Inhibition of testosterone biosynthesis by ethanol

Relation to hepatic and testicular acetaldehyde, ketone bodies and cytosolic redox state in rats

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In experiments in which liver and testis freeze-stops were performed on pento-barbital-anaesthetized rats, ethanol (1.5 g/kg body wt.) reduced plasma testosterone concentration from 13.1 to 3.2 nmol/litre. 4-Methylpyrazole abolished the ethanol-induced hepatic and testicular increase in the lactate/pyruvate ratio, and the testicular acetaldehyde level, but did not diminish the reduction in plasma testosterone concentration. In testes, but not in liver, ethanol decreased the 3-hydroxybutyrate/acetoacetate ratio, and 4-methylpyrazole did not prevent this effect. In experiments in which freeze-stop was performed after cervical dislocation, ethanol decreased the testis testosterone concentration from 590 to 220 pmol per g wet wt. The effects of ethanol and 4-methylpyrazole on testis acetaldehyde, lactate/pyruvate and 3-hydroxybutyrate/acetoacetate ratios were the same as found during anaesthesia. The NAD⁺-dependent ethanol oxidation capacity in testis ranged from 0.1 to 0.2 μ mol/min per g wet wt. and seemed to be inhibited by 4-methylpyrazole both *in vivo* and *in vitro*. In additional experiments, ethanol doses between 0.3 and 0.9 g/kg body wt. did not alter the plasma testosterone concentration in rats treated, or not treated, with cyanamide, which induced elevated acetaldehyde levels in blood and testes. The results suggest that ethanol-induced inhibition of testosterone biosynthesis was not caused by extratesticular redox increases, or by extra- or intra-testicular acetaldehyde *per se*. The inhibition is accompanied by changes in testicular ketone-body metabolism.

Plasma testosterone concentration has been observed to become lower during acute ethanol intoxication in mice (Badr & Bartke, 1974; Badr *et al.*, 1977, 1979), rats (Cicero & Badger, 1977; Cicero *et al.*, 1978, 1979, 1981; Ellingboe & Varanelli, 1979) and humans (Rowe *et al.*, 1974; Ylikahri *et al.*, 1974; Dotson *et al.*, 1975; Mendelson *et al.*, 1977; Seto *et al.*, 1978). Inhibited testosterone biosynthesis during acute ethanol intoxication seems to be supported by several findings *in vitro* and *in vivo*. The data *in vitro* come from incubations of decapsulated mouse testes (Badr *et al.*, 1977), mouse (Yu *et al.*, 1981) or rat (Ellingboe & Varanelli, 1979; Cicero & Bell, 1980; Cicero *et al.*, 1980; Murono *et al.*, 1980) Leydig-cell preparations, and from perfused rat testis (Cobb *et al.*, 1980, 1981). The first observation *in vivo* suggesting inhibited testosterone biosynthesis was made by Badr *et al.* (1977), who found that the testosterone concentration was lowered in the testes, in addition

to plasma, during acute ethanol intoxication. This finding has been supported by rat studies, in which acute ethanol treatment inhibited human chorionic gonadotropin-stimulated testosterone production *in vivo* (Cicero *et al.*, 1979; Ellingboe & Varanelli, 1979; Cicero *et al.*, 1981).

Inhibition of testosterone biosynthesis during ethanol intoxication could, in principle, be mediated directly, or indirectly, by extra- or intra-testicular ethanol in itself, or by its metabolism. Studies *in vitro* have varied in their estimates of the minimal ethanol concentrations needed to inhibit testosterone biosynthesis: >0.22 mM (Murono *et al.*, 1980), >10 mM (Cobb *et al.*, 1981), >30 mM (Cicero & Bell, 1980; Cicero *et al.*, 1980), >65 mM (Ellingboe & Varanelli, 1979), >650 mM (Yu *et al.*, 1981) and >1.7 M (Badr *et al.*, 1977). None of these studies guaranteed that the inhibitory effect was caused by ethanol in itself. In fact, the metabolism of ethanol, or more specifically ethanol-derived

