct inhibition of the enzymatic formation of ^{3}H-dihydrotestosterone. Instead, they proposed that cyproterone had competed with ^{3}H-testosterone for the androgenic receptors in the seminal vesicles, and thus, the subsequent decrease in the metabolite, ^{3}H-dihydrotestosterone. Stern and Eisenfeld also studied this system of androgenic receptors in the seminal vesicles in vitro and proposed that these receptors were of macromolecular size. This latter suggestion was based upon their findings that, following the addition of ^{3}H-testosterone, the

radioactivity was located in the macromolecular fraction of the supernatant from seminal vesicle homogenates. Furthermore, when plotting the reciprocal values of total ^{3}H-testosterone concentration versus the radioactivity bound to macromolecules from the seminal vesicles in the presence and absence of 1 x 10^{-7}M cyproterone, the cyproterone was shown to reduce the binding of the ^{3}H-testosterone to the macromolecules in a competitive manner. Thus, Stern and Eisenfeld reasoned that cyproterone may function by competing with testosterone for macromolecular binding sites. Similar findings concerning competition for receptor sites between labeled androgens and cyproterone in the rat ventral prostate were obtained by Fang, Anderson and Liao.[19]

As the nature of cyproterone acetate is to compete with androgens for receptor sites, some explanation is in order at this point with regards to androgen-receptor sites. The character of an androgen-receptor complex has been determined *in vivo* and *in vitro* by Mainwaring[55,56] and later by Fang and Liao.[20] Mainwaring[55] studied the nuclear and cytoplasmic binding components for ^{3}H-testosterone and its main metabolite, ^{3}H-5α-dihydrotestosterone, using several analytic procedures. Digestion of prostatic nuclei, labeled with ^{3}H-testosterone, with enzymes of narrow substrate specificity (RNAse, DNAse and trypsin) indicated that trypsin released an amount of radioactivity 12-13 times greater than that released by the other two enzymes. Mainwaring postulated, therefore, that the nuclear receptor was a protein. Fractionation of labeled prostatic nuclei into neutral, basic and acidic subfractions revealed that the greatest labeling was in the acidic subfraction and suggested that the receptor was an acidic nuclear protein.

Extraction of the receptor complex from nuclei in 1M NaCl and fractionation of the extract on an agarose column determined the molecular weight of the complex to be 100,000 - 120,000. Mainwaring suggested the precise intranuclear location of the receptor protein was either the nucleolus or chromatin. He further speculated that the overall specificity of the androgenic receptors might depend on additional receptors in the cytoplasm. From a later study, Mainwaring[56] determined, by sedimentation and chromatographic methods, that these cytoplasmic receptors were thermolabile proteins in the non-sedimentable, 105,000g supernatant fraction (i.e., cytoplasm) of prostatic cell homogenates. Mainwaring proposed that the receptor complex was an acidic protein since both cysteine and tryptophan residues were necessary to maintain the functional configuration of the receptor. Fang and Liao[20] later subclassified the cytoplasmic protein receptor complex into Complex I and Complex II, speculating that Complex I could be a precursor to Complex II. The function of the cytoplasmic androgen-receptor complex may be to metabolize testosterone as it enters the cell and to transport its metabolite to the nuclear androgen-receptor complex previously discussed.[19,20,55,56]

In light of the above discussion concerning androgen-receptor complexes and the antiandrogenic character of cyproterone acetate, one could speculate the histologic alterations of the testes in this present study were a result of cyproterone acetate inhibiting hormonally-induced, gonadal differentiation by competing for receptors in the target organ (i.e., the differentiating gonad). Such

an interaction could alter the differentiation of the potential male gonad towards one or some combination of the following: (i) differentiation towards the female type of gonad; (ii) a postponement or inhibition of the normal differentiation; or, (iii) an augmentation of the testicular differentiation by a mechanism similar to that proposed in the forementioned work of Bloch and Davidson.[2] It is only possible at this time to make conjectures on such directions since the controlling impetus behind the differentiation of the bipotential gonad has yet to be demonstrated and clarified.

Though the scope of the present study was limited to the induced histologic alterations in the developing hamster testis, the interpretation of our findings may be reasonably based upon existing knowledge of the phenomenon of hormonal interaction in body systems. In the present study, administration of cyproterone acetate to gravid hamsters prior to and up through implantation induced several gross and microscopic changes in the male neonates. As indicated in Table 1, the average body weight and average left testicular weight of the experimental group were 56% and 33%, respectively, of those values obtained for the control group. Androgens have been known for some time to cause nitrogen retention of body growth with a positive nitrogen balance.[16,24,45,86,87] If the forementioned studies[63] regarding the antiandrogenic capacity of cyproterone acetate are to be taken seriously, the decreases in body and testicular weights seen in this present study may be considered a consequence of cyproterone acetate inhibiting the

normal androgenic stimulation of metabolic factors such as nitrogen retention necessary for normal body growth.

Normal litter numbers (9 - 15 pups) were not found in any of the uterine horns of the mothers in the experimental group. As described in the "RESULTS", the gross findings of amorphic entities attached in linear fashion along the luminal walls of these horns suggested that implantation, some development and subsequent resorption of a number of fetuses had occurred. Hafez and Pincus[26] and Dickmann[14,15] demonstrated from studies in the rabbit and the rat that survival of the early stages of embryonic development was dependent upon a proper balance of estrogen and progesterone, otherwise pregnancy would be terminated. For example, in the adult, pseudopregnant, ovariectomized female rat (Holtmann strain), Dickmann[15] demonstrated that progesterone (2 mg/100 grms body weight/day) administered from the second through the twentieth day of pseudopregnancy plus estrone (1 ug/100 grms body weight/day) from the seventh through the twentieth day was necessary for the survival and implantation of morula transferred on the fourth day of pseudopregnancy. It is conceivable that the pharmacologic, total dose levels (i.e., 50 mg/100 grms body weight/2-1/2 days) of the progestin, cyproterone acetate, employed in this present study upset the proper balance of estrogen and progesterone in the gravid hamster. In this manner, the administration of cyproterone acetate could be directly responsible for the abnormal findings described in the uterine horns of the experimental group. As discussed in the "PRELIMINARY STUDIES-DOSE RESPONSE" (APPENDIX), the higher dose levels employed in the

preliminary study either prevented implantation (e.g., dose levels 70 and 75 mg) or caused resorption of all the implanted fetuses (e.g., dose level 60 mg). Here again, disruption of the proper balance of estrogen and progesterone induced these errors in implantation and embryogenesis.

The quantitative light microscopic findings (Table 2 - "APPENDIX") demonstrated there was a significant increase in the numbers of nuclei per unit microscopic area in the testes of the experimental group when compared with controls (i.e., a ratio of 2.47 to 1). From the data in Table 2 and a comparison of control and experimental tissue sections (FIGURES 5 - 9), it would appear that there was an actual increase in numbers of nuclei (and therefore, cell numbers) in the experimental tissues over the controls. Such an increase, however, is made less plausible because the experimental gonads were smaller and one-third the weight of the control gonads (Table 1). The possibility that the nuclei in the experimental tissues could be more closely packed together, giving the semblance of increased numbers must also be considered. As already indicated, Table 1 shows that the control testes weights were 3 times greater than those of the experimental group; and, Table 2 indicates that the mean number of nuclei in the experimental sections was 2.5 times greater than that for the controls. Had this value for the mean number of nuclei been closer to a factor of 3 times instead of 2.5, such findings would then suggest, in a very gross manner, that the total numbers of nuclei for the control and experimental gonads were similar. In such case then, the increase in numbers of nuclei, seen per unit microscopic area, in the experimental sections would be more apparent

than real, i.e., merely representing a compaction of tissue.

Comparison of the photomicrographs of control and experimental testicular sections (Figures 1-9), as previously described, indicates that in the experimental group, the normal pattern of gonadal differentiation was altered as a consequence of the cyproterone administration. The marked difference in periodic acid-Schiff's staining between the controls (FIGURES 1,2,5,6,7) and experimental (FIGURES 3,4,8,9) sections of testes indicates an alteration in the normal connective tissue matrix of the experimental gonads. It has already been stated that the mucoproteins and glycoproteins in the connective tissue matrix are sensitive to the periodic acid-Schiff's reaction. Thus, such alterations seen in the present study might be attributed to either an inability of the mucoproteins and glycoproteins to react with the periodic acid-Schiff's dye once they had reacted with it. In either case, an alteration has occurred at the molecular level of the connective tissue matrix to cause the different appearance in stained material when compared with controls. During the histologic processing of the tissue sections, the periodic acid is employed in order to oxidize the carbon to carbon bonds when they are present in the form of adjacent 1:2 glycol groups (CHOH - CHOH), turning them into aldehydes. Introduction of the Schiff's reagent (a colorless derivative of basic fuchsine) to these aldehyde groups permits attachment of the reagent to the aldehyde groups and reconstitution of the reagent's color. Thus, the sites of PAS reactive groupings are seen as a red to magenta color.[1,17] As little or no magenta color is seen in the connective

tissue matrix of the experimental gonads, alterations at the molecular level of the matrix must have occurred. Either the oxidation by the periodic acid was inhibited or oxidation did occur but the Schiff's reagent was blocked in some way from attaching to the aldehyde sites. A third possibility may be that the molecular bonding between the aldehyde and the Schiff's reagent was weak and would not retain the Schiff's reagent throughout the completion of the histologic processing. In any case, there was a disruption in the make-up of the connective tissue matrix consequent to the cyproterone administration.

The disorganization of the cellular elements within the seminiferous cords is attributable to alterations in the matrix similar to those discussed in the previous paragraph for the connective tissue matrix. This disorganization was not an artifact of histologic technique because the surrounding tunica propria (basement membrane) of the seminiferous cords are seen as being intact and in order.

The absence of mitotic figures from the sections of experimental gonads (FIGURES 3,4,8,9) is of particular interest for two reasons. First, the presence or absence of mitotic figures (in a growing tissue) can be considered a gross indication of the level of cellular metabolic activity.[5,13,57,58,59,67,76,90] Second, the presence of mitotic figures gives some gross indication as to the state of progressive spermatogenic development.[30,33] It may be concluded that the administration of cyproterone acetate induced a negative effect upon the normal process of cell division in these

testes either by an inhibition of chromosomal division or a failure of the normal nutritive support by the Sertoli cells for continued spermatogenesis. DNA synthesis occurs during a limited period of the cell cycle designated "S". The absence of mitotic figures in the experimental tissues of the present study may be a consequence of cyproterone acetate inhibiting the occurrence of the "S" phase or preventing the subsequent mitotic stages, post-"S" phase. Quantitative measurements of the nuclear DNA by Dr. Cecile Leutchenberger (See "PERSONAL COMMUNICATION" - APPENDIX) indicated no significant difference in the nuclear DNA content of individual cells of the control and experimental tissues. This finding suggests that the inhibitory action of cyproterone acetate occurred prior to the "S" phase. Determination of the cellular specifics of such interaction warrants further studies. For example, those cell stages of spermatogenesis where DNA synthesis and mitosis occur (i.e., spermatogonial and primary spermatocytic stages) might be investigated more throughly with regards to their cellular metabolic and physiologic levels.

