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Shirley J. Stein

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TIME-RELATED CHANGES IN
RAT ANDROGEN BINDING PROTEIN FOLLOWING
A STERILIZING DOSE OF 3-CHLORO-1,2-PROPANEDIOL

by

Shirley J. Stein

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Submitted to the
Faculty of The Graduate College
in partial fulfillment
of the
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Western Michigan University
Kalamazoo, Michigan
August 1979

TIME-RELATED CHANGES IN RAT ANDROGEN
BINDING PROTEIN FOLLOWING A STERILIZING
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Shirley J. Stein, M.S.

Western Michigan University, 1979

Androgen binding protein (ABP) is produced by the Sertoli cell, regulated by FSH and androgens, secreted into the testicular fluid, and transported into the caput epididymis where it is thought to play a role in sperm maturation. In this study, the response of ABP to a lesion created chemically by administration of a single, oral 60 mg/kg dose of alpha-chlorohydrin was examined in ninety day old male rats. Total testicular ABP content increased progressively for four days after treatment and there after declined whereas, epididymal ABP concentration decreased immediately. Serum testosterone showed no significant variation from controls, however, serum FSH and LH levels did change from controls. Serum FSH levels remained generally unchanged until day ten and was increased on day twenty, whereas LH increased slowly, reaching maximal concentration on day ten. The overall effect of the above stated changes suggests that ABP production continues following treatment with alpha-chlorohydrin.

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First and foremost I would like to thank my husband, Bruce, without whose patience and understanding I could not have continued for the five long years that it has taken me to complete this degree while working full-time. Secondly, I am grateful for all the help that Dr. Leonard Beuving has given me as an advisor for my graduate work and Dr. Thomas J. Lobl for guiding me through my laboratory research. I would also like to give my thanks to the Fertility Research Unit of The Upjohn Company for allowing me to use their facilities for my laboratory work and for the helpful advice I received from many of their personnel, in particular Sue Porteus who taught me the techniques involved in the testosterone assay and Bill Burr for doing the hematologic slides. I would like to thank Dr. C.W. Bardin and coworkers at The Population Council for performing the ABP radioimmunoassay of my samples, and Debbie Piper for her secretarial assistance.

Shirley J. Stein

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TABLE OF CONTENTS

CHAPTER		PAGE
I	LITERATURE REVIEW	1
	3-Chloro-1,2-propanediol (α -Chlorohydrin)	1
	Chemistry	1
	Biology	2
	General effects	2
	Cellular site of action	3
	Biochemistry	5
	Mechanism of action	5
	The Sertoli Cell	9
	Androgen Binding Protein	10
	Location	10
	Physiochemical characteristics	11
	Gonadotropic influences	11
	Effect of age on FSH and TP actions	12
	Testosterone receptor	13
	Effect of age on ABP production	13
	Other causes for variance in ABP levels	14
II	INTRODUCTION	16

CHAPTER		PAGE
III	MATERIALS AND METHODS	17
	Animals	17
	Chemicals	17
	Experimental Protocol	17
	Homogenization of Tissue and Preparation of Cytosol Fractions	17
	Quantitation of Protein Content	19
	Assay for ABP by SS-PAGE	19
	Immunoactive Assay for ABP	21
	Assay for the Gonadotropins - LH and FSH	21
	Assay for Testosterone	21
	Statistical Analyses	22
IV	RESULTS	23
	Body and Organ Weights	23
	Histology	23
	ABP Production As Measured by SS-PAGE	28
	ABP Production As Measured by Radioimmunoassay	28
	Hormonal Response	35
	Testosterone Response	35
V	DISCUSSION	
	Body and Organ Weight Changes	38
	Comparison With Ductal Ligation	38
	Comparison With Other Dosage Levels	39
	Effect of Age	40

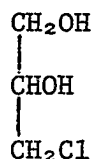
CHAPTER		PAGE
	Possible Causes of Observed ABP Changes	40
	Correlation of Testicular Weight With Changes in ABP Content	42
	Difference Between Active-Binding ABP and Immunoactive ABP Measurements	42
	Gonadotropin Changes	43
	Testosterone	45
VI	CONCLUSION	46

LITERATURE REVIEW

3-Chloro-1,2-propanediol (α -Chlorohydrin)

Chemistry

Chlorohydrins are a class of biologically active compounds which have a carbon bearing a chlorine adjacent to a carbon bearing a hydroxyl group. Alpha-chlorohydrin is a simple member of this class. It exhibits potent male antifertility activity (Coppola and Saldarini, 1974; Ericsson and Baker, 1970; and Paul et al, 1974). Its structure is drawn below. Surprisingly, no derivative of this compound has



been found to be more active than α -chlorohydrin itself (Paul et al, 1974; Ericsson and Youngdale, 1970; Cooper et al, 1974; Jackson and Robinson, 1976).

As can be seen from the structure, it looks like a mono-chlorine-substituted glycerol. This similarity has lead several researchers to propose that α -chlorohydrin is a glycerol antagonist (Coppola, 1969; Edwards et al, 1975 and 1976; Mohri et al, 1975). Since glycerol is a major precursor in the gluconeogenic pathway, an action by α -chlorohydrin to inhibit this process might have a major effect on the sperm's metabolic activity. This hypothesis is discussed in more detail in the section on the biochemical mechanism of action, p. 5.

Studies of 1-amino analogs of α -chlorohydrin (Coppola and Saldarini, 1974 and Paul et al, 1974) led to the finding that all the antifertility potency appeared to reside in the levorotatory isomer ($LD_{50} > 500$ mg/kg/day). In contrast, the dextrorotatory compound had an LD_{50} of 50 mg/kg.

The results of these studies stimulated the resolution of the isomers of d,l- α -chlorohydrin. Jackson and Robinson (1976) reported the successful synthesis of R(-)- α -chlorohydrin and examination of its biological activity. This isomer showed no antifertility action when given to mature male rats at a dose equivalent to a known sterilizing dose of racemic α -chlorohydrin. However, a single oral dose of 100 mg/kg was lethal to 6/6 rats, whereas only 5/12 succumbed to a similar dose of the racemic mixture.

The S(+)- α -chlorohydrin isomer was later successfully synthesized and tested by Jackson et al (1977). This isomer was found to act as was anticipated from the activity found in the R(-) analog, i.e. the S(-)-isomer was nearly twice as active as the racemic compound in causing temporary infertility and in causing sterility via a lesion of the caput epididymis of rats without the severe toxic side effects.

Biology

General effects

The general biological effects of α -chlorohydrin which have been observed in many of the species studied thus far are a low dose effect which causes reversible infertility and a higher dose effect which

causes functional sterility. In the adult male rat the reversible effect occurs at low oral doses of 7-8 mg/kg/day administered for 3-4 days; and greater than 35 mg/kg/day or one 45 mg/kg dose results in an epididymal occlusive lesion (Ericsson, 1970). The exception to these general biological effects is that α -chlorohydrin has not been found to inhibit fertility of mice and rabbits (Samojlik and Chang, 1970).

Cellular site of action

The cellular site of action of α -chlorohydrin is still unknown. As mentioned above, histological changes in the caput portion of the epididymis occur at doses well below a sterilizing dose (Gunn et al, 1969). In response to a sterilizing dose (or more), gross morphological changes can be seen. The testis will become enlarged, pale and firmer during the second and third days following treatment. However, spermatogenesis will continue until testicular swelling inhibits it through pressure degradation of the germinal epithelium (Ericsson, 1970).

No observable morphological evidence of endothelial damage, stasis or platelet thrombosis have been found, such as might be expected if the lesion was secondary to vascular occlusion (Ericsson, 1970; Samojlik and Chang, 1970). No changes in the weights of androgen-dependent organs other than the epididymis have been demonstrated after administration of α -chlorohydrin, suggesting that androgen secretion is not impaired. Thus examination of the gross morphological changes of the testis and epididymis gives no clue as to which cells

in the male reproductive system are effected following administration of low doses of α -chlorohydrin.

Morris and Jackson (1978) measured gonadotropic changes following α -chlorohydrin administration in order to see if there was any correlation of their changes with changes in androgen levels. Circulating FSH levels rose dramatically four days after treatment and remained elevated thereafter. In contrast, serum LH levels remained unchanged for four days and then rose to a maximum level on day seven; thereafter slowly declining. However, they reproduced the previously mentioned observation that there is no change in the weights of androgen-dependent organs following administration of α -chlorohydrin. Thus, any theory at this time which suggests that changes in gonadotropic concentrations are either caused by or a reflection of an effect of α -chlorohydrin upon androgen-dependent cells is purely speculative.

Dixit et al (1975) attempted to demonstrate that α -chlorohydrin exerted an anti-androgen effect on the testis and epididymis by administering a low dose of α -chlorohydrin chronically to dogs and then measuring changes in total RNA, protein, sialic acid and cholesterol content in these organs. They found, among other things that cholesterol concentration in the testis increased. This led them to conclude that since cholesterol is one of the primary substrates for androgen synthesis, α -chlorohydrin may be anti-androgenic in nature and acts directly on the testis and epididymal biochemistry.

A plausible answer to this discrepancy was suggested by Davis and Coniglio (1967) in their study on the changes in lipid and fatty acid composition of rat testis from normal rats and in rats treated

with cadmium chloride, triethylene melamine (an antispermatogenic drug) and in cryptorchid rats. They found an increase in testicular cholesterol concentration but not in the total amount of cholesterol of cryptorchid rats. They suggest that the atrophy of the germinal epithelium causes cholesterol to accumulate in the testes. Thus they speculate that the increase in cholesterol may be due to the release of cholesterol from an atrophied germinal epithelium into the testis.

Biochemistry

Mechanism of action

Researchers investigating the mechanism of action following administration of a reversible, antifertility producing dose of α -chlorohydrin have tended to focus their attention upon morphological changes in the epididymis or measurable changes in the spermatozoa.

The first hypothesis suggested for a possible mechanism of action was Ericsson's theory that α -chlorohydrin caused damage to the epididymal arterial supply. This has since been discredited by Gunn and Gould (1970) and Hoffer, et al (1973) neither of whom found destruction of the epididymal arterial supply when studying the ultrastructural pathology of the capillaries of the initial segment of the rat epididymis after administration of a sterilizing dose of α -chlorohydrin.