acetaldehyde (Badr *et al.*, 1977; Cobb *et al.*, 1978; Cicero & Bell, 1980; Cicero *et al.*, 1980), and ethanol-induced NAD⁺-deficiency (Ellingboe & Varanelli, 1979), have been suggested as primary causes for the inhibition. The basis for these suggestions was provided by observations that acetaldehyde directly inhibited testosterone biosynthesis and that the addition of NAD⁺ reversed the ethanol-induced inhibition. The role of competition for NAD⁺ is also supported by a previous observation by Van Thiel *et al.* (1974), who demonstrated that ethanol competitively inhibited the NAD⁺-dependent retinol oxidation in testis homogenates. In line with these considerations, Cicero *et al.* (1981) demonstrated in a recent study *in vivo* that pyrazole, which inhibits ethanol metabolism, decreased the ethanol-induced inhibition of testosterone production.

In the present study *in vivo* the role of acetaldehyde and ethanol-induced redox changes in testicular steroidogenesis was investigated by using the freeze-stop technique. To dissociate effects caused by ethanol itself from effects caused by its metabolism, 4-methylpyrazole, a rather specific and potent inhibitor of hepatic alcohol dehydrogenase (EC 1.1.1.1; Reynier, 1969), was used.

Materials and methods

Rats

Male rats belonging to a 'mixed strain' developed by Eriksson & Rusi (1981) were used in the first series of liver freeze-stop experiments. Male Long-Evans rats, bred in our laboratory (originating from rats purchased from Simonsen, Gilroy, CA, U.S.A.), were used in the other experiments. All animals were 2.5–3.5 months of age at the time of the experiments. Except for one series of experiments in which some animals received a special addition in the diet (see under 'General procedures'), the rats were given a standard laboratory diet (Astra-Ewos AB, Södertälje, Sweden) and water *ad lib*. No ethanol had been previously given to the rats.

General procedures

All experiments were performed between 08.00h and 11.00h. In the first series of experiments animals were divided into three groups: a control group that received no injections, an ethanol-treated group injected intraperitoneally with 1.5g of ethanol/kg body wt. as a 10% (w/v) solution in 0.9% NaCl, and a 4-methylpyrazole-plus-ethanol-treated group injected intraperitoneally with 10mg of 4-methylpyrazole/kg body wt. (Labkemi AB, Gothenburg, Sweden) as a 0.1% (w/v) solution in 0.9% NaCl 30min before the 1.5g of ethanol/kg injection. Pentobarbital (Nembutal R; Abbot S. A., Brussels, Belgium) at 40mg/kg body wt. was

injected intraperitoneally as a 1% (w/v) solution in 0.9% NaCl to all three groups of rats 1.75h after ethanol administration. Tail blood samples were taken 2h after ethanol administration. Immediately after blood sampling, one of the testes was frozen *in situ* by means of aluminium clamps precooled in liquid N₂. Immediately after this procedure part of the liver was freeze-clamped *in situ*. After liver freeze-stop an additional blood sample was taken by heart puncture.

In the second series of experiments a fourth group was added that received the 4-methylpyrazole but not the ethanol. Otherwise ethanol and 4-methylpyrazole treatments were the same as in the first series of experiments. No pentobarbital anaesthesia was, however, used. Instead, rats were cervically dislocated (2h after ethanol administration) after the tail-blood sampling and one of the testes was quickly excised and freeze-clamped. Elapsed time between dislocation and freeze-stop was 13s on average. The other testis was also excised (within 30s) and freeze-stopped by rapid immersion in liquid N₂.

In the third series of experiments rats were divided into two main groups, one receiving normal diet and one receiving cyanamide (as calcium carbimide; Dipsan R; Lederle Inc., Montreal, Que., Canada), a potent inhibitor *in vivo* of hepatic aldehyde dehydrogenase (EC 1.2.1.3) (Deitrich *et al.*, 1976), ground into the diet (0.2g/kg of diet: equivalent to a daily consumption of about 9mg/kg body wt.) for 5 days before the experiments. Ethanol doses of 0, 0.3, 0.6 and 0.9g/kg body wt. were administered by gastric intubation as 10% (w/v) solution in 0.9% NaCl to both groups of rats. Tail blood samples were taken 0.5, 1 and 2h after ethanol administration. After the last tail blood sample, rats were cervically dislocated and one of the testes was quickly excised and freeze-clamped. Additional blood samples were then taken by decapitation.

