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DELAYED PUBERTAL DEVELOPMENT OF THE MALE REPRODUCTIVE TRACT ASSOCIATED WITH CHRONIC ETHANOL INGESTION

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Abstract—Little is known concerning the sensitivity of the reproductive tract to ethanol as a function of development. The present study was conducted to evaluate the action of chronic ethanol ingestion on sexual maturation of the male. Mice were given free access to liquid diets containing 5% (v/v) ethanol for either 29 or 43 days, starting at age 20 days. Controls were given liquid diets in which isocaloric sucrose replaced the ethanol. Daily diet consumption and peak blood ethanol levels were highest during the first 2 weeks of treatment, dropping thereafter to adult levels of approximately 680 ml/kg body weight and 160 mg/dl respectively. Plasma testosterone levels were depressed by ethanol throughout treatment, the reduction being somewhat greater when measured during week 6 of treatment (average = 74% inhibition) as compared to either week 2 (36%) or week 4 (25%). Average weights of testes, epididymides and seminal vesicles were depressed by 24% ($P < 0.002$), 16% ($P < 0.005$) and 13% (NS), respectively, after 29 days. Testicular development was also impaired in ethanol-treated animals after 29 days. Tunica albuginea thickness and seminiferous tubule diameter were decreased (by 31%, $P < 0.05$; and 16%, $P < 0.01$ respectively), whereas desquamation of immature germ cells and inactive tubules were increased (325 and 780% respectively; $P < 0.01$). Quality of spermatogenesis was poorer in ethanol-treated animals ($P < 0.05$). Also observed were decreased sperm motility (62% inhibition, $P < 0.01$) and capacity to fertilize (decreased by 67%, $P < 0.01$), and an increase in the incidence of morphologically abnormal spermatozoa (by 163%, $P < 0.001$). Semen volume was lower (reduced by 57%, $P = 0.05$), as was the total number of motile ejaculated spermatozoa (reduced by 81%, $P < 0.05$). After 43 days treatment, improvement was noted in all indices of fertility except for the number of motile ejaculated spermatozoa. Significant differences persisted only for dysmorphic spermatozoa and volume and sperm count of electroejaculated semen. These data suggest that ethanol ingestion during pubertal development can delay several aspects of sexual maturation in the male.

The effects of ethanol on various aspects of reproductive physiology are substantial. Chronic alcohol abuse by males has been correlated with hypogonadism, feminization, sexual impotence and altered reproductive hormonal homeostasis (for reviews, see Refs. 1–4). Similarly, women alcoholics have been afflicted with amenorrhea and infertility, and have reported stressful labor during childbirth [2, 5]. Further, the teratogenicity of ethanol is well-documented. The fetal alcohol syndrome, a characteristic pattern of craniofacial, growth and urogenital anomalies accompanied by mental retardation, in addition to a number of other "fetal alcohol effects", has been described in children born to mothers (usually alcoholic) who drank heavily during pregnancy (for reviews, see Refs. 6–8). Evidence (although controversial in nature) has also been gathered which suggests that ethanol may be mutagenic when chronically ingested by the male, at least under certain conditions [9–11]. Animal studies have further shown that ethanol inhibits both *in vitro* [12] and *in vivo* [13] fertilization, possibly by inhibiting essential sperm processes which precede sperm penetration of the oocyte, such as capacitation or the acrosome reaction [12, 14].

To date, most studies of ethanol-induced infertility have been conducted with males, both clinically and in laboratory animals. Many of these investigations have focused on the acute and chronic effects of ethanol on hormonal status, in an attempt to elucidate the etiology of ethanol-associated male reproductive disorders. Much information has been gathered concerning *in vivo* and *in vitro* effects of ethanol on the male reproductive endocrine system. Among the reported effects are reduced luteinizing hormone and testosterone [15–18], increased prolactin [19] and androstenedione [20, 21], decreased activities of steroidogenic enzymes, including 17β-hydroxysteroid dehydrogenase [21] and 5α-reductase [20], and decreased numbers of testicular gonadotropin receptors [22, 23]. Alcohol exerts direct

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effects at all levels of the hypothalamic–pituitary–gonadal axis.

Risk factors associated with alcohol-related male reproductive pathology, such as duration and quantity of alcohol consumed, age of consumption and/or period of development (e.g. adolescence), have not been identified in clinical studies. Inadequate data collection and faulty patient recollection are probable factors for the lack of information in this area. The conduct of controlled experiments with laboratory animals may be a more plausible approach to the identification of risk factors associated with alcohol-related reproductive impairment. Such studies in adult males have demonstrated that the extent of male reproductive tract pathophysiology is dependent upon both the level and the duration of ethanol exposure [18, 24], and that ethanol may be exerting these effects by direct action at the level of the testes [25–27], as well as the hypothalamic–pituitary axis [28–30].

However, periods of development which may differ with regard to sensitivity to the effects of ethanol on male reproductive capacity have not been identified. Such information would be of particular importance when alcohol use by adolescent males is considered. A 1978 survey [31] indicated that nearly 80% of high-school-age youths drink alcohol; 31% were classified as heavy users, and nearly one-half of these individuals had begun drinking at age 13.

A previous investigation [32] indicated that chronic ethanol ingestion by sexually immature rats delays at least certain aspects of sexual maturation (i.e. balanopreputial separation) in these animals. Also observed were elevated basal follicle stimulating hormone (FSH) levels and an exaggerated FSH response to castration, suggesting immaturity of both hypothalamus–pituitary and testes.

Many animal studies that examined the effect of chronic ethanol ingestion on male reproductive function have been conducted with sexually immature (preadolescent) animals [33–35]. Ethanol treatment periods included, but were not restricted to, pubertal development. Moreover, such studies examined reproductive function after only a single duration of treatment. Under these conditions, it has been difficult to separate observed hormonal or reproductive tract changes due to direct effects of ethanol on pre-existing reproductive potential, from those alterations in the biochemical and physiological events associated with the process of sexual maturation. It was the purpose of the present study to address this question by evaluating several indices of reproductive tract integrity in mice that had been treated with ethanol throughout puberty. The results strongly suggest that chronic ethanol ingestion delays processes related to sexual maturation.

METHODS

Chemicals

Ethanol (95%, v/v, USP) was obtained from the institutional central supply facility at the University of Illinois Health Sciences Center. Testosterone standards [1α,2α-^{3}H(N)]testosterone (45 Ci/mmol) and rabbit testosterone antiserum were products of New England Nuclear (Boston, MA). Dextran T-70 was purchased from Pharmacia, Inc. (Piscataway, NJ). Activated charcoal (untreated powder), thiourea, fructose, *p*-nitrophenylphosphate, sorbitol dehydrogenase (EC 1.1.1.14; from sheep liver), nicotinamide adenine dinucleotide (reduced form; NADH), human chorionic gonadotropin (hCG) and bovine serum albumin (BSA, fraction V) were from the Sigma Chemical Co. (St. Louis, MO). Hematoxylin and eosin Y dyes were from Chroma-Grubler Biological Products, distributed by the Robez Surgical Instrument Co., Inc. (Washington, DC). Lacmoid dye was a product of Matheson, Coleman & Bell (Norwood, OH). Pregnant mare's serum (PMS; Gestyl) was purchased from Diosynth, Inc. (Chicago, IL). Mucolexx was obtained from Lerner Laboratories (Stamford, CT). Chocolate-flavored Carnation Slender was purchased from a local grocer. Vitamin supplement (Catalog No. 904654) was a product of ICN Nutritional Biochemicals (Cleveland, OH). All other chemicals were of the highest quality commercially available.