As no data have been found in the literature with respect to the influence of cyproterone acetate as a synthetic progestin upon mitoses and DNA content in tissues, studies by other workers on such effects by progesterone itself should be discussed for comparison. Lloyd[54] was the first to show that progesterone could stimulate cell-division of the uterine epithelial tissues. He demonstrated this by injecting oophorectomized rabbits with 0.5 mg. progesterone per day for three days and subsequently observing the number of mitoses evident in the uterine epithelial tissues. The ratio of mitoses in progesterone-treated to control tissues was 50:1.

Of more recent date, von Berswordt-Wallrage and Turner[80,81] published their data concerning the influence of progesterone upon placentomata formation as measured by total DNA content per 100 grams of uterine horns. The animals they studied were ovariectomized rats. Their study demonstrated that progesterone in graded doses stimulated a graded increase in the mean total DNA per 100 grams of experimentally traumatized uterine horns. At their maximum dose level of 6 milligrams of progesterone per day for 4 days, the mean total DNA content for the experimental groups was 2.4 times greater than the control groups'. Von Berswordt-Wallrabe and Turner attributed this phenomenon to an increase of the polyploidy normally seen in placentomata formation. Thus, the increase in total DNA was representative of nuclear multiplication rather than of cellular multiplication.

Additional investigations along similar lines have been those of Duncan,[18] Leroy,[47] Leroy and Manavian[48] and Leroy and Galand.[49] By employing the Lison histophotometer and the Feulgen reaction, Leroy and co-workers were able to estimate the DNA content in set groups of uterine epithelial and stromal nuclei. Their results indicated that progesterone (2.5 mg/day/rat), injected for four days, induced an increase in the mean nuclear DNA per 100 uterine epithelial cells when compared to the controls. However, the mean nuclear DNA per 100 stromal cells was not altered by the steroidal treatment.

Hamilton[27] reported that chromatin taken from an estrual uterus (rat) had an increased chromatin template activity, an increased ratio of total proteins to DNA and a decrease in histone to DNA ratios

when compared with diestrual uterus. It is tempting at this point to draw a correlation between the high levels of serum progesterone present during estrus[21] and Hamilton's "chromatin phenomena" if, in fact, progesterone is responsible for increasing the mean nuclear DNA and stimulating cellular division.

In the human, Heller, et al.[32,34] proposes that exogenous progesterone suppresses spermatogenesis by a direct effect upon the testis. In their studies, progesterone (50 mg/day), administered to normal males, suppressed spermatogenesis as assayed by sperm counts of the ejaculate and testicular biopsies. However, there was no concomitant suppression of gonadotropins nor conversion of the compound to estrogen nor assimilation by it of estrogenic activity. As these observations seemed to rule out any alteration of the usual, regulatory mechanisms of spermatogenesis, Heller et al.,[34] proposed the site of action to be in the testis itself. Later studies by Heller and Clermont[30] indicated that whereas androgens and progestagens inhibited the rate of development of the spermatids and the spermatozoa in the adult human, the associated spermatogonia and primary spermatocytes in the seminiferous tubules were not influenced.

The studies described in the above paragraphs indicated that progesterone stimulated cellular division in uterine epithelial tissues and also stimulated an increase in the mean nuclear DNA per 100 uterine epithelial cells studied. However, in human testes, administration of progesterone did not interfere with the rate of development of the germ cells (i.e., spermatogonia). In addition, the investigations of Neumann et al.[61,62,63,64] and Forsberg and

Jacobsohn[22,23] previously discussed, showed that post-implantation administration of cyproterone acetate did not modify the normal differentiation of the male, neonatal gonads. They further proposed that since the anti-androgenic capacity of the cyproterone did not alter the normal differentiation of the male gonads, differentiation of the male gonads was not under the direction of androgens (e.g., from the mother).

In contrast to the above four observations, the present study has demonstrated the opposite effects to be found in neonatal testes, consequent to the administration of the cyproterone to gravid hamsters prior to and up through implantation. That is to say our findings indicated that cellular division was inhibited in the testes, germ cells were altered in development and appearance, the mean total DNA content per whole testis or per 100 cell nuclei was not significantly changed (See "PERSONAL COMMUNICATION-APPENDIX"), and the normal differentiation of the male, neonatal gonads was modified. Other systemic tissues, such as liver and small intestines, were not effected implying that these effects of the cyproterone acetate were probably limited to the testes. Examination of gonadal tissues on days 12 through 15 of gestation (FIGURES 12-19) did not provide additional information concerning these modifications of gonadal differentiation by cyproterone acetate.

The histologic observations in the present study are significant to the interests of experimental embryology in that they set a precedent in demonstrating that normal differentiation of the intact male gonad can be altered by exogenous compounds.

Furthermore, the schedule by which the compound was administered to the gravid mothers indicates that a specific time in gestation is critical to the introduction of the compound in order to obtain the present results. Clarification of the physiologic and cellular mechanisms behind this specific phenomenon warrants further study and should lead to the development of methods for controlling the phenotypic direction of the bipotential gonad.

CONCLUSION

Administration of cyproterone acetate to gravid hamsters prior to and up through implantation has been shown to alter the gross and microscopic morphology of the neonatal testes. The most striking change in their microscopic morphology compared to control testes is seen as the absence of mitotic figures within the seminiferous cords and the interstitial tissue areas surrounding the cords. The inhibitive influence of cyproterone acetate upon the normal occurrence and rate of progressive mitoses of the spermatogonia and primary spermatocytes warrants further study in light of the implication of exogenous alteration of normal, male gonadal differentiation by an antiandrogen.

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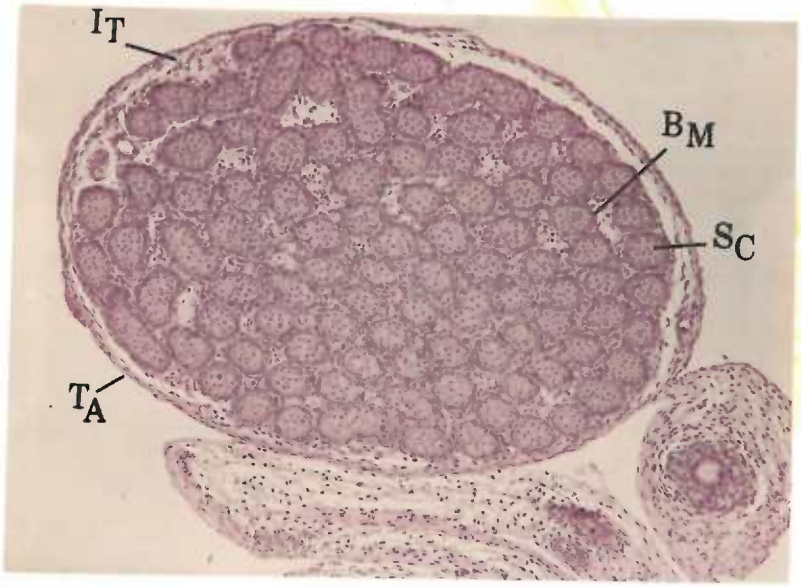
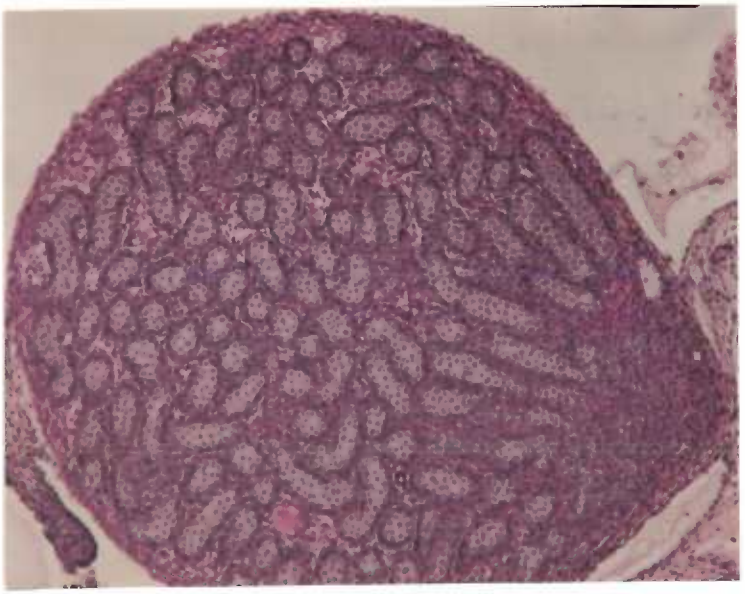
PHOTOMICROGRAPHS

FIGURE 1 (transverse view) and 2 (sagittal view). Periodic acid-Schiff and hematoxylin stained sections of right hamster testes from day-old control neonates. The mothers of these newborn had been injected with a total dose of 1.0 cc of 2:25 benzyl benzoate-castor oil. The total dose of this transfer vehicle had been administered, preimplantation, over an interval of five injections. The PAS magental stain is most evident in the tunica albuginea (TA), the interstitial tissue (IT), and in the basement membranes (tunica propria) (BM) of the seminiferous cords (SC). Differences seen in these figures between their magental intensities are attributed to the individual variations in adipose and connective tissue proportions. (100x)

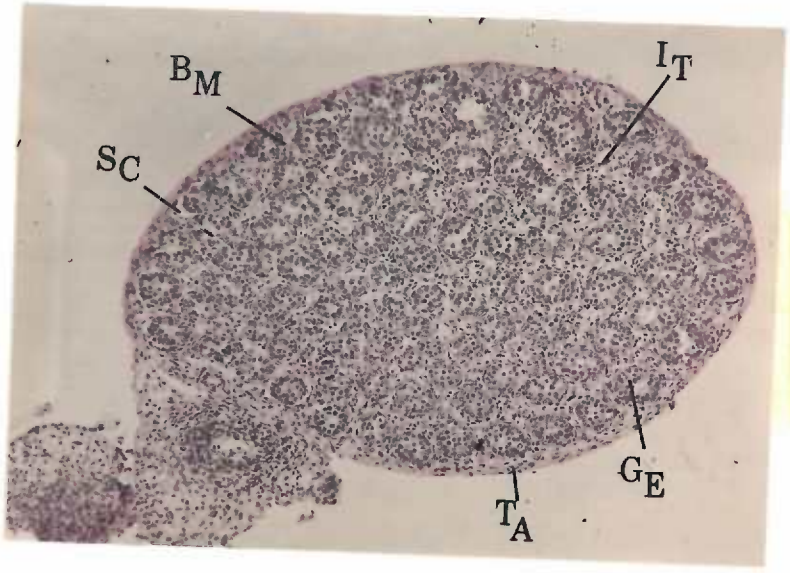


1



2

FIGURE 3 (transverse view) and 4 (sagittal view). Periodic acid-Schiff and hematoxylin stained sections of right hamster testes from day-old neonates whose mothers had been injected with a total dose of 50 mg of cyproterone acetate dissolved in 1.0 cc of 2:25 benzyl benzoate-castor oil. This total dose had been administered, preimplantation, over an interval of five injections. The intensity of the PAS magental stain is greatly reduced in comparison to that of the control sections. The hematoxylin stain in contrast is seen as being more intense. The seminiferous cords (SC) are disrupted in their continuity of germinal elements (GE). (See Figures 1 and 2 legend for explanation of TA, IT and BM.) (100x)



