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HORMONAL IMBALANCE AND ALTERATIONS IN TESTICULAR MORPHOLOGY INDUCED BY CHRONIC INGESTION OF ETHANOL*

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Abstract—Physical correlates of chronic ethanol consumption and measures of *in vitro* fertilization were examined in male C57Bl mice after a treatment period of 34 days with a total liquid nutriment diet which contained 5%/95% (v/v) ethanol. This diet represented relatively low levels of ethanol ingestion by the C57Bl mouse, as evidenced by the low peak levels of blood ethanol (160 mg/100 ml), the lack of behavioral signs of intoxication during treatment, and the absence of overt signs of physical dependence after withdrawal from the ethanol-containing diet. Plasma testosterone levels of experimental animals were depressed during the treatment period, as compared to testosterone levels of pair-fed controls. No evidence of hepatic injury was observed following the treatment period. Although fertility, as measured by the ability of spermatozoa to penetrate mouse ova *in vitro*, was unaffected by the chronic ethanol treatment, signs of testicular dysfunction were evident. Abnormal testicular morphology included disruption of the basement membranes of the seminiferous tubules, decreased tubular diameter, and desquamation of immature germ cells into the lumina of the tubules. The present study provides convincing evidence of the adverse effects upon male reproductive functions of relatively low levels of ethanol, when administered chronically, under controlled conditions.

Adverse effects of chronic ethanol consumption upon subsequent reproduction function have been observed since the early part of this century (for review, see Ref. 1). Among the first studies with experimental animals was the work of Stockard and Papanícolaou [2, 3], which showed decreased fertility of ethanol-treated guinea pigs, and decreased viability of subsequent offspring. Similar findings were made by Cole and Davis [4], who determined that chronic administration of ethanol to male rabbits rendered them subfertile. Moreover, birth defects were noted among progeny sired by the ethanol treated males. Badr and Badr [5] attributed an increased prenatal mortality of mice sired by males treated subchronically with ethanol to an ethanol-induced dominant lethal mutation.

In man, maternal consumption of ethanol during pregnancy can give rise to children with varying degrees of neurological, cardiac, urogenital and craniofacial disorders. Jones and Smith [6] have described the pattern of anomalies which are present in children born to alcoholic mothers as the Fetal Alcohol Syndrome (FAS). Thus, evidence has been accumulated in experimental animals and man, which suggests that chronic ethanol consumption can produce not only sterility, but can also act as a teratogen in the case of the Fetal Alcohol Syndrome, and as a mutagen, exemplified by defective offspring or dominant lethal mutations induced by ethanol consumption by the male.

Although numerous studies have been conducted recently which have been directed toward elucidating the mechanisms responsible for the adverse effects of ethanol on reproductive function in the female and their relation to the FAS [7–10], very few studies have been initiated which describe reproductive dysfunction subsequent to chronic ethanol ingestion by the male.

Recent studies in our laboratory [11] indicate that consumption of relatively low doses of ethanol by male C57Bl mice results in alterations of male reproductive function. This alteration was manifested by decreased litter size and increased postnatal mortality of the progeny. Decreased litter size could have been due to decreased ability of spermatozoa from ethanol-treated males to penetrate ova, and/or ethanol-induced damage to the chromatin material in the male gametes, resulting in the production of a possible dominant lethal mutation [5]. The present study is an attempt to distinguish between these possibilities by examining various measures of male fertility subsequent to chronic exposure of male C57Bl mice to an ethanol-containing liquid nutriment diet. Moreover, since one of the objectives of our laboratory was to develop an animal model which describes the effect of chronic ingestion of ethanol upon subsequent male reproductive tract function, various physiological correlates of ethanol consumption were also examined.

MATERIALS AND METHODS

Testosterone standard solutions and [1α,2α-[^{3}H](N)]testosterone (60 Ci/mmole) were obtained from the New England Nuclear Corp. (Boston, MA).

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Rabbit testosterone antiserum, bovine serum albumin, nicotinamide adenine dinucleotide (reduced form), malic dehydrogenase, lactic dehydrogenase, aspartic acid, L-alanine, sodium α-ketoglutarate and *p*-nitrophenylphosphate were products of the Sigma Chemical Co. (St. Louis, MO). Pregnant mare's serum (Gestyl) and human chorionic gonadotropin were purchased from Organon, Inc. (West Orange, NJ) and Parke-Davis (Detroit, MI), respectively. Carnation Slender (chocolate-flavored) was obtained from a local grocer. All other reagents used were of the highest quality commercially available.

Chronic administration of ethanol. Thirty male C57B1 mice (22–27 g) were obtained from Jackson Laboratories (Bar Harbor, ME). Initially, the animals were permitted an acclimation period of 7 days in our animal facility. The facility was maintained on a 14/10 hr light/dark cycle at 22 ± 0.5°. On day 8, all animals were given free access to a solution of chocolate-flavored Carnation Slender which contained 3 g/liter vitamin supplement (ICN Pharmaceuticals, Cleveland, OH) and sucrose (77 g/liter) which was isocalorically equivalent to 5%/95% (v/v) ethanol, for a period of 2 days, to acclimate the animals to the liquid diet as their only food source. The composition of the diet (in g/liter) was as follows: protein, 35.21; carbohydrate, 185.50; and fat, 16.00; when present, ethanol comprised 28% of the total caloric content of the diet. The mice were subsequently divided into two groups. Twenty mice (experimental group) were given free access to a liquid nutriment diet in which the sucrose was replaced by 5%/95% (v/v) ethanol. The remaining animals (control group) were maintained on the diet which contained sucrose. Consumption of diet by the control animals was restricted to the average daily consumption of diet by the experimental animals. Controls were thus pair-fed to the experimental group.

Experimental and control animals ingested their respective diets for 34 days, after which all mice were given free access to laboratory chow and water. During the acquisition period, consumption of diet was measured twice daily (4 hr after the beginning of the light period, and 2 hr before the onset of darkness). Body weights were recorded every two days.

Measurement of physical dependence and tolerance induced by chronic ingestion of ethanol. Withdrawal symptomatology was assessed monitoring seizure activity, body weight and rectal temperature [12] at 2-hr intervals for a period of 8 hr after removing the animals from the liquid diets (the animals were removed from their diets at 9:00 a.m.). Twenty-four hr after removal of the liquid diets, tolerance to ethanol was assessed by measuring the decrease in rectal temperature and the sleep-time induced by an acute injection of ethanol (3 g/kg, i.p.). Sleep-time was taken as the interval between the time at which the loss of righting reflex was noted and the time at which the animal righted itself three times within a 30-sec test period.

