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ROLE OF NICOTINAMIDE ADENINE DINUCLEOTIDE IN ETHANOL-INDUCED DEPRESSIONS IN TESTICULAR STEROIDOGENESIS

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Abstract—It is rapidly becoming accepted, without direct evidence, that a change in the NAD^+/NADH ratio in the testes produced by the metabolism of ethanol is the principal mechanism involved in its now well-established effects on testicular steroidogenesis. The purposes of the present studies were 2-fold: (1) to examine whether, in fact, *in vivo* or *in vitro* ethanol exposure alters the NAD^+/NADH ratio in the testes; and (2) to examine the validity of previous reports in which it was found that NAD^+ prevented the effects of ethanol on testicular steoidogenesis under *in vitro* conditions. With regard to the first objective, we found that a large dose of ethanol (2.5 g/kg) markedly reduced gonadotropin-stimulated testicular steroidogenesis *in vivo* in the male rat, but it did not alter the NAD^+ and NADH concentrations in the testes. Similarly, extremely high ethanol concentrations (200 mM) substantially suppressed hMG-stimulated testosterone biosynthesis in *in vitro* Leydig cell preparations, but no change in NAD^+ concentration occurred; NADH levels were very low in the Leydig cell preparations (less than 2% of NAD^+ levels), but did not appear to change as a function of ethanol exposure. Finally, in contrast to previously published results, we found that NAD^+ (1 mM) did not prevent the *in vitro* effects of ethanol on cAMP-stimulated testicular steroidogenesis. Consequently, our results fail to support the hypothesis that acute *in vivo* or *in vitro* ethanol administration inhibits the biosynthesis of testosterone by altering the NAD^+/NADH ratio in the testes.

It has been demonstrated conclusively that ethanol inhibits testicular steroidogenesis under *in vivo* and *in vitro* conditions in the male of several species, including man [1–9]. However, there is an increasing body of evidence which suggests that ethanol *per se* is not a gonadal toxin, but that it must be metabolized to exert its effects on testicular steroidogenesis [3, 5, 8–19]. There are two consequences of the metabolism of ethanol which could be involved: the generation of the highly reactive and toxic metabolite, acetaldehyde; or a change in the NAD^+/NADH ratio in the testes. Although a large number of studies have indicated that acetaldehyde is a potent inhibitor of testicular steroidogenesis and, therefore, could mediate the effects of ethanol [2, 5–9, 12, 15], many investigators have concluded recently that a change in the NAD^+/NADH ratio in testes may be the primary mechanism involved [3, 13, 14, 16–19]. The evidence supporting this hypothesis is based upon three observations: (1) several key enzymes involved in testicular steroidogenesis are NAD^+-dependent [20]; (2) the metabolism of ethanol produces a change in the NAD^+/NADH ratio in several organs [19, 21]; and (3) NAD^+ has been reported to overcome the *in vivo* and *in vitro* effects of ethanol on testicular steroidogenesis [3, 13, 17]. On the basis of these observations, the hypothesis that a change in the NAD^+/NADH ratio may be involved in the effects of ethanol on the biosynthesis of testosterone not only seems plausible but has been supported by several studies. Surprisingly, however, we are unaware of any experiments in which the principal assumption underlying this hypothesis has been validated: i.e. that ethanol, under *in vivo* or *in vitro* conditions, alters the NAD^+/NADH ratio in the testes. In the absence of this information, it is difficult to assess the significance of those studes in which *presumed*, but not established, ethanol-induced deficits in NAD^+ have been eliminated by the addition of the cofactor.

The present studies were carried out to determine whether, in fact, *in vivo* or *in vitro* ethanol administration alters the NAD^+/NADH ratio in the testes of the male rat. An additional objective was to replicate earlier experiments [3, 17] in which it was found that NAD^+ overcame the *in vitro* effects of ethanol on cAMP-stimulated testicular steroidogenesis, since these studies seem to provide the most direct evidence that reductions in NAD^+ are involved in the effects of ethanol on testicular steroidogenesis.