A second hypothesis is that of Wong and Yeung (1977) who suggested that α -chlorohydrin interferes with the sodium ion transport mechanism and hence fluid reabsorption in the cauda epididymis. The

effects of a dose of 9 mg/kg/day for 7 days were studied in isolated epididymal ducts in which the intraluminal sodium ions had been replaced by choline. Sodium reabsorption was found to be prevented in the fluid component which contained normal, sodium concentration. The rate of fluid reabsorption was reduced by about 50% as compared to controls. The component of fluid reabsorption which is independent of the presence of sodium ions was unaffected. Their conclusion was that this illustrated an effect of α -chlorohydrin on only the sodium-dependent component of fluid reabsorption in the rat cauda epididymis.

Alterations in spermatozoan motility and metabolism which result after treatment with α -chlorohydrin have been two of the most intensely studied areas in the search for the biochemical mode of action of α -chlorohydrin. Spermatozoan motility has been found to decrease after α -chlorohydrin treatment of rat spermatozoa both in vivo and in vitro (Brown-Woodman and White, 1974; Samojlik and Chang, 1970; and Tsunoda and Chang, 1976), in rams (Brown-Woodman et al, 1974), in swine (ibid and Johnson and Pursel, 1972), in the mouse (Tsunoda and Chang, 1976), and in sperm from human ejaculate (Homonnai et al, 1975). However, no change in spermatozoan motility has been seen in the monkey (Kirton et al, 1970 and Setty et al, 1970) at an even higher dose than used in the other species studied.

Setty et al (1970) used male Rhesus monkeys to study the effect of a sub-toxic dose (20 mg/kg b.i.d) of α -chlorohydrin upon the spermatozoa's rate of oxygen uptake; alkaline phosphatase, glycogen, lactic acid or glycerylphosphorycholine concentrations; lipid and

phospholipid levels and ion concentration. Oxygen consumption doubled. All other cellular constituent concentrations measured remained unaffected. The change in oxygen uptake indicates a change in the sperm's metabolism but does not provide any direct evidence as to the exact cause of the change in metabolism. Setty et al suggested membrane damage as a possible site of α -chlorohydrin's action.

There is, however, a substantial amount of evidence which suggests that α -chlorohydrin does alter spermatozoan metabolic activity. In contrast to Setty et al's finding respiration (measured as oxygen uptake upon incubation of spermatozoa) has been found to decrease in the rat (Samojlik and Chang, 1970 and Brown-Woodman and White et al, 1975) and in sperm ejaculated from the human and treated in vitro with α -chlorohydrin (Homonnai et al, 1975).

Glycolysis has been postulated to be inhibited in spermatozoa of animals treated with α -chlorohydrin. This conclusion is based on the fact that glucose oxidation and utilization, lactic acid accumulation and c-AMP are reduced in spermatozoa from rats and rams treated with sterilizing doses of α -chlorohydrin (Brown-Woodman and White, 1974; Homonnai et al, 1975; and Edwards et al, 1976).

Coppola (1969) suggested that the observed reduction in glycolysis was due to the similarity of α -chlorohydrin with glycerol. Additionally since glycerol is involved with phospholipid synthesis, α -chlorohydrin may act as a metabolic antagonist thus affecting phospholipid synthesis. However, he had no experimental evidence to support his hypothesis.

Edwards et al (1975) tested Coppola's hypothesis by studying

^{14}C - and ^{36}Cl -labelled α -chlorohydrin uptake into lipids of the male rat's brain, testis, epididymis, and epididymal fat pads. After injection of the ^{14}C labelled compound, radioactivity was found widely distributed in all the tissues examined. Whereas no radioactivity was found following [^{36}Cl] α -chlorohydrin. This experiment was therefore not helpful for determining the effect of α -chlorohydrin on glycolysis. Subsequent experiments by Edwards et al (1976) using [^{14}C] glycerol were equally ineffective in studying the biochemical effects of α -chlorohydrin on glycolysis.

Mohri et al (1975) conducted an experiment in which levels of various glycolytic intermediates were measured following α -chlorohydrin treatment and addition of fructose. Glyceraldehyde-3-phosphate was found to accumulate and 3-phosphoglycerate was lacking. This implicated glyceraldehyde-3-phosphoglycerate dehydrogenase (GA3Pdh) as an enzyme susceptible to α -chlorohydrin. However, when enzyme extracts were prepared, no effect upon GA3Pdh activity was seen up to a 300 mM concentration of α -chlorohydrin.

Even the metabolic degradation products of α -chlorohydrin have been examined as possible active antifertility agents or promoters of α -chlorohydrin's antifertility action (Jones et al, 1977). However, no biological activity was observed following administration of these compounds.

Thus, in summary, many studies have been conducted in an attempt to elucidate the biochemical mechanism of α -chlorohydrin. These have included studies such as: ultrastructural examination of the epididymal arterial supply pathology, changes in epididymal fluid reabsorp-

tion rate, effects on spermatozoan motility and metabolism and changes in spermatozoan glycolytic rate. However, the biochemical mechanism of α -chlorohydrin action still remains speculative.

The Sertoli Cell

For many years the elaborate shape of the Sertoli cell and its complex relationship with the germ cells hindered research aimed at elucidating its function in the testis. Results of histological studies were difficult to interpret. Thus experiments using anti-spermatogenic drugs were limited to observed pathological alterations in the germ cell. With improved techniques, light and electron microscopy, it has now been made possible to examine the role of the Sertoli cell in the testis. The accumulated evidence suggests that the Sertoli cell participates in the regulation of sperm release, maintenance of androgenic levels in the fluid surrounding the developing spermatogonia as well as providing some of the fluid necessary for transport of the spermatozoa (Fawcett, 1975). In view of this possible importance of the Sertoli cell to testicular function, future experiments should be designed to correlate the functional state of the Sertoli cell with observed changes in spermatogenesis in response to treatment of antifertility agents.

Androgen Binding Protein

Location

Recently a marker for Sertoli cell function has been discovered. It is called androgen binding protein (ABP) due to its ability to specifically bind androgens, particularly testosterone and dihydro-testosterone. The first evidence which showed that ABP originated from the Sertoli cell was obtained by Fritz and coworkers (1974) when they examined cellular extracts from rat cryptorchid testes using SXR (sex reversal translocation) mice as controls. ^3H -testosterone was found to bind to testicular protein at the same concentration in these animals as in their normal counterparts. No evidence of binding was seen in interstitial cell homogenates. Thus since testes from adult SXR mice do not contain germinal cells, it was concluded that ABP came from the Sertoli cell. Later Means et al (1975) verified Fritz's conclusion in studies utilizing the male offspring of pregnant female rats x-irradiated at day 19.5 of gestation. These males were nearly free of germ cells and were referred to as "Sertoli-cell-enriched" (SCE) rats. They have been used primarily to study the effects of FSH on the Sertoli cell and in the process of these studies, ABP has been shown to be a product of the Sertoli cell.

Androgen binding protein was first shown to be present in the rat epididymis (Ritzen et al, 1971) before it was demonstrated that ABP is produced in the testis and then transported into the caput epididymis where it is believed to be either destroyed in the lumen or absorbed by the epithelium (Hansson et al, 1975). In addition,

recent evidence suggests that ABP is present in the serum of male rats (not females) and that its plasma levels correlate with testicular rather than epididymal concentrations of ABP (Gunsalus et al, 1978).

Physiochemical characteristics

ABP (Hansson et al, 1975 review article) has been found to have a molecular weight of 90,000. It binds DHT (dihydrotestosterone) and testosterone with relatively high affinity and estradiol-17 β only slightly. Progesterone, cortisol, corticosterone, Δ^4 -androstenedione and estradiol-17 α were not found to compete. ABP is stable in a range of pH 6.5-10 but binding is destroyed below pH 5 and above pH 12. Heating up to 50°C (but not 60°) for 30 minutes has no effect on ABP's binding activity. Other physiochemical characteristics that have been measured are: a sedimentation coefficient of 4.65; a Stokes radius of 47 \AA ; and an $R_f = 0.4$ relative to bromphenol blue by polyacrylamide gel electrophoresis (PAGE). The protein rather than the carbohydrate moiety has been determined essential for binding by virtue of the fact that proteases but not DNase, RNase or neuraminidase destroy binding.

Gonadotropic influences

ABP disappears from both the testis and epididymis after hypophysectomy of mature rats (Hansson et al, 1975; ibid, 1975; Tindall et al, 1974; and Sanborn et al, 1974). If highly purified preparations of human FSH and LH are injected subcutaneously, FSH will cause marked increases in the testicular and epididymal ABP content of rats

while LH treatment causes no significant effect (Hansson et al, 1975). The effect of administration of FSH on ABP is dose-dependent. Near normal ABP levels can be maintained by pre-treatment with testosterone propionate (TP). This appears to be a specific FSH effect since no other pituitary or sex hormones have been found to increase ABP concentration (Means et al, 1976). The mechanism of its action is not yet known.

Effect of age on FSH and TP actions

As mentioned above, a "primary" dose of testosterone propionate together with FSH maintains ABP levels following hypophysectomy. While androgen alone cannot reinitiate ABP production once post hypophysectomy regression has occurred, administration of FSH alone in rats can maintain ABP production but not spermatogenesis (Hansson et al, 1975). This has been found to be an age-dependent phenomena. Steinberger et al (1977) have obtained experimental evidence which suggests that the response of the Sertoli cell to FSH declines with sexual maturation. Contrary to this Elkington et al (1977) showed that the less mature the animal, the less effect TP had on maintaining ABP levels. Furthermore the main effect of TP on mature animals was to raise testicular but not epididymal levels of ABP, whereas FSH raised both testicular and epididymal ABP levels (pmoles/organ). The conclusion which has been drawn from this is that FSH may facilitate the movement of ABP from the testis to the epididymis and that FSH acts synergistically with androgen to maintain Sertoli cell function and spermatogenesis. Also, Sanborn et al (1974) showed that the

androgen needed can either be supplied by LH stimulation of the Leydig cells or by administration of exogenous androgen.

Testosterone receptor

Means et al (1976) undertook a series of studies on hypophysectomized SCE rats to examine the testicular relationship of testosterone levels in response to exogenous administration of FSH, testosterone receptor location, and the specificity of the ABP response to testosterone. They were able to demonstrate a rapid stimulation of ABP levels in response to testosterone similar to those obtained for FSH. The SCE animals contained a cytoplasmic Sertoli cell receptor for testosterone that was saturable and specific in its binding of ^3H -testosterone. However, the total androgen bound per testis (on a per mg protein basis) was less in the SCE rats than normal rats. Therefore, they concluded that the germ cells must also contain a receptor for testosterone. This receptor was found to be specific for testosterone in that cyproterone acetate, estradiol-17 β and cortisol failed to stimulate ABP production. Subsequently, this cytoplasmic receptor for testosterone was shown to be different from ABP itself by eight different physical biochemical parameters.