Determination of plasma testosterone and blood ethanol. Starting on day 22 between 8:00 p.m. (1 hr after the onset of darkness) and 8:00 a.m. (3 hr after the beginning of the light period), 100 μl of tail blood were taken from randomly selected animals from experimental and control groups at 2-hr intervals. Three to four samples from each group were taken at each time. In addition, 25 μl of tail blood were withdrawn for blood ethanol determination (N = 5 for each time point).

Plasma testosterone content was determined by radioimmunoassay, using a slight modification of the method suggested by the New England Nuclear bulletin supplied with the Testosterone [^{3}H] Radioimmunoassay Pak. Plasma (30–45 μl) was added to the assay buffer [0.1 M sodium phosphate (pH 7.0) which contained 15 mM sodium azide, 0.15 M NaCl and 0.1% gelatin] such that the total volume was 1.0 ml. The samples were extracted with 3 ml methylene chloride–ethanol (1:1). The aqueous phase was discarded, and the solvent phase was washed sequentially by extraction with 2 ml each of 0.1 N NaOH, 0.1 N acetic acid and water. The entire organic phase was evaporated in a water bath (45°) and, after evaporation of the solvent to dryness, 0.1 ml of assay buffer was added. Assay ^{3}H-tracer (0.1 ml [^{3}H]testosterone, 50 pg/ml, 4 × 10^5 c.p.m.) and 0.1 ml rabbit anti-testosterone were added, and the mixtures were incubated at 4° for 100 min. Unbound testosterone was removed with 0.8 ml of dextran-coated charcoal [625 mg of activated charcoal (Sigma) plus 625 ml of Dextran T70 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 100 ml of assay buffer]. The dextran-coated charcoal was sedimented by centrifugation (4°) at 1500 *g* for 20 min. The supernatant fraction (0.5 ml) was added to Bray's solution [13], and the testosterone which was bound to the antiserum was determined by liquid scintillation spectrometry. Plasma testosterone was quantitated by the use of testosterone standards which were carried through the entire extraction process. Internal testosterone standards were used to correct for efficiency of extraction and for efficiency of testosterone binding to the antiserum. Values were uncorrected for dihydrotestosterone content and were expressed as ng/ml plasma.

Blood ethanol levels were measured by gas chromatography, as described previously [14]. Briefly, 25 μl blood were placed in sealed flasks which contained 0.6 N perchloric acid and 25 mM thiourea. The flasks were equilibrated at 35°, and an aliquot of the headspace over the solution was withdrawn into a gas-tight syringe and injected onto a Packard model 421 gas chromatograph equipped with a flame ionization detector and a glass column which contained Poropak Q. Peak heights were quantitated through the use of ethanol standards.

In vitro fertilization of mouse ova and evaluation of spermatozoa. In vitro fertilization of mouse ova was carried out essentially as described by Wolf *et al.* [15]. The studies were conducted 4 hr after removing the animals from the liquid nutriment. Mature female Swiss mice were superovulated with i.p. injections of 10 I.U. of pregnant mare's serum, followed 48 hr later by 10 I.U. of human chorionic gonadotropin. Fifteen hr later, the mice were killed and the ova with their cumulus masses were harvested and placed in sterilized silicone oil equilibrated with a gas mixture of N_2–O_2–CO_2 (90:5:5). This and all subsequent procedures were carried out

at 37°. Epididymal spermatozoa were obtained by making several small cuts in each cauda epididymis after it had been removed from the reproductive tract and placing the caudae epididymides into 1 ml capacitation medium. Spermatozoa were allowed to migrate into the medium for a period of 10 min prior to removing an aliquot for determination of sperm content. The ova were transferred to microdishes which contained modified Krebs–Ringer bicarbonate buffer with 3 mg/ml bovine serum albumin. The dishes were inseminated with capacitated epididymal spermatozoa from either ethanol-treated or control animals, and were incubated for 26 hr. After incubation, ova were washed with modified Krebs–Ringer bicarbonate to remove excess spermatozoa, were mounted and fixed with 2.5% glutaraldehyde, and were stained with aceto-lacmoid as described by Toyoda and Chang [16]. Microscopic observation of either the decondensed male pronucleus or the first cell cleavage was taken as evidence of fertilization. Prior to insemination into microdishes which contained ova, epididymal spermatozoa were capacitated by incubation for 60 min in modified Krebs–Ringer bicarbonate which contained 20 mg/ml bovine serum albumin.

Epididymal spermatozoa content was determined with a Newbauer hemocytometer. For *in vitro* fertilization, 10^5 spermatozoa were inseminated into the microdishes. Forward progression of spermatozoa was quantitated by measuring the time required for individual spermatozoa to move from the center to the periphery of the microscopic field (magnification = 200).

Histological evaluation of testes and liver. Testes and livers from the experimental and control males which were used for the *in vitro* fertilization studies were immersion-fixed in Bouin's solution and formyl saline, respectively. Tissues were paraffin-embedded, sectioned (5 μm) and stained with hematoxylin and eosin Y.

Biochemical evaluation of hepatic function. Plasma levels of alkaline phosphatase (EC 3.1.3.1), glutamic oxaloacetic transaminase was measured spectrophopyruvic transaminase (EC 2.6.1.2) were measured by standard techniques [17]. Alkaline phosphatase activity was quantitated spectrophotometrically (410 nm) by measuring the release of *p*-nitrophenol from the substrate *p*-nitrophenylphosphate. Glutamic oxaloacetic transminase was measured spectrophotometrically (340 nm), by following the oxidation of NADH which occurred during the reduction of formed oxaloacetate, in the presence of added malate dehydrogenase (EC 1.1.1.37) with α-ketoglutarate and aspartate as substrates. Similarly, glutamic pyruvic transaminase was quantitated by measuring the oxidation of NADH which resulted from the formation and subsequent reduction of pyruvate, in the presence of added lactic dehydrogenase (EC 1.1.1.27), with α-ketoglutarate and alanine as substrates.