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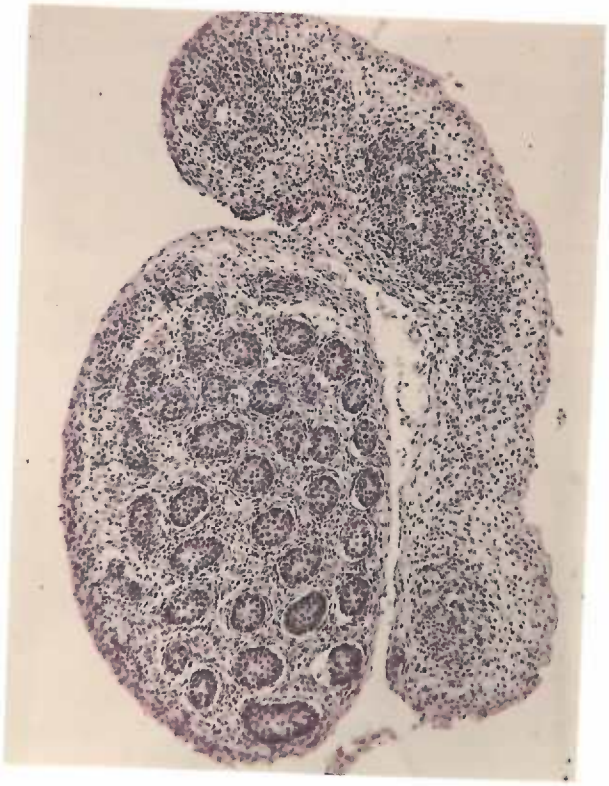


4

FIGURE 5. Higher magnification of a PAS and hematoxylin stained section from a control, right hamster testis. The interstitial cell types consist of fibroblasts (F) or mesenchymal cells. Leydig cells are not evidenced in this field, but adipose cellular outlines (AC) are demonstrated interstitially. The seminiferous cords contain spermatogonial types (A) and (In) peripherally and type (B) centrally. Sertoli cell nuclei (SCN) are distinguised from those of the spermatogonia by their indented nuclear membranes. The tunica propria (TP) surrounding each cord is PAS-positive. Mitotic figures (MF) are evident. (650x)

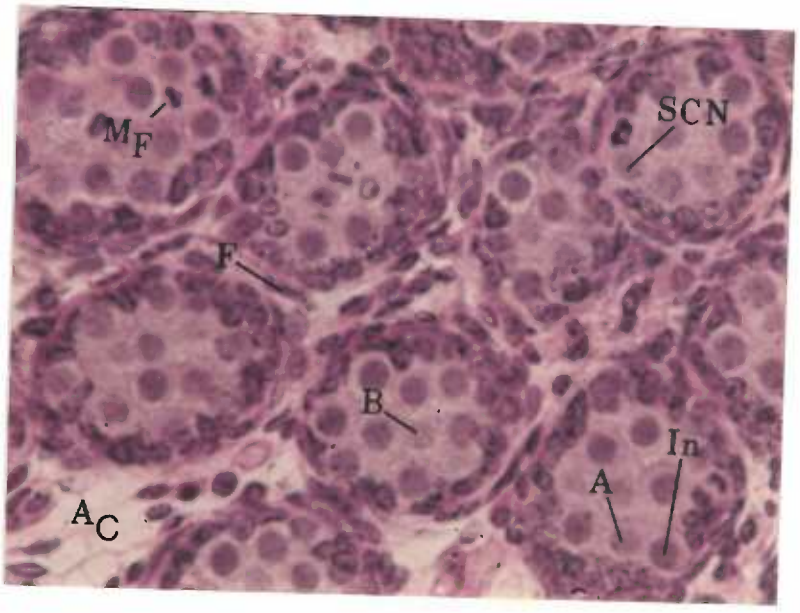
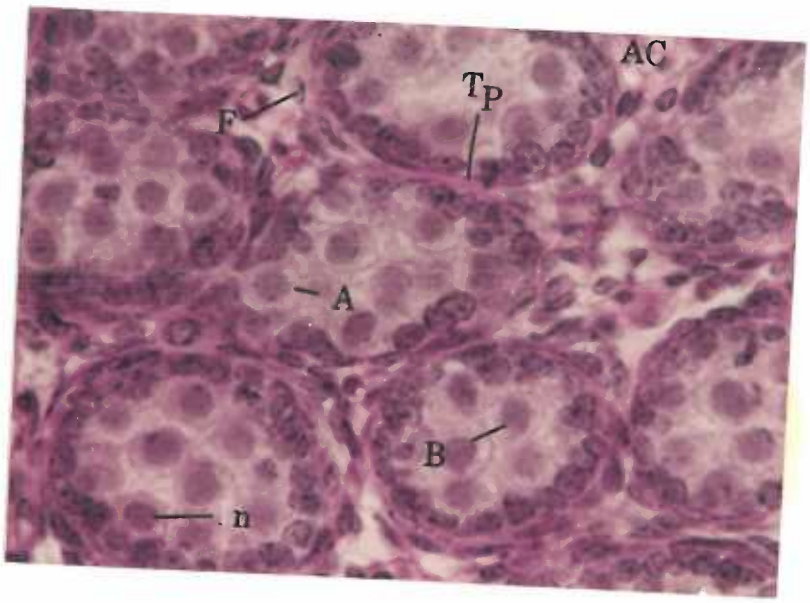

MF
SCN
F
B
In
A
AC

FIGURE 6. Periodic acid-Schiff and hematoxylin stained section of a right hamster testis from a day-old control neonate whose mother had been injected with 1.0 cc of 2:25 benzyl benzoate - castor oil. The total dose of this transfer vehicle had been administered, preimplantation, over an interval of five injections. The histologic picture is similar to that of FIGURE 5. (650x)

FIGURE 7. Periodic acid-Schiff and hematoxylin stained section of a right hamster testis from a day-old control neonate whose mother had been euthanized with ether in order to determine whether ether (as a pharmacologic agent itself) might have had any effect on the fetal tissues in this study. The picture is similar to those of FIGURES 5 and 6. (650x)



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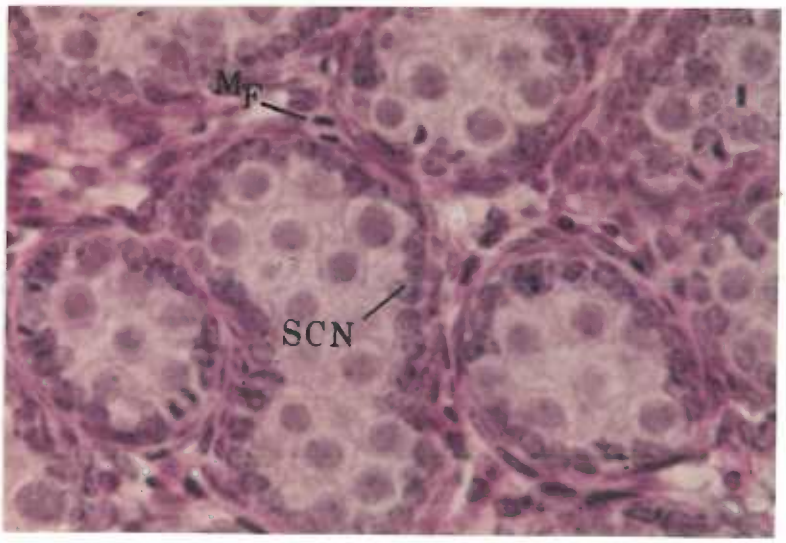

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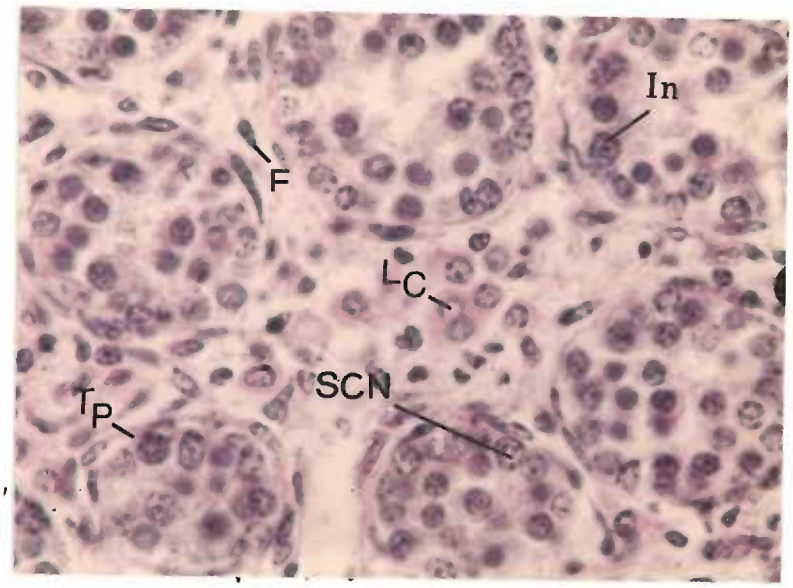

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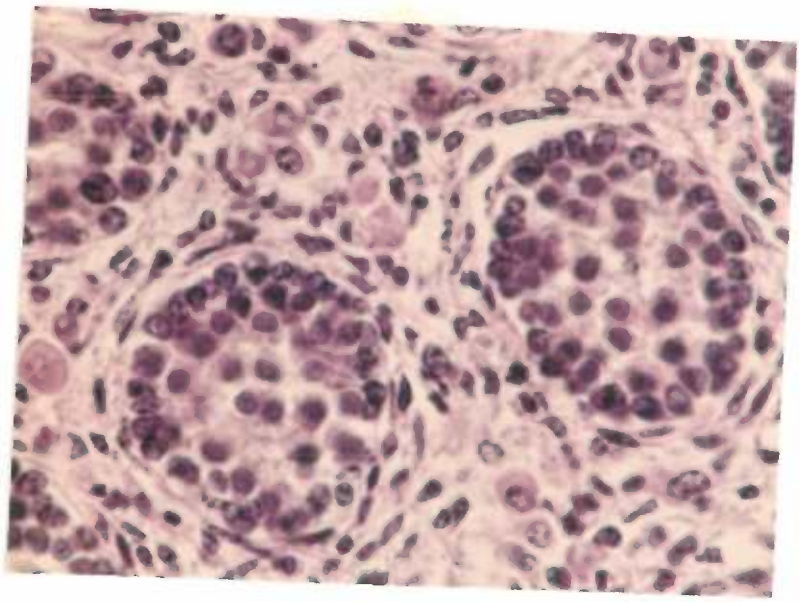
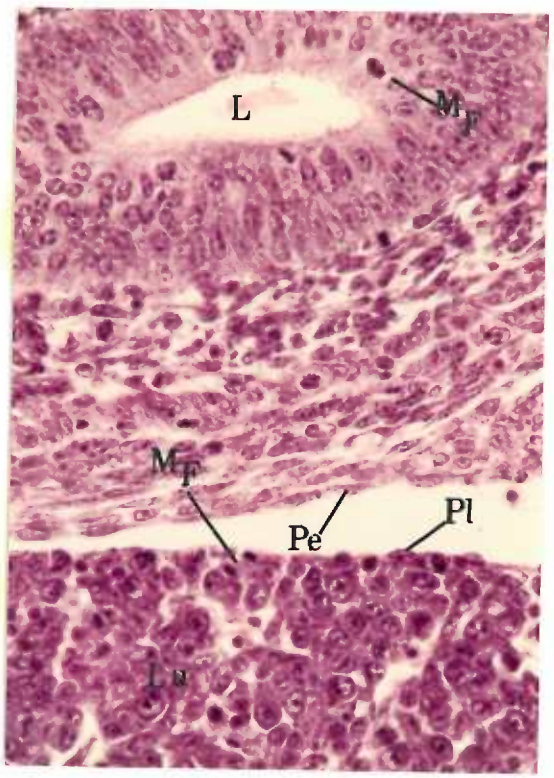