Effect of age on ABP production

It is believed that since the blood-testis barrier is a prerequisite for the formation of lumina within the seminiferous tubules and since this does not form until 16-18 days of age when the Sertoli cell's tight junctions form, passage of ABP into the epididymis is

prevented until this time. Indeed, the level of ABP in the testis and epididymis has been found to correlate with the maturational changes in the testis; starting from undetectable levels prior to the formation of the blood-testis barrier and reaching a peak coincident with the first observation of mature sperm in the testis (Tindall et al, 1974). Further support has been added to this relationship by the observance of the same pattern of age-dependence in SCE rats (Tindall et al, 1974).

Other causes for variance in ABP levels

ABP levels have been studied in several abnormal states that occur in rats. One of these states is an autosomal gene defect in which rats are fertile until approximately 100 days of age but thereafter the seminiferous tubules progressively atrophy. These rats are referred to as restricted rats (H^{re}). It has been found by Musto and Bardin (1976) that the ABP content of the testis and epididymis are less in the H^{re} rats than in normal rats at all ages studied. Furthermore, a significant decline in ABP levels was seen after a hundred days of age. This suggests either Sertoli cell dysfunction or ABP production or secretion itself is deficient. There is not sufficient information yet available to distinguish between these alternatives.

Another important testicular abnormality is cryptorchidism. Until recently, it was thought from morphological assessment that the Sertoli cells remained intact in the cryptorchid state. However, experimental unilateral or bilateral formation of the cryptorchid state with subsequent measurement of ABP levels revealed a decreasing

amount of ABP in the abdominal testis. After four weeks of cryptorchidism ABP was almost nondetectable. This suggests serious impairment of the Sertoli cell secretory function.

Finally, one other abnormal testicular condition has been studied with respect to associated ABP levels - the androgen insensitivity syndrome (testicular feminization, Tfm). ABP levels were found to be almost ten times greater in the Tfm rats than in their normal littermates when calculated per mg. of protein and as pmol/testis (Hansson et al, 1975). This shows that ABP is formed even when there is reduced response to androgen stimulation due to a diminished number of cytoplasmic receptors for androgen. The cause of these abnormally high levels of ABP are not yet known.

INTRODUCTION

Testicular androgen binding protein (ABP) is a product of the Sertoli cells (Fritz et al, 1974; Hansson et al, 1974, 1975). In normal male rats it is secreted primarily into the lumen of the seminiferous tubules and transported to the epididymis (Hansson et al, 1975; Ritzen et al, 1971). Production and secretion of ABP into the testicular fluid and epididymis are stimulated by FSH (Hansson et al, 1973; Fritz et al, 1974) and androgens (Hansson et al, 1974, 1975). As a consequence, it has been suggested that ABP is a marker of Sertoli cell secretory function (Hansson et al, 1975; Means et al, 1976).

In this study, the time-related changes in testicular and epididymal ABP concentrations following the formation of a lesion created in the caput epididymis by a sterilizing dose (60 mg/kg) of α -chlorohydrin (U-5897 or 3-chloro-1,2-propanediol) were examined. Serum levels of ABP, LH, FSH and testosterone were also measured in order to determine if there was any correlation between their changes and changes in ABP concentration in the testis and epididymis.

MATERIALS AND METHODS

Animals

Ninety day old, male Charles River Sprague-Dawley rats weighing approximately 400 g each were used for this study. The animals of each individual treatment group ($n = 5$) were kept together in a cage, given unrestricted access to food and water, and had a light cycle of 14 hr. light and 10 hr. darkness.

Chemicals

Alpha-chlorohydrin used in this study had been purchased from K and K Laboratories, Inc. and stored at ambient temperatures.

The drug was administered in an aqueous 0.25% methylcellulose solution.

Experimental Protocol

The schedule for dosing and killing the animals is described in Table I.

Homogenization of Tissue and Preparation of Cytosol Fractions

The rats were weighed and then killed by decapitation. The testis and epididymis were immediately removed, dissected free of fat and independently weighed. A piece of each right testis was cut off and fixed in Bouin's Solution for subsequent staining with hematoxylin and eosin for histological examination.

Table I
Time Response Protocol for Animals
Receiving a Single Oral Dose of
 α -Chlorohydrin (N = 5)

<u>Group</u>	<u>Treatment</u>	<u>Necropsy Time (days)</u>
1	Control ^a	20
1	60 mg./kg. ^b	20
2	60 mg./kg.	10
3	Control	6
4	60 mg./kg.	4
5	Control ^a	2
5	60 mg./kg.	2
6	60 mg./kg.	1
7	Untreated Control	0

a - These animals were given Vehicle 122 only.

b - This dose was determined from two preliminary experiments. In the first experiment 6 animals were given a single oral dose of 45 mg./kg. The percent lesions found when compared to controls (by examination of testicular weights) was low. Examination of the literature suggested that 60 mg./kg. would give lesions in 100% of the animals (Ericsson, 1970). The second experiment done on 5 animals verified this result.

Remaining testes and epididymides were frozen and stored at -80°C until tissue homogenization. Serum prepared from trunk blood was aliquoted in four, 0.6 ml portions each for subsequent LH, FSH, testosterone, and serum ABP determinations. The first three (as listed here) were stored at -20°C . The aliquot for serum ABP was stored at -80°C . The testes were homogenized using three volumes of cold TEG buffer (10 mM TRIS, 2 mM EDTA, 10% glycerol, and adjusted to $\text{pH} = 7.4$ by the addition of HCl). Homogenization was complete in 30 sec. using a Brinkman Polytron homogenizer. The homogenate was then centrifuged at $39,000 \times g$ for one hour at 4°C . The resulting supernatant was centrifuged at $104,000 \times g$ for one hour at 4°C . A 0.3 ml aliquot of the clear supernatant was removed for determination of ABP using Bardin's radioimmunoassay for serum ABP (Gunsalus et al, 1978). The remaining supernatant was stored at -80°C .

The epididymides were homogenized with seven volumes of TEG buffer following the procedure described above for the testes.

Quantitation of Protein Content

Protein content of the testis and epididymis was determined by the method of Lowry et al (1951).

Assay for ABP by SS-PAGE

Quantitation of ABP was accomplished by using steady-state polyacrylamide gel electrophoresis (SS-PAGE). The general method of Ritzen et al (1974) and Davis (1964) was used with the following modifications. Tritiated DHT (2 nM DHT per gel; $1 \text{ mCi} = 7.3 \times 10^{-3}$

mg DHT; spec. act._{EtOH} = 40 Ci/mM; New England Nuclear, Lot #772-242) was placed in a beaker and dried down. The gel solution was then placed in the beaker and stirred vigorously for 30 min. at 4°C. The ammonium persulfate solution was added to initiate polymerization. This final solution was used to fill a small glass tube (5 mm, I.D.) 3-4 mm above the 56 mm mark (to allow for shrinkage). One hundred μ l of water was then layered on top of the gel solution. The filled tube was allowed to stand overnight at 4°C before use.

A sample (150 μ l; in duplicate) to be measured for ABP content was added to a test tube containing an amount of ^3H -DHT sufficient to make a 10 nM DHT solution in 40% sucrose. These were then stored at 4°C overnight before use.

The top of the gels was rinsed with buffer, filled to the brim with buffer, and a calculated amount of sample was layered on top of the gels. Bromophenol blue was added to the upper buffer and the gels were electrophoresed for approximately 2 hrs at 1.5 mA/gel and 4°C until the dyemarker had traversed all gels.

The gels were removed from the tubes, frozen on a dry ice block in a scoopula, and then sliced transversely into 2 mm slices on a razor blade slicer. The first four slices were discarded. The next 15 were placed into individual vials containing 3 ml scintillation fluid (42 ml Liquifluor in one liter toluene), and shaken. The radioactivity was measured in a Searle Mark III scintillation counter.

Immunoactive Assay for ABP

The amount of ABP in the serum was determined by Bardin and co-workers at Hershey Medical Center using a female rabbit antisera for the first antibody and goat antisera to precipitate the bound ABP (Gunsalus et al, 1978 and Musto et al, 1978).

Assay for the Gonadotropins-LH and FSH

Plasma levels of LH and FSH were determined in duplicate by the conventional double antibody assay as described by the National Institute of Arthritis, Metabolism and Digestive Diseases of the National Institute of Health (NIAMDD), Rat Pituitary Hormone Distribution Program. NIAMDD-Rat LH-S3 and Rat FSH-S6 were used for iodination.

Assay for Testosterone

Plasma levels of testosterone were measured in duplicate by a double antibody radioimmunoassay validated by Smith and Hafs (1973). Rabbit anti-testosterone (MSU-74) was a gift from Hafs of Michigan State University (Kiser et al, 1978).

Testosterone was extracted from 0.1 ml serum samples using a benzene:hexane (1:2) solution. The extracts were stored overnight at 4°C. The assay was run using 0.2 ml of 0.01 M Tris buffer, 0.5 ml of the female rabbit antisera (1:25,000 in TRIS), 0.1 ml of labeled testosterone (~ 5,000 cpm/0.1 ml) and 0.1 ml goat antisera to precipitate the bound testosterone. Equilibrium was established overnight

at 4°C. After centrifugation at 4°C (2500 x g for 30 min), a 0.5 ml aliquot was removed from the supernatant. The radioactivity was measured in a Packard TRI-CARB scintillation counter. Serum samples from a hypophysectomized, an adrenalectomized, a castrate and a normal male rat were included as controls for the assay method.

Statistical Analyses

Calculations for the ABP SS-PAGE assay were performed by an Upjohn Company computer program adapted from one designed for ABP by Frank French of the University of North Carolina at Chapel Hill.

Calculations for the radioimmunoassay of LH, FSH and testosterone were done by another Upjohn-owned computer program based upon the Rodbard Statistical analysis method for hormone-receptor interactions.

For statistical analyses, the Student's t-test for two tailed probabilities were employed. A probability equal to or less than 0.05 was taken as the level of significant difference.