RESULTS

Both experimental and control mice consumed similar amounts of liquid nutriment diet over the 34-day period (Table 1). Moreover, the amount of diet consumed was sufficient to maintain body weight in both groups. The daily consumption of diet by experimental animals was equivalent to approximately 20 g ethanol/kg body wt. Examination of testosterone levels during the period of darkness on day 22/23 of the acquisition period (Fig. 1) revealed lower testosterone in ethanol-treated animals at all time points but one (testosterone levels of plasma collected between 4:30 and 5:30 a.m. were higher in ethanol-treated than in control animals). Testosterone levels were significantly lower in plasma from experimental animals taken between 12:30 and 1:30 a.m., as compared to controls ($P < 0.001$; two-tailed *t*-test). Blood levels of ethanol peaked at approximately 160 mg/100 ml at 10:00 p.m. and were undetectable beyond 6:00 a.m. (Fig. 1).

In contrast to the lower testosterone levels after chronic ingestion of ethanol, acute administration of ethanol (1.5 g/kg, i.p.) produced a rise in testosterone levels, compared to saline injected controls (Fig. 2). Blood ethanol levels after injection of 1.5 g/kg ethanol were similar to the blood ethanol levels during chronic alcohol ingestion (Fig. 1). When a higher dose of ethanol (3 g/kg) was administered, however, the testosterone level dropped significantly below that of saline-injected animals (Fig. 2B).

At the end of the treatment period (day 35), tail blood (100 μl) was drawn from each animal, and plasma levels of alkaline phosphatase, glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase were determined (Table 2). Increased levels of these enzymes in plasma from experimental

Table 1. Chronic ingestion of ethanol by male C57B1 mice: Average diet consumption, ethanol intake, and body weight change after 34 days of treatment*

	Daily diet consumption (ml/kg body wt)	Daily ethanol consumption (g/kg body wt)	Net weight change (g)
Control	549 ± 26 (320)		+ 0.3 ± 0.6 (10)
Experimental	550 ± 40 (624)	20.4 ± 1.6 (624)	+ 0.3 ± 0.3 (19)

* Diet consumption was measured gravimetrically, to the nearest 0.1 g. Values represent the average ± S.D. and represent diet consumption from day 3 through day 35 of treatment. Body weight was determined to the nearest 0.2 g. Values represent the average ± S.D. of the net weight change between day 1 and day 35 of treatment. One experimental animal was removed from the study on day 28, due to cessation of diet consumption, and greater than a 10 per cent loss of its initial body weight. This animal was not used in subsequent investigations. The numbers in parentheses equal the number of determinations.

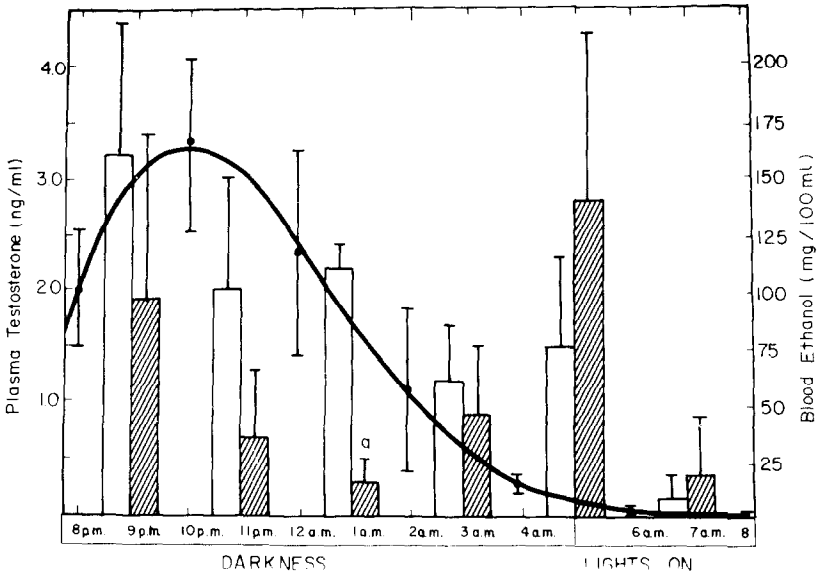


Fig. 1. Plasma testosterone and blood ethanol levels as a function of time during chronic ingestion of ethanol. On day 22/23 of ethanol treatment, tail blood was withdrawn for measurement of testosterone and ethanol, as described in Materials and Methods. Vertical bars represent testosterone levels (left ordinate). Control levels (open bars) are compared with experimental values (hatched bars). Ethanol levels (right ordinate) are represented by the closed line (●). Values represent the average ± S.E. for five determinations at each time point for ethanol levels, and three to five determinations at each time point for testosterone levels. The letter "a" indicates that testosterone levels of experimental animals differed significantly from those of controls ($P < 0.001$).

animals as compared to controls would have suggested hepatic damage was induced by ethanol treatment. One-way analysis of variance, however, indicated no significant difference in plasma enzyme levels for ethanol-treated, pair-fed or laboratory chow-fed animals ($P > 0.1$).

Signs of withdrawal, such as seizure activity, decreased rectal temperature [12], and decreased body weight (R. A. Anderson and B. Tabakoff, unpublished observations), were not severe in experimental animals after they were removed from the ethanol diet (Table 3). Measurement of rectal temperature at 2-hr intervals for up to 6 hr after removing the animals from the diets revealed no significant difference between experimental and control groups. At 8 hr after withdrawal, a slight but significant ($P = 0.05$) decrease in rectal temperature was observed in ethanol-treated as compared to pair-fed control animals. Significant decreases in body weight were observed in experimental animals as compared to controls at all times examined, the maximum weight loss being noted at 6 hr post withdrawal. No behavioral signs of withdrawal (i.e. seizure activity) were noted in any of the animals, up to 8 hr after removal of the diets. Although ethanol-treated animals were not tolerant to the decrease in rectal temperature induced by an acute injection of ethanol (3 g/kg, i.p.), tolerance of the experimental

Table 2. Biochemical evaluation of hepatic function after chronic ingestion of ethanol*

Enzyme	Enzyme activity (nmoles substrate utilized·min·ml plasma) Pair-fed control	Chow-fed control	Ethanol-treated
Alkaline phosphatase	22.9 ± 8.0	11.2 ± 1.8	16.6 ± 2.6
	$F = 1.509$, $df_1 = 2$, $df_2 = 7$; not significant ($P > 0.1$)		
Glutamic oxaloacetic transaminase	64.6 ± 10.4	41.0 ± 6.1	34.3 ± 6.9
	$F = 3.100$, $df_1 = 2$, $df_2 = 8$, not significant ($P > 0.1$)		
Glutamic pyruvic transaminase	43.9 ± 20.4	44.0 ± 8.2	25.3 ± 4.8
	$F = 1.824$, $df_1 = 2$, $df_2 = 8$, not significant ($P > 0.1$)		

* Plasma enzyme levels of experimental animals and pair-fed controls were determined after 34 days of treatment, as described in Materials and Methods. Plasma enzyme levels of chow-fed controls were also determined. Values represent the average ± S.E. of three to five determinations.

