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# DEMONSTRATION OF A FUNCTIONAL BLOOD-TESTIS BARRIER TO ACETALDEHYDE

## EVIDENCE FOR LACK OF ACETALDEHYDE EFFECT ON ETHANOL INDUCED DEPRESSION OF TESTOSTERONE *IN VIVO**

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**Abstract**—*In vitro* studies have shown that acetaldehyde is a more potent inhibitor of testicular steroidogenesis than ethanol. The present study examined the *in vivo* role of acetaldehyde in ethanol-induced reduction of testosterone by (1) determining the levels of acetaldehyde to which the testes were exposed subsequent to acute ethanol administration to mice; and (2) examining the effect of ethanol on testosterone in animals subsequent to drug pretreatment which decreased or increased ethanol-derived acetaldehyde. Ethanol-induced (3 g/kg) depression of testosterone was dependent upon gonadotropin stimulation. The increase in hCG-induced testosterone was suppressed ($P < 0.01$) in ethanol- as compared to saline-treated animals [39.8 ± 2.6 (S.E.M.) vs 28.1 ± 2.3 ng/ml]. Pargyline (100 mg/kg) or cyanamide (8.4 mg/kg) increased ($P < 0.05$) plasma and testicular acetaldehyde, while having no effect on the testosterone response to ethanol. Similarly, 4-methylpyrazole (25 mg/kg) reduced blood and testicular acetaldehyde to nondetectable levels, while having no effect on testosterone. Testicular acetaldehyde was lower ($P < 0.001$) than plasma levels (14 ± 2 vs 2.0 ± 0.2 μM). This functional blood-testis barrier to acetaldehyde could be explained by testicular aldehyde dehydrogenases in the mitochondria ($K_m$ for acetaldehyde = 1.5 μM) and in the cytosol ($K_m$ = 123 μM) whose maximal activities totaled to more than 25-fold greater than that of testicular alcohol dehydrogenase (ADH). ADH was concentrated in the Leydig cells, while aldehyde dehydrogenase was evenly distributed in the testis. Ethanol prevented further hCG-induced rises in testosterone rather than inhibiting testosterone production to below pre-ethanol values. The above data argue against a significant role of acetaldehyde in the *in vivo* response of testosterone to ethanol. Ethanol appears to impair gonadotropin–testicular receptor interaction *in vivo*.

Reduced testosterone is among several manifestations of reproductive dysfunction secondary to chronic ethanol ingestion by males; this phenomenon has been demonstrated both clinically and in laboratory animals (for reviews, see Refs. 1–4). In view of the importance of testosterone in the maintenance of male reproductive homeostasis, ethanol-induced depression of testosterone may be related to male reproductive failure subsequent to alcohol abuse. The mechanism by which ethanol lowers testosterone has not yet been fully resolved, although several foci have been suggested as sites at which ethanol exerts its effects in reducing androgen levels. For example, under specific conditions of ethanol treatment, increased hepatic levels of 5α-reductase have been demonstrated [5]. Increased activity of this enzyme may lead to increased disposition of testosterone and, hence, to lower circulating levels of this androgen. Although hepatic involvement cannot be excluded as contributing to reduced testosterone, it is unlikely that it represents the primary mechanism by which ethanol lowers levels of this steroid, since clinical studies have failed to show a strong relation between testosterone levels and the extent of ethanol-induced hepatopathology [6–8]. Moreover, acutely administered ethanol depresses testosterone [9–12], an effect not readily explained by hepatic enzyme induction.

Recent studies have demonstrated that ethanol exerts direct inhibitory effects at the levels of the hypothalamus-pituitary [13–16] and of the testis [17–20]. Inhibition of either luteinizing hormone release or testicular steroidogenesis would exert a depressant effect on testicular testosterone production. Studies that have examined all components of the hypothalamic–pituitary–gonadal axis have suggested that the testis is somewhat more sensitive to ethanol than the hypothalamus-pituitary [11, 21–23]. Ethanol has inhibited testosterone production in a variety of *in vitro* testicular preparations, including the perfused testis [19], testicular homogenates [20],

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testicular microsomal preparations [24] and isolated Leydig cells [17, 20, 25].

Acetaldehyde (the primary metabolite of ethanol) is also an effective inhibitor of testicular testosterone production at concentrations several-fold less than ethanol concentrations required to elicit the same inhibitory effect [20, 25–27]; several investigators have thus proposed that acetaldehyde may be the active agent responsible for ethanol-induced reduction of steroidogenesis. However, little is known regarding the accessibility of the testicular compartment to acetaldehyde following ethanol administration *in vivo*. Additionally, although studies by our laboratory and other investigators [9, 10, 28] have clearly indicated reduced testosterone levels subsequent to *in vivo* ethanol administration to mice, the role of acetaldehyde in eliciting this effect has not been critically evaluated. It was the purpose of the present study to measure testicular acetaldehyde levels subsequent to *in vivo* ethanol administration to mice, to quantify testicular acetaldehyde formation and disposition *in vitro*, and to examine the testosterone response to treatments which either elevate or reduce acetaldehyde subsequent to ethanol administration. The results from these studies strongly indicate that (1) a functional blood-testis barrier to ethanol-derived acetaldehyde exists in the mouse; and (2) acetaldehyde plays little, if any, role in the *in vivo* testosterone response to administered ethanol.

## MATERIALS AND METHODS

Sexually mature Swiss Webster mice (28–30 g) were utilized for all studies and obtained from a local supplier. They were group-housed (five per cage) under controlled lighting (14/10 hr light/dark cycle; lights on at 5:00 a.m.) and temperature (22 ± 2°). Animals were acclimated to these conditions for at least 7 days prior to experimentation and were maintained on laboratory chow and water *ad lib*. Ethanol (95%, v/v) was provided by the University supply facility. Acetaldehyde was a product of the Aldrich Chemical Co (Milwaukee, WI) and was glass-distilled prior to use. Human chorionic gonadotropin (hCG), hydrogen cyanamide, pargyline hydrochloride, 4-methylpyrazole, nicotinamide adenine dinucleotide ($NAD^+$), nicotinamide adenine dinucleotide reduced form (NADH), nicotinamide adenine dinucleotide phosphate ($NADP^+$), sodium pyruvate, cytochrome *c* (horse heart), disodium succinate hexahydrate, collagenase (*Clostridium histolyticum*, type IV), dehydroepiandrosterone, nitro blue tetrazolium and testosterone were purchased from the Sigma Chemical Co (St. Louis, MO). Radiolabeled [1$\alpha$,2$\alpha$-$^3$H(N)]testosterone (49.0 Ci/mmole), rabbit anti-testosterone antibody and testosterone standards for radioimmunoassay were from the New England Nuclear Corp. (Boston, MA). All other reagents were of the highest quality commercially available.