In addition to these main experiments, pilot tests in control animals compared the effects of 0.9% NaCl injections versus no treatment, and of freeze-clamping versus direct immersion in liquid N₂ (after dislocation).

Blood procedures

Tail blood was haemolysed with ice-cold water. The hemolysates were used directly for ethanol and acetaldehyde analyses. Blood taken by heart-puncture or after decapitation was centrifuged at 800g for 15min at 4°C. Plasma was removed and stored at -17°C for 4–5 weeks before testosterone analysis.

Freeze-stop procedures

The freeze-clamped livers and testes were pulverized in a mortar, and the tissue powder was suspended in ice-cold 0.6M-HClO₄, shaken and the

precipitate was centrifuged at 4000 *g* for 15 min at 4°C. Supernatants were used for ethanol, acetaldehyde, lactate, pyruvate, acetoacetate and 3-hydroxybutyrate analyses. Thiourea (25 mM) (E. Merck AG, Darmstadt, Germany) was added to the supernatants used for determining liver and testis concentrations of ethanol and acetaldehyde. The testes obtained by immersion in liquid N₂ were cut into two halves. One half of each testis was stored for 1–2 days at –17°C until alcohol and aldehyde dehydrogenase analyses. The other halves were stored for 6–7 weeks at –70°C before testosterone analysis.

Metabolite measurements

Ethanol and acetaldehyde were measured with a Perkin–Elmer F40 gas chromatograph, using the head-space technique described by Eriksson *et al.* (1977). This technique was also used for acetoacetate and 3-hydroxybutyrate, which were measured as acetone as previously described (Eriksson, 1972). Lactate and pyruvate were assayed enzymically by a method described by Hohorst *et al.* (1959). Enzymes and coenzymes were supplied by C. F. Boehringer, Mannheim, Germany.

Testosterone measurements

Plasma testosterone was measured as described by Ismail *et al.* (1972) with a radioimmunoassay kit supplied by Nordiclabb, Oulu, Finland. Testicular testosterone concentrations were measured as described by Hammond *et al.* (1978). Briefly, testis pieces (about 100 mg) were homogenized in water on ice and steroids were extracted with diethyl ether/ethyl acetate. The organic phases were chromatographed on 1 ml Lipidex-5000 columns, and testosterone was quantified from the appropriate fraction by a specific radioimmunoassay system (Jänne *et al.*, 1974). The recovery was monitored by inclusion of [³H]testosterone into each sample, and samples with recovery of less than 85% were re-analysed. Hence no correction for recovery was needed. The interassay coefficient of variation for testosterone assay was monitored by inclusion of a known serum sample into each series of 15 testis samples, and found to be 11%.

Determination of enzyme activities

Testes were homogenized in 4 vol. of 0.25 M-sucrose containing 1% (v/v) Triton X-100 (BDH Chemicals, Poole, Dorset, U.K.). The capacity of testis homogenates to utilize acetaldehyde in the presence of NAD⁺ was determined at 37°C in 50 mM-sodium/potassium phosphate buffer, pH 7.4, supplemented with 4 mM-NAD⁺ and an initial acetaldehyde concentration of 0.75 mM. The reaction was started by addition of 0.3 ml of homogenate and the final volume was 3.2 ml. Samples (0.5 ml) were

withdrawn at 2 min intervals, pipetted into serum bottles containing 3 M-HClO₄ (0.1 ml), and analysed for their acetaldehyde concentration by head-space gas chromatography (Eriksson *et al.*, 1977). Blanks with NAD⁺ omitted from the reaction mixture were subtracted from the results.