Animals

Sexually mature male C57B1/6J mice (age 60 days; 28–30 g), obtained from Jackson Laboratories (Bar Harbor, ME), were mated with female Swiss–Webster mice (28–30 g), purchased from Harlan Sprague–Dawley (Madison, WI). Pregnant mice were individually housed in an institutional facility ($22 \pm 1°$, 14/10 hr light/dark cycle; lights on at 5:00 a.m.). Progeny were weaned and sexed at 18 days of age. Males were housed individually. On the afternoon of the same day, all males were given free access to a liquid diet (chocolate-flavored Carnation Slender), containing 3 g/liter vitamin supplement and sucrose (68.8 g/l) in an amount isocaloric to that of 5% (v/v) ethanol. Detailed composition of the diet has been presented elsewhere [18].

Experimental design

At age 20 days, animals were divided into ethanol-treated and control groups. Littermates were divided between the two groups. The ethanol-treated group was given free access to a diet that contained 5% (v/v) ethanol (28% of the total caloric intake was derived from ethanol); the control group was given diet in which an isocaloric amount of sucrose replaced the ethanol. Diet consumption of the control animals was restricted to the average diet consumption of the ethanol-treated group on the previous day.

Animals were maintained on their respective diets for either 29 or 43 days. The shorter treatment period included early pubertal development and the nominal age at which sexual maturity is attained [36, 37], whereas the longer treatment period included late pubertal development. During treatment, diet consumption was measured daily. Growth was estimated by measuring body weight daily. Animals whose body weights were less than 80% of the average body weight for the control group were removed from the study and were not included in the tabulation of data. This procedure minimized possible nutritional contributions to the observed data. Blood ethanol and plasma testosterone profiles of randomly selected animals (five to seven per time point) were

determined on days 10/11, 24/25 and 38/39. Blood for both assays was not derived from the same animal, due to the small size of the animals and the relatively large volume of blood required for both assays (125 μl; see below). Blood was collected from each animal no more than twice for each assay within a 6-hr period on any given day.

On the morning of either day 30 or day 44, animals were removed from their diets and given free access to laboratory chow and water. Twenty-four hours after withdrawal, semen samples from randomly selected animals (seven per group) were obtained by electroejaculation (see below). Twenty-four hours after electroejaculation, animals were killed by cervical dislocation, and the right halves of their reproductive tracts were removed. The testes, epididymides and seminal vesicles from seven randomly selected animals per group were removed and were weighed.

Testes were immersion-fixed in Bouin's fluid, while caudae epididymides were placed into 1.0 ml of *in vitro* sperm capacitation medium (see below). Small cuts were made in the epididymides, and spermatozoa were allowed to disperse into the medium for 10 min, after which epididymal tissue was removed. Aliquots of the sperm suspension were taken for determination of count, motility and morphology as described previously [24]. The remainder of the sperm suspension was capacitated for 60 min, followed by insemination of sperm aliquots into culture dishes containing mouse oocytes (see below).

Electroejaculation and semen analysis

Semen was obtained by electroejaculation with a bipolar rectal probe as described by Anderson *et al.* [38]. Coagulae produced from the procedure were weighed, and the fluid portion of the ejaculates was analyzed for volume, sperm count, sperm motility, fructose concentration and acid phosphatase content.

Ejaculate volume was estimated from the fractional length of a 25-μl capillary pipette occupied by the fluid portion of the collected semen. Spermatozoal content was estimated with a Neubauer hemacytometer after diluting the semen with 9 vol. of buffer, consisting of 0.12 M NaCl, 4.3 mM KCl, 1.2 mM $MgSO_4$, 2.0 mM glucose and 16 mM (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.4. Percent motility was determined by direct microscopic evaluation. The number of motile sperm per ejaculate was derived from the sperm count, percent motility and ejaculate volume. All semen analyses were performed within 10 min of semen collection.

Testicular histology

After overnight fixation in Bouin's fluid, testes were dehydrated and embedded in paraffin (m.p. 56°). Sections (5 μm) were deparaffinized, rehydrated, and stained and counterstained with Lillie–Mayer hematoxylin and eosin Y respectively [39].

Morphometric and other histological analyses were performed with brightfield microscopy. Linear measurements were made with a calibrated eyepiece micrometer (accurate to the nearest μm); these included tunica albuginea thickness (measured at seven sites per testis) and seminiferous tubule diameter (ten tubules per testis were measured). Average tunica albuginea thickness and seminiferous tubule diameter for each animal were used for subsequent data analysis. Germ cell desquamation was estimated from the frequency of seminiferous tubule lumina (300 examined per testis) that contained immature germ cells (mainly primary spermatocytes). The frequency of inactive tubules (i.e. those lined with only spermatogonia and Sertoli cells) and the quality of spermatogenesis (rated on a scale of 1–5, 1 representing complete spermatogenesis; see Ref. 18) were evaluated in 300 tubules per testis.

Evaluation of epididymal spermatozoa

Epididymal (cauda) spermatozoa content and motility were estimated as described for ejaculated spermatozoa (see above). Spermatozoa were also evaluated for their ability to fertilize mouse oocytes, as previously described [17]. Briefly, cumulus masses containing oocytes were harvested from the ampullae of ovaries from mice superovulated by injection of 10 IU PMS, followed 48 hr later by 10 IU hCG. After a 60-min incubation in capacitation medium (modified Krebs–Ringer bicarbonate, containing 20 mg/ml BSA), 0.1 ml epididymal spermatozoa (5×10^5) were inseminated into culture dishes containing the cumulus masses in 0.1 ml *in vitro* fertilization medium (modified Krebs–Ringer bicarbonate, containing 3 mg/ml BSA). Incubations were carried out under silicone oil in an atmosphere of 5% O_2, 5% CO_2, 90% N_2, at 37° for 24 hr. Cells were mounted and stained with acetolacmoid and examined with phase contrast optics for signs of fertilization (2-celled stage of development or, rarely, the presence of male pronucleus). Results were expressed as the percentage of oocytes that were fertilized. Fragmented oocytes (<10%) were not included in the evaluation).

A portion of the epididymal sperm suspension was fixed in Mucolexx (formaldehyde base) and smeared onto microscope slides. The samples were air-dried, stained with Lillie–Mayer hematoxylin, dehydrated with ethanol, cleared in xylene, and covered with a liquid coverslip, as previously described [24]. Spermatozoa were examined with an oil immersion objective (97×) and scored for morphological anomalies, including those of the head and tail, and for the presence of sperm precursors (mainly primary spermatocytes and spermatids), cytoplasmic droplets and decapitated spermatozoa. Two hundred spermatozoa from each animal were examined. Results were expressed as the percentage of spermatozoa examined with one or more of the above anomalies.

