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Title: A STUDY OF THE EFFECT OF 1,2-ALPHA-METHYLENE-
6-CHLORO-DELTA-4,6-17-ALPHA-HYDROXYPROGESTERONE
ACETATE ON THE ADULT MALE RAT INCLUDING CHANGES IN
THE CELL ASSOCIATIONS OF THE GERMINAL EPITHELIUM

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SH 714 (1,2-alpha-methylene-6-chloro-delta-4,6-17-alpha-hydroxyprogesterone acetate) synthesized by Schering AG is an highly antiandrogenic, nonestrogenic compound. The present investigation was undertaken to determine the compound's effect upon the cell associations of the germinal epithelium in the mature rat. From several preliminary dosage level studies, 16 mg per animal per day was chosen for this study and administered for ten or twenty days. Somatic and accessory reproductive organ changes due to SH 714 demonstrated a significant decrease in the weights of the testes, adrenals, seminal vesicles, and ventral prostates. There was no change, however, upon thyroid, pituitary, or total body weights when compared with control animals. The greatest effect upon the

germinal epithelium for this dosage level of SH 714 was exhibited in the 20 day animals. It was postulated that SH 714 acted upon the more advanced steps (eight through 19) of spermiogenesis, thus inhibiting the maturing spermatids.

A Study of the Effect of 1,2-alpha-methylene-6-chloro-
delta-4,6-17-alpha-hydroxyprogesterone Acetate on
the Adult Male Rat Including Changes in the Cell
Associations of the Germinal Epithelium

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INTRODUCTION

The primary purpose in studies of fertility control has been to promote temporary sterility as a means of contraception. The anti-spermatogenic action of steroidal hormones has recently become the focus of studies in induced antifertility. Several publications have reviewed the natural and experimental control of mammalian spermatogenesis via steroidal hormones (Segal, 1964; Austin and Perry, 1965; Heller, Lalli and Rowley, 1966). Such inhibitory compounds have been described in the rat (Pincus, 1965), a common subject of these studies, and in man (Heller et al., 1966).

Synthetic gestagens are shown to have a greater or lesser degree of hormonal function than endogenous steroids in the male (Heller et al., 1958, 1959, 1960; Huis in't Veld, Louwerens and van der Spek, 1960, 1961). The mechanism by which the synthetic steroids act is varied. They may: (a) act as a competitive antagonist to the endogenous compound, (b) enhance the given activity of a native hormone to the exclusion of others, (c) inhibit the production or release of essential gonadal steroids, or (d) act directly on the testes

(Heller et al. , 1959; Pincus , 1967).

Revived interest in the clinical value of progesterone initiated the synthesizing of several compounds analogous to progesterone. Such compounds have included a variety of methylated, demethylated, hydroxylated, unsaturated, halogenated, and esterified derivatives of the parent progesterone. These synthetic progestational steroids have shed new light upon the treatment of endocrine infertility as well as the management of contraception. The ideal progestational compound should: (a) be highly potent, (b) produce no side reactions, (c) be orally effective, (d) lack anabolic effects, (e) lack toxicity, and (f) be free of inherent estrogenicity or androgenicity. The compound should furthermore be effective at very low dosage levels.

In 1954 Colton synthesized the first oral hormone of a progestational nature. This compound, Norethynodrel, later combined with mestranol, was found to be effective as a contraceptive agent in laboratory animals as well as in human females (Pincus et al. , 1956). Since then, many other similar compounds in the form of oral progestins, progestagens, gestagens, or progestational agents have been developed and clinically evaluated for use in birth control in the female.

It has become evident that not only close clinical observation but extensive biochemical and endocrine evaluation of each compound is necessary. The purpose is two-fold: (1) to facilitate a more

comprehensive understanding of the mode of action of these compounds and (2) to evaluate temporary or permanent organic damage, if any.

Heller et al. , (1958, 1959, 1960) demonstrated the effects of some synthetic and native progestagens upon the germinal epithelium, gonadotropin secretion, and 17-ketosteroid secretion in normal men. A comparison was made between the physiologic effects of three commercially marketed progestins, Nilevar, Norlutin, Enovid, and progesterone.

The first of these synthetic progestins, Nilevar (17-alpha-ethyl-19-nortestosterone), was known to inhibit ovulation and produce a progestational-like endometrium (Rock, Pincus and Garcia, 1957) in women. Its high effectiveness, even when administered orally, was due to the dichotomy of its androgenicity and anabolism. Seven normal subjects orally received 30 mg per day to determine its effects upon spermatogenesis. Decreases in sperm output and spermatozoan motility were noted in four weeks. Azoospermia, absence of libido, lowered potentia, and an alarming decrease in testicular size were observed in all subjects at the end of the eighth week. Also found were decreases in gonadotropin and 17-ketosteroid secretions. Histologically the testicular morphology was extremely altered in comparison to that present before treatment. For example, the seminiferous tubules decreased in diameter; there was hypoplasia

of all germinal elements; and the disappearance of Leydig cells with a simultaneous appearance of mesenchymal cells became evident.

In considering the mechanism involved, this seemed to center about the reduction of the gonadotropin secretion. Gonadotropins had been detected in pretreatment urine, but not in post-treatment urine.

This decrease of gonadotropins apparently was not due to an estrogenic increase since pretreatment and post-treatment urinary estrogen levels were unchanged. Furthermore, a suggestion of estrogen suppression was evident in several subjects. This was to be expected since a major portion of the male estrogen was derived from the Leydig cells and possibly the cells of Sertoli. In addition, the reduction of the 17-ketosteroids was attributed to the loss of Leydig cells.

These effects due to the administration of Nilevar were interpreted not as actual testicular damage, but as a return to the prepuberal state. Hence, the appearance of interstitial mesenchymal cells, reduced hormonal levels, reduced seminiferous tubule diameters, and the presence of spermatogonia as the principal germinal elements were reminiscent of the juvenile state. Six months following withdrawal of medication, sperm counts reached a status distinctly above pretreatment levels. This occurrence appeared to be similar to the "testosterone rebound" phenomenon noted in clinical treatment of nonfertile men (Heller and Maddock, 1947). A rapid recapitulation of the puberal process occurred in the other germinal

elements. The presence of plaque-like gynecomastia, often accompanying natural puberal processes, was noted in some of the subjects.

Another synthetic progestin studied was Norlutin (17-alpha-ethynyl-19-nortestosterone) which exhibited properties similar to Nilevar in women (Rock et al., 1957). Each of five normal males ingested 30 mg per day of Norlutin. Azoospermia was reached within six weeks. As with the Nilevar treatment there was a loss of libido, potentia, and testicular size. There was also a decrease of 17-ketosteroid levels. Two subjects developed gynecomastia during the treatment. Testicular morphology resembled that seen in the Nilevar administration.