*Drug treatments.* Sterile saline was used as a vehicle for hCG, cyanamide, pargyline, 4-methylpyrazole and ethanol administration. All drugs were administered by i.p. route, with treatments being initiated between 9:00 and 11:00 a.m. in every experiment to minimize possible circadian contributions to the data. Unless otherwise indicated, the injection schedule was designed such that blood was taken for testosterone measurements either 60 min or 150 min after hCG treatment and 90 min after either ethanol (3 g/kg) or control (saline) injection. Tail blood (20–100 $\mu$l) was collected into heparinized capillary pipettes for determination of plasma testosterone, while 50 $\mu$l blood was collected for measurement of blood ethanol and acetaldehyde levels. Animals were killed by cervical dislocation, and their testes were removed, decapsulated, weighed and homogenized in 9 vol. of 1.0 N HCl containing 25 mM thiourea (to prevent nonenzymic acetaldehyde formation [29]). The homogenates were subsequently processed for measurement of testosterone, ethanol and acetaldehyde (see below). The time required from animal sacrifice through homogenate preparation was 60–90 sec.

*Ethanol and acetaldehyde measurement.* Blood samples were immediately dispensed into stoppered 25 ml Erlenmyer flasks which contained 1 ml of 1.0 N HCl with 25 mM thiourea. Testicular homogenates (1 ml) were placed in flasks immediately after homogenate preparation. After equilibration of the flasks at 44° for 10 min, the ethanol and acetaldehyde content of 5 ml of headspace over the solution was determined by gas chromatography, as previously described [28, 29], with a Tracor model 560 gas chromatograph, equipped with a flame ionization detector and a 6 ft glass column, packed with Poropak Q resin. Injection port, column and detector temperatures were 180°, 120° and 200°, respectively, with a carrier gas (helium) flow rate of 60 cc/min. Ethanol and acetaldehyde were quantified by comparing the detector response (peak height) of the samples with that of known concentrations of ethanol and acetaldehyde. All measurements were made within 4 hr of sample collection. Sensitivity of the assay was 0.25 nmole.

*Measurement of blood content of testicular homogenates.* The assay for contaminating blood present in testicular homogenates was based upon the difference spectrum of oxygenated and deoxygenated hemoglobin [30] and has been described previously [29]. Samples were made alkaline with $NH_4OH$, and the difference in absorbance at 555 nm and 577 nm was recorded in samples containing 10 mg sodium dithionite, as compared with samples containing no dithionite. The absorbance in testicular homogenates was compared with a standard curve of absorbance difference as a function of known volumes of whole mouse blood.

*Testosterone determination.* Plasma and testicular testosterone levels were determined by radioimmunoassay as previously described [10, 28]. Recovery of testosterone standards added to plasma or testicular samples was in excess of 90% (range = 91.4 to 106%). The sensitivity was 15 pg testosterone per assay. Intra-assay variability was less than 10%. Testicular homogenates were centrifuged at 12,000 *g* for 10 min to remove particulate material prior to testosterone extraction. Testicular samples and plasma from hCG-treated animals were diluted with assay buffer (0.1 M sodium phosphate, 0.15 M NaCl,

0.1% gelatin and 0.15 mM sodium azide, pH 7.0) prior to solvent extraction.

*Measurement of* in vitro *testicular ethanol and acetaldehyde metabolism.* Ethanol oxidation by whole testicular homogenates or by Leydig cell or tubular components (see below) was measured by gas chromatographic determination of acetaldehyde formation. Reaction mixtures consisted of testicular homogenate (equivalent to 8–100 mg tissue, wet weight), 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 10 mM nicotinamide, 1 mM $NAD^+$, 20 mM semicarbazide, ethanol (0.03 to 5.0 mM) and 25 mM thiourea, pH 7.4, in a total volume of 1.0 ml. Incubations were carried out in stoppered flasks at 34° for 10–60 min, after which time the reactions were terminated by the addition of 0.2 ml of 6.0 N HCl. The acetaldehyde content of the headspace over the reaction mixture was determined by gas chromatography (see above) following a 10-min equilibration period at 44°.

Acetaldehyde metabolism by whole testicular homogenates or by Leydig cell or tubular components was measured by determining the rate of acetaldehyde disappearance. Reaction mixtures were in stoppered flasks, and consisted of testicular tissue (equivalent to 50 mg tissue, wet weight), 50 mM HEPES, acetaldehyde (12.5 to 500 $\mu$M) and 0.5 mM 4-methylpyrazole, pH 7.4, in a total volume of 2.0 ml. Incubations were carried out at 34° for 10–25 min after which reactions were terminated by the addition of 0.4 ml of 6.0 N HCl. Acetaldehyde remaining in the reaction was quantified by gas chromatography (see above).

*Enrichment of testicular Leydig cell and tubular components.* Interstitial cells were enzymically dispersed and separated from the seminiferous tubules by a slight modification of the method of Dufau and Catt [31]. Decapsulated testes (0.6 g) were placed in 10 vol. of oxygenated buffer consisting of 0.25 M sucrose, 25 mM HEPES, 0.7 mg/ml collagenase and 1 mg/ml bovine serum albumin, pH 7.4, previously equilibrated at 34°. The suspension was incubated at 34° for 20 min in a shaking water bath, after which 20 ml of cold 25 mM HEPES containing 0.25 M sucrose, pH 7.4 (HEPES/sucrose buffer), was added. The resultant dispersed suspension was allowed to stand at ambient temperature for 5 min, thus permitting the seminiferous tubules to sediment. The supernatant fraction containing Leydig cells was aspirated and centrifuged at 2000 *g* for 2 min; the Leydig cell preparation was resuspended in 5 ml of HEPES/sucrose buffer and homogenized with a glass–Teflon homogenizer. The tubules were resuspended in 20 ml HEPES/sucrose buffer and allowed to sediment (5 min). The supernatant fraction was aspirated, and the tubules were resuspended and homogenized in 5 ml of HEPES/sucrose buffer.

Prior to homogenization, the viability of the Leydig cell preparation was evaluated histologically [32] by measuring 3$\beta$-hydroxysteroid dehydrogenase activity in the presence of nitro blue tetrazolium (0.2 mg/ml), dehydroepiandrosterone (10 $\mu$g/ml), 1 mM $NAD^+$ and HEPES/sucrose buffer. Incubations were carried out at 34° for 1 hr. The presence of enzyme activity was inferred from the presence of Leydig cells containing deposits of blue-violet material. The above procedure yielded an enriched Leydig cell preparation containing approximately $1 \times 10^7$ Leydig cells/g testis, accounting for approximately 40% of the total cellular content. All Leydig cells showed histological evidence of 3$\beta$-hydroxysteroid dehydrogenase activity; however, approximately 25% of the cells were stained by the nitro blue tetrazolium in the absence of substrate, suggesting a lack of absolute specificity of the assay method for this enzyme. Less than 2% of the total Leydig cell content was present in the seminiferous tubule preparation.