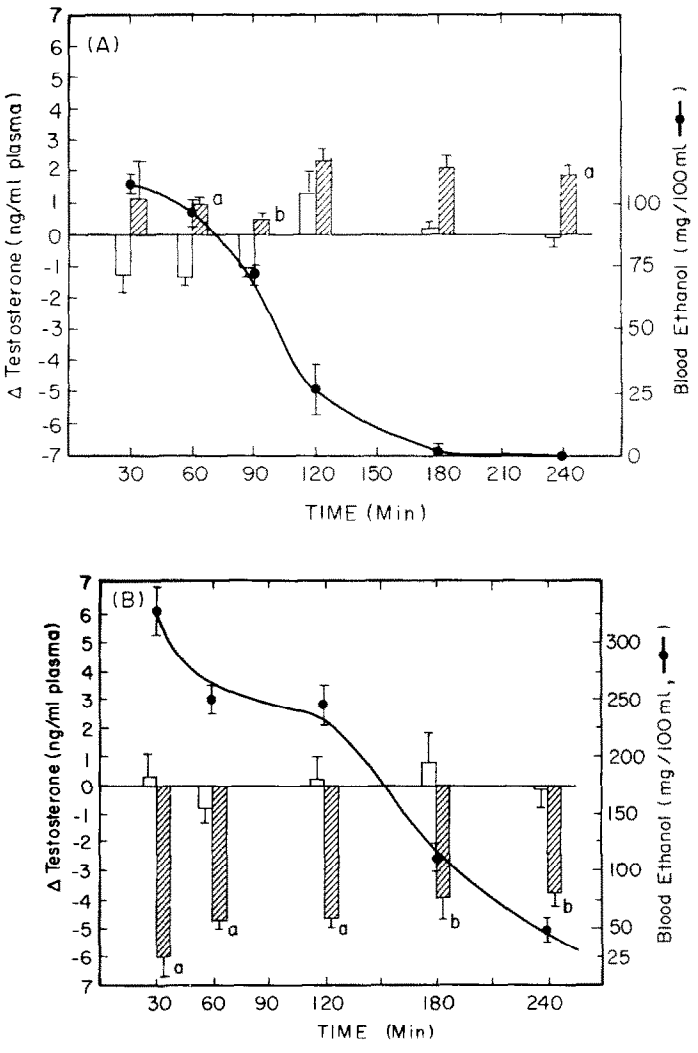


Fig. 2. Effect of acute ethanol injection on plasma testosterone. Panel A: Effect of low ethanol dose. Plasma testosterone levels were determined at various times after i.p. injection of either 1.5 g/kg ethanol (cross-hatched bars) or saline (open bars), as indicated. The values (left ordinate) at each time point are the average change ± S.E. in plasma testosterone (N = 7) from the average levels (3.2 ± 0.8 ng/ml; N = 30) obtained prior to injection. Also represented are the average ± S.E. (N = 7 at each time point) blood ethanol values (●) reported as mg/100 ml. The letter "a" indicates that changes differ significantly from controls, $P < 0.005$; the letter "b" indicates that changes differ significantly from controls, $P < 0.001$. Panel B: effect of high ethanol dose. Plasma testosterone was determined after i.p. injection of either 3 g/kg ethanol (cross-hatched bars) or saline (open bars), as described in the legend to panel A. The average plasma testosterone determined prior to injection was 6.3 ± 0.6 ng/ml (S.E.; N = 20). Also presented are the average ± S.E. (N = 7 for each time point) blood ethanol levels (mg/100 ml). The letter "a" indicates that changes differ significantly from controls, $P < 0.001$; the letter "b" indicates that changes differ significantly from controls, $P < 0.005$.

group was indicated by a significant decrease ($P < 0.025$, two-tailed *t*-test) in sleep time (Table 4).

At the level and duration of exposure to ethanol used in the present study, chronic ingestion of ethanol did not affect fertility, as measured by *in vitro* fertilization of mouse ova. Moreover, no difference in sperm motility was noted between ethanol-treated and control animals, as measured by velocity of forward progression. Epididymal sperm count, however, was significantly higher ($P < 0.01$, two-tailed *t*-test) in experimental animals as compared to controls (Table 5).

Histological examination of the testes revealed a trend toward a pathologic condition in ethanol-treated, as compared to pair-fed control mice (Figs. 3–5). The basement membranes of the seminiferous tubules from the ethanol-treated animals had a frayed appearance, whereas those from controls appeared intact (Fig. 3). In addition, seminiferous tubules from experimental animals contained a larger number of desquamated immature germ cells in the lumina of the tubules (Fig. 4). The frequency of occurrence of desquamation (4.4 ± 3.1 and 2.8 ± 0.9 per cent for experimental and control testes, respec-

Table 3. Assessment of physical dependence after chronic ingestion of ethanol*

	(A) Rectal temperature (°) Hours after withdrawal				
	2	4	6	8	N
Pair-fed control	34.9 ± 0.2	36.1 ± 0.3	35.2 ± 0.2	35.8 ± 0.2	4
Ethanol-treated	35.1 ± 0.3	35.6 ± 0.3	35.0 ± 0.2	35.0 ± 0.2	6
df (N−2)	8	8	8	8	
t	0.458	1.828	0.426	2.316	
	(P > 0.1)	(P > 0.1)	(P > 0.1)	(P = 0.05)	

	(B) −Δ body weight (g) Hours after withdrawal			
	4	6	8	N
Pair-fed control	0.5 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	8
Ethanol-treated	1.0 ± 0.09	1.5 ± 0.1	1.3 ± 0.1	15
df (N−2)	21	21	21	
t	2.716	2.877	3.259	
	(P < 0.05)	(P < 0.01)	(P < 0.005)	

* After 34 days of treatment, animals were removed from the liquid diets and were given free access to laboratory chow and water (9:00 a.m., day 35). Rectal temperatures and body weights were determined at the times indicated. Values represent the average ± S.E. Body weights of experimental and control groups were 26 ± 0.3 g (N = 19) and 25 ± 0.5 g (N = 10), respectively. Rectal temperatures of experimental and control groups at the time of withdrawal from their diets were 35.5 ± 0.2° (N = 6) and 34.8 ± 0.3° (N = 4), respectively.

tively), however, was not statistically different (P = 0.106; chi-squared analysis). Finally, the intertubular space was greatly increased, and the tubular diameter was significantly smaller [186 ± 18 and 209 ± 19 μM (S.D) for experimental and control tubules, respectively; $t = 4.696$, df = 58, $P < 0.001$] in testes from ethanol-treated males as compared to controls (Fig. 5).