METHODS

Animals and materials. Male Sprague–Dawley derived rats, 55–60 days of age, were utilized in all studies. In most of the studies reported in this paper, the rats were purchased from Harlan Sprague–Dawley, Inc., Indianapolis, IN, but in a few experiments animals were obtained from the Charles River Breeding Laboratories, Inc., Wilmington, MA. The

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rats were maintained in our laboratory on a 12-hr light : dark cycle (22–24°) for at least 1 week following their delivery. The following chemicals were purchased from the Sigma Chemical Co., St. Louis, MO: β-nicotinamide adenine dinucleotide (NAD^+), NADH, testosterone, dextran, Norit A activated charcoal, dibutyryl-cyclic adenosine monophosphate (cAMP), 3-isobutyl-1-methyl-xanthine (IBMX), beef serum albumin (BSA) and human chorionic gonadotropin (hCG). Collagenase (Type 1) was obtained from Worthington Biochemicals, Freehold, NJ, and medium 199, with Earle's modified salts, was purchased from GIBCO, Grand Island, NY. High pressure liquid chromatography (HPLC) grade water, acetonitrile (ACN) and ethyl acetate were obtained from the Baker Chemical Co., St. Louis, MO. [^{3}H]Testosterone (93.9 Ci/mmole) was purchased from the New England Nuclear Corp., Boston, MA. The human menopausal gonadotropin (hMG) used in these studies was the Second International Reference Protein and was provided by the National Institute for Medical Research, Mill Hill, London. The standard testosterone antibody used in our laboratory was generated in sheep to an 11-α-succinyl testosterone-BSA hapten and was provided by Dr. W. G. Wiest, Washington University School of Medicine, St. Louis, MO. In some studies, results with this antibody were compared to those obtained with a testosterone antibody, generated to a similar hapten, which was purchased from Micromedic, Inc., Fort Collins, CO.

Preparation of enzymatically-dispersed cells and in vitro *drug treatment.* Collagenase-dispersed cells (>90% Leydig cells) were prepared from the testes of drug-naive rats by modification of the method of Dufau *et al.* [22] which has been described elsewhere [5]. In those studies in which hMG was used to stimulate testicular steroidogenesis, intact enzymatically-dispersed cells were used, whereas in those studies using cAMP the cells were lysed by homogenizing them in Krebs–Ringer bicarbonate buffer (pH 7.6) containing 1 mg/ml glucose (hereafter designated as KRBG). Intact cells were suspended in medium 199-BSA (1.0%) at a concentration of 20×10^6 cells/ml, whereas lysed cells (40×10^6 cells/ml) were resuspended in KRBG. Aliquots of the cell suspensions were incubated in a final volume of 2 ml with the following additions (all concentrations are final): IBMX (115 μM), 0.75 mM cAMP (lysed cells) or 5 mIU hMG (intact cells), and, when appropriate, with ethanol (10 to 400 mM), acetaldehyde (0.025 to 0.5 mM), or NAD^+ (0.25 to 2.0 mM). In most studies, however, 1 mM NAD^+ was used since this concentration provided a 1000-fold excess of NAD^+ (normal concentrations of NAD^+ were less than 50 μM in the Leydig cell suspensions—see Fig. 2) and, in contrast to higher concentrations, it produced little or no inhibition of cAMP-stimulated testicular steroidogenesis. The incubation mixture was pipetted into 20 ml plastic scintillation vials which were thoroughly oxygenated (O_2:CO_2, 95%:5%) and tightly capped. The vials were then incubated at 37° in a shaking water bath for 2 hr. The vials were removed to an ice-water bath, aliquots were centrifuged (1600 *g* for 20 min), and the supernatant fluids were frozen at −20° until testosterone radioimmunoassays (RIAs) were carried out. Extreme care was taken to minimize the loss of ethanol and acetaldehyde during the incubation, and the concentrations of the drugs in the incubation media were determined as described elsewhere [5]. For the determination of NAD^+ and NADH concentrations in intact Leydig cells, 200 μl of 0.1 N HCl (for NAD^+) or 0.1 N NaOH, containing 0.5 mM cysteine (for NADH), was added to 200 μl of the Leydig cell suspensions. After standing at 4° for 12–18 hr, concentrations of NAD^+ and NADH were determined by enzymatic cycling as described elsewhere [23].