Analytical methods

Blood ethanol measurement. Blood (25 μl) was collected from the tail vein into heparinized capillary pipettes and immediately expelled into 25-ml flasks containing 1 ml of 0.1 N HCl and 25 mM thiourea [27] and stoppered. Samples were quantified by gas chromatographic analysis of 2.0-ml aliquots of the headspace of the flasks, as previously described [27]. Although blood ethanol was measured within 4 hr of blood collection, samples were stable at 4°, and could be stored for as long as 4 days without evidence of

nonenzymic ethanol oxidation [40]. Blood ethanol levels were quantified from standard curves of ethanol concentration versus detector response (peak height) and were expressed as mg/dl blood. Intra- and interassay variabilities were 3.2 and 6.2% respectively. Recovery of ethanol standards added to mouse blood was 98% (range = 97–104%; N = 5).

Measurement of plasma testosterone. Testosterone in plasma (approximately 50 μl) derived from 100 μl of blood obtained from the tail vein was determined by radioimmunoassay, as described previously [17]. Recovery of testosterone standards added to plasma was in excess of 90% (range = 91.4–106%; N = 7). The sensitivity of the assay was 15 pg/assay. Intra- and interassay variabilities were 7.5 and 9.0% respectively.

Fructose determination. The fructose concentration of the diluted semen sample was determined from the initial rate analysis of the decrease in absorbance at 340 nm which occurred in the presence of sorbitol dehydrogenase and NADH; this was compared with that obtained with fructose standards [41]. Blank reactions contained only the diluant buffer in place of the semen. Glucose did not interfere with the assay at the concentration used in the buffer [41].

Determination of acid phosphatase. Acid phosphatase was measured by a standard clinical procedure [42], with 7 mM *p*-nitrophenylphosphate as substrate at pH 5.0. After 30 min of incubation at 37°, the rate of release of *p*-nitrophenol was estimated spectrophotometrically at 410 nm after adjusting the pH of the reaction medium to 9.0. Blank reactions contained semen samples that had been placed prior to assay in a boiling water bath for 7 min. Results were quantified by using a molar absorptivity of 18,315 for *p*-nitrophenol at 410 nm. Enzyme activity was expressed as mIU acid phosphatase (nmoles *p*-nitrophenol released/min) per ml semen.

Statistical analyses. Body weights and diet consumption were compared between ethanol-treated and control groups by means of the unpaired *t*-test. Association between plasma testosterone and blood ethanol levels was evaluated with Pearson's product-moment correlation coefficient [43]. Ejaculate sperm count, total motile sperm and epididymal sperm content were subjected to logarithmic transformation, whereas data concerning *in vitro* fertilization, frequency of germ cell desquamation and inactive tubules were subjected to arcsin transformation prior to parametric analyses [43]. Results were reported as the back-transform of the average transforms, together with the 90% confidence limits. All other data were reported as averages ± standard errors of the mean, unless otherwise indicated. Data were analyzed by two-way analysis of variance and by the Newman–Keuls multiple range test [44], with the exception of the quality of spermatogenesis, which was evaluated with the nonparametric Mann–Whitney U test [43], and sperm dysmorphology, which was evaluated by Chi-square analysis. Differences were considered significant at the 0.05 level of confidence.

RESULTS

Diet consumption was variable during the first 2 weeks of treatment. Average daily ethanol ingestion rose from 39 ± 6 g/kg body weight on treatment day 1 to a peak of 44 ± 7 g/kg on day 11. Thereafter, consumption decreased and remained rather constant at approximately 25–30 g/kg for the duration of the study. Average peak blood ethanol levels appeared to vary as a function of average diet (ethanol) consumption (Table 1) on the 3 days for which blood ethanol levels were available ($r = 0.841$, df = 19; $P < 0.01$).

Three animals (two ethanol-treated, one control; 6% of the total) were removed from the study during the first week of treatment, due to low diet consumption with concomitant reduction in growth. For the remaining animals, body weight gain was comparable for both ethanol-treated and control groups. Average body weight increased by approximately 100% in both groups subsequent to 29 days of treatment. Animals maintained on liquid diets for an additional 2 weeks exhibited further increases in body weight of approximately 20% for both groups ($P > 0.1$, two-tailed *t*-test). Hematocrits determined from all animals at the end of 29 days of treatment revealed no effect of ethanol treatment (0.44 ± 0.03 and 0.44 ± 0.04 for ethanol-treated and control groups respectively; N = 14 for each group).

Except for one time point (day 10/11, 8:30 p.m.), average testosterone levels were lower in ethanol-treated as compared to control animals at all times for which measurements were made (Figs. 1–3). Significant reductions were seen twice on day 10/11 when ethanol-treated animals were compared with their respective controls at each time point. When testosterone levels on day 24/25 were similarly compared, one significant difference between ethanol-treated and control groups was noted (Fig. 2). On the other hand, two-way analysis of variance revealed an ethanol effect on plasma testosterone for each of the 3 days during which values were obtained, when all time points within a given day were considered ($F^1_{40} = 13.39$, $F^1_{37} = 147.1$ and $F^1_{34} = 62.8$; $P < 0.001$, for days 10/11, 24/25 and 38/39 respectively). Testosterone levels of ethanol-treated animals were depressed ($P < 0.05$) relative to those of the control group at all time points on day 38/39 of treatment (Fig. 3).

Interestingly, no correlation was seen between plasma testosterone and blood ethanol levels. Correlation coefficients for average plasma testosterone and average blood ethanol were −0.86, 0.017, and 0.31 on days 10/11, 24/25 and 38/39 respectively (df = 3 for days 10/11 and 24/25, df = 2 for day 38/39; $P > 0.1$ for each day). Correlations between percent inhibition of plasma testosterone (ethanol-treated vs controls at each time point) and blood ethanol levels were similarly poor (0.62, −0.77 and −0.59 for days 10/11, 24/25 and 38/39 respectively; $P > 0.1$). The lack of association between blood ethanol and inhibition of plasma testosterone levels in ethanol-treated as compared to control animals was even more apparent when data from all three sampling days were pooled ($r = 0.095$, df = 12; $P > 0.1$).

Table 1. Ethanol consumption, peak blood ethanol levels and body weight gain during diet acquisition*

Treatment day	Ethanol consumption (g/kg body wt)	Peak blood ethanol concentration† (mg/dl)	Body weight increase‡ (g)	
			Pair-fed control	Ethanol-treated
10	43 ± 2 (28)	343 ± 70	3.4 ± 0.9 (19)	2.7 ± 0.7 (28)
24	25 ± 2 (28)	119 ± 39	9.2 ± 1.7 (19)	8.6 ± 1.8 (28)
30			10 ± 1 (19)	11 ± 3 (28)
38	29 ± 1 (14)	193 ± 32	13 ± 1 (10)	12 ± 2 (14)
44			14 ± 1 (10)	14 ± 3 (14)

* At age 20 days, animals were given either free access to liquid diets containing 5% (v/v) ethanol (ethanol-treated) or were pair-fed liquid diets in which isocaloric sucrose replaced the ethanol (control). Values represent the average ± standard deviation, with the number of determinations indicated in parentheses.

† Blood was drawn from the tail veins of seven randomly selected animals on each of the indicated treatment days for blood ethanol determinations (see Methods).

‡ Body weights on day 1 (age 20 days) were 10.0 ± 1.0 and 9.7 ± 1.0 g for the control and ethanol-treated groups respectively.