Table 4. Assessment of tolerance after chronic ingestion of ethanol

(A) Sleep time*	
Group	Sleep time (min)
Control	22.7 ± 4.6
Ethanol-treated	4.7 ± 3.7
df (N−2)	10
t	3.083
	(P < 0.025)

(B) Decreased rectal temperature† −Δ Temperature (°)					
	Time (min) after ethanol administration				
	15	45	75	105	135
Control	2.5 ± 0.2	3.1 ± 1.1	2.0 ± 1.2	2.1 ± 1.2	2.0 ± 1.0
Ethanol-treated	3.1 ± 0.1	3.0 ± 0.4	2.2 ± 0.2	1.8 ± 0.2	1.0 ± 0.5
df (N−2)	5	5	5	5	5
t	2.570	0.016	0.187	0.227	0.954
	(P = 0.05)	(NS)	(NS)	(NS)	(NS)

* Sleep time was measured after administration of ethanol (3 g/kg body wt, i.p.) to either ethanol-treated or naive control animals, as described in Materials and Methods. Values represent the average ± S.E. for six animals from each group.

† Rectal temperatures were determined at various times after an acute injection of ethanol (3 g/kg body wt, i.p.) to either ethanol-treated or naive control animals. Values are expressed as the average decrease (°) ± S.E. from initial rectal temperatures. Three and four determinations were made per time point, for control and experimental, respectively. NS indicates no significant difference (P > 0.1) between experimental and control animals.

Table 5. *In vitro* measures of fertility*

Group	Sperm count ($\times 10^{-6}$)	Sperm motility (μm/sec)	Fertilization (%)	No. of ova examined
Pair-fed control	15 ± 5	91 ± 6	54 ± 1	50
Ethanol-treated	42 ± 4	89 ± 12	60 ± 6	105
df (N−2)	5	5	df = 1†	
t	4.38 (P < 0.01)	0.16 (P > 0.1)	$\chi^2 = 0.285$ (P > 0.1)	

* Epididymal sperm count (suspension volume = 1 ml) and sperm motility of experimental and control animals were evaluated as described in Materials and Methods. Fertilization was measured as the number of penetrated ova or two-cell stages divided by the total number of ova examined (see Materials and Methods). Values represent the average ± S.E.

† Chi-square analysis was performed on *in vitro* fertilization.

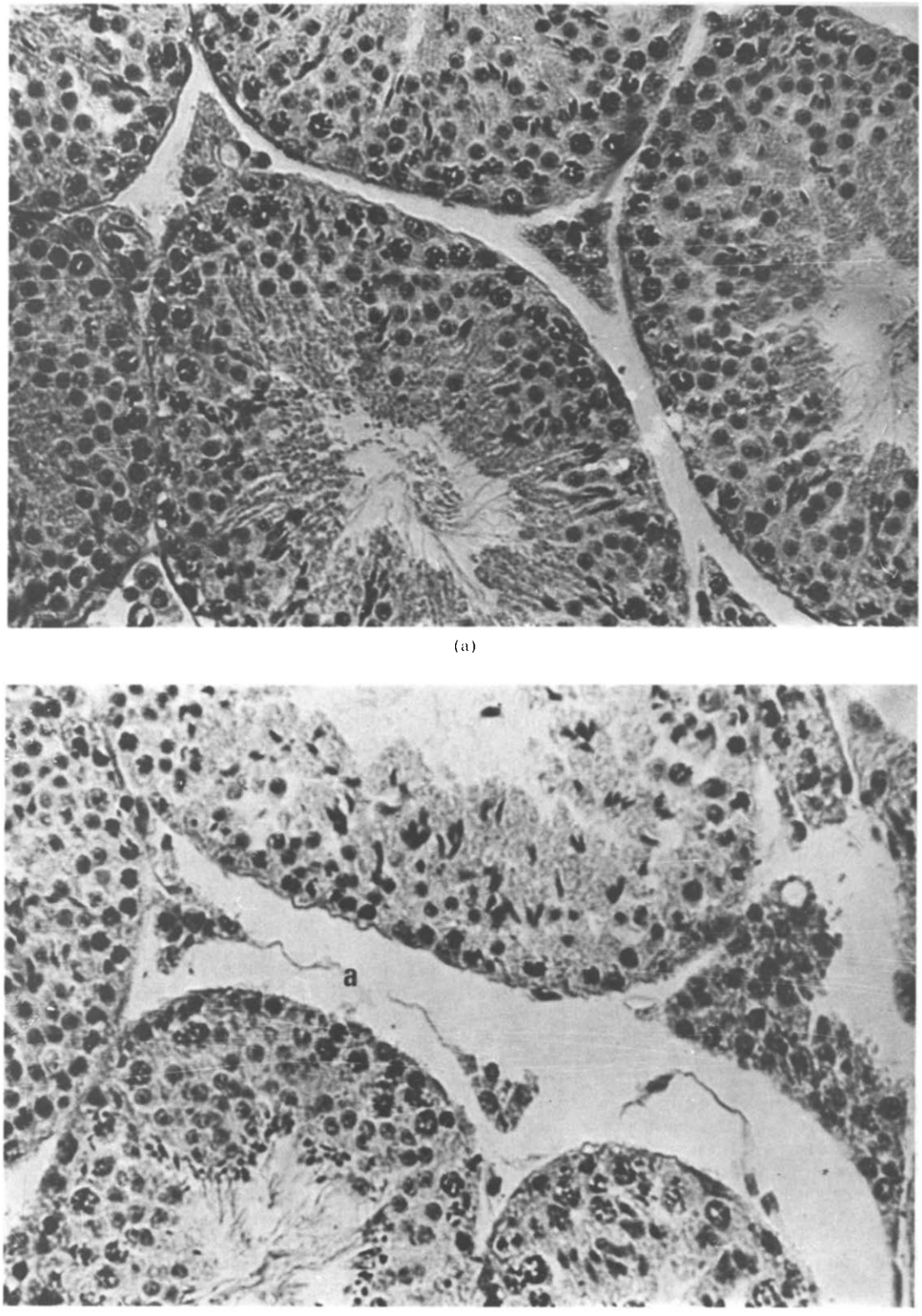
DISCUSSION

Chronic ingestion of relatively low levels of ethanol by the male resulted in hormonal imbalance (Fig. 1) and testicular dysfunction (Figs. 3–5). Fertility, however, as measured by *in vitro* fertilization (Table 5), remained unaffected. The average daily consumption of 20.4 g ethanol/kg body wt agreed well with the value reported previously by our laboratory [11]. The contention that the animals ingested relatively low levels of ethanol is supported by the low blood ethanol levels observed (Fig. 1). Furthermore, the experimental group was not subjected to continuous exposure to ethanol since blood ethanol levels were undetectable during most of the light period.