RESULTS

Body and Organ Weights

In a pilot study where 90 day old male rats were treated with a single dose of 45 mg/kg α -chlorohydrin, no significant difference between control (n = 5) and treated (n = 6) testicular and epididymal weights was observed six days after treatment. However, a single 60 mg/kg dose given in a second study showed testicular weights to be significantly increased in the treated animals (n = 5) as compared with controls (n = 5) five days post treatment. Epididymal weight was unaffected. Therefore a single oral dose of 60 mg/kg of α -chlorohydrin was chosen for this study.

Table II summarizes the observed changes in body, testicular and epididymal organ weights. No final body weights were found to be statistically different from controls. Testicular weights generally increased to the tenth day and thereafter declined. The epididymal weights remained essentially unchanged through the first ten days of the experiment, after which they decreased in weight as compared to controls.

Histology

The first histopathological effects observed following administration of a lesion producing dose of α -chlorohydrin were seen in testicular tubules ten days after treatment (see Fig. 1). Germinal elements are in various stages of degeneration. Multi-nucleation has occurred

Table II
Effect of Sterilizing Dose of α -Chlorohydrin on
Body, Testis, and Epididymal Weight^a of Male Rats (n = 5)

Days Post Treatment	Body Weight (g)				% Testis Wt/Body Wt (x 10 ⁻³)		% Epididymal Wt/Body Wt (x 10 ⁻³)	
	Initial Control	Treated	Final Control	Treated	Control	Treated	Control	Treated
0			432±26.8		8.32±0.43		3.20±0.34	
1		438±24.2		421±27.1		9.54±1.60		3.08±0.29
2	417±34.7	451±26.6	394±38.6	444±28.6	8.85±0.74	10.3±0.92*	3.09±0.43	2.98±0.418
4		450±20.0		453±25.7		10.9±1.32**		2.76±0.19
6	433±12.4		450±12.3		7.78±0.68		2.75±0.26	
10		394±43.1		431±30.2		12.5±3.42**		2.47±0.47**
20	402±4.8	389±9.9	440±57.7	449±25.5	8.32±1.61	6.26±1.00**	2.84±0.41	1.85±0.20**

a = Mean ± S.D. Statistical difference from pooled control: * = $P \leq 0.05$, ** = $P \leq 0.01$.

The 20 day treatment group was shown to be significantly different from all other treatment groups both in testicular and epididymal weight changes. The day 10 post-treatment group was significantly different from treated groups day two and one as well as day 20.

Fig. 1



Plate A: Section of testis from a control rat. The seminiferous tubule shows full spermatogenesis. (X 100)

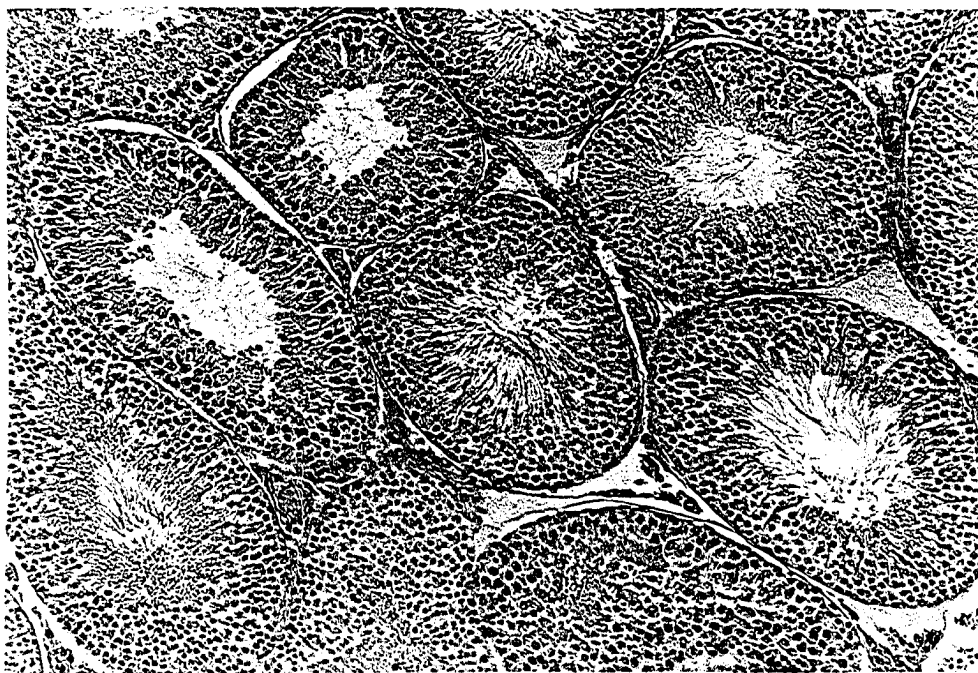


Plate B: Section of testis from an untreated control rat. (X 100)

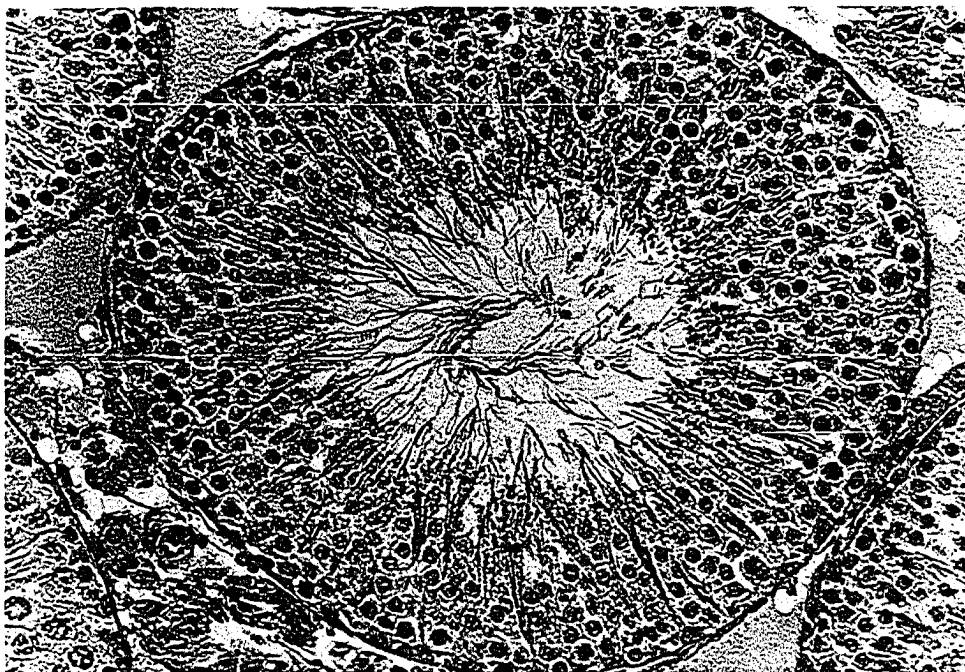


Plate C: Section of testis four days after α -chlorohydrin treatment. (X 250)

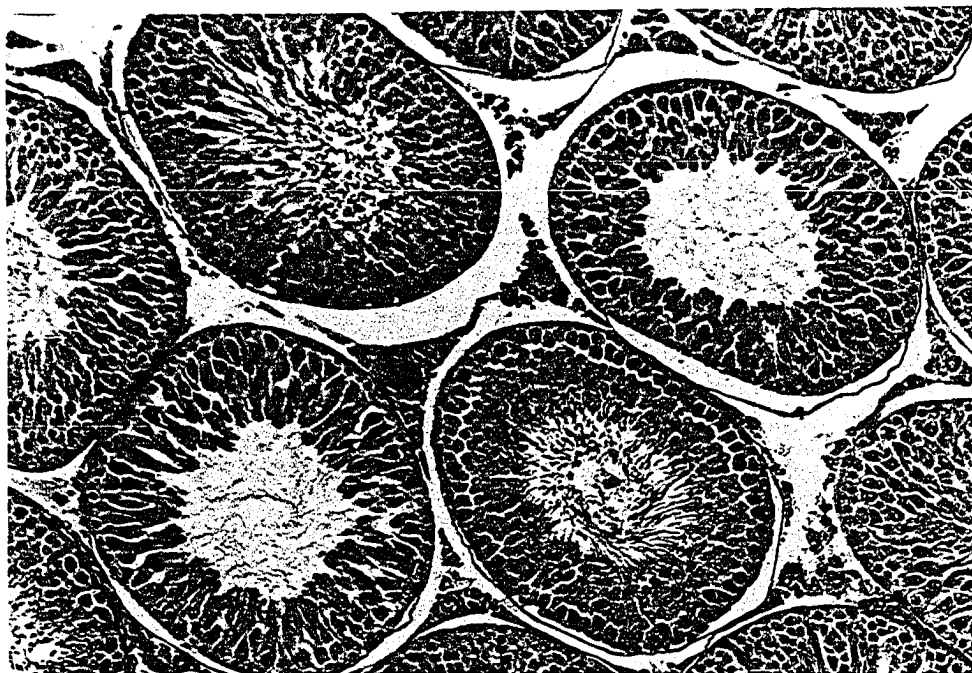


Plate D: Section of testis six days after α -chlorohydrin treatment. (X 100)

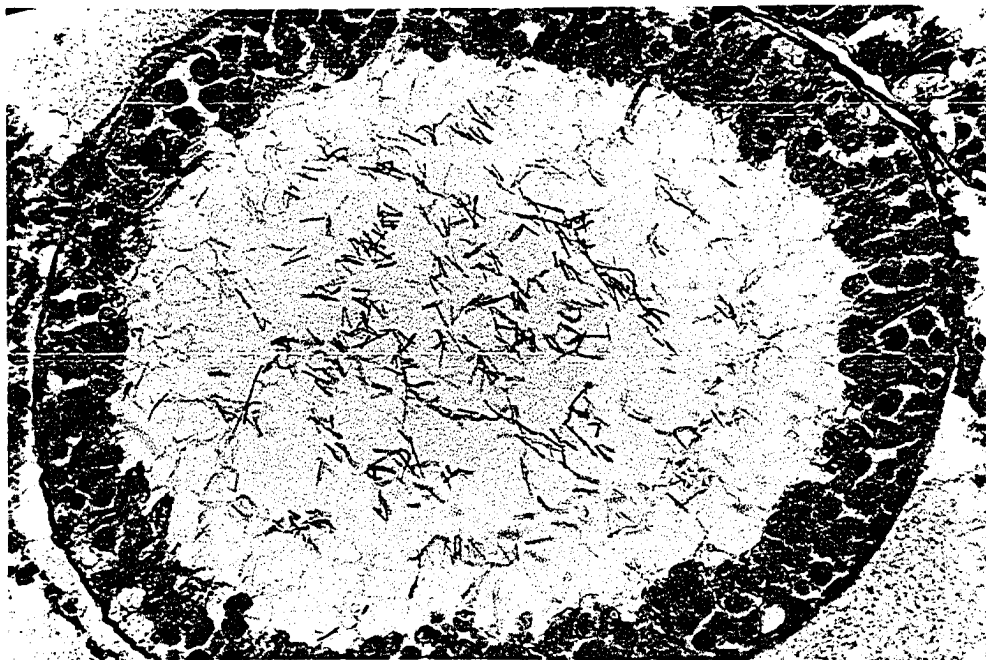


Plate E: Section of testis ten days after α -chlorohydrin treatment. (X 250) Note the irregular pattern of spermatogenesis.