9

FIGURES 10 (control) and 11 (experimental). Hematoxylin and eosin stained sections of transverse sections of liver and small intestines taken from 14 day-old hamster fetuses. The mothers of the fetuses in the experimental group had been injected with a total dose of 50 mg of cyproterone acetate dissolved in 1.0 cc of 2:25 benzyl benzoate - castor oil. This total dose had been administered, preimplantation, over an interval of five injections. Mitotic figures (MF) are seen in the columnar cells lining the lumen (L) of the small intestine and in the tissue mass of the liver (Lu). The peritoneal lining of the small intestine (Pe) and the liver (Pl) is seen to encapsulate the respective organs. There is no histologic distinction between the control and experimental tissues. (500x)



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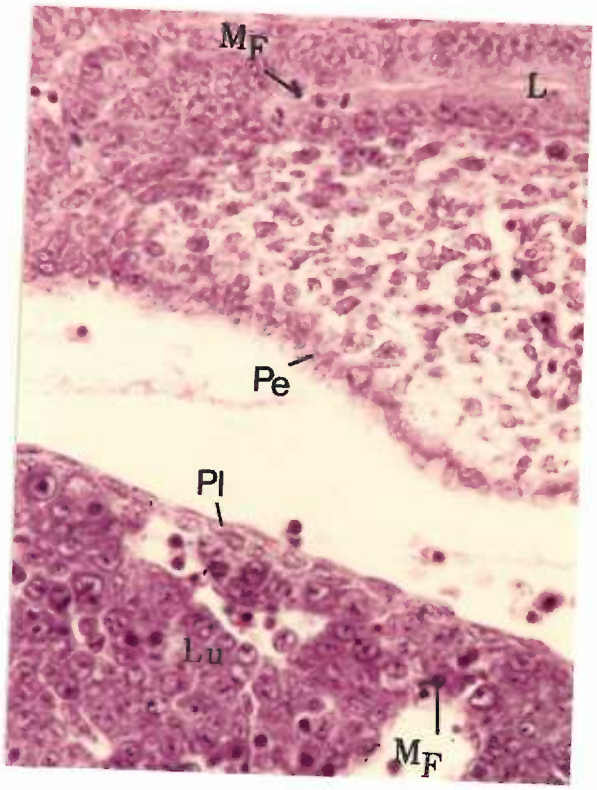
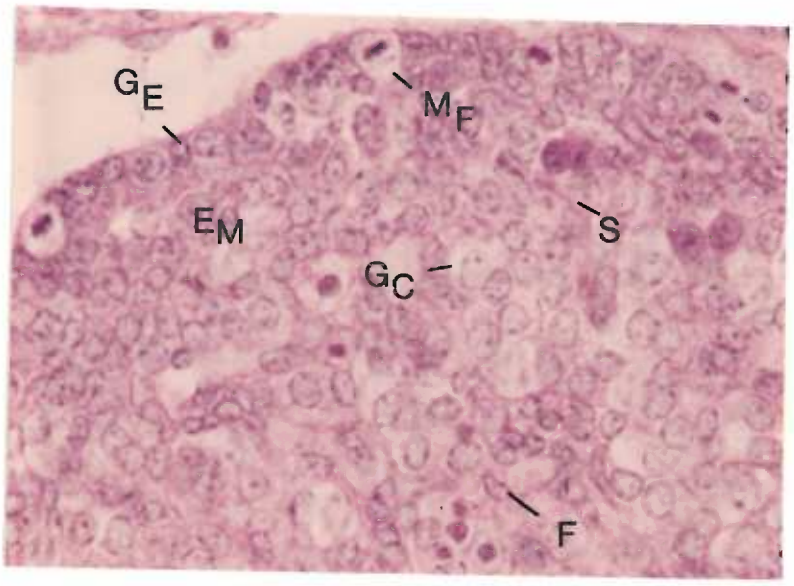


11

FIGURES 12 (control)and 13 (experimental). Periodic acid-Schiff and hematoxylin stained sections of transverse sections of right hamster gonads from 12 day-old hamster fetuses. The mothers of the fetuses in the experimental group had been injected with a total dose of 50 mg of cyproterone acetate dissolved in 1.0 cc of 2:25 benzyl benzoate - castor oil. This total dose had been administered, preimplantation, over an interval of five injections. The histologic picture seen in both photomicrographs is that of the indifferent gonad. Underlying the germinal epithelium (GE) is the epithelial mass (EM). Primordial germ cells (GC) and mesenchymal or fibroblastic cells (F) compose the major elements of the epithelial mass. PAS positive strands (S) represent intercellular material separating primitive sex cords (see page15). Mitotic figures (MF) are seen in the germinal epithelium of the control section. The magenta color of the PAS reaction is more evident in the control section than the experimental section. The nuclei in the experimental gonad are hyperchromatic compared to the control nuclei. (800x)



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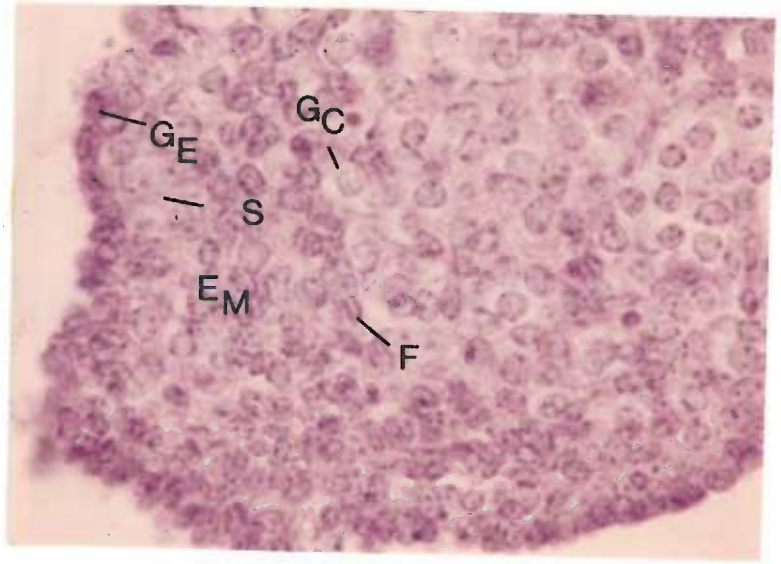
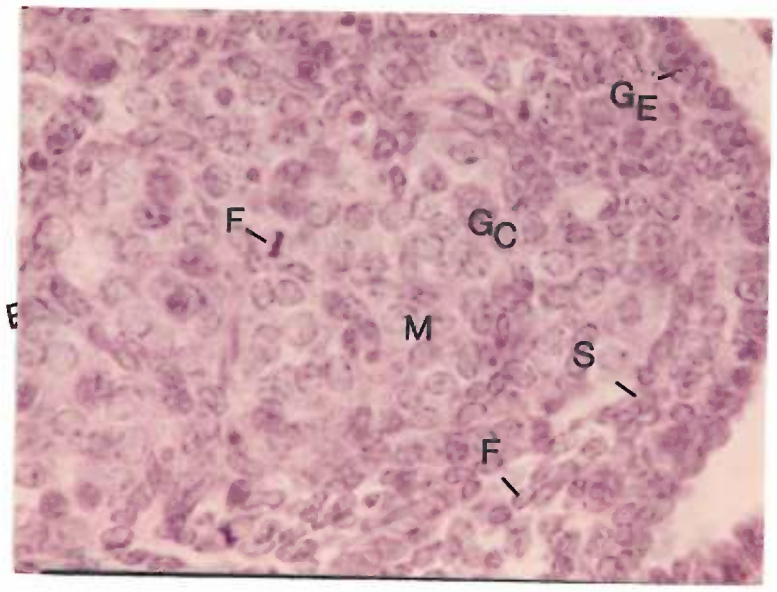


13

FIGURES 14 (control) and 15 (experimental). Periodic acid-Schiff and hematoxylin stained sections of transverse sections of right hamster gonads from 13 day-old hamster fetuses. The mothers of the fetuses in the experimental group had been injected with a total dose of 50 mg of cyproterone acetate dissolved in 1.0 cc of 2:25 benzyl benzoate - castor oil. This total dose had been administered, preimplantation, over an interval of five injections. The histologic picture is still that of the indifferent gonad as described in Figures 12 and 13. There is seen, in addition, an increase in the presence of fibroblastic cells with their elongated nuclei (F). Mitotic figures (MF) are absent from the experimental gonad and there is a more striking difference in PAS staining between the control and experimental gonads. (800x)



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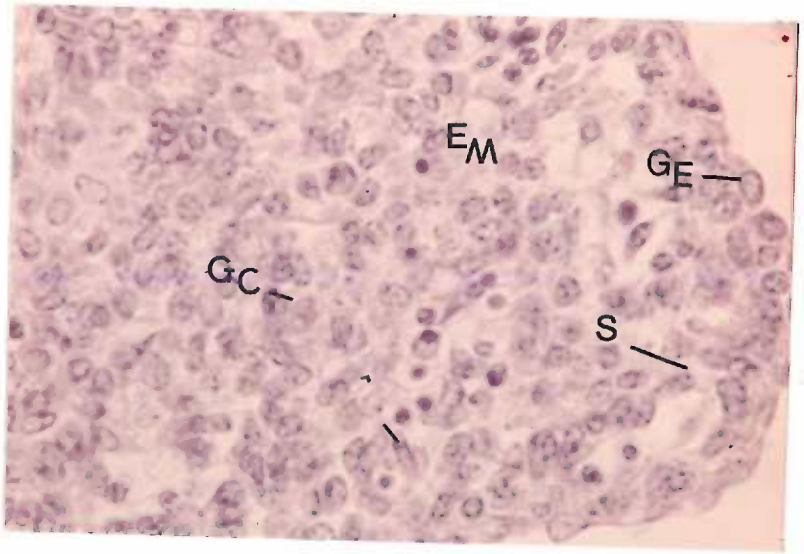
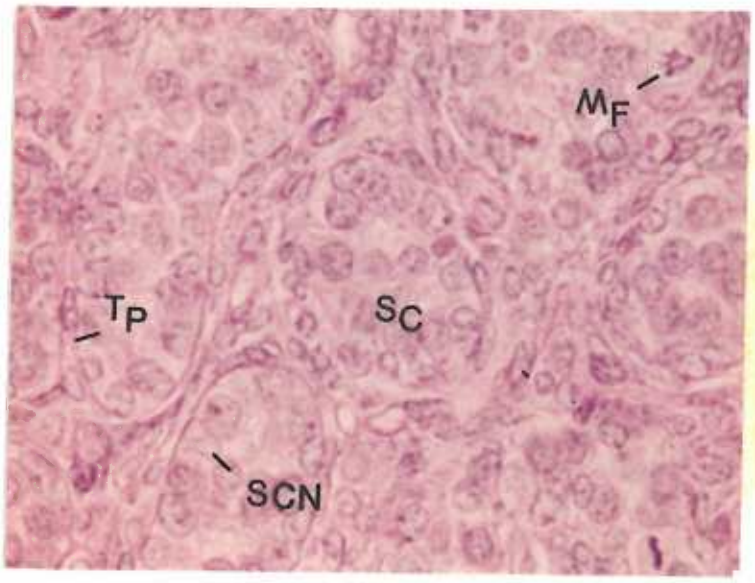