The mechanism here was also similar to Nilevar. Absence of gonadotropins post-treatment implied the alterations were due to an inhibition of gonadotropins by Norlutin. However, unlike the effect of Nilevar, an increase in urinary estrogens in each subject post-treatment was found. Following the proposals of the "utilization hypothesis" (Heller et al., 1952), estrogen alone could account for the decrease of post-treatment gonadotropins. This idea was later substantiated by Apostolakis (1961). Post-treatment, normal hormonal levels were reattained, and in some subjects the rebound phenomenon of sperm counts was again found as with Nilevar. Parada, Napp, and Voigt (1960), in their work with Norethisterone (Norgestrel), were unable to demonstrate any drop in gonadotropin

excretion. Furthermore, they were unable to find any significant changes in the measurements of estrogen excretion.

Enovid (17-alpha-ethynyl-17-hydroxy-delta-5,10-estrene-3-one) also induced progestational-like endometria, inhibited ovulation, and was anabolic in women (Rock et al., 1957). As with Norlutin, each of five normal subjects orally received 30 mg per day. Within five to six weeks, spermatogenesis had been suppressed to zero. The time required for suppression was less than that of Nilevar or Norlutin. A concomitant effect upon the germinal elements again was evident. 17-ketosteroid levels fell, and gynecomastia was present in four of the five subjects. Histologically, a hyalinization of the basement membrane of the seminiferous tubules was noted and attributed to the higher amount of estrogen present in Enovid (1.5%) than in Norlutin (less than 0.2%) and Nilevar (0%).

The mechanism here seemed obvious in light of the reduced gonadotropin levels and the greatly increased estrogen circulation. Upon withdrawal of medication, there were returns to the pretreatment hormonal levels, sperm counts of a rebound phenomenal nature, and normal testicular morphology.

Progesterone itself was administered intramuscularly to nine subjects at the arbitrary dosage of 50 mg per day. After four weeks of injection, sperm counts fell. Azoospermia was attained by ten weeks. Whereas an anabolic effect of the previous progestins had

been noted, it was not noted with progesterone. Physic and physical alterations were similar to those of the synthetic progestins. However, testicular morphology was not impaired to the extent produced by the shorter duration of the synthetic progestins. All elements of the germinal epithelium were present, including the cells of Leydig. The levels of 17-ketosteroids were reduced slightly. Within six months post-treatment, sperm counts showed complete recovery.

Whereas the mechanism of the synthetic progestins was of the conventional steroid manner, i. e., apparent suppression of spermatogenesis by first suppressing the gonadotropin secretion, progesterone neither suppressed gonadotropins, nor was estrogenic, nor converted to estrogen. However, spermatogenesis was reduced, even if not completely. Therefore the mechanism by which progesterone suppressed spermatogenesis could have been a direct effect on the testes (Nelson and Patanelli, 1960). Grunt and Walker (1960) have demonstrated that progesterone, in doses of 100 mg or 500 mg per day for seven days, had essentially no effect on the seminal vesicles of castrated rats.

Several reasons have been hypothesized for the significant reductions of 17-ketosteroid levels found in all four groups above (Heller et al., 1958, 1959, 1960). It has been suggested that since estrogen levels remained unchanged or were elevated during therapy, the Leydig cell function was not disturbed. A paucity of information

has existed concerning the nature of the Leydig cells. It may have been that one entity of the cells was inhibited in its production of 17-ketosteroids while a second entity was not inhibited in its production of estrogen. Brooks and Prunty (1957) attributed the 17-ketosteroidreduction of Nilevar to be of an adrenal origin. Huis in't Veld et al. , (1958, 1960, 1961) in their studies of a related compound, Orgasteron (17-alpha-methyl-19-nortestosterone), in human subjects, found a definite decrease in urinary excretion of 17-ketosteroids and 17-hydroxycorticosteroids due to daily administration of 25 mg of Orgasteron for three or more weeks. There was no observable effect on the testicular intersitial tissue. These effects were attributed to the following mechanisms: (a) Orgasteron inhibited the interstitial cell stimulating hormone production, and/or (b) Orgasteron influenced the metabolism of the adrenocortical hormones. Patrono and Nicolosi (1962) intramuscularly administered progesterone in a Holtorff and Kock test (modified by Landau) to 20 normal men and 34 normal women. The findings for increases in 17-ketosteroids were positive in only two of the men and none of the women. However, Singer and others (1963) have found that progesterone, in the intact rat, stimulated aldosterone production (perhaps as a precursor) and yet inhibited the production of corticosterone. They also have found that hypophysectomy caused no changes in these results.

A progestational steroid recently synthesized by Dr. R.

Wiechert (Schering AG) is compound SH 714. According to the criteria of Edgren, Jones and Peterson (1967), SH 714 may be considered a "true" progestagen. That is, SH 714 has an activity spectrum comparable to that of native progesterone. Generically, this compound is a cyproterone acetate. Its chemical name is 1,2-alpha-methylene-6-chloro-delta-4,6-prenadiene-17-ol-3,20-dione-17-acetate.

Preliminary studies by Schering AG, Berlin, West Germany, 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. (Schering AG, 1965)

It was the opinion of Neumann and Elger (Schering AG, 1965) that since cyproterone acetate also inhibited exogenous testosterone (in contrast to noninhibition by estrogens), one was dealing here with a competitive antagonism. That is, there was present a competitive action on androgen receptors within the target organs. It was further demonstrated that neither an appreciable inhibition on the pituitary gland nor an estrogenic effect was the causative factor in the mechanism of action (Junkmann and Neumann, 1964). This problem will be further discussed.

For the range of dosages studied, Schering AG (1965) noted that within two to three weeks following withdrawal of medication all male rats showed a complete reversibility of the antiandrogenic effects of SH 714. This was substantiated by successful matings (Junkmann and Neumann, 1964). The toxicity of SH 714 was low, the LD₅₀ being greater than two gm per Kg of body weight orally in the mouse.

Some of the side effects which developed with the other progestagens previously discussed were encountered with SH 714 by Schering AG (1965). For example, in preliminary studies with the guinea pig, Winkler and Harkness (1964), demonstrated that daily administration of one mg of SH 714 per kg of body weight for 28 days caused a transient decrease of the 17-hydroxycorticosteroid excretion in the urine. However, since a "rebound phenomenon" occurred after cessation of medication, it was suggested that there was no permanent change in adrenal activity. Hamada, Neumann, and Junkmann (1963) further noted that rats receiving ten mg per animal per day for three weeks showed a decrease in adrenal weight. It was their opinion that this phenomenon suggested some adrenal inhibitory effects at this dosage level.