Alcohol dehydrogenase activities were measured spectrophotometrically (366 nm) in the soluble fraction of the homogenates (after 30 min centrifugation at 35 000 *g* at 4°C). NADH production was determined at 37°C in a buffer containing 74.7 mM-semicarbazide, 74.7 mM-sodium pyrophosphate, 8.9 mM-glycine, pH 7.4, supplemented with 1.6 mM-NAD⁺, with or without 2.9 mM-4-methylpyrazole, and with (total activity) or without (endogenous activity) 14.3 mM-ethanol. The reaction was started by addition of 0.3 ml of supernatant and the final volume was 3.5 ml. Alcohol dehydrogenase activities were calculated by subtracting values obtained with 4-methylpyrazole present from values obtained without 4-methylpyrazole addition.

Statistical analyses

The results are given as means ± s.d. Group mean comparisons were made with Student's *t*-distribution (Snedecor, 1961). Pearson *r*-coefficients (Senter, 1969) were used for correlation analyses.

Results

Effects of ethanol on plasma testosterone, and liver and testis metabolite concentration determined during anaesthesia

In the first series of experiments, the ethanol group showed a markedly decreased ($P < 0.001$) plasma testosterone concentration (3.2 ± 2.4 nM; $n = 22$) relative to the controls (13.1 ± 7.1 nM; $n = 10$). 4-Methylpyrazole treatment did not prevent the ethanol-induced testosterone decrease (3.3 ± 2.4 nM; $n = 7$). Pilot studies with control rats showed that the 0.9% NaCl injection alone had no effect on plasma testosterone concentration.

That 4-methylpyrazole at least partly inhibited ethanol metabolism is demonstrated in Table 1. Higher ethanol and lower acetaldehyde concentrations were observed in the blood, liver and testes of the 4-methylpyrazole-plus-ethanol-treated rats compared with the ethanol-treated group. In fact, blood and testis acetaldehyde levels were not significantly different from zero after 4-methylpyrazole treatment. The average testis acetaldehyde concentration during normal ethanol oxidation was only 14% of the corresponding blood acetaldehyde concentration and about one-third ($n = 7$) of the individual testis acetaldehyde values were below the detection level (< 2 nmol per g wet wt.).

Ethanol increased the hepatic redox (free NAD⁺/free NADH) ratios in both the cytoplasm (indicated

Table 1. *Effects of ethanol on metabolite concentrations determined during anaesthesia*

Values are means $\pm$ s.d. from tail blood, freeze-stopped livers and testes. Samples were taken 2 h after the ethanol (1.5 g/kg body wt.), 2.5 h after 4-methylpyrazole (10 mg/kg body wt.) and 0.25 h after the pentobarbital (40 mg/kg body wt.) injections.

Metabolite	Metabolite concn. (nmol/ml of blood or nmol/g wet wt. of tissue)		
	Control (n = 10)	Ethanol (n = 22)	4-Methylpyrazole + ethanol (n = 7)
Blood			
Ethanol		22 500 $\pm$ 5400‡	32 600 $\pm$ 3100†
Acetaldehyde		28 $\pm$ 24‡	0 $\pm$ 2†
Liver			
Ethanol		16 100 $\pm$ 4600	24 800 $\pm$ 4100†
Acetaldehyde		109 $\pm$ 45	8 $\pm$ 7†
Lactate	1380 $\pm$ 430	1110 $\pm$ 330	530 $\pm$ 140*†
Pyruvate	202 $\pm$ 73	64 $\pm$ 31*	69 $\pm$ 17*
3-Hydroxybutyrate	232 $\pm$ 118	222 $\pm$ 66	226 $\pm$ 119
Acetoacetate	163 $\pm$ 44	104 $\pm$ 23*	271 $\pm$ 80†
Testis			
Ethanol		20 400 $\pm$ 7500	29 700 $\pm$ 4500†
Acetaldehyde		4 $\pm$ 4‡	2 $\pm$ 4
Lactate	1840 $\pm$ 480	1400 $\pm$ 340	1580 $\pm$ 370‡
Pyruvate	96 $\pm$ 30‡	55 $\pm$ 11*	84 $\pm$ 14†
3-Hydroxybutyrate	491 $\pm$ 80‡	284 $\pm$ 113*	255 $\pm$ 96*
Acetoacetate	52 $\pm$ 15‡	86 $\pm$ 20*	108 $\pm$ 33*‡

* $P < 0.001$, compared with the control group.