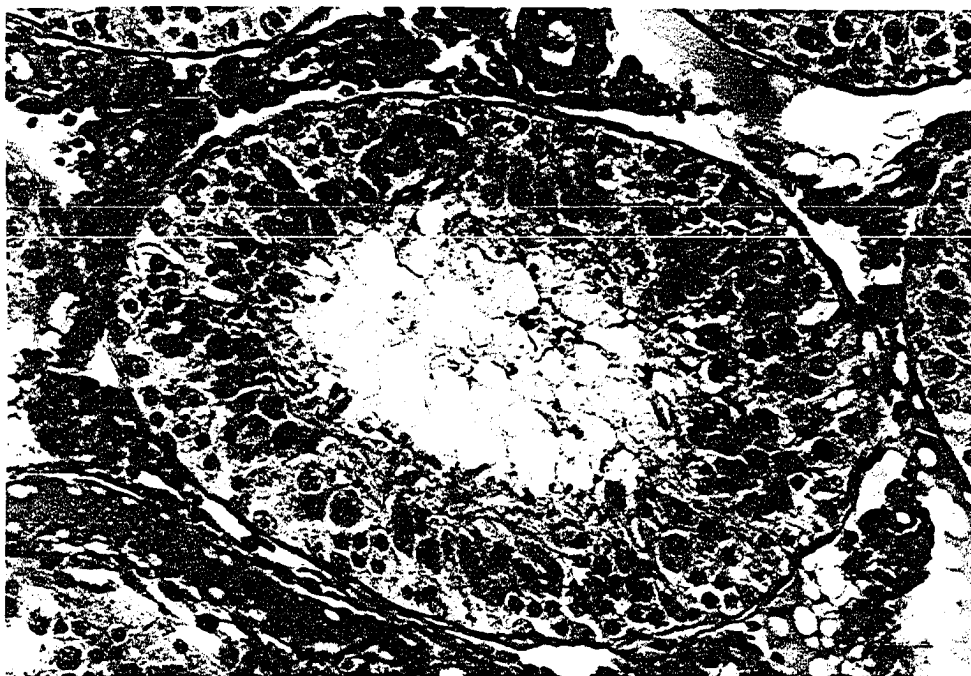


Plate F: Section of testis twenty days after α -chlorohydrin treatment. (X 250) Cellular debris is accumulating in the lumen.

in some spermatogenic cell types. There appears to be more space between cells and less uniformity amongst the cells of the various cell types. By the twentieth day after treatment, necrosis in the seminiferous tubules is apparent. There is cellular debris in the lumen.

ABP Production As Measured by SS-PAGE

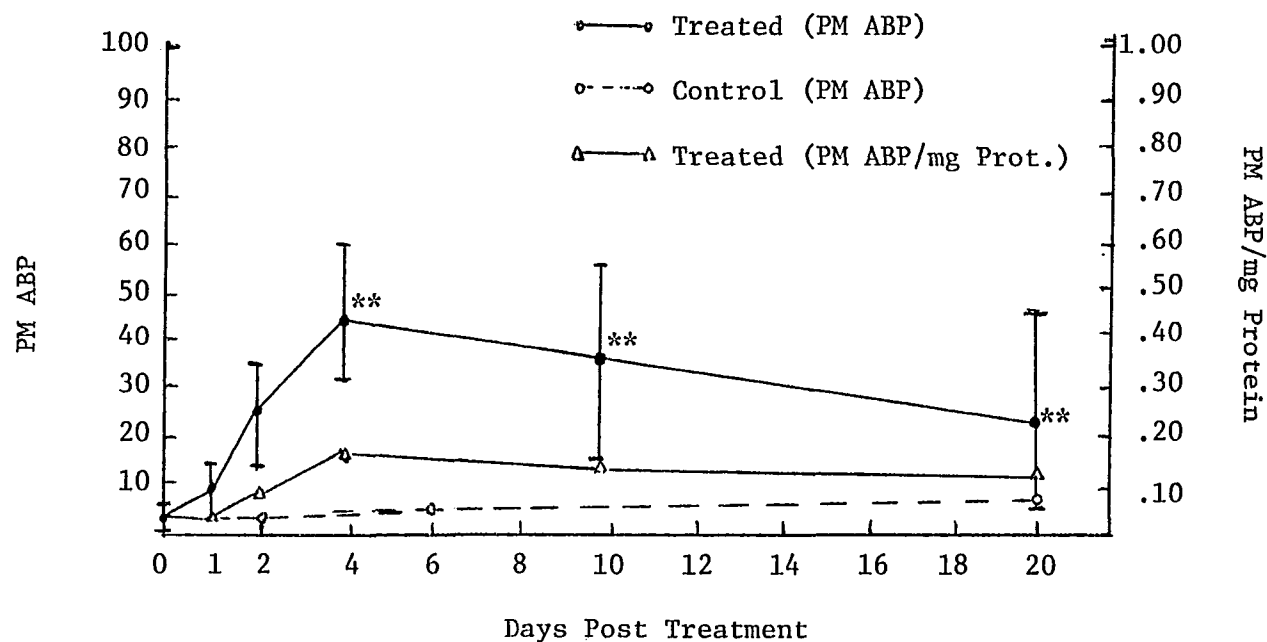
ABP in the testis initially rises to a maximal concentration on day 4 and thereafter declines (see Fig. 2). The picomoles of ABP per milligram of testicular protein and the total picomoles of ABP in the testes samples follow the same pattern as the molar concentration of ABP in the testis (see Fig. 3). By day 4 the total testicular ABP content had increased 15 fold from day zero and thereafter decreased approximately 50% by day 20. The total amount of testicular ABP in controls remained essentially constant. On the other hand, epididymal levels of ABP (total pM in sample) decreased from day 0 to day 20 by 240 fold (see Fig. 4). The molar concentration of ABP follows the same pattern as total ABP in the epididymis following treatment (see Fig. 5).

ABP Production As Measured by Radioimmunoassay

Immunoactive ABP concentrations reflected the same pattern of ABP alterations in the testis and epididymis as the active binding measurements of ABP (SS-PAGE) with one exception. Testicular ABP was found to peak at day 10 by the radioimmunoassay measurement whereas ABP peaked at day 4 when measured by SS-PAGE (see Fig. 6 and 7). Also,

Fig. 2

Content of ABP in the Testis Following a Single 60 mg/kg Dose of α -Chlorohydrin in 90-day-old Male Rats (N = 5; Mean \pm S.D.)



* = $P \leq 0.05$; ** = $P \leq 0.01$ Treated days 20, 10 and 4 are significantly different from controls (PM ABP). In addition, day 20 is significantly different from days 10, 4 and 1; day 10 is also different from day 1 and days 4 and 2 are significantly different from day 1 (in PM ABP).
 Note: Values of ABP content (PM/mg Protein) were $\cong 0.01$ for all control groups.

Fig. 3

Molar Concentration of Testicular ABP Following
a Single 60 mg/kg Dose of α -Chlorohydrin in 90
day-old Male Rats (N = 5; Mean \pm S.D.)