With regards to effects on libido, studies by Neumann and Hamada (1963) and Junkmann and Neumann (1964) 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. It was demonstrated that this feminization could not be attributed to an estrogenic activity of SH 714 since the compound had not shown any such activity in the Allen-Doisy test. This side effect also did not seem to be related to the progestational action of SH 714 since a series of other steroids with about the same progestational activity (via the Clauberg test) did not show this effect. For example, the free alcohol of SH 714, 6-chloro-delta-6-1,2-alpha-methylene-17-alpha-hydroxyprogesterone, possessed no progestational activity and yet had a strong antimasculine and antiandrogenic effect on the fetuses. However, another compound, 19-nor-17-alpha-hydroxyprogesterone acetate, exhibited strong progestational activity but had no antimasculine effect on fetuses. Furthermore, progesterone itself had no antimasculine effect on fetuses. An explanation for this latter compound's lack of effect was that it could have been inactivated too rapidly during fetal metabolism. It was postulated (Neumann and Hamada, 1963; Junkmann and Neuman, 1964) that the possible cause for the intrauterine feminization may have been similar to the clinical picture of testicular feminization seen in humans.

The characteristics found in that malformation were: (a) chromosomally male, (b) undescended testes, (c) female external genitalia, (d) feminine voice and psyche, (e) short, dead-end vagina,

and (f) normal development of the breasts. For the mechanism here, an androgen or testosterone refractory state (i. e., resistance to androgen or testosterone) is postulated (Hamada et al., 1963). That is to say, the antiandrogenically active compound occupies the receptors of the native androgens, thereby inhibiting the action of the androgens (Neumann and Kramer, 1964).

A second approach to this mechanism was postulated by Neuman and Elger (Schering AG, 1965). An enzyme deficiency was " . . . suspected in the sense of a reaction incapacity" of the androgen receptor. Hence, in the human patients with this malformation, there was no reaction to the administration of androgens such as was found in normal patients.

The apparently conflicting observations that SH 714 exhibited androgenicity as well as antiandrogenicity may be better understood by mentioning a similar mechanism of estrogens. Estradiol can be degraded to estriol which shows weak estrogen activity. However, if estradiol is simultaneously administered, the estriol will inhibit the estradiol activity, creating an antiestrogenic effect (Schering AG, 1965).

A deduction of embryological significance can be made from the above observations. Since the antiandrogenic compound SH 714 has no estrogenic effects, and since its feminizing effects are related to its androgen antagonism, then it may be possible for the fetal

androgens of the male testes alone to inhibit the development of accessory female structures such as the mammary glands.

In one demonstration of SH 714 in humans, it was reported (Schering AG, 1965) that following one 250 mg tablet of the compound per day for three to six weeks, all subjects showed a loss of libido and potency. However, it was also noted that no gynecomastia developed as was the situation with treatments of Enovid, etc.

Experiments with isotope labeled SH 714 (carboxy-¹⁴C labeled) in rats demonstrated a relatively slow excretion of the ¹⁴C containing compound. This excreted amount reached a peak of 30 % about 24 hours after intravenous administration (Schering AG, 1965).

Preliminary bioassays of SH 714 performed by Schering AG may be summarized by stating that

...this compound in a dosage of 3 mg per day for 3 weeks caused a 3 fold reduction in ventral prostate weight of the intact adult male rat. The compound was found to be strongly progestational, being 250 times more active than progesterone in the Clauberg test, weakly estrogenic, being less than 1/1000 as active as estradiol in the vaginal smear method of Allen and Doisy, and weakly androgenic, being less than 1/640 as active as testosterone propionate in the chicken-comb test according to the method of Dorfman. However, it should be noted that the compound did cause some shortening of the urethra in male fetuses when given to mothers in dosages of 1 to 30 mg per animal per day on the 16th to 19th day of gestation. This action was described as one of 'intrauterine feminization'. (Bridge and Scott, 1964, page 100)

In a more recent study (Schering AG, 1965) SH 714 was found to be 350 times more active (subcutaneously administered) as progesterone in the Clauberg test. When SH 714 was administered orally, it was 1000 times as active.

Since limited work has been done to demonstrate the relationship of the chemical structure of SH 714 to its activity, one can best postulate what it might be on the basis of the known activity of related compounds. Zaffaroni and Bowers (1964) speculate it is 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 B-hydroxylation in the liver. This results in a deactivation of or decrease in potency of the gestagen (Bishop, 1962).

Sala (1958) 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-alpha-hydroxy-progesterone was now able to form an ester. By itself, the 17-alpha-hydroxyprogesterone was anabolically reactive since it could easily be transformed (Bishop, 1962). 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 (Loewenthal, 1959). The steric hindrance found here at the carbon 20 position has been suggested by Wiener (1961) to be one of deactivation. Third, Wiener (1961) also found that an ester formed by the introduction of a caproate at the 17-alpha-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.

Following the introduction of the hydroxyl and ester groups (in the case of SH 714, a 17-alpha acetoxylation), methylation of the six-alpha 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 of native progesterone and 17-alpha-acetoxyprogesterone was increased 30-40 times and 13 times, respectively (Miyake and Pincus, 1958; Zafforni and Bowers, 1964).

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

acetate (6- α -chloro- δ -6-17- α -acetoxyprogesterone), Brennan and Kraay (1963) 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 adrenals 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 (Ringold et al. , 1959). 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 (Ringold et al. , 1959).

SH 714 is structurally similar to chlormadione acetate. The difference lies in SH 714 having a one,two- α -methylene group attached. Apparently the alkylation of the carbon one and two position increases the antiandrogenic effect.

Junkmann and Neumann (1964) 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 pre-nane side chain caused the molecule to have no activity in comparison with SH 714.

Since SH 714 has been found to be inherently capable of performing as a progestational antiandrogen, the present investigation was undertaken to determine the compound's effect upon the cell associations of the germinal epithelium in the mature male rat. This study also considered the efficacy of SH 714 as a male contraceptive in light of previous information on progestational contraceptives in the male.

MATERIALS AND METHODS

Prior to this present study, preliminary dosage level studies of SH 714 were done (Lin, 1965, 1966) in intact male Sprague-Dawley and Harvard rats. From these initial studies a particular dosage level of 16 mg per animal per day was chosen for the present study. It was felt that during the preliminary studies, 15 mg per animal per day was optimal for causing obvious spermatogenic effects in the germinal epithelium. Therefore, the 16 mg level was chosen in slight excess of 15 mg. Propylene glycol (USP, Robinson Laboratories) was chosen as the transfer agent for SH 714. The SH 714 was dissolved in the transfer agent at a concentration of two mg SH714 per 0.1 cc propyleneglycol.