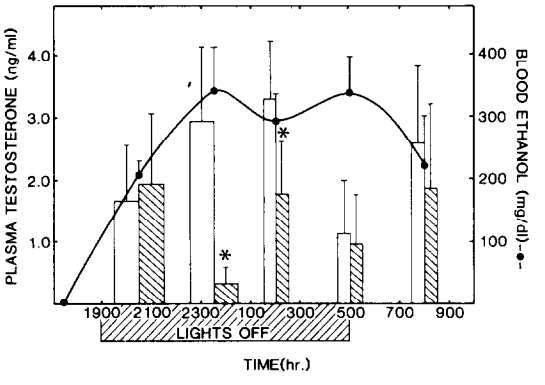

Fig. 1. Blood ethanol and plasma testosterone profiles during day 10/11 of treatment. Ethanol and testosterone were determined from samples collected at the indicated time points. Open bars represent testosterone levels in the control group; hatched bars represent testosterone levels in the ethanol-treated group. Blood ethanol levels are indicated by the closed line. Values are reported as averages ± standard deviation of five to seven determinations per time point. Asterisks at individual time points indicate significant ($P < 0.05$, Newman–Keuls multiple range test) differences between the ethanol-treated and the respective control groups. Two-way analysis of variance over all time points indicated a significant ethanol effect on plasma testosterone ($F^1_{48} = 13.39$; $P < 0.001$), an effect of time of day ($F^3_{48} = 3.26$; $P < 0.05$), and treatment/time interaction ($F^3_{48} = 5.21$; $P < 0.005$).

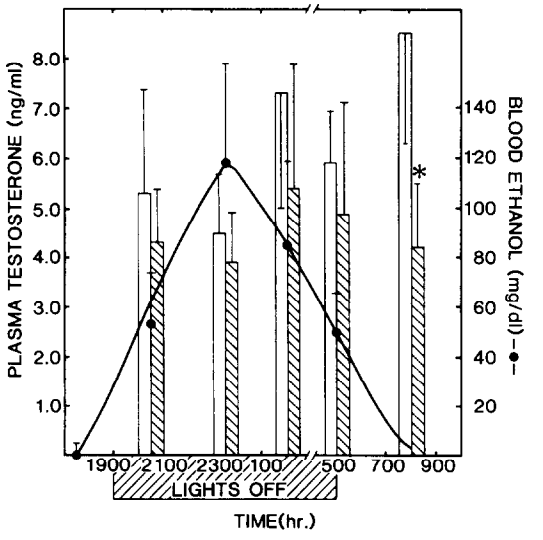

Fig. 2. Blood ethanol and plasma testosterone profiles during day 24/25 of treatment. Details are given in the legend to Fig. 1. Note scale changes in ordinates as compared to those in Fig. 1. Two-way analysis of variance over all time points indicated a significant ethanol effect on plasma testosterone ($F^1_{45} = 16.83$; $P < 0.001$), in addition to a significant time of day effect ($F^4_{45} = 3.16$; $P < 0.05$).

Animals treated with ethanol for 29 days had decreased reproductive organ weights as compared to those from controls (Table 2). Testicular weights and epididymal weights were reduced by 24 and 16% respectively ($P < 0.01$). The average seminal vesicular weight appeared to be reduced (14%) as compared to the control group, although the reduction was not significant. On the other hand, reproductive organ weights were not depressed in ethanol-fed animals as compared to their controls after 43 days of treatment (Table 2).

Substantial testicular impairment was noted following 29 days of ethanol ingestion (Table 3). The average thickness of the tunica albuginea and the average seminiferous tubule diameter were less by 31% ($P < 0.05$) and 16% ($P < 0.01$), respectively, in the ethanol-treated as compared to the control group. Increases were also noted in the frequencies of germ cell desquamation and inactive tubules. Desquamation was increased by 325% ($P < 0.01$), whereas there was nearly a 9-fold increase ($P < 0.01$) in the frequency of inactive tubules in the animals that received ethanol from ages 20 to 49 days. Additionally, the quality of spermatogenesis was poorer ($P < 0.05$) in testes from animals given ethanol for 29 days.

In contrast, no differences were observed in any of the above variables in testes from animals maintained on the ethanol diet for an additional 14 days

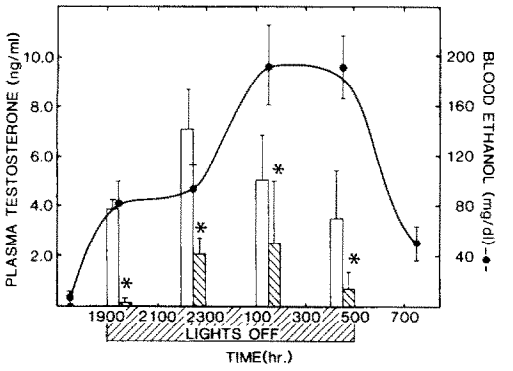

Fig. 3. Blood ethanol and plasma testosterone profiles during day 38/39 of treatment. Details are given in the legend to Fig. 1. Values are reported as averages ± standard deviations of five to six determinations per time point. Note scale changes in ordinates. Two-way analysis of variance over all time points indicated a significant ethanol effect on plasma testosterone ($F^1_{34} = 62.85$; $P < 0.001$), and a significant effect of time of day ($F^3_{34} = 10.16$; $P < 0.001$).

Table 2. Delayed reproductive organ growth due to chronic ethanol ingestion by adolescent mice*

	Weight (mg)		
Treatment	Testis	Epididymis	Seminal vesicles
29 Days			
Control	74 ± 2	26.4 ± 0.8	108 ± 8
Experimental	56 ± 4†	22.3 ± 0.8†	93 ± 6
43 Days			
Control	76 ± 7	26.7 ± 1.2‡	124 ± 6‡
Experimental	69 ± 3	27.9 ± 1.2	158 ± 10†

* Forty-eight hours after the indicated treatment periods, animals were killed, and their reproductive organs were removed and weighed. Values represent the average ± SEM of the right organ from each of seven animals per group.

† Differs from control group ($P < 0.01$, Newman–Keuls multiple range test).

‡ Two-way analysis of variance indicated a significant effect of age on weights of epididymis ($F^1_{24} = 7.01$; $P < 0.025$) and seminal vesicles ($F^1_{24} = 29.19$; $P < 0.001$).

(Table 3). Values were at or above control levels for tunica albuginea thickness, seminiferous tubule diameter and quality of spermatogenesis. While the frequencies of germ cell desquamation and inactive tubules appeared to be somewhat elevated relative to those of the control group, these differences were not significant. Improvement was noted over time for both of these indices of spermatogenesis in the control group (compare values after 29 days and 43 days; Table 3).

Similar findings were made with regard to cauda epididymal spermatozoa (Table 4). Although average sperm content in the ethanol-treated group was depressed by 50% as compared to their controls after 29 days, the difference was not significant. On the other hand, motility and fertilizing capacity of spermatozoa from animals given ethanol for 29 days were less by 62% ($P < 0.01$) and 67% ($P < 0.01$) respectively. However, these differences were no longer evident after an additional 2 weeks treatment with the same diets. While sperm content, motility and fertilizing capacity were depressed slightly (21, 14 and 5% respectively), these values were not significantly lower than those of the control group ($P > 0.1$). As with testicular morphology, maturational changes appeared to occur in the control group between 52 days and 66 days of age, with regard to sperm content, motility and fertilizing capacity.