Testosterone levels were depressed when blood ethanol levels were above 50 mg/100 ml; at lower ethanol levels, no significant effect was noted (Fig. 1). Ethanol ingestion has been shown to depress testosterone in man [18]. Decreased testosterone in the present study appears to be due to a composite of the acute and chronic influence of ethanol, since testosterone levels after an acute injection of ethanol were elevated (Fig. 2A) at blood ethanol levels approximating those associated with significantly depressed testosterone levels in chronically treated animals (Fig. 1). When higher levels of ethanol were given (Fig. 2B), testosterone levels were depressed. This apparent biphasic effect is in agreement with the work of Cicero and Badger [19], who demonstrated that low levels of ethanol elevate, and high levels of ethanol depress plasma testosterone content. Decreased testosterone may be secondary to ethanol-induced hepatic damage. Our study indicated no signs of hepatic damage, either biochemically (Table 2) or morphologically (results not shown). On the other hand, chronic ingestion of ethanol may have caused an increased level of 5α-testosterone reductase, the rate-limiting enzyme in the catabolism of testosterone, as suggested by Rubin *et al.* [20]. Since this enzyme activity was not examined in the present study, the possibility of lowered testosterone levels due to altered liver metabolism cannot be totally excluded. However, the effect of ethanol on testosterone levels seen in the present study is, at least in part, independent of changes in liver metabolism, since such changes would not be expressed within 30 min after ethanol administration (Fig. 2, panels A and B).

Ritzmann and Tabakoff [12] have shown that rectal temperatures of mice rendered physically dependent upon ethanol dropped significantly shortly after withdrawal from ethanol. Both the magnitude and duration of hypothermia correlated well with seizure activity associated with alcohol withdrawal. Moreover, mice chronically exposed to ethanol were tolerant to acute ethanol-induced hypothermia. Measurement of rectal temperature in the present study indicated little, if any, physical dependence (Table 3). Depressed rectal temperatures of experimental animals relative to those of controls (8 hr after withdrawal) were, in fact, due to a rise in body temperature of controls, rather than to a decreased rectal temperature of ethanol-treated animals. Furthermore, no behavioral manifestations of withdrawal (i.e. spontaneous, or induced seizures [12]) were noted. A decrease in body weight after withdrawal (Table 3) did, however, indicate a slight degree of physical dependence. Previous studies have shown that physically dependent mice lost approximately 10 per cent of their body weight at 8 hr after ethanol withdrawal (R. A. Anderson and B. Tabakoff, unpublished observations). The drop in body weight in the present study was maximal (approximately 6 per cent) after 6 hr (Table 4). Although acute administration of ethanol produced no significant difference in rectal temperatures between ethanol treated and control animals, a significantly decreased ethanol induced sleep time (Table 4) indicated the development of a tolerance by the experimental group.

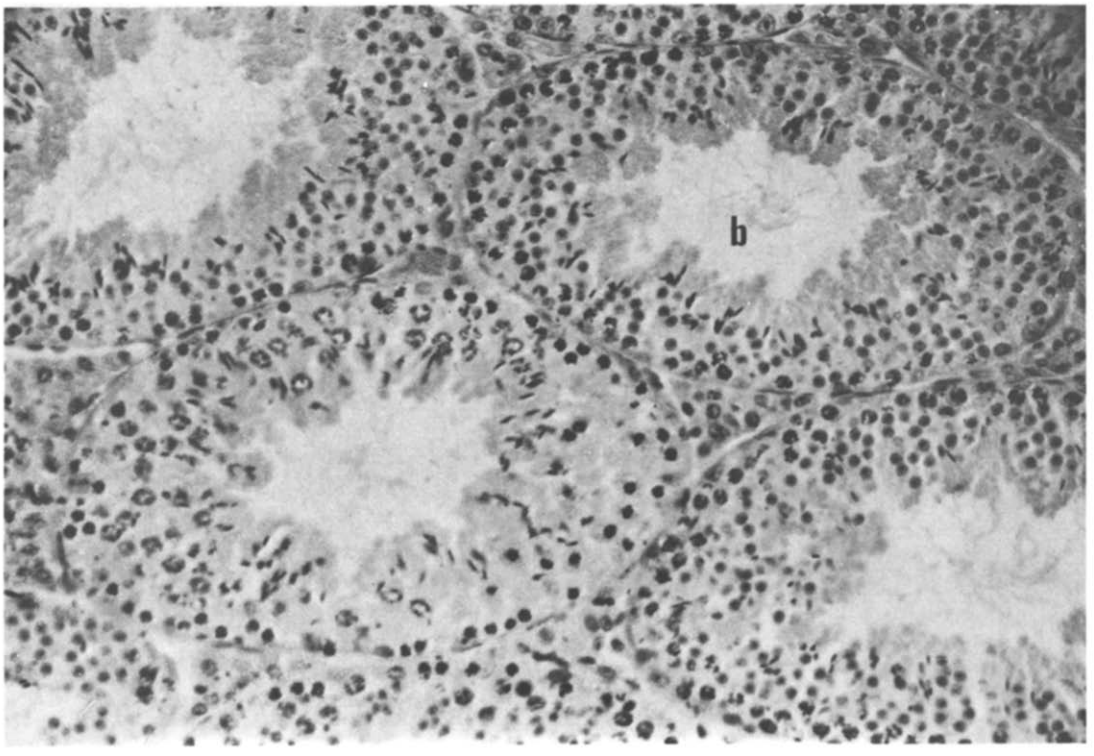
The similar frequencies of fertilization of mouse ova *in vitro* by spermatozoa from ethanol-treated and control males (Table 5) agree with our previous study [11] in which no change in fertility was evident, as measured by the frequency of conception *in vivo*. An unexpected observation, however, was the significant increase in epididymal spermatozoa in experimental animals (Table 6). Ingestion of ethanol may have resulted in altered transport of spermatozoa through the reproductive tract. Recent observations in our laboratory have suggested that the distribution of spermatozoa in the male reproductive tract is dependent upon the bi-directional transport