In vivo *drug treatment.* To assess the effects of ethanol on gonadotropin-stimulated testicular steroidogenesis *in vivo*, groups of rats were injected with saline or 5 IU hCG, subcutaneously. Half of the animals in each group were simultaneously (<15 sec) injected, intraperitoneally, with water or 2.5 g/kg ethanol. The animals were then killed 3 hr later, and blood was collected from the decapitated carcass. After standing at room temperature for at least 1 hr, sera were obtained by centrifuging the bloods at 1600 *g* for 20 min; the sera were frozen at −20° until testosterone levels were measured (see below). Blood ethanol levels were determined as described elsewhere [24]. The testes were also removed from these animals as quickly as possible (<30 sec) and frozen in liquid N_2. They were stored at −80° until they were assayed for NAD^+ and NADH as follows. The frozen tissue was homogenized at −20° in 20 vol. of 75% methanol containing 0.1 N KOH and 0.5 mM cysteine. Aliquots were then diluted another 20-fold in acid (0.01 N H_2SO_4, 0.1 N Na_2SO_4, 3 mM ascorbic acid) for NAD^+ or in alkali (0.02 N NaOH, 0.5 mM cysteine) for NADH. The acid samples were heated for 30 min at 60° and the alkaline samples for 10 min at the same temperature. Each was then analyzed for NAD^+ by enzymatic cycling [23] as in the case of the Leydig cells (see above). Since the testes represent a heterogeneous group of cells, it was possible that whole testicular measurements of NAD^+/NADH might obscure a difference occurring only in Leydig cells. To rule out this possibility, rats were treated with ethanol *in vivo* as described above, but in this case the testes were dispersed in collagenase to harvest Leydig cells. Half of the testes was dispersed only in collagenase, whereas the other half was dispersed in a collagenase medium, containing 200 mM ethanol. The latter procedure was employed in an effort to prevent a reversal of ethanol-induced alterations in the NAD^+/NADH ratio in the Leydig cells which could occur during the preparation of the cells in an ethanol-free medium. NAD^+/NADH levels were measured, as described above, in these preparations.

Determination of testosterone levels in media and sera. The levels of testosterone in sera were determined by a sensitive and specific testosterone RIA which has been described elsewhere [25]. Testosterone levels in *in vitro* incubation media were determined by RIA in unextracted dilutions of the media, in ethyl acetate extracts of the media, or in heptane : benzene [3] extracts. In several studies, ethyl acetate extracts of the media were subjected to

HPLC as follows. The dried extracts were resuspended in 150 μl of ACN, and 850 μl of water was added. The samples were transferred to a Baker-10 Extraction System; 1 ml octadecyl silane (ODS) columns were used which had been preconditioned with methanol and 15% ACN. After applying the samples under vacuum, the columns were washed twice with 1 ml of water, followed by 1 ml of 30% ACN. The columns were then eluted with 0.3 ml of 75% ACN three times. The combined eluates were dried under N_2 and dissolved in 40 μl of 75% ACN. A 20-μl sample was injected into the HPLC (Beckman 332 Gradient Liquid Chromatograph with a model 155 variable wavelength detector) which was pneumatically interfaced with an LKB 2112 Redirac fraction collector. A Beckman Ultrasphere-ODS column (5 μm particle size, 4.6 mm × 250 mm) was employed, and the gradient was constructed as follows: during the first 20 min, the ACN concentration increased linearly from 10 to 49% and then remained at 49% for 10 min. The gradient was again increased linearly to 75% ACN over 7.5 min, at which time it was held constant for 2.5 min and, then, increased to 100% over 2.5 min. The flow rate was 0.9 ml/min initially, but it was increased to 1.1 ml/min at 7.5 min. Fractions (0.5 min, 550 μl) were collected from 19 min to 46 min into the gradient. The column was allowed to equilibrate at 10% ACN for at least 15 min between injections. Testosterone standards were used to establish its retention time (≈26.6 min), and the steroid was detected by ultraviolet absorption at 230 nm. Trace amounts of labeled testosterone were used to calibrate the fraction collector. Retention times were checked daily prior to the injection of samples by chromatographing authentic testosterone standards. The HPLC fractions were dried under N_2 and assayed for testosterone by RIA (see above). Although the testosterone peak overlapped somewhat with estradiol, the testosterone antisera we employed showed no cross-reactivity with estradiol.