*Subcellular fractionation of the testis.* Testicular subcellular fractions were prepared by differential centrifugation. A 20% (w/v) homogenate was prepared in 0.25 M sucrose containing 25 mM HEPES, pH 7.4, with the aid of a glass–Teflon homogenizer (hand-held, seven passes). The resultant homogenate was centrifuged at 300 *g* for 10 min to remove large cellular debris and intact germ cells. Nuclear, mitochondrial and microsomal fractions were isolated by centrifugation at 1,000 *g* for 10 min, 10,000 *g* for 20 min and 100,000 *g* for 60 min respectively. The 100,000 *g* supernatant was taken as the cytosolic fraction. All pellets were resuspended in the original homogenate volume of 0.25 M sucrose containing 25 mM HEPES, pH 7.4, and dialyzed against two changes of 1000 volumes of the same buffer (18 hr total dialysis time). Activities of succinic dehydrogenase (succinate: cytochrome *c* oxidoreductase, EC 1.3.99.1), 17$\beta$-hydroxysteroid dehydrogenase (17$\beta$-hydroxysteroid: NADP oxidoreductase, EC 1.1.1.51) and lactic dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27) were measured as markers for mitochondria, microsomes and cytosol respectively.

*Enzyme assays.* Succinic dehydrogenase was measured spectrophotometrically essentially as described by Kuff and Schneider [33]. Reaction mixtures contained subcellular fraction (10–30 mg protein), 30 mM potassium phosphate, 1 mM KCN, 0.4 mM $CaCl_2$, $5 \times 10^{-5}$ M cytochrome *c*, 0.2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) and 1 mM succinate, pH 7.4. Reaction blanks contained no succinate. Enzyme activity was quantified by measuring the rate of increase in absorbance at 550 nm, assuming a millimolar absorptivity of 29.5.

The microsomal enzyme, 17$\beta$-hydroxysteroid dehydrogenase, was measured according to the method of Bergmeyer [34]. Reaction mixtures contained subcellular fraction (1.0 to 5.0 mg protein), 100 mM Tris–HCl, 1 mM $NADP^+$, 17 $\mu$M testosterone, 1% dimethyl sulfoxide (solvent for testosterone) and 0.2% CHAPS, pH 9.0. Enzyme activity was quantified by measuring the rate of increase in absorbance at 340 nm. Reaction blanks contained no testosterone.

Lactic dehydrogenase and alcohol dehydrogenase (alcohol: NAD oxidoreductase, EC 1.1.1.1) activities were measured by established enzymic assays [35, 36]. Lactic dehydrogenase activity was quantified by measuring the rate of decrease in absorbance at 340 nm in the presence of subcellular fraction (1.0 to 3.0 $\mu$g protein), 0.2 mM NADH and 5 mM pyruvate, pH 7.0, while alcohol dehydrogenase

activity was quantified by measuring the rate of increase in absorbance at 340 nm in the presence of subcellular fraction (3.0 to 8.5 mg protein), 1 mM $NAD^+$, 50 mM ethanol, 0.2% Triton X-100 and 20 mM semicarbazide, pH 7.4. Aldehyde dehydrogenase (aldehyde: NAD oxidoreductase, EC 1.2.1.3) activity was measured by following the rate of increase in absorbance at 340 nm in the presence of subcellular fraction (0.1 to 0.25 mg protein), 30 mM sodium phosphate (pH 7.4), 1 mM $NAD^+$, 0.2% Triton X-100 and 10 mM acetaldehyde. Reaction blanks for the above enzyme assays contained no substrate.

*Kinetic evaluation of testicular alcohol and aldehyde dehydrogenase activities.* Maximal velocities and apparent Michaelis constants of ethanol and acetaldehyde oxidation were obtained from double-reciprocal plots [37] of either initial reaction velocities (determined spectrophotometrically for subcellular fractions) or the rate of acetaldehyde formation or disappearance (determined by gas chromatographic analysis for testicular homogenates) as a function of substrate concentration (range of ethanol concentrations, 0.05 to 5.0 mM; range of acetaldehyde concentrations, 3.0 $\mu$M to 10.0 mM). Curves were fit to the data by linear regression analysis.

*Protein estimation.* Protein content of testicular homogenates, cellular fractions and subcellular fractions was determined by the method of Lowry *et al.* [38]. Crystalline bovine serum albumin was used as protein standard.

*Statistical analyses.* The effects of various individual drug treatments on testosterone levels were evaluated by one-way analysis of variance. Specific differences were identified by the Newman–Keuls multiple range test [39]. The effects of drug treatments in combination with ethanol on testosterone levels were evaluated by two-way analysis of variance [39]. Association of plasma and testicular levels of acetaldehyde with plasma and testicular testosterone concentration was evaluated with Pearson's product-moment correlation coefficient [40]. Linear regression analysis [39] was used to evaluate the significance of differences in the y intercepts of double-reciprocal plots of hCG dose as a function of plasma testosterone in ethanol- and saline-treated animals. Differences were considered significant at the 0.05 level of confidence.

Table 1. Ethanol-induced suppression of plasma testosterone: Requirement for gonadotropin stimulation and influence of treatment sequence*

| Treatment | | | |
|---|---|---|---|
| 1st | Time interval (min) | 2nd | Plasma testosterone (ng/ml) |
| Saline | | | 0.42 ± 0.17† (9) |
| Ethanol (3 g/kg) | | | 0.50 ± 0.28† (9) |
| hCG (5 I.U.) | 60 | Saline | 39.8 ± 10.6‡ (10) |
| hCG | 60 | Ethanol | 28.1 ± 7.3§ (10) |
| Saline | 30 | hCG | 37.3 ± 15.6‡ (16) |
| Ethanol | 30 | hCG | 13.3 ± 4.4‖ (8) |

* Animals were given either one or two treatments, with a time interval between treatments, as indicated. Blood was taken for testosterone measurement at 90 min after either saline or ethanol treatment. Values represent the average ± standard deviation, with the number of animals indicated in parentheses. Two-way analysis of variance (first four treatment groups) indicated a significant hCG effect ($F^1_{34} = 270.0$; $P < 0.001$), a significant ethanol effect ($F^1_{34} = 6.04$, $P < 0.05$) and ethanol–hCG interaction ($F^1_{34} = 6.04$; $P < 0.05$).

†–‖ Values with different superscripts indicate significant differences ($P < 0.01$, Newman–Keuls multiple range test).

## RESULTS

Ethanol-induced depression of plasma testosterone levels was dependent upon gonadotropin stimulation (Table 1). Ethanol had no effect upon basal testosterone levels in the absence of gonadotropin stimulation ($P > 0.1$). However, testosterone levels of animals given 3 g/kg ethanol 60 min after gonadotropin stimulation (5 I.U. hCG) were 29% lower than those of saline-treated animals receiving gonadotropin stimulation ($P < 0.01$). The magnitude of the ethanol effect was dependent upon the order in which the drugs were administered. A greater effect was observed when ethanol administration preceded gonadotropin stimulation, which resulted in a 64% depression in testosterone levels relative to those of saline-treated controls ($P < 0.01$).