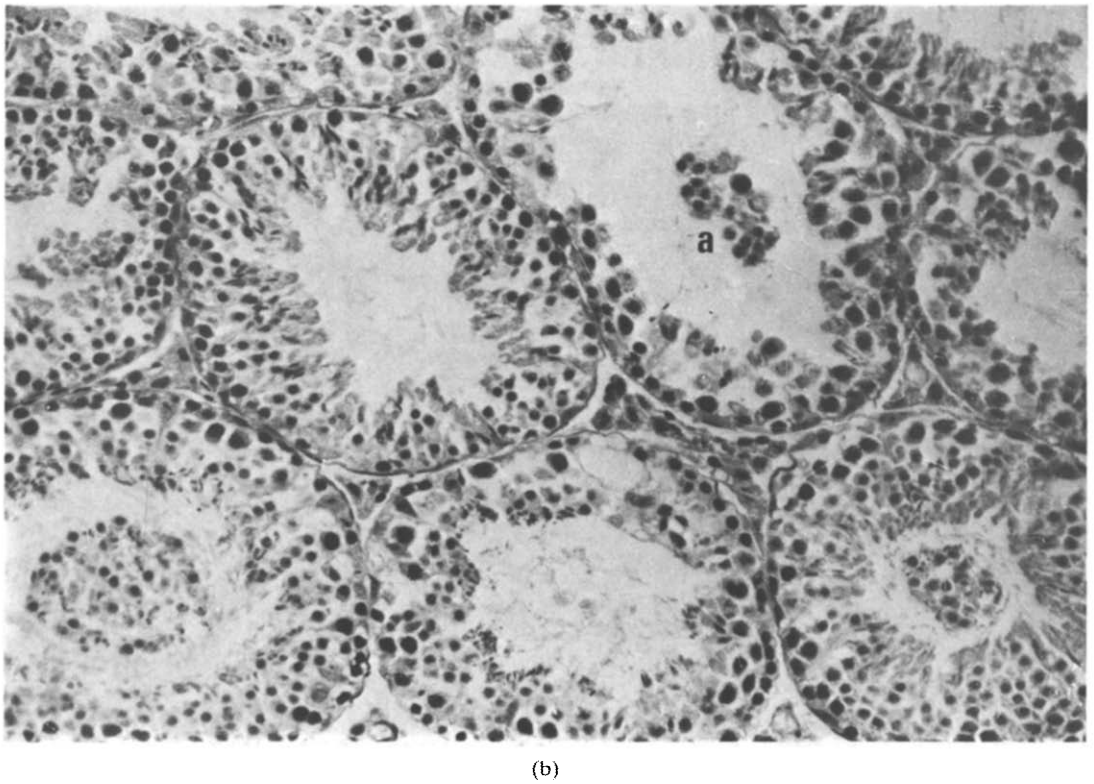
(a)

(b)

Fig. 3. Alterations of testicular morphology induced by chronic ethanol ingestion. Damage to the basement membranes of the seminiferous tubules. Panel a: cross-section of a control testis (magnification × 680). The basement membrane is regular and intact, and the intertubular space is reduced from that of the ethanol-treated testes (see below). Panel b: a similar section of seminiferous tubules of an experimental testis. Note the frayed appearance of the basement membrane (a), and the relatively large intertubular space (magnification × 680).

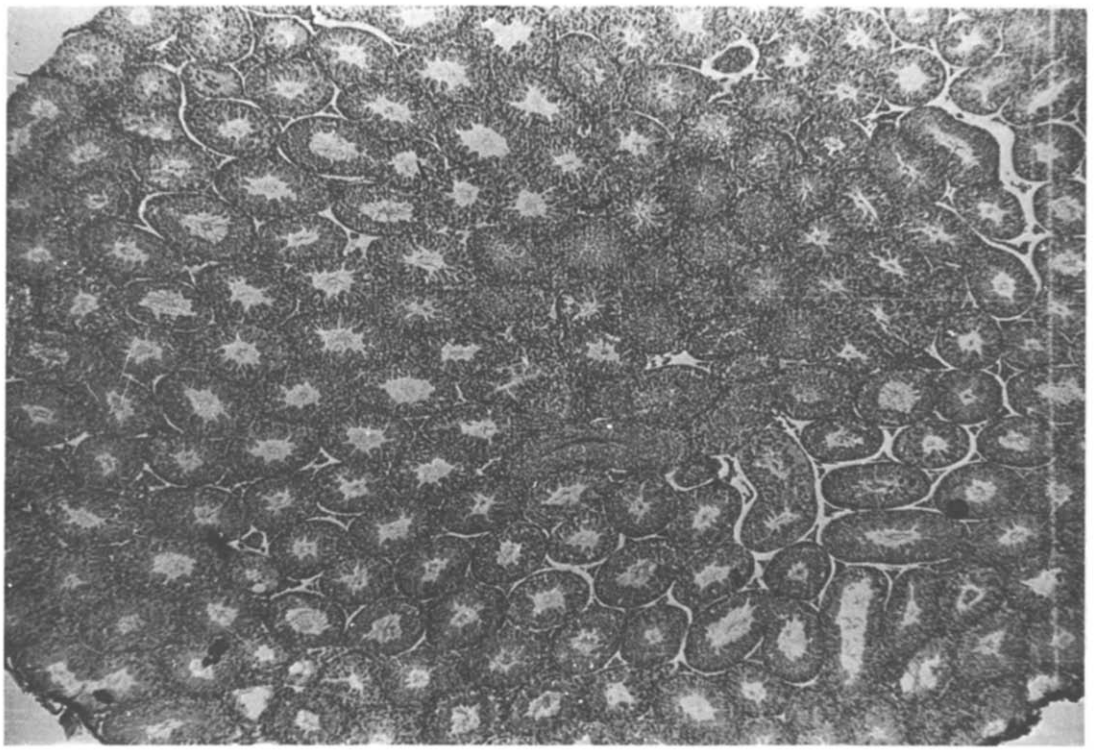


(a)