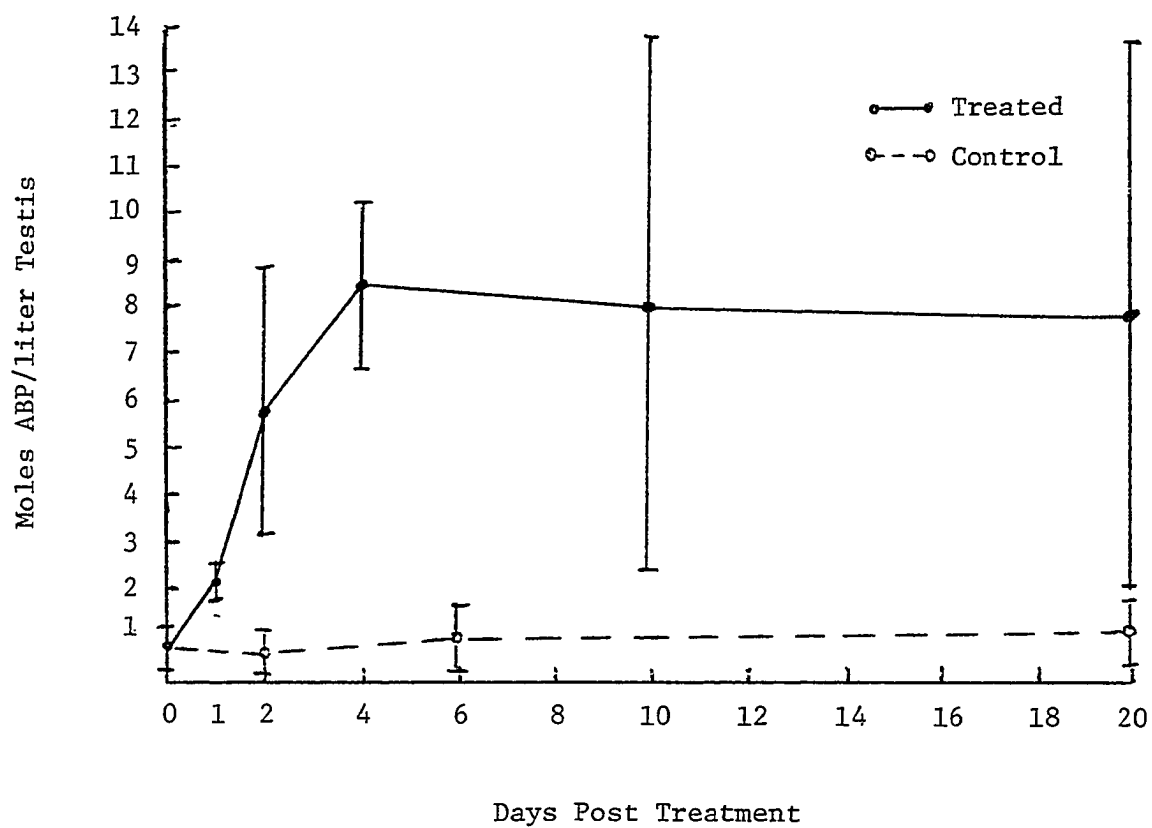
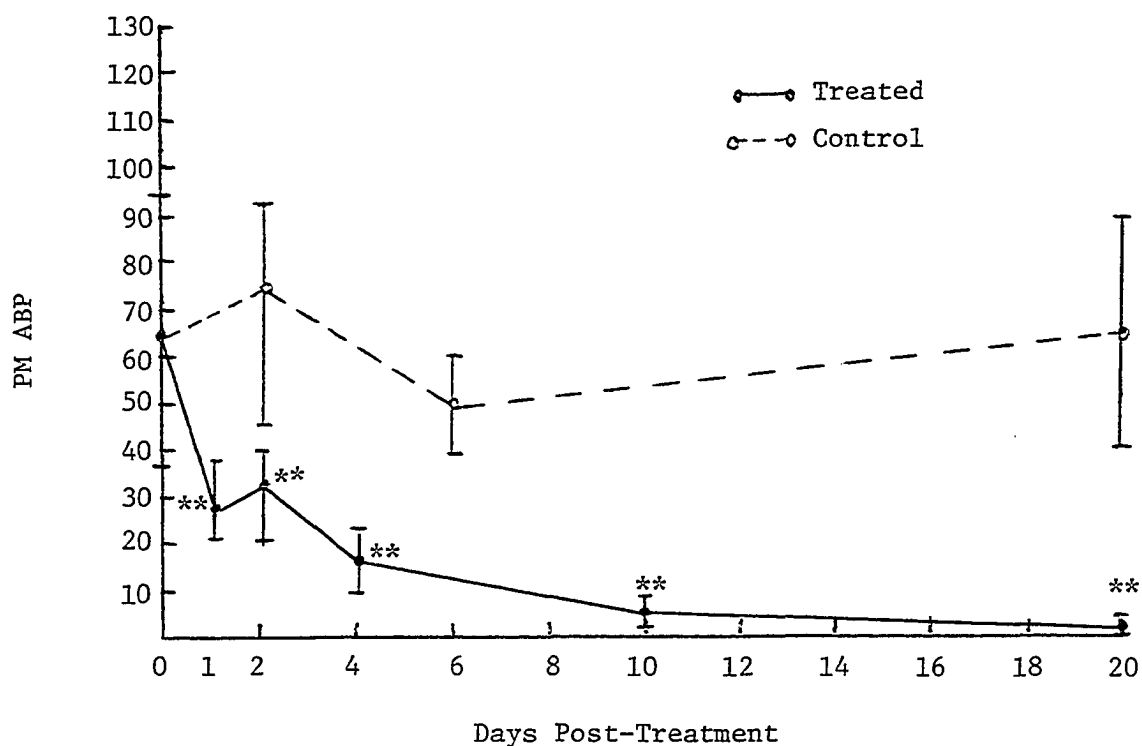


Fig. 4

Total EPIDIDYMAL ABP Following a Single
Oral 60 mg/kg Dose of α -Chlorohydrin in
90 day-old Male Rats (N = 5; Mean \pm S.D.)



* = $P \leq 0.5$; ** = $P \leq 0.01$ Total epididymal ABP concentration of all treated groups is significantly different from controls. In addition levels of days 20 and 10 are different from epididymal levels of days 2 and 1 (treated).

Fig. 5

Molar Concentration of Epididymal ABP Following
a Single Oral 60 mg/kg Dose of α -Chlorohydrin in
90 day-old Male Rats (N = 5; Mean \pm S.D.)

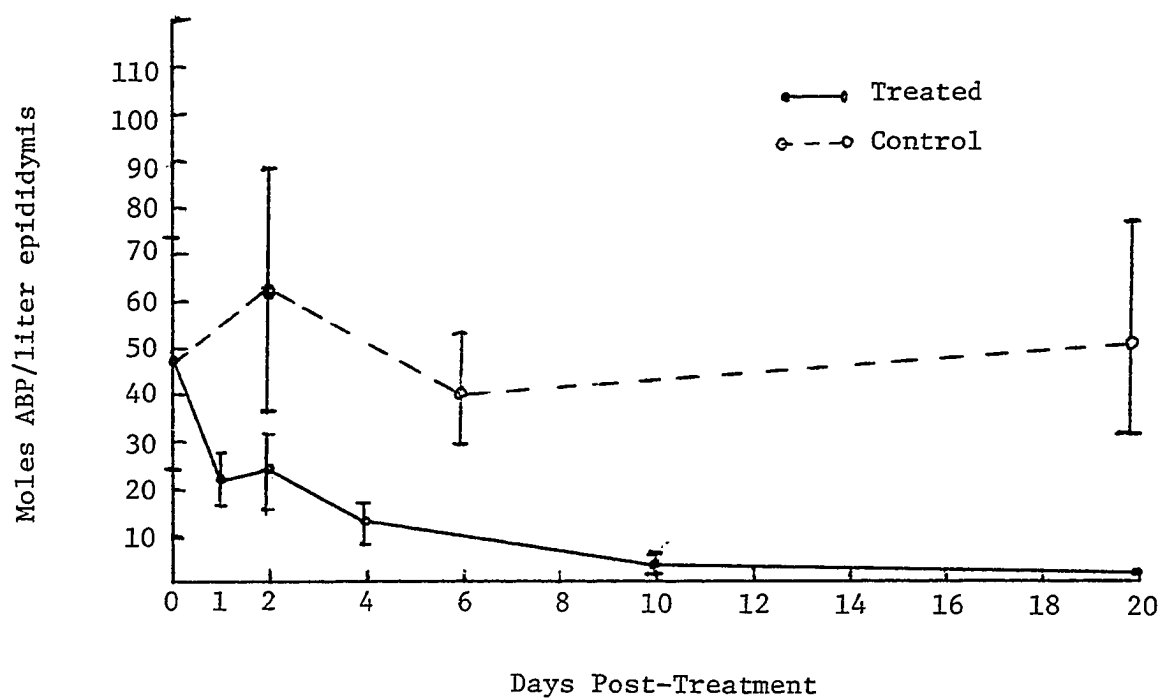


Fig. 6

Total ABP in the Testis and Epididymis Following a Single Oral 60 mg/kg Dose of α -Chlorohydrin in 90 day-old Male Rats (N = 5; Mean \pm S.D.)

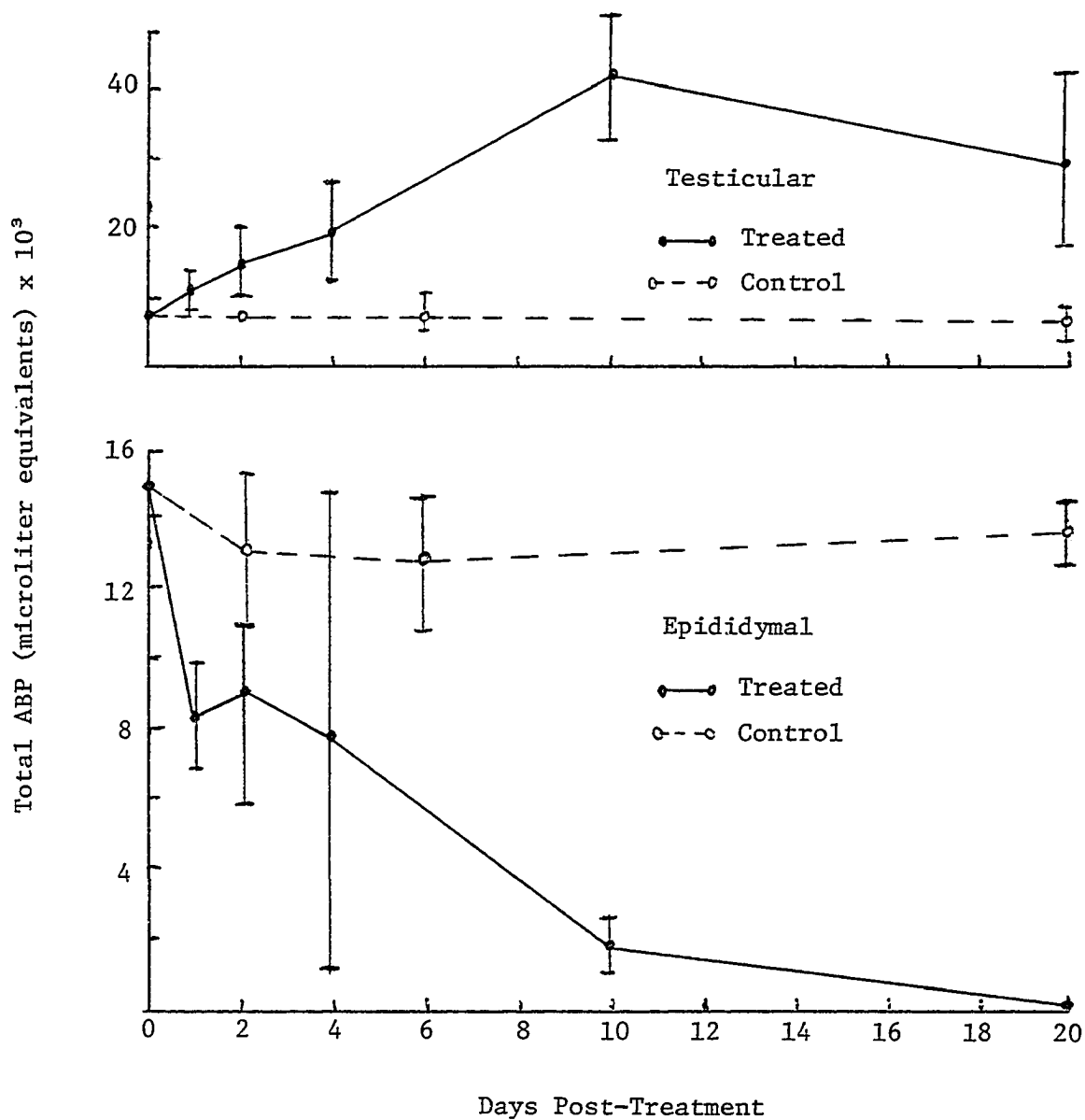
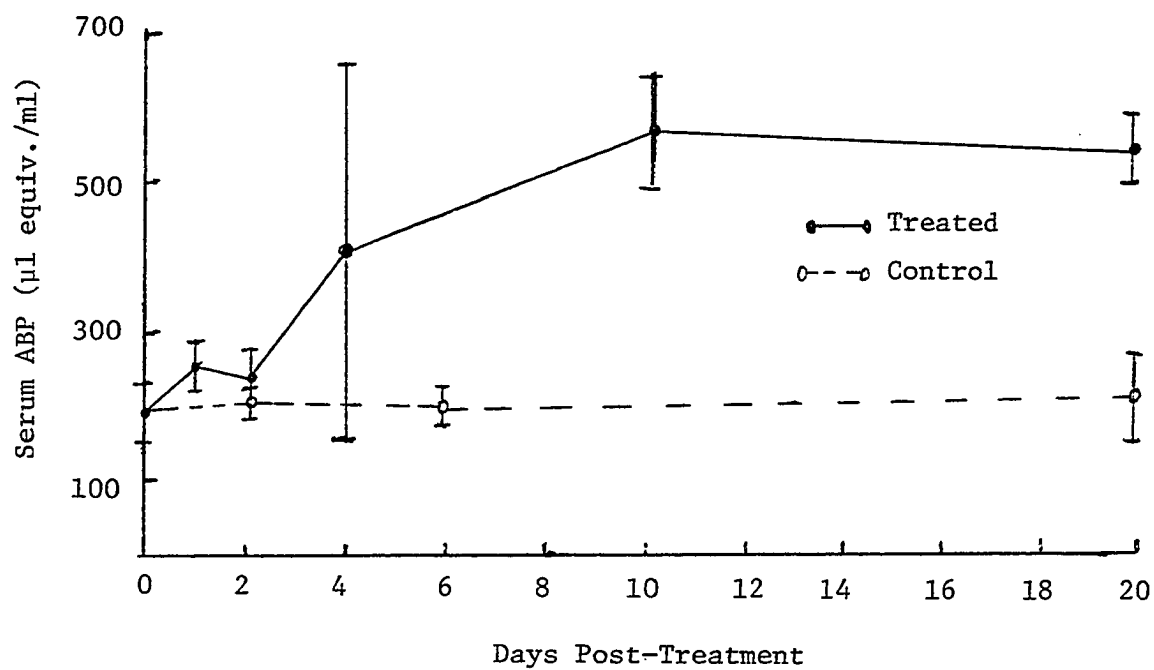