Both pargyline and cyanamide have been shown to elevate blood acetaldehyde levels subsequent to ethanol administration [41–43]. However, neither drug influenced the effect of ethanol administration on plasma testosterone levels (Table 2). Ethanol administration reduced hCG-induced testosterone levels in saline-, cyanamide- (8.4 mg/kg) and pargyline- (100 mg/kg) pretreated animals by 38%, 42% and 33% respectively. Although pargyline treatment elevated plasma testosterone, there was no pargyline–ethanol interaction with regard to testosterone levels. Similarly, the alcohol dehydrogenase inhibitor 4-methylpyrazole [44, 45] failed to alter the testosterone response to ethanol. Ethanol depressed testosterone levels in 4-methylpyrazole-pretreated (25 mg/kg) animals by 34% (Table 2). Cyanamide and pargyline pretreatment resulted in significantly higher, while 4-methylpyrazole pretreatment produced significantly lower, plasma acetaldehyde concentrations subsequent to ethanol administration. In contrast, drug pretreatments had no effect on blood ethanol levels 90 min after ethanol administration. Blood ethanol concentrations in saline-, cyanamide-, pargyline- and 4-methylpyrazole-treated animals were 49 ± 13 (S.D.) mM (N = 10), 48 ± 3 mM (N = 8), 44 ± 2 mM (N = 5) and 52 ± 10 mM (N = 20) respectively.

Similar drug effects were noted on testicular testosterone and acetaldehyde levels (Table 3). There was a significant ethanol effect on testicular testosterone in each drug treatment group ($P < 0.001$), but no drug–ethanol interaction was noted. Cyanamide and pargyline pretreatment resulted in significant increases ($P < 0.01$), while 4-methylpyrazole produced a significant decrease ($P < 0.01$) in tes-

Table 2. Effect of pargyline, cyanamide and 4-methylpyrazole pretreatment on ethanol-induced suppression of hCG-induced plasma testosterone*

| Treatment | Plasma testosterone (ng/ml) | Blood acetaldehyde ($\mu$M) |
|---|---|---|
| Saline, Saline | 37 ± 9 (10) | |
| Saline, Ethanol (3 g/kg) | 23 ± 13† (10) | 14 ± 8‡ (10) |
| Pargyline (100 mg/kg), Saline | 49 ± 4§ (5) | |
| Pargyline, Ethanol | 33 ± 11† (4) | 169 ± 33‡ (5) |
| Cyanamide (8.4 mg/kg), Saline | 38 ± 6 (6) | |
| Cyanamide, Ethanol | 22 ± 7† (6) | 117 ± 25‡ (6) |
| 4-Methylpyrazole (25 mg/kg), Saline | 41 ± 15 (7) | |
| 4-Methylpyrazole, Ethanol | 27 ± 4† (7) | 0‡ (15) |

* Forty-five minutes after the indicated first treatment, animals were administered 5 I.U. hCG, followed 60 min later by either ethanol (3 g/kg) or saline. Ninety minutes thereafter, blood was collected for testosterone and acetaldehyde determinations. Values represent the average ± standard deviation of the number of animals indicated in parentheses.

† Two-way analyses of variance indicated a significant ethanol effect after all drug pretreatments ($P < 0.01$), but no drug–ethanol interaction.

‡ All pretreatments resulted in significantly different acetaldehyde levels ($P < 0.05$, Newman–Keuls multiple range test). No association existed between plasma testosterone and blood acetaldehyde concentration (Pearson's product-moment correlation coefficient = 0.580; $P > 0.1$).

§ Value differs from saline, saline group ($P < 0.05$, Newman–Keuls multiple range test).

ticular acetaldehyde levels. On the other hand, there was no significant association between testicular acetaldehyde and testosterone levels. Drug treatments had no significant effect upon testicular ethanol levels. Testicular ethanol levels 90 min after ethanol administration to gonadotropin-stimulated animals pretreated with saline, 4-methylpyrazole, cyanamide and pargyline were 47 ± 8 mM (N = 5), 54 ± 12 mM (N = 10), 40 ± 10 mM (N = 4) and 45 ± 6 mM (N = 5) respectively.

Testicular acetaldehyde was significantly lower ($P < 0.001$, two-tailed *t*-test) than blood acetaldehyde subsequent to ethanol administration in saline, cyanamide and pargyline treatment groups (Tables 2 and 3). The blood content of testicular homogenates was 4.3 ± 1.6 $\mu$l/g testis, wet weight (N = 5). Blood contamination of testicular homogenates contributed little to the measured testicular ethanol and acetaldehyde content in saline-treated animals (<3%). The contribution of blood to measured testicular acetaldehyde levels was somewhat higher in pargyline- (9%) and cyanamide- (14%) treated ani-

Table 3. Lack of association of testicular acetaldehyde with depressed testicular testosterone subsequent to ethanol treatment*

| Treatment | Testicular testosterone (ng/g tissue) | Testicular acetaldehyde ($\mu$M) |
|---|---|---|
| Saline, Saline | 1322 ± 84 (13) | |
| Saline, Ethanol (3 g/kg) | 1208 ± 106† (8) | 2.0 ± 0.6 (8) |
| Pargyline (100 mg/kg), Saline | 1326 ± 142 (5) | |
| Pargyline, Ethanol | 1176 ± 126† (4) | 8.2 ± 1.6‡ (5) |
| Cyanamide (8.4 mg/kg), Saline | 1318 ± 84 (4) | |
| Cyanamide, Ethanol | 1064 ± 266† (4) | 3.5 ± 1.1‡ (5) |
| 4-Methylpyrazole (25 mg/kg), Saline | 1326 ± 146 (5) | |
| 4-Methylpyrazole, Ethanol | 941 ± 270† (5) | 0‡ (10) |

* The drug treatment schedule was identical to that described in Table 2. At 90 min after either ethanol or saline injection, animals were killed, and their testes were removed, decapsulated and processed for testosterone and acetaldehyde measurements, as described in Methods. Values represent the average ± standard deviation of the number of determinations indicated in parentheses.

† Two-way analyses of variance indicated significant ethanol effects for all drug pretreatment groups (pargyline, $F^1_{26} = 10.08$; cyanamide, $F^1_{25} = 11.39$; 4-methylpyrazole, $F^1_{27} = 15.71$, $P < 0.005$). However, no drug–ethanol interactions were present (pargyline, $F^1_{26} = 0.04$; cyanamide, $F^1_{25} = 0.68$; 4-methylpyrazole, $F^1_{27} = 3.41$; $P > 0.05$).

‡ All drug pretreatment groups had significantly different testicular acetaldehyde concentrations relative to that of saline pretreatment group ($P < 0.01$, Newman–Keuls multiple range test). No association was present between testicular acetaldehyde and testosterone levels (Pearson's product-moment correlation coefficient = 0.633, $P > 0.1$).