15

FIGURES 16 (control) and 17 (experimental). Periodic acid-Schiff and hematoxylin stained sections of transverse sections of right hamster testes from 14 day-old hamster fetuses. The mothers of the fetuses in the experimental group had been injected with a total dose of 50 mg of cyproterone acetate dissolved in 1.0 cc of 2:25 benzyl benzoate - castor oil. This total dose had been administered, preimplantation, over an interval of five injections. Primitive sex cords enclosing primordial germ cells are now seen as seminiferous cords (SC) lined by a tunica propria (TP) or basement membrane. Within the seminiferous cords, the nuclei of spermatogonium type A and Sertoli cells are demonstrated. Fibroblastic cell nuclei (F) are the prominent structures in the interstitial areas. The decrease in PAS staining and the hyperchromaticity of nuclei are well evidenced in the experimental testis section. (800x)



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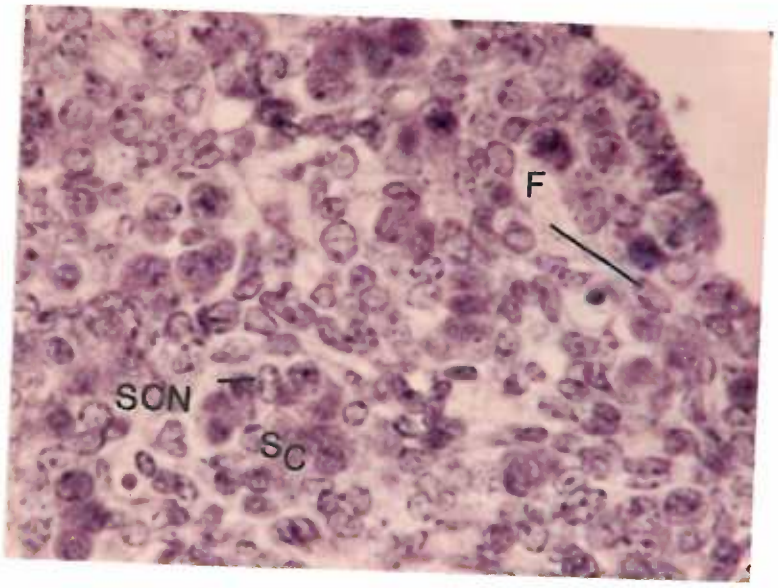
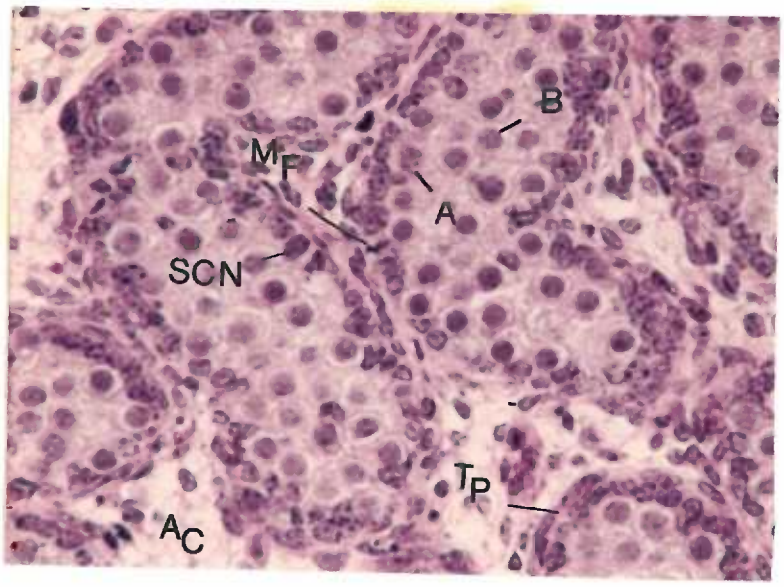


17

FIGURES 18 (control) and 19 (experimental). Periodic acid-Schiff and hematoxylin stained sections of transverse sections of right hamster testes from 15 day-old hamster fetuses. The mothers of the fetuses in the experimental group had been injected with a total dose of 50 mg of cyproterone acetate dissolved in 1.0 cc of 2:25 benzyl benzoate - castor oil. This total dose had been administered, preimplantation, over an interval of five injections. These figures were taken to demonstrate the initial tortuosity of the seminiferous cords not previously seen. The tortuosity is indicative of the growth and lengthening of the seminiferous cords within a space limited by the tunica albuginea (not shown) encapsulating the testis. Within the tunica propria (TP) of the seminiferous cords are seen the nuclei of spermatogonia types A, In and B and Sertoli cells (SCN). Adipose cellular outlines (AC) are seen in the interstitial areas along with nuclei of fibroblastic cells (F). Mitotic figures (MF) are seen only in the control section. Disorganization within the seminiferous cords, hyperchromaticity of the nuclei, lack of mitotic figures and the decrease in PAS staining characterize the experimental testis section. (650x)



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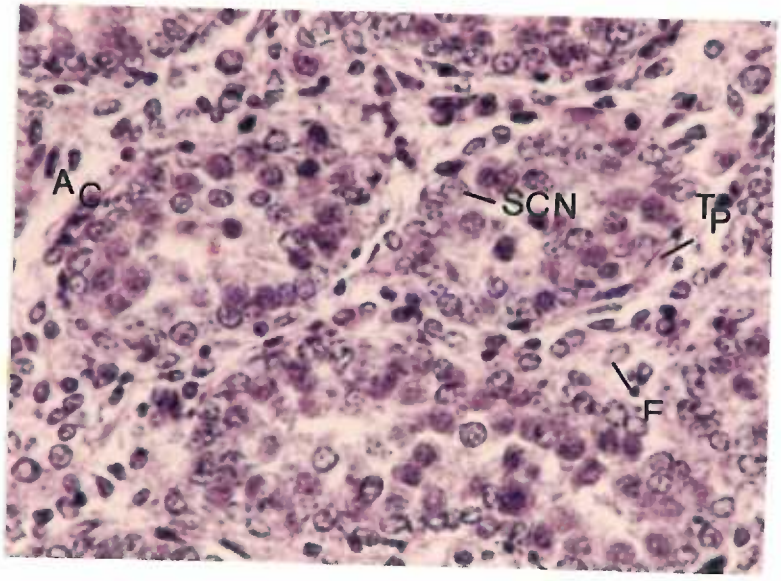

19

APPENDIX

PUP GROUPS	NUMBER OF PUPS	AVERAGE BODY WGHT (grms)	AVERAGE LEFT TESTIS WGHT (grms)	LEFT TESTIS PER BODY WGHT (grms/grms)	TOTAL DNA (ugrms) PER LEFT TESTIS
controls (C)	15 SD=	2.75 ±0.28	.0018 ±.00047	.0007 ±.00013	14.9 ±4.0
cyproterone acetate (CA)	23 SD=	1.55 ±0.34	.0006 ±.00019	.0004 ±.00016	14.5 ±5.5
CA / C	--	0.56	0.33	0.57	0.98
t value	--	11.673	9.106	5.601	--

df = 36
t Table value = 3.589
p level = .001

TABLE 1. HAMSTER PUP BODY AND TESTICULAR WEIGHTS AND DNA VALUES

The above data summarize the comparison of body and left testis weights and DNA per left testis values obtained in this study. The data for both the control and experimental groups were acquired from day-old hamster neonates whose mothers had been injected, pre-implantation, with a total of 1.0 cc of transfer agent benzyl benzoate - castor oil (controls) or a total of 50 mg of cyproterone acetate in 1.0 cc of transfer agent (experimental). The p level was derived by application of the "T" test to the original data.

	SLIDE	FIELD	NUCLEI COUNTED	AVERAGE RANGE	MEAN	CONTROL MEAN/ SH 714 MEAN
CONTROL TESTES SECTIONS FROM NEONATAL HAMSTERS	Q1c	a	1083			
		b	962			
	Q2c	a	1327	810-1327	1064.0	
		b	1153			
	Q3c	a	1049			
		b	810			
						$\frac{1}{2.47}$
SH 714 TESTES SECTIONS FROM NEONATAL HAMSTERS	Q1t	a	2421			
		b	2666			
	Q2t	a	2008	2008-3274	2627.3	
		b	2891			
	Q3t	a	2518			
		b	3274			

TABLE 2. COMPARISON OF CONTROL AND TREATED VALUES FOR NUCLEI NUMBERS

The above datum compares numbers of nuclei counted per unit area in microscopic fields of constant magnification as described in the "MATERIAL AND METHODS". Per unit area there are more nuclei in the SH 714 sections than the controls. P-levels were not calculated as the control and experimental values are of obvious difference and significance. Further interpretation is undertaken in the "DISCUSSION".

PRELIMINARY STUDIES - DOSE RESPONSE

As previously mentioned in the "INTRODUCTION" the studies of Neumann et al.[61-64] and Forsberg et al.[22,23] employed the administration of 10 milligrams of cyproterone acetate per day per kilogram body weight to a variety of animals. Their findings have already been discussed. The initial steps for our present study included a determination of a dose level for the gravid hamster that would permit a duplication of two phenomena found in the above mentioned studies: (i) the maternal ability to implant following SH 714 treatment and (ii) an inability of the mother to deliver a litter naturally. Since there was a lack in the literature concerning the matter of dose-response levels for SH 714 in the hamster, a set of total dose levels (45, 50, 60, 70 and 75 mg SH 714/100 grms body weight) was empirically determined. Each dose level was subsequently administered to two mated female hamsters per dose over five equally spaced injection periods, commencing with the 70th hour post-copulation and then each succeeding twelve hours. The 70th hour represents a developmental time at which the embryo is in the morula stage. This is the last developmental stage before implantation of the hamster embryo begins (112th hour, post-copulation).[28]

On day 15.5 of gestation, the uterine horns of the hamsters which had not delivered offspring were examined. Where a litter had been delivered, or when a litter was found within the uterine horns, the male gonads were removed and prepared for histologic examination in the same manner described for the present study under "MATERIALS AND METHODS - LIGHT MICROSCOPY."

With the three higher dose levels (60, 70 and 75 mg/100 grms body weight), the animals were either unable to implant their embryos or had reabsorbed their implantation. The latter observation was made from the gross findings of amorphic entities linearly spaced and attached to the lumen wall of the uterine horns. The 45 milligram dose level did permit the animals to implant and deliver small numbered litters.

However, at the 50 milligram dose level, the females could implant but were unable to deliver their young by natural means. This dose level did prevent some implantation and full gestation of some of the embryos as the litter number was consistently lower (1-4) when compared to controls (9-15). As with the high dose levels and the 45 milligram level, resorption of some fetuses was evident in the uterine horns.

The following chart compares the several effects of the dose levels employed.