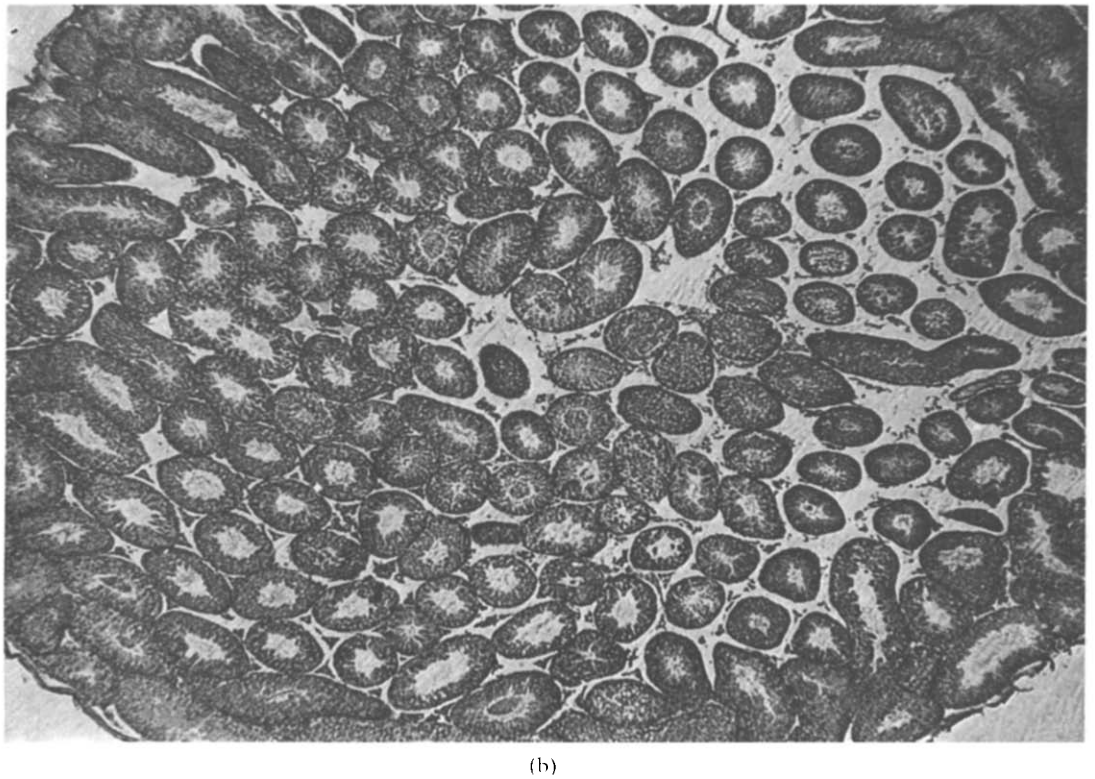


(b)

Fig. 4. Alterations of testicular morphology induced by chronic ethanol ingestion: desquamation of immature germ cells into the lumina of the seminiferous tubules. Panel a: a cross-section of a control testis (magnification × 272). The lumina contain only mature spermatozoa (b). Panel b: A similar section of seminiferous tubule of an experimental testis (magnification × 272). Note the presence of large numbers of immature germ cells (mainly spermatids and primary spermatocytes) in the lumina (a).



(a)



(b)

Fig. 5. Alterations of testicular morphology induced by chronic ethanol ingestion: increased intertubular space. Panel a: Cross-section of control testis (magnification × 42). Panel b: Cross-section of experimental testis (magnification × 42). Note the increased intertubular space in the experimental testis (panel b) as compared to the control testis (panel a).

of spermatozoa both proximally and distally to the epididymis [21]. Spontaneous motility of the vas deferens is thought to be modulated by norepinephrine from sympathetic fibers in the muscular sheath [22]. Zankl and Leidl [23] found that depletion of norepinephrine by a sympatholytic agent simulated vasoligation, causing an increase in epididymal sperm concentration. Acetaldehyde (the primary oxidation product of ethanol) is a sympathomimetic agent which releases catecholamines from the adrenals. In contrast to our findings, an ethanol-induced increase in circulating norepinephrine should result in a lower epididymal sperm content. However, Schneider [24] has demonstrated that chronic exposure to acetaldehyde depletes catecholamine levels in the adrenals. A depletion of catecholamines in the vas deferens due to chronic ingestion of ethanol may explain the higher epididymal sperm content found in the present study. The validity of the hypothesis is, of course, subject to further experimentation.

Chronic ingestion of relatively low levels of ethanol caused testicular damage (Figs. 3–5). These findings are in agreement with the studies of Van Thiel *et al.* [25] and of Klassen and Persaud [26]. There were, however, major differences in experimental design between their studies and those presented here.

In the studies of Van Thiel *et al.*, sexually immature rats were used, whereas the present study utilized adult mice. A direct comparison of ethanol intake between the two studies cannot be made, since diet consumption was not reported by Van Thiel *et al.* Moreover, the diet used (that of Lieber and De Carli [27]) was subsequently shown to result in hepatic damage when chronically administered to female rats [28]. Testicular atrophy observed in their study may have been due to not only the direct effect of ethanol, but also to changes secondary to liver injury. The diet used in the present study produced no detectable hepatic dysfunction.

Klassen and Persaud [26] also observed testicular degeneration in adult rats chronically exposed to ethanol. However, the amount of ethanol in the liquid diet was far higher (58 per cent of total calories) than that used by Van Thiel *et al.* (36 per cent) or in the present study (28 per cent). Moreover, damage to the kidneys, spleen and liver, in addition to severe weight loss, was also apparent. Many of the histopathological findings associated with the testes could have been due to malnutrition and a subsequently poor general physical condition of the animals. The observations made in the present study provide more supportive evidence for the adverse effect of chronic consumption of relatively low levels of ethanol, *per se*, on testicular function.

Previous work from our laboratory indicated that male mice chronically exposed to ethanol subsequently sired litters with significantly fewer offspring per litter when compared to pair-fed controls [11]. These findings could have been due to subfertile matings and/or maternal resorption of dead or weakened fetuses. Subfertile matings would suggest spermatozoal alterations which adversely affect their ability to fertilize. However, spermatozoa from ethanol-treated and control males were equally effective in fertilizing ova *in vitro* (Table 5). Our observations concerning testicular dysmorphology (e.g. desquamation of immature germ cells into the lumina of the seminiferous tubules) indicate that longer exposure to low levels of ethanol may ultimately lead to subfertility and/or sterility. Confirmation of these speculations, however, is dependent upon further experimentation.

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