Two groups of Sprague-Dawley male rats, averaging two hundredforty grams in weight, were injected with eight mg of SH 714 twice a day, including Sunday. One group was treated for ten days, the second for 20 days. The daily injections of an animal were dispersed along the area of the back and the nape of the neck. Body weights were taken both prior to treatment and at autopsy. Basal metabolic rates were determined via a Scholander respirometer. Daily observations of the animals' somatic condition were made.

The animals were killed with ether. The testes, seminal

vesicles, ventral prostates, adrenals, thyroids, and pituitaries were removed and weighed. Gross observations were made of the areas subcutaneous to the injection sites. The testes were fixed in Zenker's formol solution and sections cut at a thickness of six microns.

Periodic acid solution, Schiff's reagent, and Harris' hematoxylin were employed for microscopic studies (Rowley and Heller, 1966).

Sections of the testes were randomly chosen to quantitate the cell associations of spermatogenesis. Fifty seminiferous tubules per section were studied and classified according to the 14 cell associations as described by Clermont and Perey (1957).

RESULTS

The microscopic study of the germinal epithelium in control and propylene glycol treated animals of the ten and 20 day groups indicated an apparent similar histologic picture in both control and carrier treated animals (Figures 1, 2, 4, 5). The morphology of the seminiferous tubules in the animals receiving 16 mg of SH 714 per day for ten days did not demonstrate any significant change from that seen in the controls (Figure 3). However, the germinal epithelium in those animals receiving 16 mg of SH 714 per day for 20 days exhibited some atrophy (Figure 6). Several cross-sections of seminiferous tubules from this latter group appeared comparable to the controls. Other tubule sections were seen as having undergone antispermiogenesis. In these latter cross-sections, the more advanced steps (eight through nineteen) of spermiogenesis were either absent from their respective cell associations or apparently reduced in numbers when compared with control sections.

During each of the preliminary studies, treatments (where either sesame oil or propylene glycol was the carrier) indicated that animals receiving greater than or equal to 12 mg per day of SH 714 developed scabs at the injection sites. In the present study, even though the injection sites were constantly varied, these scabs

inevitably appeared in all animals receiving the eight mg of SH 714 per animal b. i. d. In the areas surrounding the scabs, body hair fell out leaving only bare skin. The scabs and hairlessness were absent in the control and propylene glycol treated animals. At autopsy, the gross, subcutaneous appearance of these areas was normal except for the presence of scar tissue. Sections were not made.

Adverse neuromuscular effects due to injections of SH 714 were not demonstrated. This was macroscopically determined by noting that all animals maintained normal control over their body movements. However, it was also noticed that the body hair of both the propylene glycol and SH 714 in propylene glycol treated animals became coarser and duller in appearance than that of the control animals.

During the treatment and at autopsy, wheals due to subcutaneous accumulation of the injected material were not found. Thus it was assumed that all the injected material had been absorbed.

At autopsy, the gross appearance of the viscera and the glands removed was normal in all animals except those on SH 714 treatment. In these latter animals, there was a reduction in the size of some glands. Glandular weights were recorded. The average weights and standard errors are shown in Table 1. Several of the seminal vesicles of both the ten and 20 day SH 714 groups contained very little or no seminal fluid. Some animals of the 20 day SH 714 group

Table 1. Summarization of data obtained in a study of SH 714 (animal groups, treatments, average basal metabolic rates, body and organ weights with their Standard Errors are included)

	Ten Day Group			Twenty Day Group		
	Control	Propylene Glycol	SH 714 in Propylene Glycol	Control	Propylene Glycol	SH 714 in Propylene Glycol
Number of rats	8	8	8	8	8	7
Treatment per animal (b. i. d.)	0 cc	0.4 cc	8 mg	0 cc	0.4 cc	8 mg
Initial body weight (gm)	232 ± 7.9	251 ± 8.0	216 ± 5.4	238 ± 7.1	245 ± 9.3	244 ± 9.6
Final body weight (gm)	223 ± 8.7	259 ± 7.9	228 ± 5.5	280 ± 8.7	275 ± 8.0	251 ± 8.9
Basal metabolic rate (Kcal per m ² -hr)	40.8 ± 4.9	38.7 ± 1.8	42.2 ± 2.9	56.4 ± 2.0	58.9 ± 8.6	* 63.2 ± 18.1
Testes weight (mg)	1825 ± 41.8	* 1977 ± 28.9	* 1680 ± 14.7	2170 ± 31.7	2171 ± 26.2	* 1420 ± 30.9
Adrenal weight (mg)	37 ± 2.6	* 47 ± 1.7	* 19 ± 1.0	48 ± 3.0	* 40 ± 3.6	* 17 ± 1.0
Seminal vesicles (mg)	406 ± 34.9	* 435 ± 35.1	* 172 ± 14.9	600 ± 30.2	* 560 ± 25.9	* 114 ± 10.4
Ventral prostate (mg)	287 ± 24.4	* 335 ± 29.0	* 133 ± 23.1	435 ± 26.2	438 ± 16.1	* 40 ± 13
Thyroid (mg)	16 ± 0	15 ± 0	15 ± 0	15 ± 0	13 ± 0	15 ± 0
Pituitary (mg)	10 ± 0	10 ± 0	9 ± 0	10 ± 0	10 ± 0	9 ± 0

* indicates 0.95 or greater probability in the t-test for confidence

possessed ventral prostate glands too small to be measured. The ventral prostate glands present in other animals of the same group weighed less than one tenth as much as those present in either the control or propylene glycol treated animals. Thyroid and pituitary weights were consistent for all groups.

Body weights and basal metabolic rates are given in Table 1. The ten day controls unexplainably lost weight, whereas their corresponding propylene glycol and SH 714 groups gained weight. The 20 day controls did gain weight. Their corresponding treated animals gained weight also, but to a lesser degree. The basal metabolic rates in the ten day group were shown to be consistent throughout the control, transfer agent, and SH 714 treated animals. However, the rates determined for the SH 714 animals in the 20 day group were shown by the t-test to be significant in their difference from the controls. It is noted, however, that the corresponding comparison of thyroid weights in the 20 day group did not demonstrate any significant difference between the SH 714 and control animals.