After 29 days treatment, a higher fraction of epididymal spermatozoa from ethanol-treated animals had one or more morphological anomalies as compared to their controls (163% higher incidence; $P < 0.001$). Significant increases in the number of head and tail anomalies, as well as increased incidence of decapitation, cytoplasmic droplets and sperm precursors were present after 29 days treatment (Table 5).

After 43 days treatment, the frequency of spermatozoa with one or more abnormalities was lower, although it remained increased by 31% ($P < 0.001$) as compared to the control group. On the other hand, anomalies that were not greater than those of the control group included the incidence of sperm precursors, cytoplasmic droplets (a measure of sperm maturation; [45]) and decapitated spermatozoa (Table 5). The percentage of abnormal spermatozoa remained constant in control animals from ages 52 to 65 days.

The primary seminal (ejaculate) deficiency resulting from ethanol ingestion was that of volume (Table 6). Significant ($P = 0.05$) reduction (57%) of semen volume in the ethanol-treated as compared to the control group was seen after 29 days treatment. Small decreases in sperm count, percent motility and acid phosphatase concentration were seen after 29 days, although these apparent differences were not significant. Total motile sperm, on the other hand, derived from semen volume, sperm count and percent motility, was depressed ($P < 0.05$) by 81%, from an average of 3.4×10^3 for the control group to 0.6×10^3 for ethanol-treated animals. Coagulum weights, percent motility and fructose concentrations appeared to remain unchanged, whereas acid phosphatase may have increased somewhat. Interestingly, sperm count was depressed significantly in ethanol-treated animals after 43 days (75% reduction, $P < 0.01$). Additionally, total motile ejaculated spermatozoa remained depressed by 90% ($P < 0.01$) in ethanol-treated versus control animals.

DISCUSSION

Few studies have been directed toward the evaluation of biochemical or physiological effects of ethanol, administered either acutely or chronically, during male adolescent development. With regard to reproductive status, only two other studies, to the authors' knowledge, have purposely utilized adolescent male animals [32, 46]. These studies, however, provided little information concerning the amount of ethanol ingested and/or the blood ethanol profiles that were associated with the observed

Table 3. Delayed testicular development in ethanol-treated pubertal mice*

Treatment	Tunica albuginea thickness (μM)	Seminiferous tubule diameter (μM)	Germ cell desquamation (%)	Inactive tubules (%)	Spermatogenesis (1–5)
29 Days					
Control	13.4 ± 2.4	193.0 ± 2.1	3.1 (1.1–6.0)	0.5 (0.1–1.2)	1.0
Ethanol-fed	8.6 ± 1.0†	161.8 ± 4.2‡	15.9‡ (8.4–25.1)	4.4‡ (1.3–9.3)	1.7§
43 Days					
Control	12.0 ± 1.0	184.0 ± 4.4	0.6‖ (0.2–1.4)	0.3‖ (0.05–0.7)	1.0
Ethanol-fed	14.0 ± 0.7	173.4 ± 7.4	2.2 (0.8–4.2)	0.6 (0.1–1.4)	1.0

* After either 29 or 43 days treatment, animals were removed from their respective diets and given free access to laboratory chow and water. Forty-eight hours thereafter, animals were killed, and the right testes were removed, fixed, and prepared for microscopic examination (see Methods). Values were derived from six animals per treatment group. Linear measurements were reported as average ± standard error of the mean. Data concerned with germ cell desquamation and inactive tubules were subjected to arcsin transformation prior to statistical analysis and were expressed as the back-transformation of the average transform, with 90% confidence limits indicated in parentheses. Spermatogenesis was reported as the average score (1 representing complete spermatogenesis).

† Value differs from that of respective control group ($P < 0.05$, Newman–Keuls multiple range test).

‡ Value differs from that of respective control group ($P < 0.01$, Newman–Keuls multiple range test).

§ Value differs from that of respective control group ($P < 0.05$, Mann–Whitney U-test).

‖ Two-way analysis of variance indicated a significant age effect ($F^1_{20} = 22.89$, $P < 0.001$, and $F^1_{20} = 7.30$, $P < 0.025$, for germ cell desquamation and inactive tubules respectively).

Table 4. Epididymal spermatozoa content and viability after ethanol ingestion by sexually immature mice*

Treatment	Spermatozoal content ($\times 10^{-5}$)	Spermatozoal motility (%)	*In vitro* fertilizing capacity (% fertilization)
29 Days			
Control	33.6 (14.3–78.6)	45 (32–57)	64 (56–70)
Ethanol-fed	13.8 (4.5–42.1)	17† (13–21)	18† (6.2–35)
43 Days			
Control	95.8‡ (53.2–172.2)	43 (39–47)	74‡ (66–82)
Ethanol-fed	91.1 (77.8–106.9)	37 (31–43)	71 (58–82)

* Forty-eight hours after the indicated treatments, sperm content, motility and fertilizing capacity were measured in spermatozoa recovered from the right cauda epididymis from each of seven animals per treatment group, as described in Methods. Data regarding sperm content were subjected to logarithmic transformation, whereas data regarding sperm motility and *in vitro* fertilization were subjected to arcsin transformation prior to further analysis. Values are expressed as averages, with the 90% confidence limits indicated in parentheses.

† Value differs from that of respective control ($P < 0.01$, Newman–Keuls multiple range test).

‡ Two-way analysis of variance indicated a significant age effect ($F^1_{24} = 13.86$ and 25.32 for sperm content and fertilizing capacity respectively; $P < 0.025$).

changes. Moreover, in one of the studies [46], animals experienced substantial reduction in body weight gain (37%) as compared to control animals during week 2 of treatment. Nutritional factors therefore, cannot be excluded in their studies. Neither of the above studies was directed toward examination of the reproductive tract.

In the present study, treatment was carried out for sufficient duration so as to include the age of onset of sexual maturity (29 days treatment) in the mouse [36, 37], as well as late pubertal development, during which time male mice become fully reproductively competent (43 days treatment). The longer treatment period was based upon *in vivo* mating experiments, from which it was determined that successful matings occurred consistently only with males whose ages were at least 60 days.*

* R. A. Anderson and B. R. Willis, unpublished observations.

Table 5. Increased spermatozoal dysmorphology associated with chronic ethanol ingestion during pubertal development*

Treatment	Frequency of anomaly (%)					
	Head	Tail	Decapitated	Cytoplasmic droplet	Sperm precursors	Abnormal spermatozoa (1 or more anomalies)
29 Days						
Control	5.9	8.8	6.8	0.3	0.4	19.0
Ethanol-fed	25.9†	18.2†	9.1‡	2.4†	5.4†	50.3†
43 Days						
Control	7.0	6.0	6.5	0.5	0.9	18.0
Ethanol-fed	11.6†	8.1‡	5.6	0.6	1.5	23.0†

* Forty-eight hours after the indicated treatment, spermatozoa were recovered from the right cauda epididymis from each of seven animals per treatment group. Sperm were fixed, stained and examined for morphological anomalies, as described in Methods. Values were calculated based upon examination of 1400 spermatozoa per treatment group.

† Value differs from that of respective control group ($P < 0.001$, χ^2 analysis).

‡ Value differs from that of respective control group ($P < 0.05$, χ^2 analysis).