Fig. 7

Molar Concentration of Serum ABP^a Following
a Single Oral 60 mg/kg Dose of α -Chlorohydrin
in 90 day-old Male Rats (N = 5; Mean \pm S.D.)



a - As measured by Bardin's radioimmunoassay technique.

the increase in testicular ABP content was found to be only 6-fold by the radioimmunoassay method as compared to 15-fold for the SS-PAGE method. Epididymal ABP decreased from day 0 to day 20 by only 101 fold as compared to the 240 fold decrease observed by the SS-PAGE method of measurement.

Hormonal Response

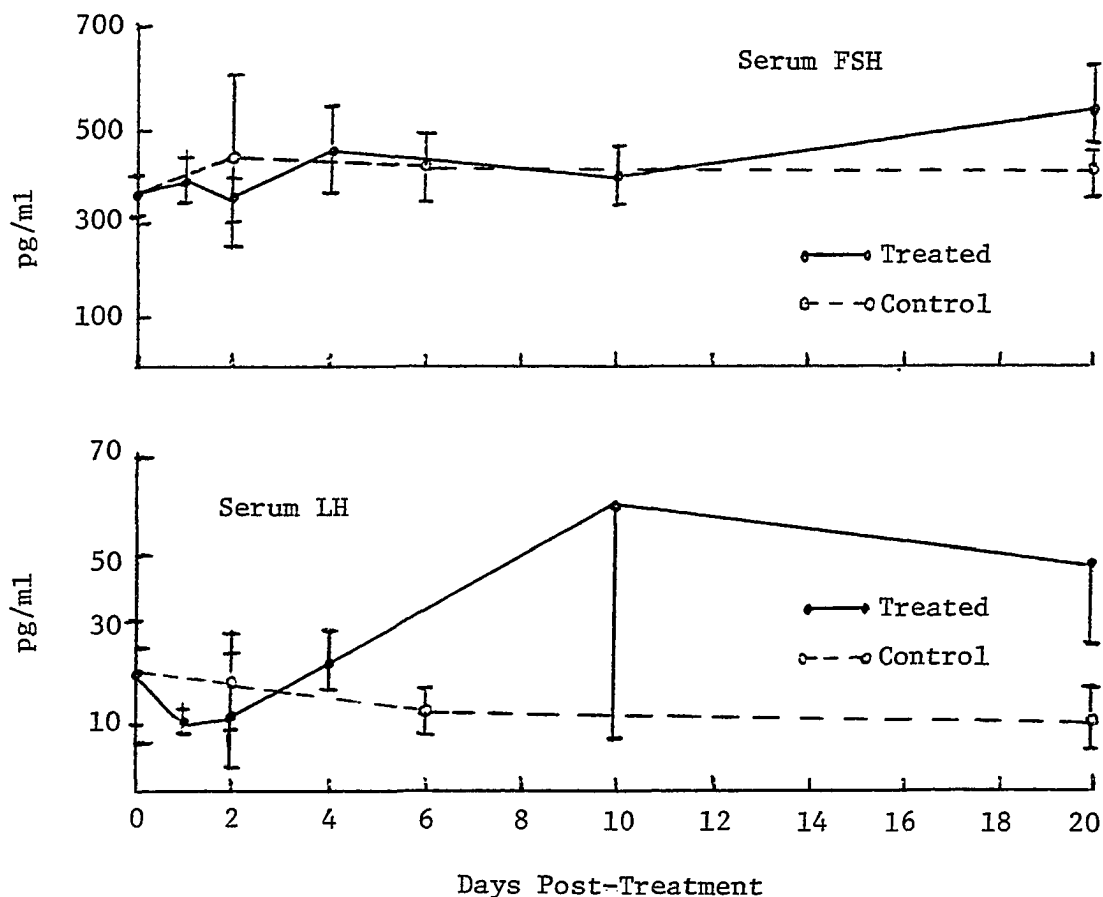
There were notable differences between the way the gonadotropins responded following α -chlorohydrin treatment (Fig. 8). Serum FSH levels remained generally unaffected until day ten and thereafter increased significantly until day twenty. Serum LH levels remained unchanged until day four when they then increased dramatically to a maximum on day ten. This was followed by a subsequent leveling off of serum LH concentration.

Testosterone Response

Serum testosterone levels were not found to be significantly different from controls at any time after α -chlorohydrin treatment (Fig. 9).

Fig. 8

Serum FSH and LH Changes Following
a Single Oral 60 mg/kg Dose of α -
Chlorohydrin in 90-day old Male
Rats (N = 5; Mean \pm S.D.)



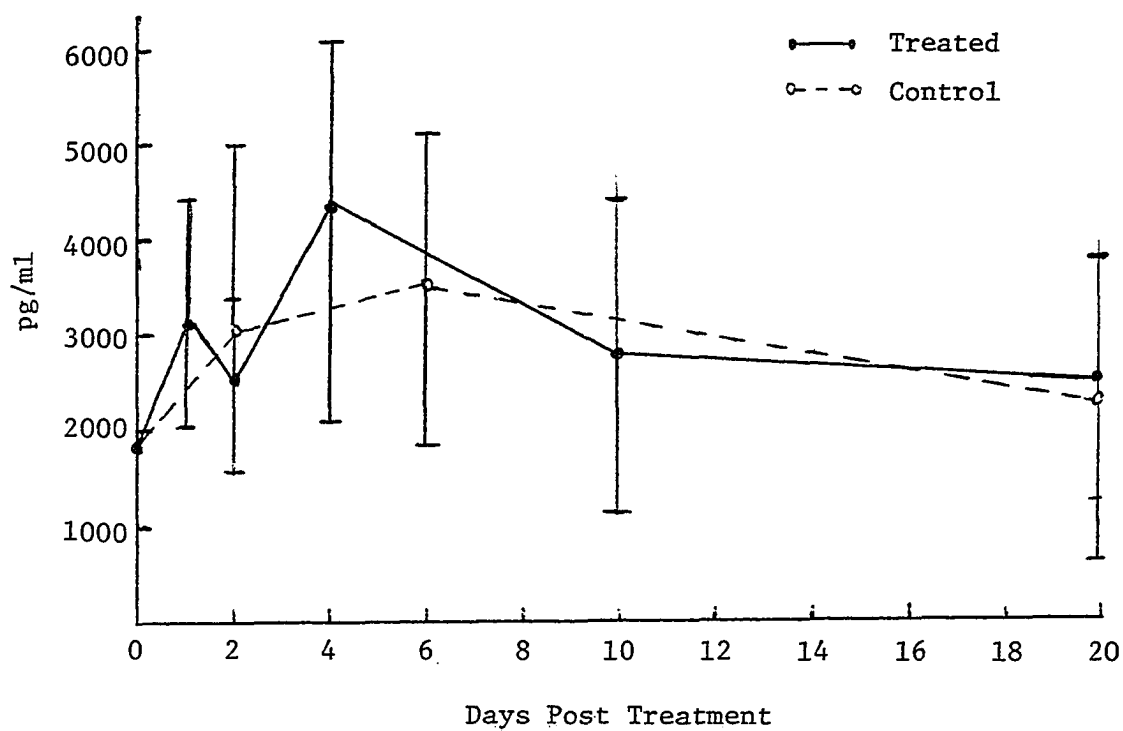
* = $P \leq 0.05$; ** = $P \leq 0.01$

Serum FSH: Day 20 is significantly different from treated days 10, 2 and 1. And day 4 is different from day 2.