DISCUSSION

Jackson, Fox and Craig (1961) discussed the timing for spermatogenesis in the rat. They mentioned that the overall timing for rat spermatogenesis was about 65 days. Jackson et al. (1961) arrived at this overall length by modification of the data of Leblond and Clermont (1952). In that original data, 27 days had been hypothesized as the length of time required for the development of mature spermatozoa from Type A spermatogonia. However, with the use of more sophisticated techniques developed for this kind of a study, e.g., the use of tritiated thymidine, 48 days has become the basis for the testicular phase of spermatogenesis in the rat. An additional 15 days was required to transport the maturing spermatozoa from the rete testis to the vas deferens (Jackson et al., 1961). Consequently, the numbers 63 and 65 were reached for the full period. Of the four phases of the Type A spermatogonia (stem cells, second, third, and fourth generations), the second generation was chosen to represent the starting point for the above count of 63 days. This designation was necessary because of the discrepancy concerning how long the stem cell phase was present. The problem here basically involved the dynamics of the quantitative analysis of spermatogonial renewal (Clermont, 1962).

With the above discussion in consideration, one is able to

relate more precisely the effects of antifertility agents to the locus or loci most affected in the germinal epithelium. Earlier in this dissertation several ideal qualities for a temporary antifertility agent were mentioned. At this time one additional one may be brought in. That is, an ideal antifertility agent should be "convenient" in the male. This is to say that the agent should furnish the temporary sterility via immediate action upon the germinal epithelium. When the mechanism involved is dependent upon an indirect effect in the testes by way of the pituitary for example, temporary sterility may be reached. However, it would be at the cost of the time required for the agent to affect the pituitary, then indirectly the testicles; and, the time required for the effect upon the spermatogonia to be carried through the spermatogenic phases. If a sterile effect is to be temporary, it becomes necessary for the spermatogonial element to remain intact even though its subsequential maturation to spermatozoa becomes altered. Spermatogonial renewal (Clermont, 1962, 1966) assumes that following several mitotic generations, the majority of the Type A spermatogonia enter into the meiotic phases of spermatogenesis while the rest continue to mitotically reproduce. Thus, an unlimited means is provided by which there will always be stem cells. It is also obvious that an antifertility agent having an antispermatogonial effect would promote permanent rather than temporary sterility. In the rat, an antifertility agent with the above mechanism

would require some 70 days to be effective. In the human male, the agent would require approximately the same amount of time to be effective. Indubitably then, an immediate action upon the germinal epithelium and preferably so upon the more mature stages of the epithelium becomes a criterion of the ideal antifertility agent.

As discussed previously, progesterone by itself was thought to cause antispermatogenic effects by a direct influence upon the testes (Heller et al., 1958, 1959, 1960). If this is the mechanism involved, increasing the activity of the basic progesterone by modification of its chemical structure should be paralleled by a more pronounced effect upon the testes. This effect upon the testes may be found primarily in the Leydig cells and secondarily in the germinal epithelium. On the other hand, the effect may be thought of as being directly upon some faction of the germinal epithelium. In this present study as well as in the preliminary studies (Lin, 1965, 1966), SH 714, being 350 times more active than progesterone (Schering AG, 1965), focused its antispermatogenic effect upon the more mature elements of the germinal epithelium.

Several approaches were attempted to best illustrate the anti-spermatogenic effect of SH 714. Quantitations of the different kinds of cell associations were made and merely resulted in confirming data that designated the approximate length of time a particular stage of rat spermatogenesis would be present (Jackson et al., 1961). A

second approach was to base the evidence of the compound's effect on the morphologic appearances of the seminiferous tubules.

In the present as well as the preliminary studies, the morphologic picture of the seminiferous tubules in the control (Figures 1, 4) as well as in the propylene glycol treated animals (Figures 2, 5) were the same. The tubule sizes, the visual comparisons of the numbers of mature spermatozoa within the lumina of the tubules, and the amount or extent of sloughing were seen as being quite similar. Since all tissues had been cut at a thickness of six microns, handled in similar fashion prior to and during staining, and observed under the same magnifications, these subjective deductions were considered to be representative.

In the SH 714 treated animals, a more antiandrogenic and anti-spermiogenic picture was seen morphologically. In the present study, the group treated with the compound in propylene glycol for ten days was not much changed from the controls with regards to the tubule sizes and amount or extent of sloughing (Figure 3). However, a visual comparison of the density of mature spermatozoa in the lumina of the seminiferous tubules presented a slightly different picture. In the case of the ten day treated groups, the density of the spermatozoan tails in all SH 714 treated animals was less than that found in control and transfer agent groups. A site of effect was postulated as being in some step of spermiogenesis and not in the

spermatogonial or spermatocytic stages. Observation of the 20 day treated animals seemed to confirm this.

In the latter treated group, a more marked distinction was found between the controls or propylene glycol treated groups and the SH 714 treated group. With regards to the size or diameters of the seminiferous tubules, those of the control and propylene glycol treated animals were similar. Those of the compound treated group, however, appeared to have shrunk. As previously mentioned, an antiandrogenic agent would be expected to cause some atrophy of the seminiferous tubules since they are androgen dependent. Disuse or use below a normal level would promote an atrophic condition. In a preliminary study (Lin, 1966) a dosage level of 20 mg per day for 25 days caused atrophy of the seminiferous tubules (Figure 8) when compared with those of the controls (Figure 7). However, other side effects were so drastic upon the animals, that this high dosage was not repeated for this study. The dosage for the current study apparently caused an initial atrophy of the seminiferous tubules. The tubular shapes were more rounded than those of other groups. Consequently the size was more compact and the diameter smaller in all 20 day SH 714 treated animals.

The density of spermatozoan tails within the lumina of the tubules of the 20 day SH 714 treated group appeared markedly less than that found in the controls. Though the possible effect of

improper handling of the tissues as a causative factor cannot be ruled out, nevertheless, since this histologic picture was consistent in the animals of this particular group, the phenomenon was attributed to SH 714. An increase in the numbers of tubules containing sloughed germinal epithelial elements was also observed. Due to the absence of the more mature steps of spermiogenesis, as will be further discussed, the increased sloughing of germinal elements was attributed to SH 714. The type of germinal elements being sloughed ranged collectively from the zygotene spermatocyte to the more mature spermatozoan. The sloughing evident in the tubules of the controls also included this range of germinal elements but not to the extent seen in the compound treated group.

From the present as well as the preliminary studies it is suggested that the site of the antispermatogenic effect of SH 714 was in the spermiogenic steps of maturation following the cap phase (Clermont and Perey, 1957). More explicitly, the antispermiogenic effect of SH 714 was apparent in steps eight through nineteen of spermiogenesis. Whereas steps one through seven of spermiogenesis with their associated spermatogonia and spermatocytes were present in the tubules, their mature spermiogenic steps were either absent or reduced in number. The reduction in number would indicate that the antispermiogenic effect was not yet complete in that particular tubule at the time of autopsy.