Table 6. Semen analysis of mice subsequent to chronic ethanol ingestion*

Treatment	Volume (μl)	Coagulum weight (mg)	Sperm count ($\times 10^{-5}$/ml)	Motility (%)	Fructose (mM)	Acid phosphatase (mIU/ml)
29 Days						
Control	7.0 ± 1.7	8.0 ± 0.8	15.7 (6.0–41.6)	31 (16–49)	17.0 ± 3.8	60.2 ± 7.3
Ethanol-fed	3.0 ± 0.7†	9.9 ± 1.5	10.6 (4.6–24.6)	20 (8–35)	25.1 ± 6.2	44.0 ± 8.4
43 Days						
Control	17.0 ± 2.1§	11.1 ± 2.0	71.1§ (49.9–101.4)	22 (8–39)	25.0 ± 6.3	61.4 ± 5.2
Ethanol-fed	8.0 ± 1.9‡	7.1 ± 1.1	17.9‡ (6.5–48.9)	19 (7–36)	19.3 ± 3.2	77.0 ± 13.6

* Twenty-four hours after removal of their respective diets, semen was collected from all animals by electroejaculation, as described in Methods. Data regarding semen volume, coagulum weight, seminal fructose and acid phosphatase were expressed as average ± standard error of seven determinations per group. Data regarding sperm content and motility were subjected to logarithmic and arcsin transformations, respectively, prior to statistical analyses; averages are presented, with 90% confidence limits indicated in parentheses.

† Value differs from that of respective control group ($P = 0.05$, Newman–Keuls multiple range test).

‡ Value differs from that of respective control group ($P < 0.01$, Newman–Keuls multiple range test).

§ Two-way analysis of variance indicated a significant age effect ($F^1_{24} = 19.38$, $P < 0.001$, and $F^1_{24} = 5.56$, $P < 0.05$, for volume and sperm count respectively).

In contrast to previous studies with adult animals [18], diet consumption (normalized for body weight) was not constant throughout the treatment period (Table 1). Diet (and, hence, ethanol) consumption was substantial during the first 2 weeks (ages 20–34 days), dropping thereafter to levels approximating those consumed by adults [18]. These findings were not unexpected, since increased diet ingestion occurred during a period of rapid growth and development [36].

The data strongly suggest that mice subjected to chronic ethanol treatment from ages 20 to 49 days have not achieved the reproductive competency of their age-matched controls. Developmental impairment was observed with regard to testicular histology (Table 3), spermatozoal motility (Table 4), spermatozoal morphology (Table 5), ability of spermatozoa to fertilize *in vitro*, and possibly sperm production (content; Table 4) and accessory sex gland secretions (Table 6), as well as lowered reproductive organ weights. Underlying factors for deficiencies observed after 29 days of ethanol treatment may include delayed sexual maturation, degenerative changes of preexisting reproductive function, or a combination of the two factors. Although the latter two possibilities cannot be entirely excluded on the basis of the present data, their significance is minimized by the apparent normalization of the various indices of reproductive function in animals maintained on the same ethanol treatment regimen for an additional 2 weeks. If the impairments in reproductive function observed after 29 days treatment were due to ethanol-induced degeneration of pre-existing reproductive potential, then one would

have expected an exacerbation of the impairment of reproductive function after 49 days treatment, rather than its normalization.

The exact extent to which delayed sexual maturation occurred is not possible to ascertain from the present study, since only two time points were evaluated. However, a separate study (unpublished), in which testicular weights were measured as a function of age, suggested that after 29 days ethanol-treated animals lagged behind their controls with respect to testicular growth by approximately 13 days.

The apparent failure of 43 days of ethanol treatment to further compromise reproductive function is not unexpected, since similar treatment of adult mice produced only subtle changes in testicular morphology [18], and had little or no effect upon sperm motility, morphology or ability to fertilize oocytes *in vitro* [24]. Longer treatment periods, however (i.e. 10 weeks), produced moderate pathological changes in these variables [18, 24].

The effects of ethanol on indices of reproductive function appeared to be relatively specific. Gross estimates of body growth and nutrition (i.e. body weights, hematocrits) were not affected by ethanol treatment (see text, Results), minimizing the contribution of possible nutritional deficiency [47] to the observed data.

As expected, plasma testosterone levels of control animals increased during the course of treatment (Figs. 1 and 2), which corresponded to the period of pubertal development in the mouse [36, 37]. Testosterone levels were depressed throughout ethanol ingestion, suggesting that testes (and/or the hypothalamic–pituitary axes) from sexually maturing animals may be qualitatively similar to those from the adult with regard to sensitivity to reduction of steroidogenesis by ethanol [25, 26, 48, 49]. However, also in agreement with previous studies with adult mice [17, 18], blood ethanol levels were poorly correlated with plasma testosterone. Since blood ethanol levels closely parallel the alcohol levels of the testes [50], these data indicate that ethanol may be exerting an indirect effect on *in vivo* steroidogenesis, possibly by interfering with gonadotropin-testicular receptor interaction [27, 51]. Previous studies in the mouse [27] indicated that consistent reduction in plasma testosterone by acute administration of ethanol is dependent upon prior administration of human chorionic gonadotropin, which has luteinizing hormone (LH)-like activity. Ethanol appears to have an active role in preventing a gonadotropin-induced rise in testosterone production. A dependence upon spontaneous gonadotropin stimulation for ethanol-induced testosterone depression, *in vivo*, could explain the poor correlation between blood ethanol and inhibition of plasma testosterone levels, and the greater inhibition relative to controls of plasma testosterone by ethanol on treatment day 38/39, as compared to effects seen in younger animals (Figs. 1–3). At age 58 days (day 38/39 of treatment), LH release and levels should be increased to very near adult levels [37]. The profiles of plasma testosterone observed throughout the treatment period argue against reduced plasma testosterone as a primary mechanism for the effects of ethanol seen after 29 days treatment in the present study, since the greatest reduction of testosterone (day 38/39; Fig. 3) occurred when androgen-responsive reproduction variables of ethanol-treated animals were approaching those of their controls. Moreover, minimal effects on testosterone were noted (Fig. 2) during a period in which substantial reproductive impairment was evident (ages 44–49 days).

Depressed testosterone may represent an acute, rather than a chronic, ethanol effect. Studies in the mouse showed that ethanol-induced depression of testosterone levels in adult males is readily reversible after similar treatment for as long as 10 weeks [18]. Plasma testosterone levels in ethanol-treated animals did not differ from those of their controls when measured 12 hr after ethanol withdrawal. The possible acute nature of depressed testosterone observed in the present study is supported by the significant increase in seminal vesicle weight seen in ethanol-treated animals subsequent to 43 days treatment (Table 1). Increased growth of the seminal vesicles during puberty is closely associated with increased androgen levels [52]. Normal or above-normal plasma testosterone levels during periods of low ethanol intake may be sufficient to support accessory gland growth, since the state of the seminal vesicles more closely reflects long-term androgen status, rather than transient fluctuations [53]. Due to the nocturnal feeding habit of mice, ethanol consumption between 9:00 a.m. and 5:00 p.m. is generally less than 20% of the total daily intake, resulting in blood ethanol levels ranging from nondetectable to less than 50 mg/dl [17]. Such low levels would not be expected to depress plasma testosterone, regardless of the exact mechanism by which ethanol may elicit this effect. In fact, low doses of ethanol, given acutely, result in elevated plasma testosterone [15, 17]. At present, these possibilities remain speculative. Measurement of plasma testosterone during periods of low ethanol consumption (e.g. during the lights-on period) will help to clarify this question.