Table 4. Kinetic constants of testicular ethanol and acetaldehyde metabolism*

| | Alcohol dehydrogenase | | Aldehyde dehydrogenase | |
|---|---|---|---|---|
| | $K_m$ ($\mu$M) | $V_{max}$ [nmoles · min$^{-1}$ · (g testis)$^{-1}$] | $K_m$ ($\mu$M) | $V_{max}$ [nmoles · min$^{-1}$ · (g testes)$^{-1}$] |
| Homogenate | 570 ± 208 | 11.6 ± 1.4† | 126 ± 5 | 370 ± 13 |
| Mitochondria | | | 1.5 ± 0.4 | 13 ± 3 |
| Cytosol | 216 ± 17 | 11.3 ± 1.2‡ | 123 ± 20 | 284 ± 45 |

* Ethanol and acetaldehyde metabolism in testicular homogenates was measured by gas chromatography, while alcohol dehydrogenase and aldehyde dehydrogenase activities in subcellular fractions were determined spectrophotometrically. Further details are given in Methods. Values represent the average ± S.E.M. of determinations from three separate preparations.

† Ethanol (5 mM) oxidation was 91.4% inhibited by 0.5 mM 4-methylpyrazole.

‡ Ethanol (0.5 to 50 mM) oxidation was completely inhibited by 0.5 mM 4-methylpyrazole.

mals, due to the higher blood acetaldehyde levels in these groups. These data suggest a functional blood-testis barrier to acetaldehyde subsequent to *in vivo* ethanol administration, possibly enzymic in nature. This possibility was supported by the finding of a relatively high capacity of testicular homogenates to metabolize acetaldehyde, as compared to their capacity to oxidize ethanol (Table 4).

Aldehyde dehydrogenase activity was present in mitochondrial and cytosolic fractions of the testes, with the major part of the activity confined to the cytosol (Fig. 1). Testicular alcohol dehydrogenase was localized entirely in the cytosol (Fig. 1) and was completely inhibited by 0.5 mM 4-methylpyrazole. Testicular ethanol oxidation by whole homogenates could be accounted for by the cytosolic alcohol dehydrogenase activity (Table 4). Although separate aldehyde dehydrogenase enzymes were present in the mitochondria and cytosol (as evidenced by the different maximal reaction velocities and Michaelis constants in the two fractions), maximal acetaldehyde oxidation by whole testicular homogenates appeared to be dependent primarily on the cytosolic aldehyde dehydrogenase (Table 4).

The distribution of ethanol oxidizing activity between the Leydig cell and seminiferous tubule fractions indicated that the highest concentration of alcohol dehydrogenase was in the Leydig cells, although activity was also present in the seminiferous tubule fraction (Fig. 2). Ethanol (5 mM) oxidation by these fractions was more than 90% inhibited by 0.5 mM 4-methylpyrazole. In contrast, acetaldehyde metabolism was rather evenly distributed between the two testicular compartments, suggesting an ubiquitous distribution of aldehyde dehydrogenase within the testis.

A more detailed analysis of the effect of ethanol on gonadotropin-induced levels of plasma testosterone was conducted by measuring the testosterone response to various doses of hCG in animals receiving either ethanol or saline. Mice were given hCG at doses ranging from 0.2 I.U. to 10 I.U., followed

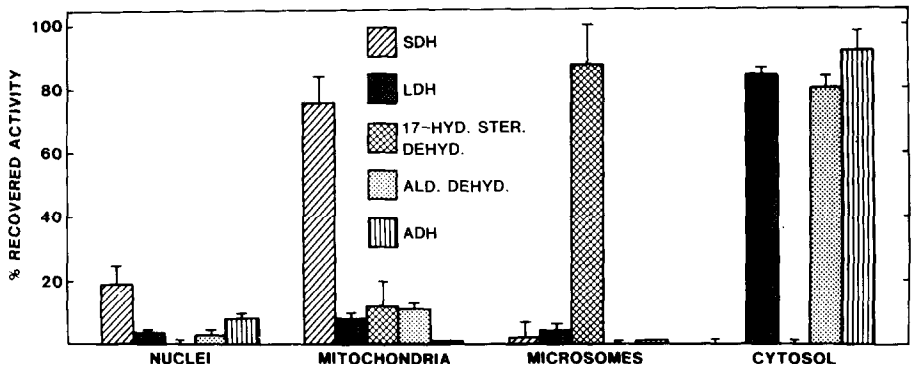


Fig. 1. Subcellular distribution of testicular alcohol and aldehyde dehydrogenases. Nuclear, mitochondrial, microsomal and soluble fractions of 20% (w/v) testicular homogenates were isolated as described in Methods. Activities of succinic dehydrogenase (SDH), lactic dehydrogenase (LDH) and 17$\beta$-hydroxysteroid dehydrogenase (17-hyd. ster. dehyd.) were used as markers for mitochondria, cytosol and microsomes respectively. These activities and those of aldehyde dehydrogenase (ald. dehyd.) and alcohol dehydrogenase (ADH) were measured as described in Methods. Values are expressed as the average percentage of total recovered activity present in each fraction, with the upper 90% confidence limits indicated, for three separate preparations. Total activity [expressed as $\mu$moles substrate utilized · min$^{-1}$ · (g testes)$^{-1}$] for each enzyme was as follows: succinic dehydrogenase, 1.32 ± 0.07; lactic dehydrogenase, 5.58 ± 0.19; and 17$\beta$-hydroxysteroid dehydrogenase, 0.054 ± 0.008.

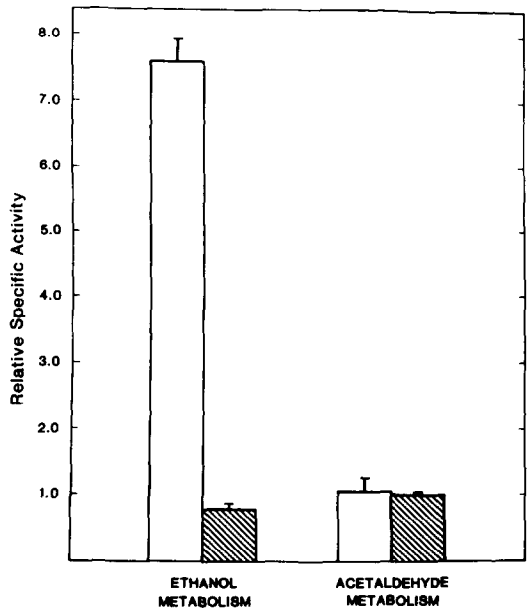


Fig. 2. Cellular distribution of testicular ethanol and acetaldehyde metabolism. Leydig cell and seminiferous tubule fractions were isolated as described in Methods. The relative specific activity of ethanol and acetaldehyde metabolism by Leydig cells (open bars) and seminiferous tubules (hatched bars) were derived from the ratio of each fraction's specific activity [measured as nmoles product formed (substrate utilized) $\cdot$ min$^{-1}$ $\cdot$ (mg protein)$^{-1}$] to the specific activity of the whole testicular homogenate. Values represent the average $\pm$ standard deviation of four separate cellular preparations. Homogenate specific activities were calculated from the data in Table 4, using a homogenate protein content of 93 $\pm$ 9 mg/g testis (N = 5).