RESULTS

Effects of ethanol and acetaldehyde on cAMP-stimulated testicular steroidogenesis. The effects of ethanol and acetaldehyde on cAMP-stimulated testicular steroidogenesis in lysed Leydig cells are shown in Fig. 1. As can be seen, both ethanol and acetaldehyde markedly suppressed the biosynthesis of testosterone, but acetaldehyde was considerably more potent (>1000 times) than ethanol. The concentration of acetaldehyde producing the half-maximal suppression of cAMP-stimulated testicular steroidogenesis was approximately 90 μM, compared to the 140 mM for ethanol (as determined by log-probit plots of the data shown in Fig. 1).

Effects of ethanol on NAD+/NADH levels in testes. To determine whether *in vivo* or *in vitro* ethanol administration altered the NAD+/NADH ratio in the testes, two studies were carried out. In the first, enzymatically-dispersed cells, prepared from the testes of drug-naive rats, were incubated in the presence and absence of 200 mM ethanol; testicular steroidogenesis was stimulated by 5 mIU hMG. At the end of the 2-hr incubation period, testosterone levels in the media and NAD+/NADH levels in the intact cells were determined as described in Methods. In the second study, rats were injected with saline or hCG (5 IU); half of the animals in each group were simultaneously (<15 sec) injected with water or 2.5 g/kg ethanol. Three hours later, the rats were killed, and testosterone levels and the testicular contents of NAD+ and NADH were determined (see Methods). The concentration of ethanol used in the *in vitro* studies and the dose employed in the *in vivo* studies were selected, on the basis of previous work, to provide substantial decreases in hMG and hCG-stimulated testicular steroidogenesis (Refs. 5 and 2 respectively).

The results of these two studies are presented in Fig. 2. As shown in this figure, ethanol completely prevented hCG-stimulated testosterone production

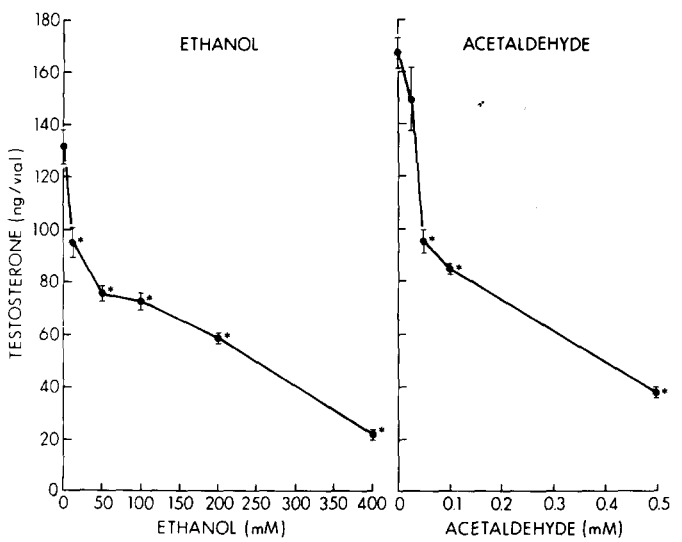

Fig. 1. Effects of ethanol and acetaldehyde on cAMP (0.75 mM)-stimulated testosterone production (ng/vial) in lysed Leydig cells prepared from drug-naive rats (see Methods). Values are means (± S.E.M.) of three experiments carried out in quadruplicate. Key: (*) significantly ($P < 0.01$) lower when compared to controls.