Serum LH: Day 20 is significantly different from days 1 and 2. Day 10 is different from all days except day 20.

Fig. 9

Serum Testosterone Response Following a Single Oral 60 mg/kg Dose of α -Chlorohydrin in 90-day old Male Rats (N = 5; Mean \pm S.D.)



DISCUSSION

The purpose of this study was to look for changes in ABP concentration in the testis and epididymis of male rats in response to a sterilizing dose of α -chlorohydrin. In addition, alterations in testicular morphology, testis and epididymis weights, and serum FSH, LH and testosterone concentrations were measured to see if changes in these parameters were associated with changes in ABP.

Body and Organ Weight Changes

As mentioned in the results section, neither body weights nor epididymal weights were found to be altered by treatment with α -chlorohydrin. On the other hand, testicular weights increase until day ten and then decrease until day 20. This change in testicular weights may be due to the build-up in fluid and cellular debris caused by the lesion in the caput epididymis. The subsequent decrease occurs after the build-up in pressure has caused substantial damage to the seminiferous tubules thereby preventing normal tubular fluid and cellular secretory products from being released into the testicular lumen.

Comparison With Ductal Ligation

Smith in 1962 looked at changes in testicular weight and morphology following ligation of the ductuli efferentes. The changes he found in the testicular weight and morphology followed the same pattern of changes observed following α -chlorohydrin treatment. The only

major difference was the time the response occurred. Changes in response to ductal ligation occurred much more rapidly than those observed after α -chlorohydrin treatment.

Comparison With Other Dosage Levels

Studies which used doses of α -chlorohydrin higher than 60 mg/kg report more rapid testicular and epididymal changes than those observed in this study. For example, an 80 mg/kg dose (Morris and Jackson, 1978) produced a maximal increase in testicular weight by day four as compared to the day ten maximum found in this study. Studies using a lower dose of 40 mg/kg for 3 days (Samojlik and Chang, 1970) resulted in total inhibition of spermatogenesis by day 20. A lower dose of 30 mg/day given for 1-3 consecutive days resulted in decreased testis weight, unchanged epididymal weight and evidence of sterility by the end of the first week after treatment (Ericsson, 1970). Thus when testicular and epididymal effects are being studied after treatment by α -chlorohydrin, the amount of drug administered is an important consideration. The comparisons described above suggest that only the difference between the general pattern of testicular and epididymal weight changes, not quantitative or even absolute time response changes, can be directly compared between different dosage levels and schedules of drug.

Effect of Age

The effect of an animal's age upon its testicular, epididymal and serum ABP and upon hormonal concentrations can be extremely important. The serum ABP concentration in the rat has been shown to decline from birth to 5 days of age and thereafter rise until 18 days of age. After that time the serum ABP levels decline until approximately day 60 when they level off and remain relatively constant until approximately 120 days of age in the adult animal (Gunsalus et al, 1978). Experimentally, ABP has been first detected in the epididymis at 18 days of age in the male rat (Tindall et al, 1974 and Means et al, 1975). These observations are compatible with the hypothesis that ABP is released into the blood prior to formation of the blood-testis barrier. Consequently, 90 day old male rats were chosen for this experiment because any changes observed in testicular ABP levels could be attributed to α -chlorohydrin treatment and not to age-related changes in ABP concentrations.

Possible Causes of Observed ABP Changes

When considering the causes for the rise in testicular ABP and concurrent decrease in epididymal levels of ABP after a lesion-forming dose of α -chlorohydrin has been administered, three plausible explanations emerge. First, the drug merely causes a lesion in the initial segment of the caput epididymis thereby blocking further ABP transport into the epididymis and consequent build up of ABP in the testis. A second possible explanation would be that the drug causes

damage to the Sertoli cell resulting in a rapid release of ABP into the testis and into the blood. However equally likely would be a combination of the two theories - a lesion is formed and Sertoli cell damage also occurs.

The second alternative can be disregarded when the results of testicular concentrations of ABP are compared with epididymal levels. If the Sertoli cell were damaged in such a way as to cause ABP to leak out into the lumen of the seminiferous tubule, ABP concentration would increase and then decline as the Sertoli cell's function is altered. Furthermore, ABP levels in the epididymis would also rise and fall concomittant with the testicular levels if no lesion were formed to prohibit transport into the epididymis. The latter was shown not to occur.

Morphological evidence shows that a lesion forms in the caput epididymis in response to a sterilizing dose of α -chlorohydrin (Ericsson, 1970; Cooper, Jones, and Jackson, 1973; and Hoffer, Hamilton, and Fawcett, 1973). Pathological damage is observed within 2-4 hours in the cells lining the caput epididymis at 140 mg/kg. By 2-3 days the lumen of the initial segment is full of debris which blocks the normal passage of sperm down the remaining length of the epididymis (Hoffer, 1973).

In this study, the fact that testicular levels of ABP continue to increase until the fourth day and thereafter decline, but never to a level below that of controls, suggests that the Sertoli cell is still producing ABP albeit at lower levels than at day four.

These results suggest that there is some mechanism which regulates

the production and secretion of ABP. This may be the result of some type of regulatory control mechanism and/or the influence of a change in FSH levels upon ABP production. Thus it is likely that the concentration of ABP does increase in the testis in response to the lesion blocking its transport into the epididymis but thereafter declines due to some regulatory control of the ABP-producing system.

If the Sertoli cell itself had been structurally damaged, then after the initial increase in ABP levels caused both by the lesion and the leakage from the possibly damaged cell, testicular levels of ABP would have been found to decrease below the levels observed in the controls due to the lack of ABP synthesis in the structurally damaged cell.

Correlation of Testicular Weight with Changes in ABP Content

An attempt was made to correlate the change in testicular weight with the changes in total testicular ABP on a particular day. Linear regression analysis yielded $r = 0.6041$ and $r^2 = 0.3649$ which indicates that a change in the testicular weight was associated with a change in total testicular ABP only about one-third of the time which is not often enough to suggest that there is any actual correlation between these two events.

Difference Between Active-Binding ABP and Immunoactive ABP Measurements

This study was designed to measure the time-dependent changes in testicular and epididymal levels of ABP. But since Bardin and

co-workers (Gunsalus et al, 1978) reported measuring ABP (by RIA) in the serum of rats, it was of interest to see if changes in testicular ABP concentration (as measured by SS-PAGE) could be associated with a corresponding change in serum ABP (as measured by RIA). As noted in the results, serum ABP increased to a maximum concentration on the tenth day after treatment, whereas testicular ABP increased to a maximum level by day four. Thus since a temporal correlation between these two different methods of measuring ABP content is not readily apparent, no association between changes in serum ABP levels and testicular ABP levels was made on the basis of this experiment. Future experiments need to be designed to show whether or not results obtained from SS-PAGE and RIA can be correlated.

Gonadotropin Changes

Very few of the studies investigating the relationship between ABP and the Sertoli cell have also included measurement of the gonadotropin and testosterone levels. Primarily in vitro data, results from hypophysectomized and castrate animals, and FSH and/or testosterone challenge to a SCE animal have been used to implicate the role FSH, LH and testosterone play in spermatogenesis (Hansson et al, 1975; Means et al, 1976; Tindall et al, 1974).

Morris and Jackson (1978) have studied the gonadotropin response after a single, oral 80 mg/kg dose of α -chlorohydrin and have attempted to draw some conclusions about the functional relationship of the gonadotropins and spermatogenesis. The pattern of change which they observed in testicular and epididymal weights, and serum LH and FSH

was somewhat similar to the changes found in this study. They caution that the functional status of the Sertoli cell was not evaluated in their study. Therefore, future experiments should ensure the functional capacity of the Sertoli cell, perhaps by the simultaneous measurement of ABP.

This experiment simultaneously measured the response of ABP, the gonadotropins, and testosterone to a sterilizing dose of α -chlorohydrin in the normal male rat.

The rise to a maximal serum FSH level occurred later than the maximal rise in total testicular ABP content (day 20 compared to day 10, respectively). The reason for this temporal difference cannot be explained on the basis of this experiment alone. Therefore, future research will need to focus on elucidating the relationship between the Sertoli cell, FSH and ABP.

An independent and temporal relationship between changes in serum LH and FSH levels following treatment by α -chlorohydrin (80 mg/kg) has been previously described by Morris and Jackson (1978). In their study changes in LH were delayed as compared to those seen for FSH, whereas in this study, the rise in serum LH levels occurred prior to the rise in serum FSH levels. It is possible that the difference in dosage levels (80 mg/kg compared to 60 mg/kg) between the two experiments might explain part of the difference in gonadotropin time-related response. However, neither this experiment nor the one conducted by Morris and Jackson provide sufficient evidence which explains the reason for the temporal difference between serum LH and FSH response following α -chlorohydrin treatment.

Testosterone

Serum testosterone concentration was not found to be significantly changed as compared to control values after α -chlorohydrin treatment. Thus, since androgen production appears to be unaltered, the Leydig cells may not be affected from exposure to α -chlorohydrin. Serum testosterone concentration was not found to be significantly changed as compared to control values after α -chlorohydrin treatment. Thus, since androgen production appears to be unaltered, the Leydig cells may not be affected from exposure to α -chlorohydrin (Morris and Jackson, 1978). However, serum LH levels were found to increase to day ten and LH is known to stimulate testosterone secretion. Therefore, a corresponding rise in testosterone levels as LH levels increase would be expected. The fact that the LH-testosterone association was not observed suggests that the Leydig cell function may indeed be altered. Future research aimed at elucidating the relationship between the Leydig cells and other testicular components will hopefully be able to determine whether or not α -chlorohydrin treatment affects the Leydig cells.

CONCLUSION

This experiment demonstrates that in the mature, adult male rat a single oral dose of at least 60 mg/kg is sufficient to cause a lesion in the initial segment of the caput epididymis. The change in weight of the testis and epididymis and the change in morphology are similar to those observed after ductuli efferentes ligation. Furthermore, the resulting changes in testicular, epididymal and serum ABP content closely follow the pattern of change that is expected as a result of blockage in the initial segment of the caput epididymis. The observed changes in ABP levels also indicate, along with the morphology, that the Sertoli cell continues to produce ABP even after spermatogenesis has been altered as a result of the α -chlorohydrin treatment. The observed gonadotropin and testosterone level changes serve to support this hypothesis, although they themselves are not direct and conclusive evidence.

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