† $P < 0.001$, compared with the ethanol-treated group.

‡ $P < 0.001$, compared with corresponding hepatic means.

Table 2. *Effects of ethanol on redox ratios determined during anaesthesia*
Conditions are as in Table 1.

Redox ratio	Redox ratio		
	Control	Ethanol	4-Methylpyrazole + ethanol
Liver			
Lactate/pyruvate	7.0 $\pm$ 1.3	23.3 $\pm$ 15.6*	8.0 $\pm$ 2.9
3-Hydroxybutyrate/acetoacetate	1.5 $\pm$ 0.9	2.3 $\pm$ 0.8	0.9 $\pm$ 0.7†
Testis			
Lactate/pyruvate	19.9 $\pm$ 4.0‡	26.3 $\pm$ 7.5*	18.6 $\pm$ 2.1†‡
3-Hydroxybutyrate/acetoacetate	10.2 $\pm$ 3.2‡	3.4 $\pm$ 1.8*	2.9 $\pm$ 1.7*

by the lactate/pyruvate ratio) and the mitochondria (indicated by the 3-hydroxybutyrate/acetoacetate ratio) (Table 2). Again, 4-methylpyrazole proved its efficiency in inhibiting intermediary effects of ethanol metabolism by blocking the ethanol-induced hepatic redox increases. In testes, the control redox ratios were considerably higher than in the liver. As in the liver, ethanol did increase the lactate/pyruvate ratio in the testis, and 4-methylpyrazole blocked this effect. The possibility that the testis lactate/pyruvate changes merely reflected hepatic changes was,

however, indicated by the correlation ($r = 0.614$, $P < 0.01$) between the liver and testis lactate/pyruvate ratios. In contrast with the hepatic and testicular lactate/pyruvate ratio, and hepatic 3-hydroxybutyrate/acetoacetate changes, the testis 3-hydroxybutyrate/acetoacetate ratio decreased during ethanol oxidation and, furthermore, 4-methylpyrazole did not prevent this effect. The fall in testis 3-hydroxybutyrate/acetoacetate ratio was the combined result of decreased 3-hydroxybutyrate and increased acetoacetate concentrations (Tables 1 and

Table 3. *Effects of ethanol in testis*

Values are means from freeze-stopped testes after cervical dislocation (no anaesthesia). Other conditions are as in Table 1, except that significance here denotes $P < 0.05$.

Parameter	Control (<i>n</i> = 10)	Control + 4-methylpyrazole (<i>n</i> = 9)	Ethanol (<i>n</i> = 10)	4-Methylpyrazole + ethanol (<i>n</i> = 10)	Concentration unit (per g wet wt. of testis)
Testosterone	590 ± 350	690 ± 210 (<i>n</i> = 8)	220 ± 150*	180 ± 110*	pmol
Ethanol			26.2 ± 6.6	33.2 ± 5.2†	μmol
Acetaldehyde			1 ± 2	2 ± 2	nmol
Lactate	900 ± 170	810 ± 120	920 ± 170	930 ± 180	nmol
Pyruvate	49 ± 10	71 ± 26*	41 ± 14	69 ± 22*†	nmol
Lactate/pyruvate	16.9 ± 2.3	13.8 ± 6.4	23.3 ± 9.3*	16.0 ± 7.0	
3-Hydroxybutyrate	800 ± 310	792 ± 428	445 ± 154*	484 ± 216*	nmol
Acetoacetate	125 ± 70	127 ± 70	123 ± 45	173 ± 66	nmol
3-Hydroxybutyrate/ acetoacetate	8.0 ± 4.4	7.1 ± 4.2	4.1 ± 2.3*	3.3 ± 1.8*	