60 min later by 3 g/kg ethanol. Plasma testosterone was measured immediately prior to, and 90 min following, ethanol administration. When double-reciprocal plots were constructed of hCG dose vs plasma testosterone (measured 90 min after ethanol administration), ethanol appeared to act as a competitive inhibitor of testosterone with regard to administered gonadotropin (Fig. 3). A linear log (dose)–response of testosterone as a function of administered hCG was obtained when testosterone was determined 60 min subsequent to gonadotropin injection (i.e. prior to ethanol administration; Fig. 4). When the change in testosterone levels was measured between 60 min and 150 min subsequent to hCG injection, testosterone levels continued to rise in saline-treated animals given up to 2.0 I.U. hCG (Fig. 5); essentially no change in testosterone was observed in mice given 5.0 I.U. hCG, while decreased testosterone was noted in animals given 10 I.U. hCG. In contrast, essentially no change in testosterone occurred in ethanol-treated animals at any dose level of hCG (Fig. 5). Small decreases in plasma testosterone from pre-ethanol values were seen only in animals given 2.0, 5.0 and 10 I.U. hCG, representing changes of 14, 17 and 12% respectively. The latter value can be compared to a 20% decrease in plasma testosterone during the same time interval in saline-treated animals given 10 I.U. hCG. Thus, the data suggest that ethanol may be acting to prevent subsequent changes in testosterone, rather than inhibiting its synthesis and release to levels below those observed prior to ethanol administration.

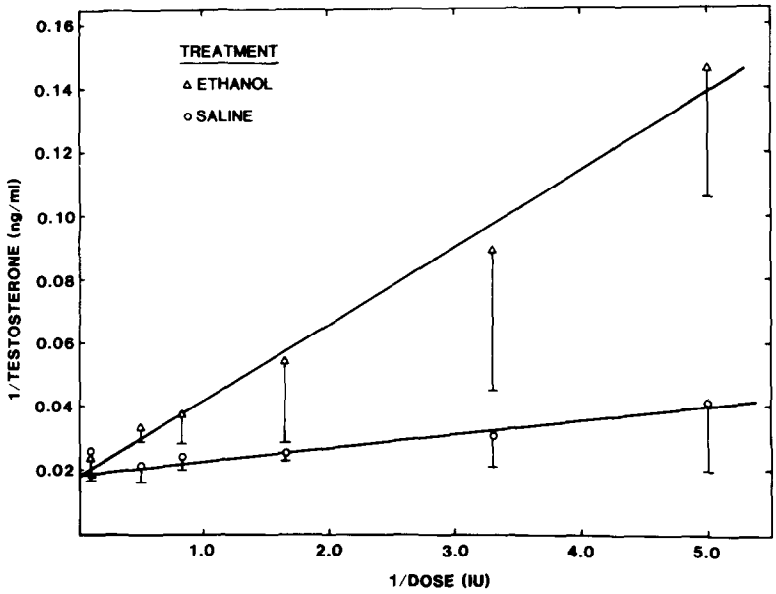


Fig. 3. Inhibition of gonadotropin-induced plasma testosterone as a function of hCG. Animals were injected with the indicated dose of hCG (measured in I.U.) followed 60 min later by ethanol (3 g/kg) or saline. Ninety minutes after ethanol/saline administration, blood was taken for testosterone measurement. Data are presented as a double-reciprocal plot of testosterone as a function of hCG dose. Values are expressed as the average (N = 4 for each data point) reciprocal testosterone level, with the lower 90% confidence limits indicated. Linear curves were fit to the data by the method of least squares. Correlation coefficients ($r$) for the ethanol- and saline-treated groups were 0.992 and 0.994 respectively. Linear regression analysis indicated no significant difference between the ordinate intercepts of the ethanol- and saline-treated groups ($t = 0.442$, $df = 8$; $P > 0.1$).

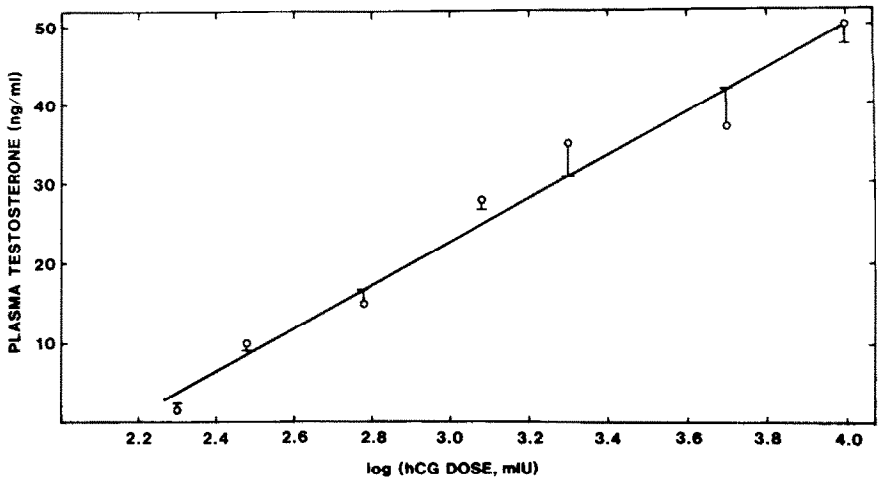


Fig. 4. Dose–response of testosterone levels as a function of hCG. Plasma testosterone levels were determined 60 min after administration of either 0.2, 0.3, 0.6, 1.2, 2.0, 5.0 or 10 I.U. hCG. Data were expressed as the average ± standard error (N = 8 for each point) testosterone level as a function of log(hCG) dose. A linear curve was fit to the data by the method of least squares ($r = 0.984$).

## DISCUSSION

Acetaldehyde is a more potent inhibitor of *in vitro* testicular steroidogenesis than ethanol [20, 25–27], suggesting that acetaldehyde may be responsible for ethanol-induced depression of testosterone *in vivo*. Previous studies have provided little information regarding the accessibility of the testicular compartment to acetaldehyde after ethanol ingestion, nor have they provided direct evidence of a negative association of *in vivo* acetaldehyde levels with those of testosterone. The present study has addressed these concerns.