Total SH 714 given per 100 grams body weight	Occurrence of implantation	Fetuses seen in resorption	Ability to deliver naturally	Neonatal Testicular development
75 mg	absent	--	--	--
70 mg	absent	--	--	--
60 mg	present	all	absent	--
50 mg	present	some	absent	abnormal
45 mg	present	some	present	abnormal

From the histologic observations made upon the male gonads from the fetuses of the mothers treated with the 50 milligram level, as well as the maternal findings mentioned above, this particular dose level was thus selected for a more extensive study of its effect upon testicular histology.

INSTITUT SUISSE DE RECHERCHES
EXPÉRIMENTALES SUR LE CANCER

SCHWEIZERISCHES INSTITUT FÜR
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SWISS INSTITUTE FOR EXPERIMENTAL
CANCER RESEARCH

1005 LAUSANNE, BUGNON 21, TÉL. (021) [redacted] 20 27 31/32

July 20, 1971 55

REGISTERED

Dr. Samuel LIN
Department of Anatomy
University of Oregon
Medical School
3181 S.W. Sam Jackson Park Road

PORTLAND, Oregon 97201
USA

Dear Dr. Lin,

Enclosed please find the DNA data in individual cells of your material.

As you will see we did not find any significant differences in the DNA content from cell to cell for the various groups. The mean value in arbitrary units would be for each of the groups approximately 1.5. It is also of interest that there do not seem to be any significant differences in the size distribution of the nuclei for the different groups.

I would be interested to know what your own interpretation of these DNA data is in regard to your own data.

With best regards also to Dr. Moore.

Sincerely,

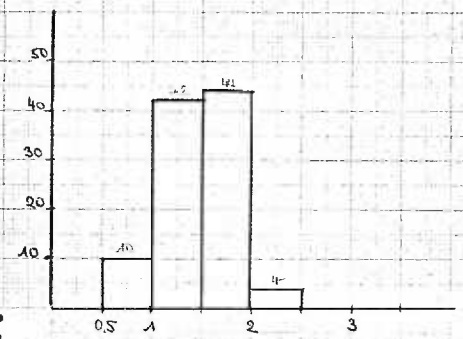
Prof. Cecile Leuchtenberger
Head, Department of Cytochemistry

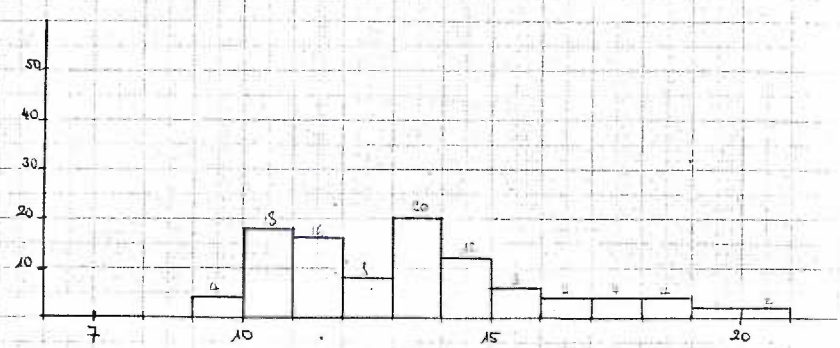
Encl.

AMOUNT OF DNA AND NUCLEAR SIZES OF INDIVIDUAL CELLS* OF TESTICULAR TISSUE FROM NEWBORN MALE RATS (24 HOURS AFTER BIRTH) IN CONTROL AND EXPERIMENTAL GROUPS.

*N = ~~150~~ 100 (Number of cells measured.)

159A





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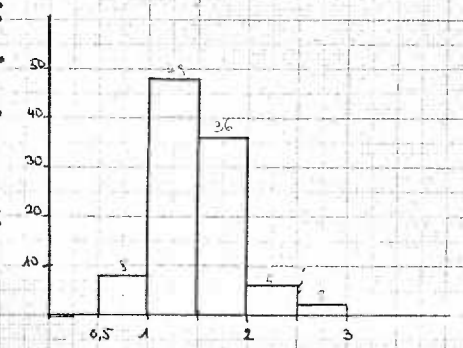

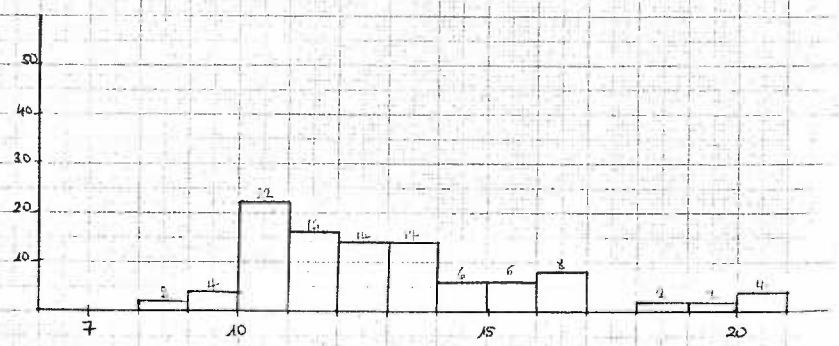

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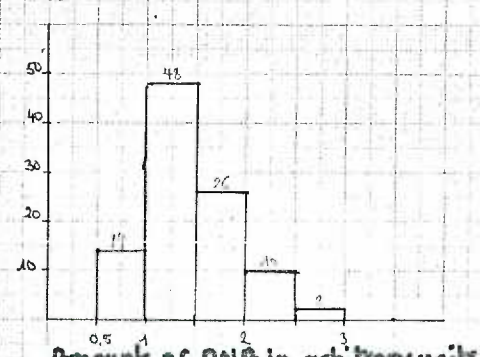
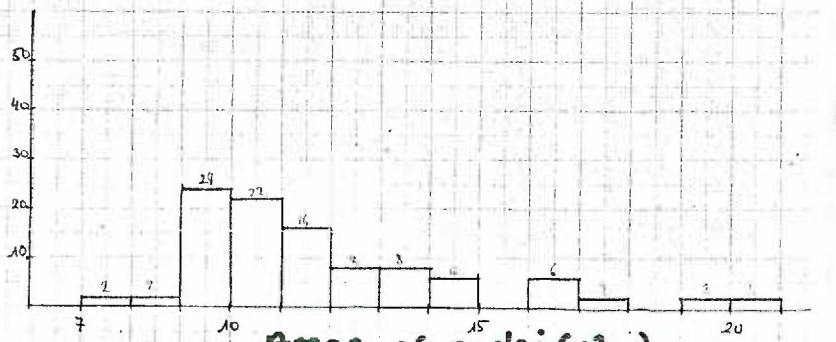




FREQUENCY IN PERCENT.

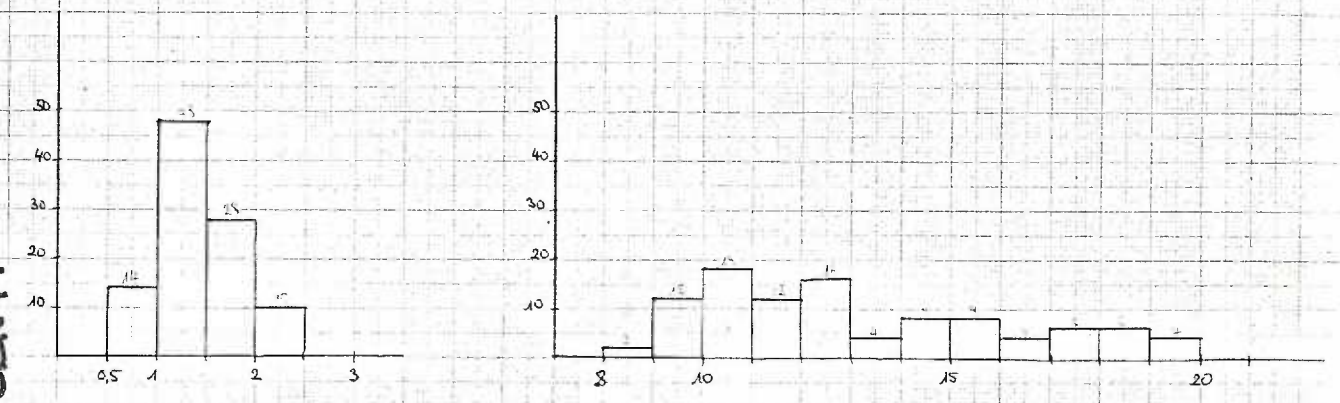
Amount of DNA in arbitrary units.

Areas of nuclei (μ^2)

AMOUNT OF DNA AND NUCLEAR SIZES OF INDIVIDUAL CELLS* OF TESTICULAR TISSUE FROM NEWBORN MALE RATS (24 HOURS AFTER BIRTH) IN CONTROL AND EXPERIMENTAL GROUPS.

* N = 100 (Number of cells measured.)

160 A



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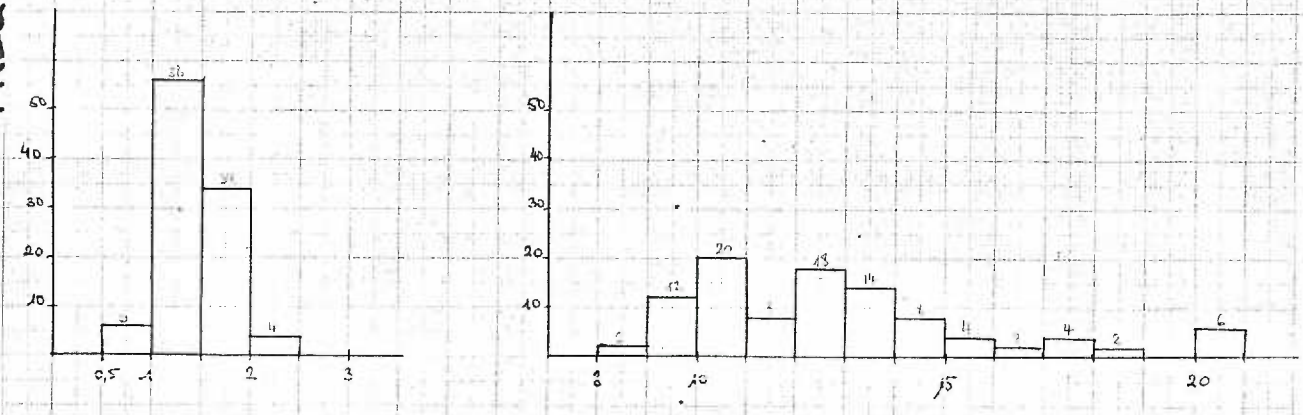


FREQUENCY IN PERCENT.

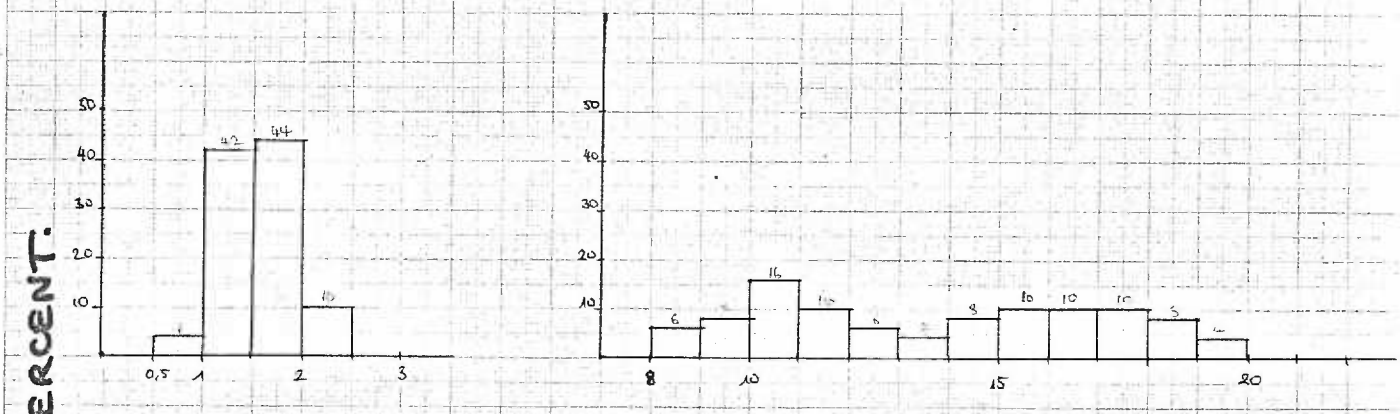
Amount of DNA in arbitrary units.