2). The increased acetoacetate thus seemed to be the result of increased testicular 3-hydroxybutyrate oxidation. The role of the hepatic ketone-body metabolism in determining testicular ketone-body concentrations was demonstrated by the positive correlations between hepatic and testicular acetoacetate (control: $r = 0.723$, $P < 0.05$; ethanol: $r = 0.667$, $P < 0.001$; 4-methylpyrazole + ethanol: $r = 0.930$, $P < 0.01$) and 3-hydroxybutyrate (control: $r = 0.372$, not significant; ethanol: $r = 0.763$, $P < 0.001$; 4-methylpyrazole + ethanol: $r = 0.880$, $P < 0.01$) concentrations. In addition, testicular acetoacetate concentration correlated positively in the ethanol-treated group ($r = 0.444$, $P < 0.05$) and ethanol-plus-4-methylpyrazole-treated group ($r = 0.622$, not significant) but negatively in the control group ($r = -0.751$, $P < 0.05$) with plasma testosterone concentration. Testosterone concentration did not correlate with any other metabolite concentration measured in any of the groups.

Effect of ethanol on testicular testosterone and metabolite concentration determined after cervical dislocation

The results obtained in the first series of experiments could, in theory, have been partially affected by the 15 min period of pentobarbital anaesthesia. Thus experiments were designed in which cervical dislocation followed by rapid freeze-stop were used. To check if 4-methylpyrazole had any effects of its own, an additional control group receiving only 4-methylpyrazole was included. In the pilot studies with control rats it was observed that the lactate/pyruvate ratio tended to be higher in testes freeze-stopped by immersion in liquid N₂ than in testes freeze-clamped directly (13 s) after dislocation. Thus only the testes obtained by direct freeze-clamping were used in the metabolite experiments.

Testicular testosterone concentrations are listed in Table 3. In accord with the plasma changes seen in

the first series of experiments, ethanol markedly decreased the testicular testosterone concentration, and 4-methylpyrazole did not prevent this effect. Neither did 4-methylpyrazole alone have any effect on testosterone concentration.

4-Methylpyrazole plus ethanol treatment increased the ethanol concentration more than ethanol treatment alone in both blood (from 22.0 ± 5.3 to 29.2 ± 4.8 mM, $P < 0.01$) and in testes (Table 3). Significant acetaldehyde levels were not detected in blood or testes in any of the groups (in contrast with the previous experiments, in which pentobarbital anaesthesia was used).

As demonstrated in Table 3, the testicular lactate/pyruvate ratio increased and 3-hydroxybutyrate/acetoacetate ratio decreased during ethanol oxidation, and 4-methylpyrazole blocked the former but not the latter effect (in accordance with the first series of experiments). 4-Methylpyrazole treatment alone had no significant effect on these redox metabolites. In these experiments no significant ethanol-induced increase of acetoacetate was observed in testes. The acetoacetate also did not correlate significantly with the testosterone concentration (control: $r = -0.245$; 4-methylpyrazole: $r = -0.525$; ethanol: $r = 0.001$; 4-methylpyrazole + ethanol: $r = 0.199$).

The acetaldehyde elimination capacities are listed in Table 4. No group differences were observed. Neither were any significant differences observed in the total alcohol dehydrogenase activities in the presence of a surplus of ethanol added *in vitro* (Table 4). The highest endogenous alcohol dehydrogenase activity was observed in the ethanol-treated group, which obviously was due to the remaining presence of a fraction (0.45 mM) of the ethanol *in vivo*. In spite of the same (or even higher) residue of ethanol *in vivo* also in the testis preparations from animals treated with 4-methylpyrazole plus ethanol, only low endogenous

Table 4. *Effects of ethanol and 4-methylpyrazole on testicular aldehyde dehydrogenase and alcohol dehydrogenase activities*

Aldehyde dehydrogenase (ALDH) activities were determined (as acetaldehyde elimination rates) in testis homogenates after addition of 0.7 mM-acetaldehyde. Alcohol dehydrogenase (ADH) activities were determined (as NADH formation rates) in the soluble fraction of the homogenates after addition of 14.3 mM-ethanol (total activity) or without added substrate (endogenous activity). Numbers in parentheses indicate numbers of rats.