The question is raised concerning the mode of action upon the latter spermiogenic steps. The mechanism by which their depletion took place may lie in a postulation that these latter steps are more androgen dependent than other germinal epithelium elements. The blockage of the androgen receptors by the methods previously discussed would then be more pronounced in these latter steps. A long term study of this particular dosage level of SH 714 would determine whether or not the less mature elements would subsequently be affected by SH 714 as well. However, it is mentioned that in a longer term study, the secondary reproductive organs would undergo an increased atrophy.

From the combined data of Leblond and Clermont (1952) and Jackson, Fox and Craig (1961), the findings and postulations of the present study apparently are valid. Jackson et al. (1961) show that a maximum of 28 days may be needed for the acrosome stage of the spermatids (steps eight through 14) and 23 days for the maturation phase (steps 15 through 19) to reach emission as mature spermatozoa. If as postulated, SH 714 at a dosage level of 16 mg per day, does act directly upon the latter steps of spermiogenesis, a 20 day treatment period would show an initial marked effect upon the seminiferous tubules.

SH 714 possibly may have caused some skin irritation in and about the injection sites. Several animals were observed scratching

these areas prior to and during the presence of these scabs.

In this study, the observation of the development of hairlessness in and around the injection areas of the SH 714 treated animals was in line with an earlier study (Schering AG, 1965), which investigated the effect of SH 714 on the sebaceous glands of mice. The study demonstrated that due to SH 714 treatment, the number and size of cells per sebaceous gland alveolus decreased. Furthermore, areas previously plucked of hair showed little or no tendency for hair regeneration. This phenomenon was in contrast to that found in animals with hairless areas but on testosterone propionate treatment. But it was postulated that the influence on hair regeneration could not be simply explained on the supposition of testosterone propionate antagonism. The possible stimulating effect of testosterone propionate on hair growth therefore, remained unclear.

With regards to the coarser or duller hair of the propylene glycol and SH 714 in propylene glycol groups, this observation contrasted those seen in a preliminary study (Lin, 1965). In that preliminary study, sesame oil was used as a carrier. The coat luster at that time appeared to have increased in those animals receiving sesame oil treatment only. The animals that received SH 714 in sesame oil developed coarser and duller coats in comparison. The mechanism involved here may have been related to the discussion of the sebaceous gland alveoli mentioned above. However, SH 714

has been found to have no effect upon acne vulgaris or seborrhea in humans (Schering AG, 1965). Therefore, a clearer understanding of the mechanism involved here has not been found.

The decreases in glandular weights of the testes, adrenals, seminal vesicles, and ventral prostates in SH 714 propylene glycol treated animals fall into place with the expected effects of an anti-androgenic agent. That is to say, all structures and systems which are functionally or morphologically androgen dependent are expected to be influenced by the presence of an antiandrogen. In studies of Provest in rats, Duncan, Lyster and Clark (1963) note that testicular weight is not greatly influenced by the quantity of interstitial tissue present. Rather, the testicular weight is more an index of the mass and functional state of the seminiferous tubules. Duncan et al. (1963) further indicate that the indices of the functional status of the interstitial tissue may be found in the accessory gland weights. Ventral prostates are noted as being more sensitive indicators than the seminal vesicles. Hamada et al. (1963) note that daily administration of ten mg of SH 714 per animal per day for 21 days to normal adult rabbits causes an atrophy of the seminal vesicles and adrenals. However, the weight of the testes remains unaffected.

With regards to resultant body weights, DeWar (1962) demonstrated that progesterone induced body weight gain in mice. He concluded that the principal mechanism involved was that of water

and nitrogen retention promoted by the progesterone. Simultaneously, there was an increase of energy expenditure. A secondary response to these retentions and increases was an increase in food uptake. Fat deposition also occurred when this response was over-adjusted. Following withdrawal of progesterone, the gains were reversible via the expedient of reducing the food as well as the water consumption below normal levels of maintenance.

Since only those compounds related to the 19-nortestosterone series showed significant accelerated body growth or anabolism in a study by Edgren (1963), one could expect a relatively minute anabolic effect from SH 714. One could furthermore postulate an antianabolic property for this compound, i. e., the antiandrogenicity developed in the native androgen receptors subsequently causes an antianabolic effect towards the endogenous testosterone.

SH 714 caused a significant increase in the basal metabolic rates of the 20 day treated animals without a concomitant increase in the thyroid weights. These values ranged from 44.8 Kcal per m^2 -hr to 77.9 Kcal per m^2 -hr.

SUMMARY

The present investigation has shown that 16 mg per day of SH 714 for 20 days caused atrophy of the seminiferous tubules and of the secondary reproductive organs in the mature rat. The mechanism was thought to be either a direct effect upon the tubules by SH 714 or an antiandrogenic effect, thus, only secondarily affecting the seminiferous tubules. Microscopic studies suggested the compound may act directly upon steps eight through nineteen of spermiogenesis. Further work in this area with SH 714 should center about a long term study of a similar nature.

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APPENDIX

Figures 1-8

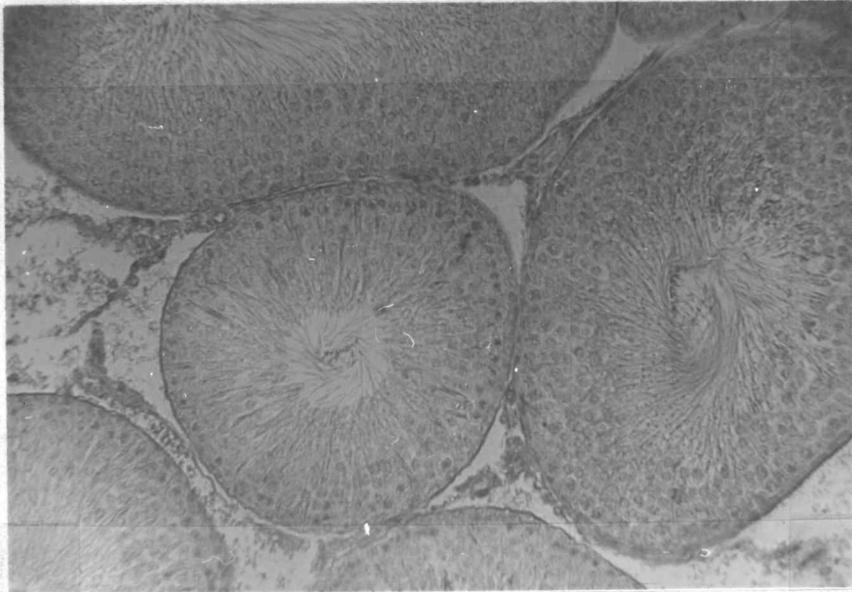


Figure 1. Seminiferous tubules of an animal used as a control for ten days. Note density of spermatozoan tails in the lumina of the tubules as well as the continuity of the germinal epithelium from the basement membranes to the lumina. x 1000.