Areas of nuclei (qμ)

AMOUNT OF DNA AND NUCLEAR SIZES OF INDIVIDUAL CELLS* OF TESTICULAR TISSUE FROM NEWBORN MALE RATS (24 HOURS AFTER BIRTH) IN CONTROL AND EXPERIMENTAL GROUPS.

* N = 100 (Number of cells measured.)

162A



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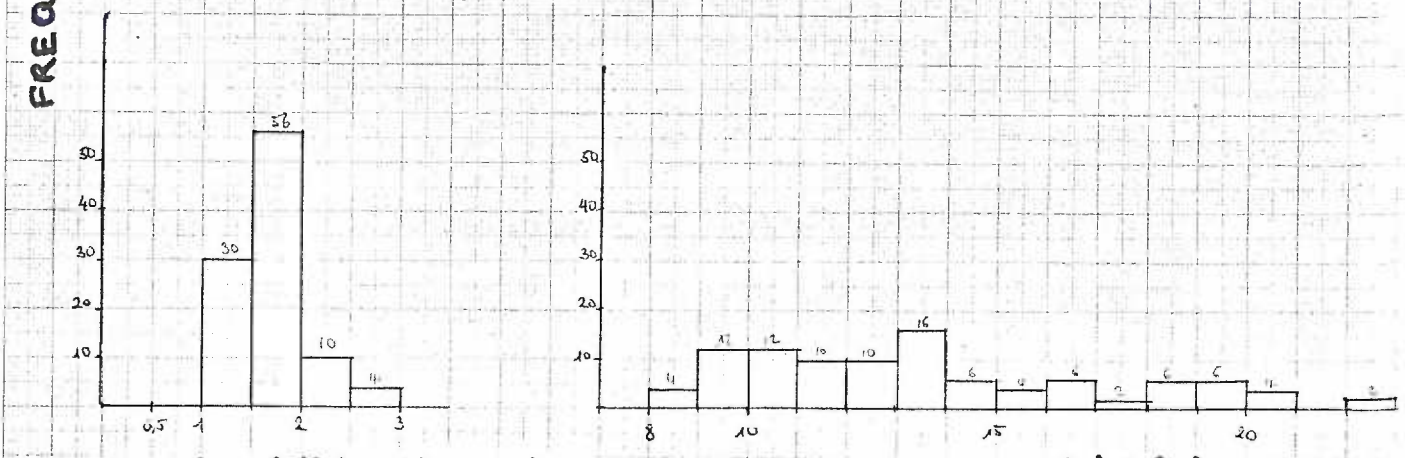


Amount of DNA in arbitrary units.

Areas of nuclei (μ^2)

PROCEDURE USED FOR EXTRACTION OF

DNA FROM TESTICULAR TISSUE

STEPS

1. 1 testis in 0.7 ml H_2O centrifuge tube
2. Freeze and thaw in dry ice-acetone bath (6x) (to promote autolysis of cells)
3. Add 1.5 ml of cold 10% trichloroacetic acid (TCA) (removal of acid soluable compounds)
4. Centrifuge at 4° C for 10 min in Sorvall centrifuge
5. Decant supernatant
6. Resuspend pellet in 1.5 ml of cold 10% TCA
   Repeat steps 4 and 5
7. Resuspend pellet in 3.0 ml 95% Etoh (removal of lipoidal compounds)
8. Repeat steps 4 through 7
9. Repeat steps 4 and 5
10. Resuspend pellet in 0.45 ml of 5% TCA (<u>room temp</u>) (removal of DNA and RNA from tissue proteins)
11. Heat in 90° water bath for 15 min, occasionally shake tubes
12. Centrifuge (room temp) for 5 min
13. Decant supernatant and <u>SAVE</u> (nuclei acids here)
14. Resuspend pellet (insoluable protein here) in 0.30 ml 5% TCA
15. Repeat steps 12 and 13
16. Combine supernatants of steps 13 and 15 (total volume 0.75 ml)
17. Assay supernatant for DNA by diphenylamine method

- based on Schneider[73]

PROCEDURE USED FOR DETERMINATION OF TOTAL DNA CONTENT EXTRACTED FROM TESTICULAR TISSUE

A. DNA Standard Solution

1. Stock solution - calf thymus DNA dissolved in 5MM NaOH (500ug/ml)

2. Working solution - 3.0 ml stock solution (500ug/ml) diluted with 7.0 ml 0.5 N perchloric acid (PCA). Heat in 70° water bath for 15 minutes. Final Concentration = 150ug/ml

B. Diphenylamine Reagent

1. Stock solution - 1.5 grms steam - distilled diphenylamine dissolved in 100 ml glacial acetic acid and add 1.5 ml concentrated H_2SO_4

2. Working solution - Add 0.1 ml aqueous acetaldehyde (16 mg/ml) to each 20 ml stock solution used on day of use

C. Steps

1. Add 0.5 ml 1N perchloric acid (PCA) to 0.5 ml nucleic acid extract (now in 0.5N PCA so that TCA does not effect final optical density)

2. 1 ml nucleic acid extract in 0.5N PCA combined with 2 ml diphenylamine reagent (see step 4)

3. Prepare test tubes with known concentrations (7.5ug/ml-105ug/ml) of "working solution" DNA diluted with 0.5N PCA, blank tube containing 0.5N PCA, tubes with unknowns from step 2

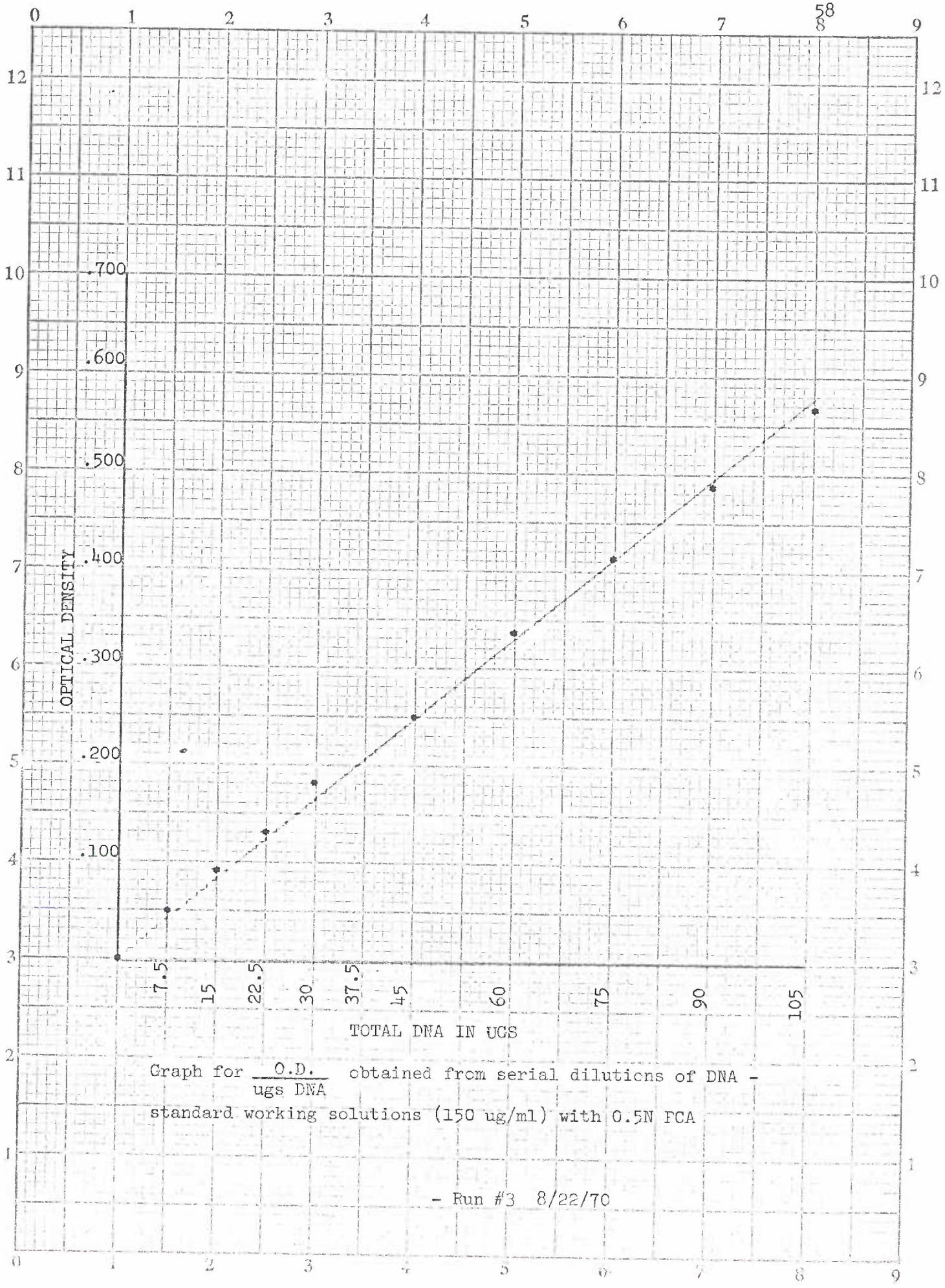
4. Incubate test tube in 30° water bath, 16 hours, to develop color intensity

5. Read optical density at 600 mu for blank, standards and unknown using Beckman DG-G spectrophotometer

6. Read DNA values for unknowns from DNA standard graph (sample next page)

- based on Burton[8]

n.b. Acknowledgement is given to the assistance of personnel in the laboratory of Dr. Demetrios A. Rigas in the DNA extraction and determination procedures.