	Activity (nmol/min per g wet wt. of testis)			
	Control	Control + 4-methylpyrazole	Ethanol	4-Methylpyrazole + ethanol
ALDH activity	770 ± 180 (9)	740 ± 270 (9)	810 ± 190 (9)	670 ± 150 (10)
Total ADH activity	126 ± 28 (5)	173 ± 60 (6)	134 ± 62 (7)	126 ± 47 (8)
Endogenous ADH activity	31 ± 17 (7)	12 ± 37 (6)	74 ± 38 (7)*	8 ± 9 (7)†

* $P < 0.025$, compared with the control group.

† $P < 0.001$, compared with the ethanol-treated group.

dehydrogenase activity was found in these preparations (Table 4), which obviously was due to the presence of some 4-methylpyrazole (or derivative) left from the treatment *in vivo*.

Effect of elevated acetaldehyde on testosterone concentrations

To further test the hypothesis that acetaldehyde is the testicular toxin acting after ethanol ingestion, an additional series of experiments was designed. The idea was to test ethanol doses that in themselves would not lower the testosterone levels, together with an acetaldehyde concentration *in vivo* which was elevated as a result of cyanamide to such a degree that acetaldehyde would appear also in testes. The results are shown in Table 5. No significant effects on plasma testosterone concentration were observed in any of the groups. There were no significant acetaldehyde concentrations observed in the testes or blood of the control animals receiving only the acute ethanol doses. In the cyanamide-treated rats an ethanol dose-dependent elevation in the acetaldehyde concentration was observed. With the highest ethanol dose (0.9 g/kg body wt.) acetaldehyde appeared also in the testes. Blood samples taken 30 min after ethanol administration displayed blood acetaldehyde concentrations of 95 ± 90 (ethanol dose: 0.3 g/kg body wt.), 207 ± 75 (0.6 g/kg) and $226 \pm 119 \mu\text{M}$ (0.9 g per kg body wt.). Since these blood acetaldehyde levels are much higher than those found 2 h after ethanol administration (Table 5), it may be concluded that earlier during ethanol intoxication testis acetaldehyde had also been higher than after 2 h. In addition, it may be noted that the elevated acetaldehyde concentrations appeared in connection with a decreased rate of ethanol metabolism, as demonstrated by the elevated ethanol concentrations in the cyanamide groups (Table 5).

Discussion

The present observation, that acute ethanol doses of 0.3–0.9 g/kg body wt. did not reduce plasma testosterone concentration, and that the ethanol dose of 1.5 g/kg did reduce it, is in agreement with previous studies on rats (Cicero & Badger, 1977; Cicero *et al.*, 1978, 1979, 1981; Ellingboe & Varanelli, 1979). However, we did not observe the increased testosterone concentration after a lower (0.75 g/kg) ethanol dose, as reported by Cicero & Badger (1977) and Cicero *et al.* (1978). Mice seem to be more sensitive than rats to this effect of ethanol, since doses of only 0.16–1.24 g/kg body wt. have been reported to decrease their plasma testosterone concentration (Badr & Bartke, 1974); the reason for their greater sensitivity is unknown. The reason for the conflicting results regarding human testosterone concentration during acute ethanol intoxication in non-alcoholic subjects is also unknown [a decrease has been reported by Rowe *et al.* (1974), Ylikahri *et al.* (1974), Mendelson *et al.* (1977) and Seto *et al.* (1978), no change by Toro *et al.* (1973), Ylikahri *et al.* (1978) and Linnoila *et al.* (1980) and a biphasic effect by Dotson *et al.* (1975)].

The present results regarding ethanol-induced decreased testosterone concentrations in freeze-stopped testes seem to eliminate increased hepatic metabolism, increased peripheral uptake and inhibited secretion from the testes as being the principal causes for decreased plasma testosterone concentration. Instead, inhibited testicular testosterone biosynthesis seems to be the main cause for the testosterone reduction. No previous reports have been published about rat testis testosterone concentration during acute ethanol intoxication. Our data are in accord with those from a previous study on mice, in which an additional ethanol dose (after 4

Table 5. *Effects of elevated acetaldehyde on testosterone concentration*

Values are mean concentrations ($n=6-8$) $\pm$ s.d. determined in tail blood, plasma of heart blood and freeze-clamped testis (wet wt.), 2h after administration of different doses of ethanol in rats without (controls) or with (cyanamide treatment) inhibited aldehyde dehydrogenase.