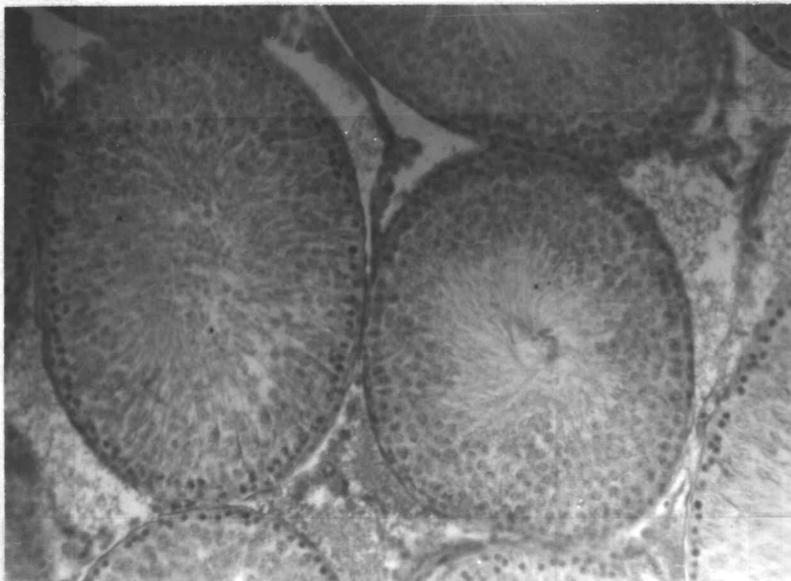


Figure 2. Seminiferous tubules of an animal treated for ten days with 0.8 cc per day of propylene glycol. Note the similarity to the morphologic picture found in the controls of the same treatment period. x 1000.

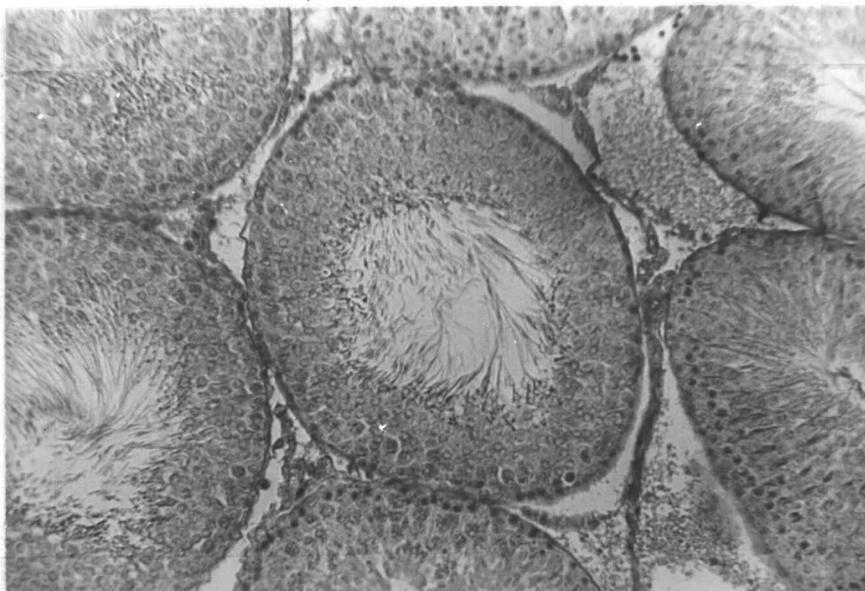


Figure 3. Seminiferous tubules of an animal treated for ten days with 16 mg of SH 714 in propylene glycol. As compared with controls, note the less dense appearance of spermatozoan tails in the lumina. x 1000.

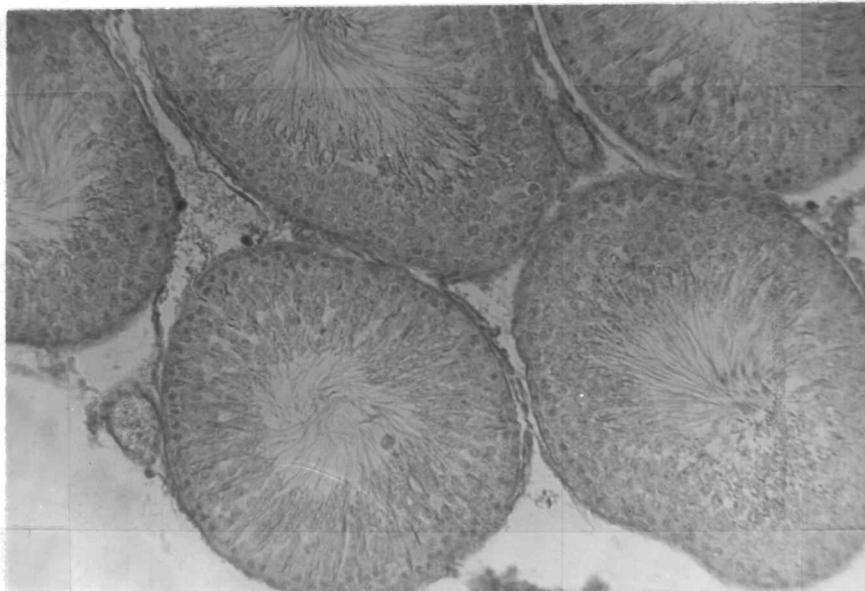


Figure 4. Seminiferous tubules of an animal used as a control for 20 days. Note density of spermatozoan tails in the lumina of the tubules as well as the continuity of the germinal epithelium from the basement membranes to the lumina. x 1000.

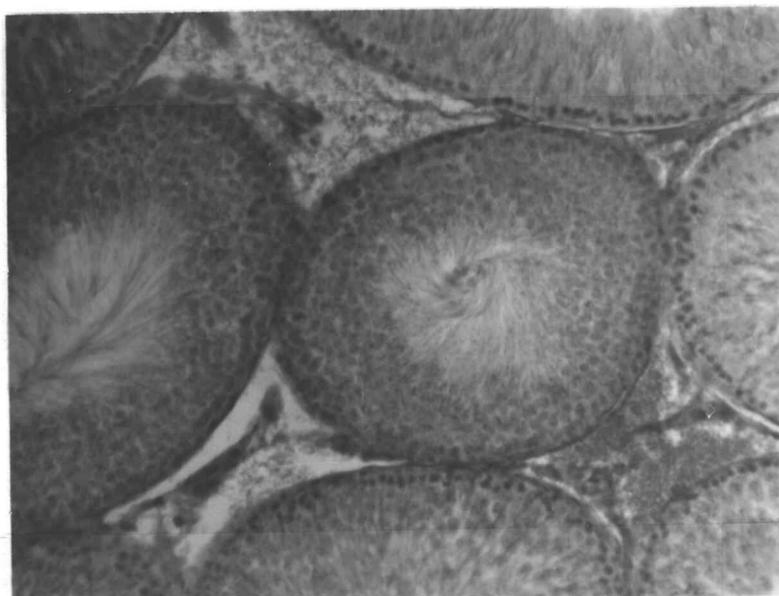


Figure 5. Seminiferous tubules of an animal treated for 20 days with 0.8 cc per day of propylene glycol. Note the similarity to the morphologic picture found in the controls of the same treatment period. x 1000.