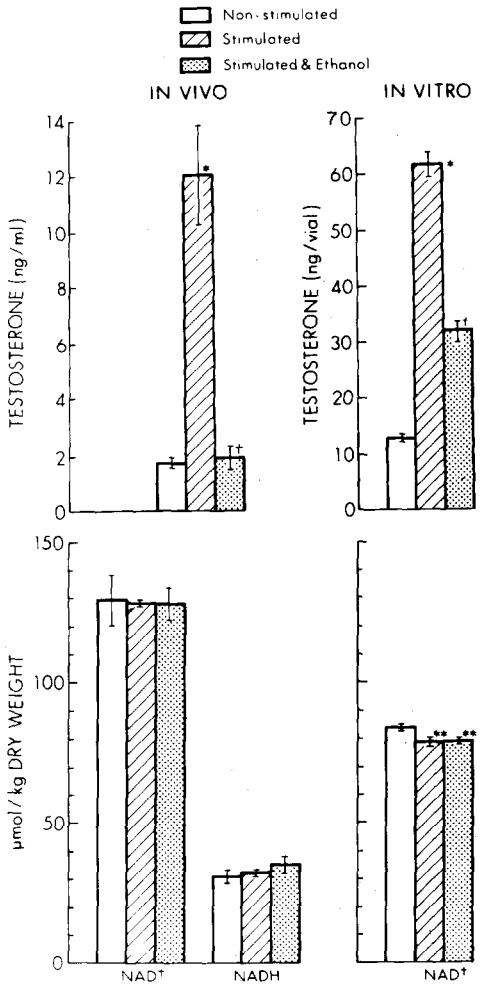

Fig. 2. Effects of ethanol on *in vivo* and *in vitro* gonadotropin-stimulated testicular steroidogenesis (top panels). Values are means (± S.E.M.) of six animals (*in vivo*) or six incubation vials (*in vitro*). In the bottom panels NAD^+ and NADH concentrations in the testes obtained from animals treated *in vivo* with ethanol are presented, as are the NAD^+ levels in ethanol-exposed Leydig cells. Values are means (± S.E.M.) of three determinations in the *in vivo* studies and six in the *in vitro* studies. See Methods for further details of the experimental design. Key: (*) significantly ($P < 0.001$) higher than the non-stimulated group; (**) significantly ($P < 0.05$) lower than the non-stimulated group; and (†) significantly ($P < 0.001$) lower than the gonadotropin-stimulated group (−ethanol).

under *in vivo* conditions (Fig. 2, top panel). However, there were no differences in the NAD^+ or NADH concentrations in whole testes (Fig. 2, bottom panel) or Leydig cells (Table 1) derived from these animals; in fact, in the Leydig cells, the NAD^+/NADH ratio tended to be higher in ethanol-treated rats than it was in saline-injected animals. In agreement with the *in vivo* studies, ethanol also significantly inhibited hMG-stimulated testicular steroidogenesis under *in vitro* conditions (Fig. 2, top panel), but there was no effect of this very high concentration of ethanol (200 mM) on NAD^+ concentrations in intact Leydig cells. In preliminary studies, it was also found that NADH concentrations were not altered by exposure of Leydig cells to ethanol, but the levels of the cofactor were so low (less than 2% of the NAD^+ levels) that they fell at the lower limits of detection in our assay system. For this reason, NADH concentrations were not measured in the studies depicted in Fig. 2.

It is interesting to note in Fig. 2 that NAD^+ concentrations were slightly reduced in hMG-stimulated Leydig cells, when compared to non-stimulated cells, but ethanol produced no additional decrease in the levels of the cofactor. A similar effect of gonadotropin stimulation on NAD^+/NADH levels was not observed in the *in vivo* studies (Fig. 2, bottom panel). The effects of ethanol on non-stimulated testosterone production have not been presented since these results simply confirmed previously published data [2, 5, 11].

Effects of NAD^+ on the suppression by ethanol of cAMP-stimulated testicular steroidogenesis. Although the preceding studies rule out any effect of ethanol on the NAD^+/NADH ratio in the testes, it has been reported [3, 17] that NAD^+ prevents the inhibition by ethanol of testicular steroidogenesis under *in vitro* conditions, presumably by reversing an ethanol-induced deficit in NAD^+. Since the studies shown in Fig. 2 clearly indicate that the fundamental assumption made in these studies was invalid, we attempted to resolve this paradox by replicating these earlier experiments. The results are shown in Fig. 3; the data presented in this figure represent the means of five separate experiments, with four incubation vials per treatment group, for a total N of 20. As can be seen, ethanol produced the expected dose-dependent decrease in cAMP-stimulated testicular steroidogenesis (see also Fig. 1). However, NAD^+ was completely ineffective in reversing the

Table 1. Effects of *in vivo* ethanol treatment on NAD^+ and NADH levels in the testes of male rats

Treatment	NAD^+*	NADH*	NAD^+/NADH ratio
Ethanol (2.5 g/kg)	0.5628 ± 0.009	0.0524 ± 0.003	10.911 ± 0.617
Water	0.5376 ± 0.017	0.0611 ± 0.005	9.082 ± 0.622

* Values are means ± S.E.M. of six determinations; the units are nmoles cofactor per mg total soluble protein. [Alternative means of expressing the data (e.g. μmoles NAD^+/NADH per kg dry wt or per 10^6 cells) produced equivalent results.] Values for cells harvested in collagenase media, with and without 200 mM ethanol, have been pooled, since no differences were observed between groups.