Graph for $\frac{\text{O.D.}}{\text{ugs DNA}}$ obtained from serial dilutions of DNA - standard working solutions (150 ug/ml) with 0.5N PCA

- Run #3 8/22/70

SAMPLE CALCULATIONS

1. Dilution of DNA-standard working solution to obtain standard graph

   a. 0.1 ml of DNA working solution (150 ug DNA/ml) diluted with 1.4 ml of 0.5 N PCA = 1.5 ml total solution

      (0.1 ml) (150 ug DNA/ml) / 1.5 ml = 15 ug DNA/1.5 ml = 10 ug DNA/ml

   b. 10 ug DNA/ml reacted with 2 volumes diphenylamine

   c. O.D. reading at 600 mu = 0.090

      0.090 = 10 ug DNA/ml = 15 ug DNA / 1.5 mu
      (plotted)

2. DNA extract from experimental testis

   a. Total DNA extract in 0.75 ml of 5% TCA

   b. 0.5 ml of extract diluted with 0.5 ml of 1N PCA (to prevent TCA from influencing O.D.)

   c. Volume in step "b." reacted with 2 volumes diphenylamine

   d. O.D. reading at 600 mu = 0.087 (tube No. 12 - Run No.3)

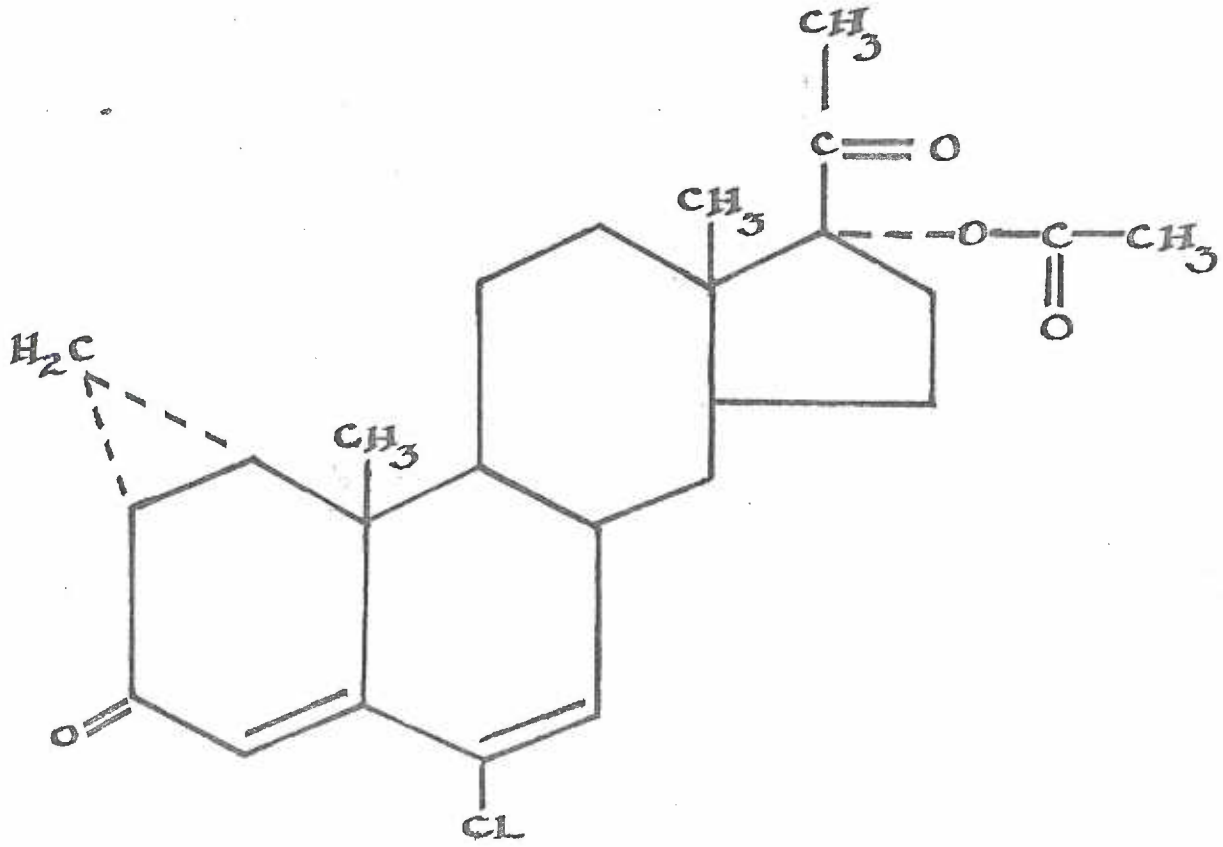
      0.087 O.D. value on standard graph (corrected for dilution in step "1.c.") corresponds to 15 ug total DNA (in 0.75 ml extract)

CYPROTERONE ACETATE

Historical Background

Cyproterone acetate (SH 714) was synthesized by R. Wiechert about 1962.[13] According to the criteria of Edgren, Jones and Peterson,[4] SH 714 may be considered a "true" progestagen. That is, SH 714 has an activity spectrum comparable to that of native progesterone.

Cyproterone acetate's chemical name is 1,2-α-methylene-6-chloro-Δ-4,6-prenadiene-17-ol-3,20-dione-17-acetate. The chemical structure is:



SH 714 was demonstrated analytically[13] to be 350 times more active (when subcutaneously administered) than progesterone in the Clauberg test (rabbit uterine weight changes). When administered orally, it was 1000 times as active as progesterone. Moreover, the compound was shown to be 1/1000 as active as estradiol in the Allen and Doisy test (rat vaginal smear changes) and less than 1/640 as active as testosterone propionate in the Dorfman test (chicken-comb length changes). Experiments with isotope labeled SH 714 (carboxy-^{14}C labeled) in rats demonstrated a relatively slow excretion of the ^{14}C containing compound. This excreted amount reached a peak of 30% about 24 hours after intravenous administration.[13]

> Preliminary studies indicated that:
>
> SH 714 is strongly antiandrogenic in rats. A dose of 200-400 gm/animal/day neutralizes the effect of 100 gm testosterone propionate/animal/day on the accessory genital glands and levator ani by 50% in castrated male rats. Daily administration of SH 714 over a period of three weeks causes a dosage dependent decrease in the weight of the seminal vesicles and ventral prostate as well as a decrease in the weight of the levator ani in normal puberal rats."[13]

It was the opinion of Neumann and Elger[13] that since cyproterone acetate could be shown to efficiently inhibit (i.e., antagonize) the effects of exogenous androgens (in contrast to noninhibition by exogenous estrogens), one was dealing with a competitive antagonism by an antiandrogen. That is, there was present a competitive action by SH 714 for androgenic receptors within the target organs. It was further demonstrated that neither an appreciable inhibition by SH 714 on the pituitary gland nor an estrogenic effect was the determining factor behind the mechanism of action

for SH 714.[6] In the early studies of the mechanism of action of SH 714, two possibilities were considered. Hamada et al.[5] proposed that SH 714 caused an androgenic refractory state by actively occupying the androgenic receptor sites in end organs and thereby causing the sites to become refractory (resistant) to androgens. Neumann and Elger[13] proposed that an enzyme deficiency was "...suspected in the sense of a reaction incapacity [of the androgenic receptors]". Further discussion on SH 714's mechanism of action is taken up on page 19 of the text of this thesis.

In their initial work, Junkmann and Neumann[6] noted that within two to three weeks following withdrawal of SH 714 all experimental male rats showed a complete reversibility of the antiandrogenic effects of SH 714. This reversibility was determined on the basis of successful matings. Furthermore, the toxicity of SH 714 was low, oral LD_{50}° being greater than two grams per kilograms of body weight.[13]

With regards to effects of SH 714 on libido, studies by Neumann and Hamada[9] and Junkmann and Neumann[6] indicated that juvenile male rats, whose mothers had received ten mg per day from day 16 to day 19 of gestation, were sexually inactive. Furthermore, these males had undergone intrauterine feminization.

The characteristics found with the intrauterine feminization were: (a) chromosomally male, (b) undescended testes, (c) female external genitalia, and (d) short, dead-ended vagina. It was demonstrated that this feminization could not be attributed to any estrogenic

activity of SH 714 since the compound had not shown any such activity in the Allen-Doisy test. This feminization effect also did not seem to be related to the progestational action of SH 714 since similar experimentation with other steroids of about the same progestational activity (via the Clauberg test) did not show this effect. For example, the free alcohol of SH 714, 6-chloro-Δ-6-1, 2-α-methylene-17-α-hydroxyprogesterone, possessed little progestational activity and yet had strong antimasculine and antiandrogenic effects on the fetuses.[6,9]

Since limited work has been done to demonstrate the relationship of the chemical structure of SH 714 to its progestagenic activity, one can speculate on this activity by considering the known progestagenic activity of related compounds. Zaffaroni and Bowers[15] speculated it was necessary to modify the basic gestagen structure (progesterone) in order to make it available for oral therapeutic and prophylactic use.

In its native state, progesterone is relatively weakly active in oral administration. Its lack of an active hydroxyl group adds to its inertness, e.g., it cannot form an ester. The carbon six position is particularly susceptible to β-hydroxylation in the liver. This results in a deactivation of or decrease in potency of the gestagen.[1]

Sala[12] found that the potency of progestational and oral effects depended upon the esterification of the 17 -α-hydroxyl function and the introduction of some group such as a methyl on the six -α position. Therefore, the primary step in the progesterone modification was to

introduce an hydroxyl group onto the molecule, e.g., at the carbon 17 position.

This introduction had several effects. First, the progesterone as a 17-α-hydroxy-progesterone was now able to form an ester. By itself, the 17-α-hydroxyprogesterone was anabolically reactive since it could easily be transformed.[1] Second, the formation of an ester by the introduction of an acetate, for example, afforded protection to the carbonyl group at carbon 20 against acidically catalyzed reactions, such as oxidation.[7] The steric hindrance found here at the carbon 20 position has been suggested by Wiener[14] to be one of deactivation. Third, Wiener also found that an ester formed by the introduction of a caproate at the 17-α-hydroxyl position changed the relatively weak compound into one with more prolonged and greater activity. Wiener further demonstrated that the structure of the ester at carbon 17 was not changed or removed when analyzed in the urine.[14]

Following the introduction of the hydroxyl and ester groups (in the case of SH 714, a 17-α acetoxylation), methylation of the six-position revealed further enhancement of the activity of the original gestagen. Not only was the carbon six position protected, but there was also an increase in the progestational activity. The activity to native progesterone and 17-α-acetoxyprogesterone was increased 30-40 times and 13 times, respectively.[8,15]

In a comparison of progesterone, medroxyprogesterone (6-α-methyl-17-α-acetoxyprogesterone), and chlormadione acetate (6-α-

chloro-Δ-6-17-α-acetoxyprogesterone). Brennan and Kraay[2] demonstrated that chlormadione acetate possessed the strongest antiestrogenic effect. They attributed the mechanism of chlormadione acetate to be that of blocking the action of the interstitial cell stimulating hormone. This postulate was derived from the observation that there was a decrease in the seminal vesicle and ventral prostate weights. The adrenal weights were depressed, but the testicular weights were only slightly affected.

A double bond introduced at the carbon six position further contributed to increasing the activity of the modified progesterone. Chlormadione acetate was shown to have seven hundred times the activity of 17-α-acetoxyprogesterone.[11] The dehydrogenation at carbon six had a greater effect than at another readily dehydrogenated position, carbon one. It was noted that dehydrogenation of both carbons one and six was not accumulative in activity, i.e., 1,6-bisdehydro-6-chloro-17-α-acetoxyprogesterone was not more active than chlormadione acetate.[11]

SH 714 is structurally similar to chlormadione acetate. The difference is that SH 714 has a one,two-α-methylene group attached. Apparently it is this alkylation of the carbon one and two position that increases the antiandrogenic effect. Junkmann and Neumann[6] found that the dealkylation of the carbon one and two position diminished the effect by one half to one third. The free alcohol form of SH 714 had one fourth to one sixth the activity of the acetate, and dechlorination of the molecule dropped its activity one sixth to one fifteenth. Complete removal of the prenane side chain caused the molecule to have no progestagenic activity in comparison with SH 714.

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