Analysis of semen collected by electroejaculation yielded equivocal results, especially with regard to ejaculated spermatozoa (Table 6). In contrast to epididymal sperm content and motility (Table 4), sperm count and sperm motility of ejaculates were depressed only slightly after 29 days treatment; although motility remained unchanged after 43 days, sperm count was depressed at this time. The failure to detect a decrease in sperm count after 29 days may be explained, at least in part, by decreased ejaculate volume. The total number of spermatozoa recovered from ejaculates produced by ethanol-treated animals was reduced by 71% ($P < 0.05$), as compared to the control group after 29 days. On the other hand, the depressed sperm count after 43 days (together with reduced semen volume) suggests that, under certain conditions, epididymal spermatozoa content (and most likely, sperm quality) may not be reflected by ejaculated spermatozoa.

Such discrepancy may be at least partially explained by ethanol-induced changes in sperm distribution throughout the reproductive tract. These changes may concentrate spermatozoa within the

epididymis and reduce their emission during ejaculation. Administration of 5% ethanol diets for either 5 or 10 weeks to adult mice [24] elevates epididymal sperm content. This increase may be due to the effects of ethanol on norepinephrine release in the vas deferens [54], which may, in turn, affect motility of this part of the reproductive tract [55]. Sympatholytic agents have been shown to be similar to vasoligation, in terms of producing increases in the epididymal sperm concentration [56]. Alternatively, chronic ethanol ingestion may impair the ejaculatory reflex. Inhibition of the ejaculatory response by ethanol has been known for some time [57, 58]. Whether chronic ethanol ingestion during pubertal development has a similar effect is unknown; sexual impotence has been reported in chronic alcoholics [59]; additionally, alcoholics have produced azoospermic [60] or oligoospermic [61] semen samples. However, the precise role of an altered ejaculatory response could not be assessed from such studies. Decreased semen volume, together with apparently normal fructose and acid phosphatase concentration, support either the possibility of an impaired ejaculatory response or decreased secretory activities of the seminal vesicles and prostate glands respectively. On the other hand, the significant increase in seminal vesicular weights after 43 days treatment (Table 2) argues against decreased secretory activity of this organ. Further experimentation is required to clarify this question.

The precise mechanism(s) for ethanol-induced delayed sexual maturation is unknown. Much of the research concerning male sexual maturation has been conducted in the rat (for review, see Ref. 62). From these studies, at least some of the physiological and biochemical events that transform the male from its prepubertal to its adult state can be postulated. Pubertal development is associated with a transient increase in testicular 5α-reductase, which may act indirectly to increase the number of LH receptors and testicular 17β-hydroxysteroid dehydrogenase activity, which are observed to occur during sexual maturation, via increased follicle stimulating hormone (FSH) release [62]. This, in turn, results in increased levels of testosterone with a concomitant decrease in androstenedione. Decreased androstenedione levels permit the development of the GnRH self-priming effect (an effect in which a primary GnRH stimulus sensitizes the pituitary to subsequent GnRH stimuli), and lead to decreased sensitivity of LH release to negative feedback inhibition by testosterone (which permits increased LH levels in the face of rising testosterone). Additionally, in the immature male, testosterone augments the stimulatory effect of GnRH on LH release [63], which allows for further increases in testosterone levels. Due to substrate inhibition, rising testosterone levels lower 5α-reductase activity to adult values. This completes the putative sequence of changes leading to sexual maturation, resulting in adult levels of testosterone, LH and FSH, with a mature hypothalamic–pituitary–gonadal axis. Since the hormonal profile leading to the maturation of the mouse is similar to that of the rat [37], the above scheme may also apply to the mouse.

Studies conducted in adult males indicate that many events related to sexual maturation may be affected by ethanol. Chronic ethanol exposure diminishes hepatic 5α-reductase activity [20], may prevent the pubertal rise in activity of the testicular form of this enzyme [64], reduces the number of testicular gonadotropin receptors [65], exerts a direct effect on gonadotropin–testicular receptor interaction (at least acutely; see Refs. 27 and 51), decreases testicular 17β-hydroxysteroid dehydrogenase [21], increases androstenedione levels [21, 66], and reduces circulating LH and testosterone levels [15–18]. Whether any one or more of these effects are observed in the pubertal male remains to be determined.

The clinical consequences of delayed sexual maturation are substantial. Not only is reproductive performance affected, but also the development of secondary male characteristics (e.g. body hair pattern, voice, musculature, behavior). A delay in these maturational changes could be psychologically, as well as physiologically, deleterious, creating depression, which could potentiate the drinking behavior of the alcohol abuser. It is hoped that the present study may serve as a basis for future clinical studies that evaluate risk factors associated with adolescent alcohol abuse as they relate to subsequent maturational processes.

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REFERENCES

1. G. G. Gordon, A. L. Southren and C. S. Lieber, *Alcoholism: Clin. expl Res.* **3**, 210 (1979).
2. E. L. Abel, *Drug Alcohol Depend.* **5**, 321 (1980).
3. D. H. Van Thiel, *Alcoholism: Clin. expl Res.* **4**, 44 (1980).
4. R. A. Anderson, B. R. Willis, C. Oswald and L. J. D. Zaneveld, *Pharmac. Biochem. Behav.* **18** (Suppl. 1), 305 (1983).
5. S. C. Wilsnack, *J. abnorm. Psychol.* **82**, 253 (1973).
6. K. R. Warren, *Critical Review of the Fetal Alcohol Syndrome.* National Institute on Alcohol Abuse and Alcoholism, Rockville, MD (1977).
7. L. Erb and B. D. Andresen, *Clin. Pediat.* **17**, 644 (1978).
8. A. P. Streissguth, *Am. J. Epidem.* **107**, 467 (1978).
9. F. M. Badr and R. S. Badr, *Nature, Lond.* **253**, 134 (1975).
10. R. A. Anderson, S. A. Beyler and L. J. D. Zaneveld, *Fert. Steril.* **30**, 103 (1978).
11. R. F. Mankes, R. LeFevre, K-F. Benitz, I. Rosenblum, H. Bates, A. I. T. Walker and R. Abraham, *J. Toxic. environ. Hlth* **10**, 871 (1982).
12. R. A. Anderson, J. M. Reddy, C. Joyce, B. R. Willis, H. Van der Ven and L. J. D. Zaneveld, *Biology Reprod.* **27**, 833 (1982).
13. C. Joyce, C. Oswald and R. A. Anderson, *Biology Reprod.* **24** (Suppl. 1), 85A (1982).
14. B. J. Rogers, in *Biochemistry of Mammalian Reproduction* (Eds. L. J. D. Zaneveld and R. T. Chatterton), p. 203. John Wiley, New York (1982).
15. T. J. Cicero and T. M. Badger, *J. Pharmac. exp. Ther.* **201**, 427 (1977).
16. T. J. Cicero, D. Bernstein and T. M. Badger, *Alcoholism: Clin. expl Res.* **2**, 249 (1978).