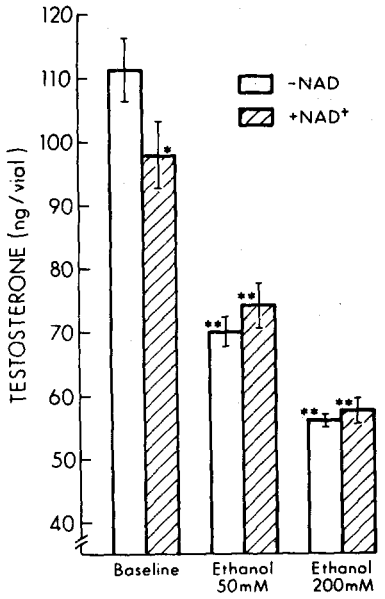

Fig. 3. Effects of NAD^+ (1 mM) on cAMP (0.75 mM)-stimulated testosterone production (ng/vial) in lysed enzymatically-dispersed cells obtained from the testes of drug-naive rats. cAMP-stimulated cells were incubated with and without NAD^+ and 0 (labeled baseline), 50 or 200 mM ethanol. Values are means ± S.E.M. of five experiments carried out in quadruplicate. Key (*) significantly ($P < 0.05$) lower than the baseline ($-NAD^+$) group; and (**) significantly ($P < 0.001$) lower than the baseline (+ or $-NAD^+$) groups.

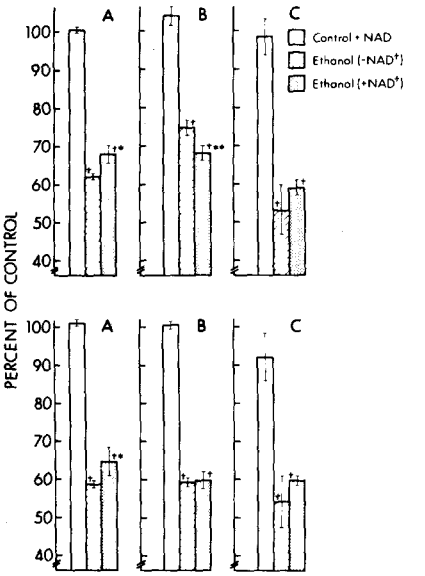

Fig. 4. Effects of NAD^+ (1 mM) on cAMP (0.75 mM)-stimulated testosterone production in lysed enzymatically-dispersed cells. cAMP-stimulated cells were incubated, with and without NAD^-, and 0 or 200 mM ethanol. Results are expressed as percentage of control (i.e. cAMP-stimulated cells incubated in the absence of NAD^+). Testosterone RIAs were carried out with the Micromedic testosterone antibody (top panel) and a testosterone antibody, generated in sheep to an 11-α-succinyl testosterone-BSA hapten, which is routinely used in our laboratory (bottom panel). Testosterone RIAs were carried out in heptane–benzene extracts of the media (column A), unextracted media (column B), or in ethyl acetate extracts of the media subjected to HPLC (column C). See Methods for details. Values are means ± S.E.M. of six determinations. Key: (†) significantly ($P < 0.001$) lower than control ($+NAD^+$); (*) significantly ($P < 0.05$) higher than ethanol ($-NAD^+$) group; and (**) significantly ($P < 0.05$) lower than the ethanol ($-NAD^+$) group.

effects of ethanol. Non-stimulated values have not been presented for the sake of clarity, particularly since similar results have been published elsewhere [5]. Moreover, in several additional studies, we employed different concentrations of NAD^+ (0.25 to 2.0 mM), and a more expanded ethanol concentration range (10 to 400 mM), but we were still unable to demonstrate any reversal of the effects of ethanol by NAD^+ (data not shown). It is interesting to note in Fig. 3 that NAD^+ slightly reduced cAMP-stimulated testicular steroidogenesis in control (i.e. 0 ethanol) Leydig cell preparations, but this was not consistently observed in all studies (see Fig. 4, for example).