Ethanol was given at a dose (3 g/kg) previously shown to reduce plasma testosterone levels in the mouse [10, 28]. In contrast to previous studies [10, 28], ethanol exerted no effect on plasma testosterone in the absence of exogenous gonadotropin stimulation (Table 1). Whether the requirement for gonadotropin stimulation would persist subsequent to different ethanol doses is uncertain. However, the dose used in the present study was either equal to or well above those previously shown to depress testosterone in rats and mice [46, 47]. In view of the reported dose-dependency of the testosterone response to ethanol [18, 25, 26, 47], it is not likely that lower ethanol doses would depress testosterone in the absence of gonadotropin stimulation. The lack of effect of ethanol on basal testosterone levels may have been due to the time at which ethanol was administered. In our previous studies [10], ethanol was given at a time corresponding to the lights-off phase of the animals' light–dark cycle; basal testosterone levels during this period were substantially higher than those observed in the present study

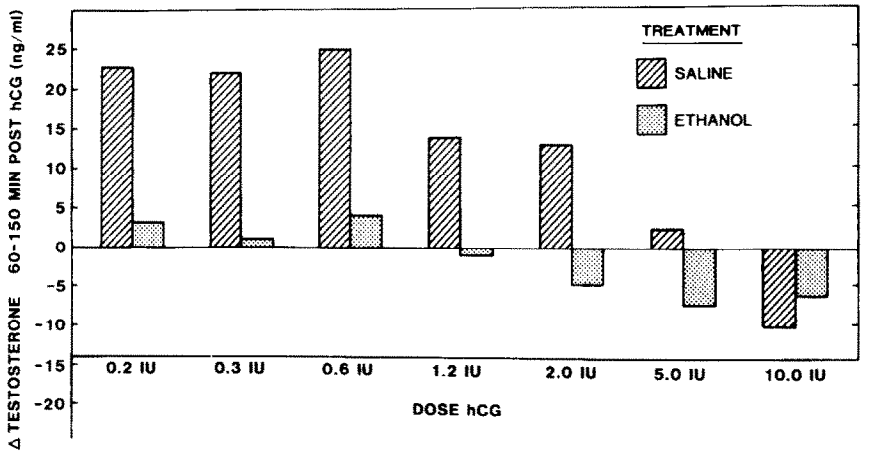


Fig. 5. Suppression of hCG-induced rise in plasma testosterone by ethanol. hCG, followed by either saline or ethanol, was administered as described in the legend to Fig. 3. Values represent the average change in plasma testosterone (ng/ml) which occurred between 0 and 90 min after saline (hatched bars) or ethanol (stippled bars) treatment. The variability associated with each average value can be estimated from the data in Figs. 3 and 4 for each dose of hCG.

($6.3 \pm 0.6$ vs $0.42 \pm 0.17$ ng/ml). Higher testosterone in the former studies most probably reflects the steroidogenic response to endogenous gonadotropin stimulation. Pretreatment with hCG allowed for a more direct assessment of the gonadal effects of ethanol, since the possible confounding effects of ethanol on endogenous gonadotropin release [13–16] (and, hence, upon gonadal function) were minimized.

Cyanamide and pargyline elevate plasma acetaldehyde subsequent to ethanol administration [41, 43], most probably due to inhibition of hepatic aldehyde dehydrogenase [42, 48]. If acetaldehyde were responsible for the depressant effect of ethanol on testosterone levels *in vivo*, cyanamide or pargyline pretreatment should have exacerbated the testosterone response to ethanol. Similarly, if acetaldehyde levels were lowered with an alcohol dehydrogenase inhibitor (e.g. 4-methylpyrazole), the testosterone response to ethanol should have been reduced. No association was evident between acetaldehyde and testosterone concentrations (Tables 2 and 3). These data do not support a role for acetaldehyde as the active agent in the acute depressant effect of ethanol on testosterone, *in vivo*. The lack of drug effect on the testosterone response to ethanol is in agreement with the recently published studies of Eriksson *et al.* in the rat [49], suggesting that the lack of association between *in vivo* acetaldehyde and testosterone is not specific to the mouse.

Santucci *et al.* [27] reported that 4-methylpyrazole reversed the effects of ethanol on gonadotropin-stimulated testosterone production by isolated Leydig cells. However, isolated Leydig cells may not be representative of Leydig cells integrated within the testes. Moreover, significant effects on steroidogenic activity of Leydig cells preparations were noted at 4-methylpyrazole concentrations greater than 0.5 mM. Whether this could be responsible for the apparent protective effect of 4-methylpyrazole remains to be determined.

The low levels of ethanol-derived testicular acetaldehyde may represent a possible explanation for the lack of association of acetaldehyde with *in vivo* testosterone levels. Even when plasma acetaldehyde was elevated 10-fold or more, testicular acetaldehyde remained below the concentrations necessary for significant inhibition of *in vitro* steroidogenesis [25–27, 46] (Tables 2 and 3).

Biomembranes are readily permeable to acetaldehyde; a blood-testis barrier to this substance would likely be enzymic. This speculation is supported by testicular acetaldehyde metabolism whose maximal activity is over thirty times higher than that of testicular ethanol oxidation (Table 4). The presence of a low $K_m$ ($\mu$M) mitochondrial and a high $K_m$ (mM) cytosolic aldehyde dehydrogenase with regard to acetaldehyde oxidation is similar to that observed in other tissues [50–53]. However, unlike other tissues, the testicular mitochondrial enzyme represents a small fraction (<5%) of the total activity. Acetaldehyde metabolism by testicular homogenates can be attributed to the sum of the mitochondrial and cytosolic aldehyde dehydrogenase activities. The $K_m$ for cytosolic aldehyde dehydrogenase is in agreement with that reported by Messiha and Hutson [54]. To our knowledge, no quantitative data are available for acetaldehyde metabolism by a low $K_m$ testicular mitochondrial enzyme.

Inhibition of *in vitro* testicular testosterone production by ethanol is dose-dependent up to concentrations of nearly 200 mM [4, 17, 20, 25, 26]. If ethanol-derived acetaldehyde were solely responsible for depressed testosterone production, the rate of its formation should increase as a function of ethanol concentration up to 200 mM. This concentration is nearly three orders of magnitude higher than the $K_m$ values for ethanol of either the hepatic alcohol dehydrogenase (ADH) isozyme EE from horse [55] or the isozymes isolated from rat liver [56]. It is thus well above that which would result in maximal rates of ethanol oxidation. Acetaldehyde formation would increase as a function of ethanol concentrations of this magnitude only in the presence of an enzyme or enzyme system with a much higher $K_m$ for ethanol oxidation. Ethanol oxidation by testicular homogenates was due almost entirely to a cytosolic ADH. Based upon its $K_m$ for ethanol and sensitivity to 4-methylpyrazole inhibition (Table 4), most of the activity appears to be derived from an EE-like isozyme, rather than from one similar to the SS isozyme [55], $\Pi$ ADH [57] or the C2 ADH (which has been localized in other mouse reproductive tissues) [58]; the latter isozymes have high $K_m$ values for ethanol, and are relatively insensitive to inhibition by pyrazole derivatives. This argues against acetaldehyde as being the primary agent responsible for ethanol-induced depression of *in vitro* testosterone production, at least in the presence of relatively high ethanol concentrations.