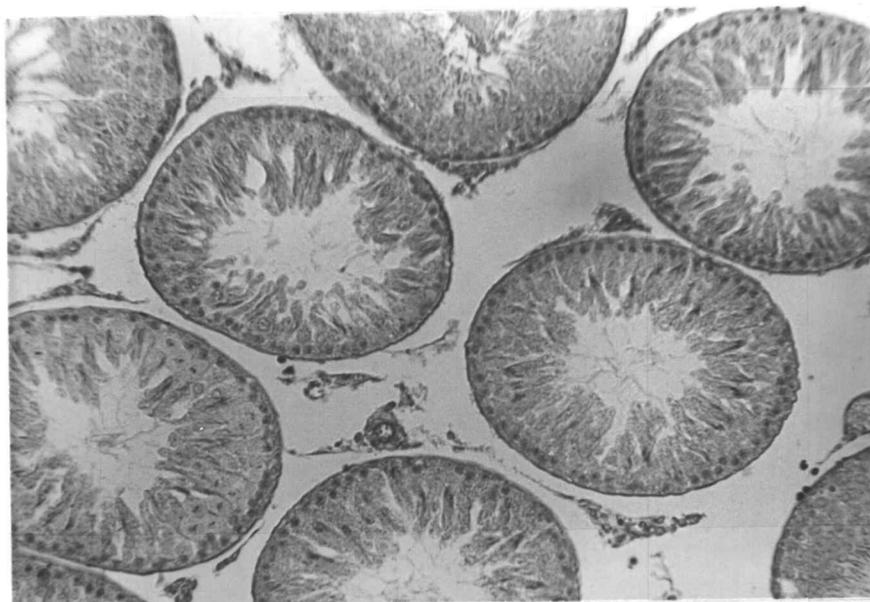


Figure 6. Seminiferous tubules of an animal treated for 20 days with 16 mg of SH 714 in propylene glycol. As compared with controls, note the absence or reduction of the later spermiogenic steps as well as atrophy of the tubule itself. Also demonstrated is the lack of continuity about the lumen periphery as compared with controls. It is in this area that the later spermiogenic steps are normally located. x 1000.

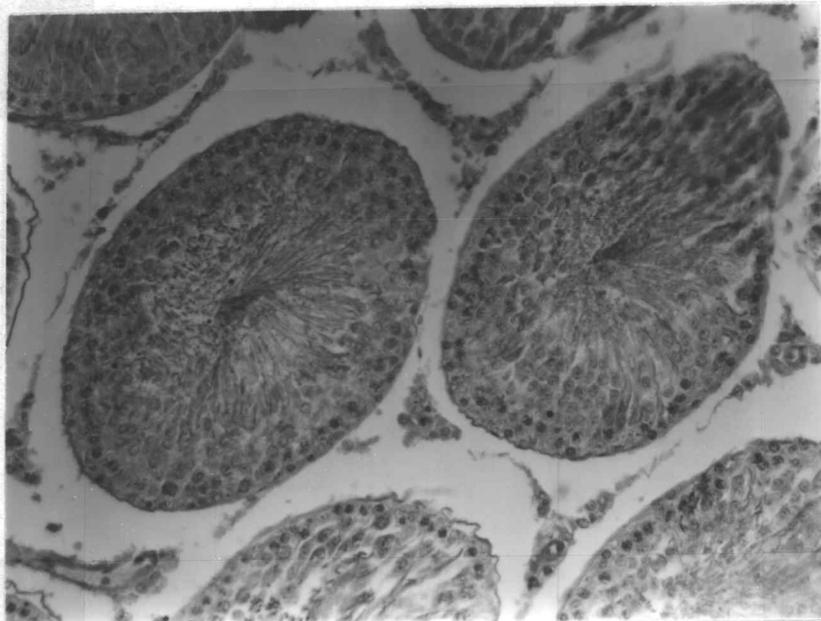


Figure 7. Seminiferous tubules of an animal used as a control for 25 days. Note density of spermatozoan tails in the lumina of the tubules as well as the continuity of the germinal epithelium from the basement membranes to the lumina. x 1600.

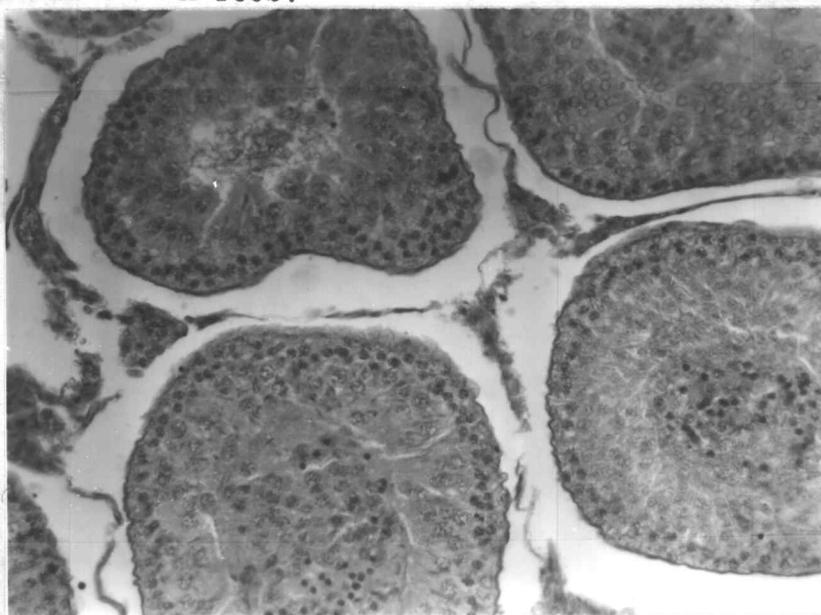


Figure 8. Seminiferous tubules of an animal treated for 25 days with 20 mg of SH 714. Note extreme atrophy of tubule, and presence of very few spermatozoa. x 1600.