Because our studies directly contradicted previously published results [3, 17], we carried out several additional experiments in an attempt to resolve this discrepancy. Since the main differences in the experiments were that different antibodies and testosterone extraction techniques were employed, we repeated the studies shown in Fig. 3, but in this case parallel testosterone RIAs, with both antibodies, were performed on unextracted dilutions of the *in vitro* incubation media, heptane–benzene extracts of the media (used by Ellingboe and Varanelli [3]) and, finally, ethyl acetate extracts of the media which were then subjected to HPLC to recover the testosterone peaks. The results are shown in Fig. 4. As can be seen, both antibodies (top panel: Micromedic antibody; bottom panel; our routine antibody) produced equivalent results under the three extraction conditions: ethanol suppressed cAMP-stimulated testicular steroidogenesis, but NAD^+ failed to overcome these effects. As shown in Fig. 4, under one extraction condition, NAD^+ seemed to slightly attenuate (5–10%) the effects of ethanol (panel A), whereas in the other two conditions, it either slightly exacerbated by 5–10% (panel B) or left unchanged (panel C) the effects of ethanol on cAMP-stimulated testicular steroidogenesis.

A final series of studies was carried out to eliminate the possibility that, although we and Ellingboe and Varanelli [3] used the same strain of rats, a difference in suppliers could be involved in the discrepancy between our two studies. In agreement with all of the studies described in this paper, we found equivalent results with either Charles River (Ellingboe and Varanelli) or the Harlan Sprague–Dawley derived rats routinely used in our laboratories (data not shown).

DISCUSSION

It has been shown conclusively that ethanol inhibits tesicular steroidogenesis under *in vivo* or *in vitro* conditions [1–9], but that it must be metabolized

to exert its effects [3, 5, 8–19]. There are two consequences of the metabolism of ethanol which could be involved: the production of acetaldehyde or a change in the NAD^+/NADH ratio in the testes.

The possibility that acetaldehyde may be involved in the effects of ethanol on the biosynthesis of testosterone has been suggested by the observation that acetaldehyde is a potent inhibitor of gonadotropin-stimulated testicular steroidogenesis under *in vitro* conditions at concentrations within the physiological range and that it is considerably more effective than ethanol (see Fig. 1 and Refs. 2, 4, 5–9, 12 and 15) which is effective only at extremely high, non-physiological concentrations. In addition, it has also been demonstrated that ethanol can be metabolized to acetaldehyde to a very small extent under *in vitro* conditions and that only those concentrations of ethanol which yield detectable levels of acetaldehyde produce significant inhibitions of testicular steroidogenesis [5]. Although these observations support a role for acetaldehyde in the actions of ethanol, they are for the most part indirect, and definitive studies have been very difficult to carry out.

The hypothesis that a change in the NAD^+/NADH ratio is the primary mechanism involved in the effects of ethanol on the biosynthesis of testosterone has recently gained widespread acceptance [3, 13, 14, 16–19]. This hypothesis is primarily based upon two related observations. First, Gordon *et al.* [13] found that the activity of an important NAD^+-dependent enzyme involved in the biosynthesis of testosterone, Δ5-3β-dehydrogenase [20], was reduced markedly in the testes of rats chronically exposed to ethanol. Since the addition of NAD^+ (1 mM) completely restored the activity of the enzyme to control levels, they suggested that the metabolism of ethanol resulted in a reduction in the NAD^+/NADH ratios in the testes, much as it does in several other organs [19, 21], and that this mechanism was responsible for the effects of ethanol on testicular steroidogenesis. Second, Ellingboe and coworkers [3, 17] showed that the inhibitory effects of ethanol on cAMP-stimulated testicular steroidogenesis *in vitro* were completely prevented by the addition of NAD^+ (2 mM). Although the results of these two studies appear to strongly support the hypothesis that an ethanol-induced depletion of NAD^+ is responsible for its effects on testicular steroidogenesis, there is a fundamental conceptual problem with them. That is, both groups of investigators make the explicit assumption that ethanol reduced testicular stores of NAD^+ which they, in turn, reversed by the addition of excess cofactor. However, since neither group directly demonstrated that ethanol lowered the NAD^+/NADH ratio in their preparations, and the present results (Fig. 2) clearly indicate that such changes do not take place, this assumption does not appear to be warranted.