17. R. A. Anderson, B. R. Willis, C. Oswald, J. M. Reddy, S. A. Beyler and L. J. D. Zaneveld, *Biochem. Pharmac.* **29**, 1409 (1980).
18. B. R. Willis, R. A. Anderson, C. Oswald and L. J. D. Zaneveld, *J. Pharmac. exp. Ther.* **225**, 470 (1983).
19. D. H. Van Thiel, C. J. McClain, M. K. Elson, M. J. McMillan and R. Lester, *Metabolism* **27**, 1778 (1978).
20. G. G. Gordon, J. Vittek, R. Ho, W. S. Rosenthal, A. L. Southren and C. S. Lieber, *Gastroenterology* **77**, 110 (1979).
21. T. J. Cicero and R. D. Bell, *Biochem. biophys. Res. Commun.* **94**, 814 (1980).
22. V. K. Bhalla, C. J. H. Chen and M. S. Gnanaprakasam, *Life Sci.* **24**, 1315 (1979).
23. M. S. Gnanaprakasam, C. J. H. Chen, J. G. Sutherland and V. K. Bhalla, *Biology Reprod.* **20**, 991 (1979).
24. R. A. Anderson, B. R. Willis, C. Oswald and L. J. D. Zaneveld, *J. Pharmac. exp. Ther.* **225**, 479 (1983).
25. C. F. Cobb, J. S. Gavaler and D. H. Van Thiel, *Clin. Toxic.* **18**, 149 (1981).
26. T. J. Cicero, R. D. Bell, J. G. Carter, M. M-Y. Chi and O. H. Lowry, *Biochem. Pharmac.* **32**, 107 (1983).
27. R. A. Anderson, J. M. Quigg, C. Oswald and L. J. D. Zaneveld, *Biochem. Pharmac.* **34**, 685 (1985).
28. T. J. Cicero, K. S. Newman, M. Gerrity, P. F. Schmoeker and R. D. Bell, *Life Sci.* **31**, 1587 (1982).
29. R. R. Schade, G. Bonner, V. C. Gay and D. H. Van Thiel, *Alcoholism: Clin. expl Res.* **7**, 150 (1983).
30. W. L. Dees and G. P. Kozlowski, *Peptides* **5** (Suppl. 1), 209 (1984).
31. J. V. Rachal, S. A. Maisto, L. L. Guess and L. L. Hubbard, in *National Institute on Alcohol Abuse and Alcoholism. Alcohol Consumption and Related Problems.* Alcohol and Health Monograph No. 1, p. 55. U.S. Government Printing Office, Washington, DC (1982).
32. J. A. Ramaley, *J. Androl.* **3**, 248 (1982).
33. M. Ficher and D. R. Levitt, *J. Steroid Biochem.* **13**, 1089 (1980).
34. G. G. Gordon, J. Vittek, A. L. Southren, P. Munnangi and C. S. Lieber, *Endocrinology* **106**, 1880 (1980).
35. Y-B. Chiao, D. E. Johnston, J. S. Gavaler and D. H. Van Thiel, *Alcoholism: Clin. expl Res.* **5**, 30 (1981).
36. P. L. Altman and D. S. Dittmer, *Biology Data Book,* 2nd Edn, Vol. 1, p. 138, p. 207. Federation of American Societies for Experimental Biology, Bethesda, MD (1972).
37. S. D. Michael, S. B. Kaplan and B. T. Macmillan, *J. Reprod. Fert.* **59**, 217 (1980).
38. R. A. Anderson, C. Oswald, B. R. Willis and L. J. D. Zaneveld, *J. Reprod. Fert.* **68**, 1 (1983).
39. R. D. Lillie, *Histopathologic Technique and Practical Histochemistry.* McGraw-Hill, New York (1965).
40. K. O. Lindros, *Alcoholism: Clin. expl Res.* **6**, 70 (1982).
41. R. A. Anderson, J. M. Reddy, C. Oswald and L. J. D. Zaneveld, *Clin. Chem.* **25**, 1780 (1979).
42. J. F. Kachman, in *Fundamentals of Clinical Chemistry* (Ed. N. W. Tietz), p. 362. W. B. Saunders, Philadelphia, PA (1970).
43. R. R. Sokal and F. J. Rohlf, *Biometry*, p. 417. W. H. Freeman, San Francisco (1981).
44. C. M. Woolf, *Principles of Biometry*, p. 101. D. Van Nostrand, Princeton, NJ (1968).
45. L. J. D. Zaneveld, in *Biochemistry of Mammalian Reproduction* (Eds. L. J. D. Zaneveld and R. T. Chatterton), p. 37. John Wiley, New York (1982).
46. A. M. Symons and V. Marks, *Biochem. Pharmac.* **24**, 955 (1975).
47. A. R. Glass, J. Anderson, D. Herbert and R. A. Vigersky, *Endocrinology* **115**, 19 (1984).
48. J. Ellingboe and C. C. Varanelli, *Res. Commun. Chem. Path. Pharmac.* **24**, 87 (1979).
49. L. Santucci, T. J. Graham and D. H. Van Thiel, *Alcoholism: Clin. expl Res.* **7**, 135 (1983).
50. B. R. Willis and A. K. Gupta, *Biology Reprod.* **24** (Suppl. 1), 91A (1981).
51. V. K. Bhalla, V. P. Rajan and M. E. Newman, *Alcoholism: Clin. expl Res.* **7**, 153 (1983).
52. M. B. Lipsett, in *Adolescent Endocrinology* (Eds. F. P. Heald and W. Hung), p. 91. Appleton-Century-Crofts, New York (1970).
53. T. Mann and C. Lutwak-Mann, *Male Reproductive Function and Semen*, p. 174. Springer, New York (1981).
54. N. C. Degani, E. M. Sellers and K. Kadzielawa, *J. Pharmac. exp. Ther.* **210**, 22 (1979).
55. W. P. Ventura, M. Freund, J. Davis and C. Pannuti, *Fert. Steril.* **24**, 68 (1973).
56. H. Zankl and W. Leidl, *J. Reprod. Fert.* **18**, 181 (1969).
57. W. H. Gantt, *Psychosom. Med.* **14**, 174 (1952).
58. H. A. Teitelbaum and W. H. Gantt, *Q. Jl Stud. Alcohol* **19**, 394 (1958).
59. F. Lemere and J. W. Smith, *Am. J. Psychiat.* **130**, 212 (1973).
60. D. H. Van Thiel, R. Lester and R. J. Sherins, *Gastroenterology* **67**, 1188 (1974).
61. K. Kucheria, R. Saxena and D. Mohan, *Andrologia* **6**, 558 (1985).
62. S. J. Nazian and V. B. Mahesh, *Archs Androl.* **4**, 283 (1980).
63. S. J. Nazian and V. B. Mahesh, *Neuroendocrinology* **29**, 313 (1979).
64. R. A. Anderson and J. F. Phillips, *Biology Reprod.* **32** (Suppl. 1), 181 (1985).
65. P. A. Gantt, P. T. Tho, V. K. Bhalla, P. G. McDonough, A. Costoff and V. B. Mahesh, *J. Pharmac. exp. Ther.* **223**, 848 (1982).
66. D. H. Van Thiel and D. L. Loriaux, *Metabolism* **28**, 536 (1979).