As in the rat [54], mouse testicular ADH is most highly concentrated in the Leydig cells (Fig. 2), in contrast to the even testicular distribution of aldehyde dehydrogenase. However, ADH is not exclusive to the Leydig cells. Almost half of the testicular activity was present in the seminiferous tubules.

The steady-state testicular acetaldehyde concentration can be predicted at a given blood ethanol and acetaldehyde level through the use of the kinetic constants derived for testicular ADH and aldehyde dehydrogenases (Table 4). If a testicular blood flow of $0.2\ \text{ml} \cdot \text{min}^{-1} \cdot (\text{g testis})^{-1}$ [59, 60], and a maximal rate of testicular ethanol oxidation of $11.3\ \text{nmoles} \cdot \text{min}^{-1} \cdot (\text{g tissue})^{-1}$ (Table 4) are assumed, the rate of acetaldehyde formation and entry would be $14.1\ \text{nmoles} \cdot \text{min}^{-1} \cdot (\text{g tissue})^{-1}$ when plasma acetaldehyde levels are 14 $\mu$M (Table 2). This is based upon the assumption that $2.8\ \text{nmoles} \cdot \text{min}^{-1} \cdot (\text{g testis})^{-1}$ enters from the blood, and $11.3\ \text{nmoles} \cdot \text{min}^{-1} \cdot (\text{g testis})^{-1}$ is formed due to the action of testicular ADH at $V_{max}$ concentration of ethanol. Testicular acetaldehyde metabolism is the difference between acetaldehyde formation and entry ($14.1\ \text{nmoles} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$) and its removal ($0.4\ \text{nmoles} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$) by testicular blood flow. Based upon these considerations, a calculated testicular acetaldehyde concentration of 2.45 $\mu$M agrees well with the observed value of $2.0 \pm 0.6$ $\mu$M (Table 2).

The maximal reaction velocity of testicular ADH suggests that the major contributor to testicular acet-

aldehyde *in vivo* is normally testicular ADH, rather than plasma acetaldehyde [11.3 out of 14.1 nmoles · $min^{-1}$ · (g testes)$^{-1}$ is due to testicular ADH]; this estimate may be somewhat high, however, since no provision has been made for possible substrate inhibition at high (50 mM) ethanol concentrations. At any rate, the above conclusion would remain valid unless substrate inhibition exceeded 75% of the maximal reaction velocity. If testicular acetaldehyde is 2.0 μM (Table 2), testicular mitochondrial aldehyde dehydrogenase can account for nearly 60% of acetaldehyde metabolism, although comprising less than 5% of the total maximal activity. As in the liver [50, 51, 61], testicular mitochondrial aldehyde dehydrogenase may represent the principal contribution to testicular acetaldehyde metabolism. Other factors not considered in the present study, such as testicular distribution of substrate and cofactor ($NAD^+$) availability, may also contribute to testicular disposition of acetaldehyde *in vivo*.

Localized high acetaldehyde concentrations may occur, due to the high concentration of ADH in Leydig cells. Metabolism of the higher acetaldehyde concentration would be more rapid; the observed acetaldehyde content in whole testicular homogenates would, therefore, be considerably less than the calculated concentration, which is based upon a homogeneous acetaldehyde distribution. The agreement between calculated and observed testicular acetaldehyde concentration argues against this possibility.

When testosterone levels were measured 150 min after hCG administration (90 min after ethanol), ethanol appeared to act as a competitive inhibitor of testosterone levels with respect to administered dose of hCG (Fig. 3). This may have been due to a more rapid attainment of near maximal testosterone response at higher doses of hCG. At lower gonadotropin doses, the maximal response was lower, and required a longer period after administration before being manifested. Ethanol appeared to maintain testosterone at levels observed prior to ethanol administration. If the dose of hCG were high enough to produce a maximal testosterone response within 60 min, subsequent administration of ethanol produced no apparent effect on testosterone 90 min thereafter, as compared to hCG-treated saline controls. On the other hand, if maximal stimulation required longer than 60 min (i.e. at relatively low doses of hCG), ethanol administration prevented the further increase in plasma testosterone between 60 and 150 min after hCG treatment; plasma testosterone in saline-injected animals continued to rise during this time interval. Hence, ethanol appeared to exert a greater effect on plasma testosterone subsequent to lower doses of gonadotropin, resembling competitive inhibition with respect to hCG.

Ethanol may prevent subsequent gonadotropin-induced changes in testosterone, rather than inhibiting pre-existing levels of testosterone production (Fig. 5). Absolute levels of plasma testosterone increased as a function of hCG in both saline- and ethanol-treated animals, indicating no effect of ethanol on pre-existing *in vivo* steroidogenic activity. It is interesting in this regard that Bhalla *et al.* [62] found that ethanol is incapable of dissociating the gonadotropin–testicular receptor complex *in vitro*, although it inhibits initial gonadotropin binding to testicular receptors.

When ethanol was administered prior to hCG stimulation, the inhibitory effect was greater than when hCG was administered prior to ethanol treatment (Table 1). These data support the contention that ethanol prevents gonadotropin-induced changes in *in vivo* steroidogenic activity, rather than inhibiting steroidogenic activity, *per se*.

Cicero *et al.* [26, 63] suggested that ethanol acted as a noncompetitive inhibitor with respect to gonadotropin stimulation. However, the timing of ethanol and gonadotropin administration was different between the present study and that of Cicero *et al.* [63]. In the present work, hCG was administered first, followed 60 min later by ethanol. Cicero *et al.* administered ethanol and gonadotropin simultaneously. Their protocol would not have detected an effect of gonadotropin dose on the testosterone response to ethanol, since (1) ethanol was always given during basal testosterone production; and (2) testosterone levels were measured at only one point after drug administration (not allowing for the detection of change in testosterone over time). On the other hand, Cicero *et al.* [26] did not detect an effect of 200 mM ethanol on basal testosterone production by Leydig cells (no added gonadotropin), which is in agreement with the *in vivo* data presented in Table 1.

The cause(s) of decreased testosterone between 60 and 150 min after administering high levels of hCG (i.e. 10 I.U.) is not entirely clear. Desensitization of Leydig cell responsiveness occurs after high gonadotropin doses but its onset is usually later than the times examined in the present study [64]. Similar decreases in testosterone have been reported in rats subsequent to high doses of gonadotropin [65]. Interestingly, the decrease in testosterone after 10 I.U. hCG (Fig. 5) was not as great in ethanol-treated as compared to saline-treated animals.

The depressant effects of ethanol on *in vivo* testosterone levels may result from an impaired expression of gonadotropin–testicular receptor interaction; this, of course, does not preclude effects of ethanol on steroidogenic events occurring subsequent to the gonadotropin–receptor interaction. A direct *in vivo* effect of ethanol on testicular gonadotropin receptors is supported by studies in which chronic ethanol ingestion resulted in reduced testicular gonadotropin receptor number [66]. Identification of the exact mechanism by which ethanol alters the steroidogenic response to gonadotropin stimulation, however, remains to be determined.

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