The reasons for the discrepancy between the present results and the earlier work by Gordon *et al.* [13] and Ellingboe and coworkers [3, 17] are not known at the present time. However, in the case of the studies by Gordon *et al.* [13], they examined only the effects of *chronic* ethanol administration on the activities of several enzymes involved in testicular steroidogenesis, whereas our studies were confined to the acute effects of ethanol on the biosynthesis of testosterone and on the NAD^+/NADH ratio in the testes. Consequently, it is quite possible that chronic ethanol administration produces a change in the levels of these cofactors, but acute administration does not. However, it seems clear that the burden of proof must be placed on Gordon *et al.* [13] to demonstrate that chronic ethanol administration changes testicular NAD^+ concentrations before their NAD^+ replacement studies can be meaningfully interpreted.

It is recognized that NAD^+/NADH ratios based on total tissue concentrations (as reported here) are much greater than free nucleotide ratios calculated from lactate/pyruvate or malate/oxaloacetate ratios, etc., and their respective equilibrium constants [26]. These differences have been ascribed to disproportionate binding of NADH by the enzymes subserved. It is, therefore, possible that if a change in the free nucleotide ratio had occurred, the total nucleotides might not change in exact parallel. Nevertheless, some change should have occurred as has been shown for the liver after ethanol exposure [19, 21].

With respect to the studies carried out by Ellingboe and Varanelli [3], we attempted to replicate their observation that NAD^+ overcame the *in vitro* effects of ethanol on cAMP-stimulated testicular steroidogenesis, but we were unable to do so in five replicate experiments carried out in quadruplicate (Fig. 3). Moreover, in an effort to resolve the discrepancy between our studies, we carried out several experiments using their antibody, their testosterone extraction technique, and their supplier of rats. In all of these studies, we were still unable to show that NAD^+ prevented the effects of ethanol on testicular steroidogenesis (Fig. 4). The only other difference between our two studies was that we employed 1 mM NAD^+ in most of our experiments, whereas Ellingboe and Varanelli [3] used 2 mM. However, as discussed in Results, we examined the effects of a range of NAD^+ concentrations (including 2 mM) and still found no effect of the cofactor on ethanol-induced depressions in the biosynthesis of testosterone. The decision to use 1 mM in our studies was made on the basis of two considerations: (1) this concentration provided a 1000-fold excess of NAD^+ in the *in vitro* Leydig cell incubation (normal levels of NAD^+ were less than 50 μM) which was certainly more than ample to overcome any effect of ethanol on NAD^+ levels; and (2) we found that 2 mM NAD^+ alone produced substantial decreases in cAMP-stimulated testicular steroidogenesis which could introduce a potentially important confounding variable if it were employed. We can offer no explanation for the differences between the present studies and those of Ellingboe and Varanelli [3].

Our finding that the NAD^+/NADH ratio did not change in the testes under *in vitro* conditions, or after an acute *in vivo* injection of ethanol, suggests that only a limited degree of ethanol metabolism occurs in the testes under *in vivo* or *in vitro* conditions in the male rat. Since we have shown previously that there is little alcohol dehydrogenase activity in the testes and that the *in vitro* metabolism of ethanol by the testes occurs to a very limited extent [5], it may not be surprising that no change

in the NAD^+/NADH ratio was found in the present studies. Of course, it is not necessary to postulate that ethanol is metabolized in the testes to change the redox state of pyridine nucleotide cofactors since this could be the secondary result of changes occuring in some other organ. For example, it has been suggested that increased levels of lactate, produced by the metabolism of ethanol by the liver, could result in an enhanced oxidation of lactate by gonadal lactate dehydrogenase resulting in a reduction in the NAD^+/NADH ratio [13]. However, the present results are inconsistent with this interpretation, since we found no effect of acute *in vivo* ethanol administration on the NAD^+/NADH ratio in the testes.

In conclusion, although it is now widely accepted that a change in the NAD^+/NADH ratio is the primary mechanism involved in the effects of ethanol on testicular steroidogenesis, the present results do not support this hypothesis.

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