Ethanol dose (g/kg body wt.)	Plasma testosterone (pmol/ml)	Testis		Tail blood	
		Acetaldehyde (nmol/g)	Ethanol (μ mol/g)	Acetaldehyde (nmol/ml)	Ethanol (μ mol/ml)
Control					
0	16 $\pm$ 12				
0.3	14 $\pm$ 9	1 $\pm$ 2	1.2 $\pm$ 0.8	0 $\pm$ 0	0.8 $\pm$ 1.2
0.6	9 $\pm$ 6	1 $\pm$ 1	1.0 $\pm$ 0.9	0 $\pm$ 0	0.4 $\pm$ 0.6
0.9	14 $\pm$ 15	1 $\pm$ 1	2.3 $\pm$ 2.1	0 $\pm$ 1	2.6 $\pm$ 2.8
Cyanamide					
0	32 $\pm$ 28				
0.3	17 $\pm$ 14	0 $\pm$ 0	0.8 $\pm$ 0.5	4 $\pm$ 3*	0.5 $\pm$ 1.0
0.6	17 $\pm$ 24	3 $\pm$ 4	3.4 $\pm$ 1.2†	48 $\pm$ 31†	3.6 $\pm$ 1.7†
0.9	13 $\pm$ 9	7 $\pm$ 6*	6.7 $\pm$ 5.0*	86 $\pm$ 38‡	7.1 $\pm$ 3.3*

* $P < 0.05$, compared with the control group.

† $P < 0.005$, compared with the control group.

‡ $P < 0.001$, compared with the control group.

days of preceding exposure to ethanol) decreased testicular testosterone concentrations (Badr *et al.*, 1977).

The conclusion from several studies *in vitro*, in which acetaldehyde concentrations of 100mM (Badr *et al.*, 1977), 100 μ M (Cicero & Bell, 1980) and 50 μ M (Cicero *et al.*, 1980) have inhibited testosterone biosynthesis, has been that acetaldehyde rather than ethanol may be the primary mediator of inhibited steroidogenesis in rats. However, the results below from the present study argue against a role *in vivo* for extra- or intra-testicular acetaldehyde in itself. 1. The testicular acetaldehyde concentration seems extremely low, in many cases absent, during normal ethanol intoxication. 2. 4-Methylpyrazole treatment had no effect on the testosterone decrease, even though it completely abolished the blood and testicular acetaldehyde concentration. 3. Elevated blood and testicular acetaldehyde concentrations at lower ethanol doses had no significant effect on plasma testosterone concentration. In addition to the exclusion of acetaldehyde as responsible for inhibited testosterone biosynthesis, the hepatic redox shifts (increased cytosolic and mitochondrial free NADH/free NAD⁺ ratios) may be excluded as extra-testicular mediators. This follows from the finding that the 4-methylpyrazole treatment abolished these redox changes (determined as changes in hepatic lactate/pyruvate and 3-hydroxybutyrate/acetoacetate ratios) without having any effect on the testosterone decreases. Taken together, the present acetaldehyde and hepatic redox data seem to show that extratesticular (hepatic) ethanol metabolism is

not the primary cause for inhibited testicular steroidogenesis.

Two previous studies, one *in vitro* (Cobb *et al.*, 1978) and one *in vivo* (Cicero *et al.*, 1981), report that pyrazole (*in vivo*) and 4-methylpyrazole (*in vitro*) attenuate the ethanol-induced inhibition of testosterone biosynthesis. The present results *in vivo*, in which 4-methylpyrazole did not prevent the lowering of plasma or testis testosterone concentration, do not support the previous findings. One possible explanation may be that both Cobb *et al.* (1978) and Cicero *et al.* (1981) investigated human chorionadotropin-stimulated steroidogenesis. Thus the highly increased testosterone biosynthesis may have become sensitized to competitive inhibition by testicular ethanol metabolism. Another possibility would, in principle, be that our 4-methylpyrazole treatment, although inhibiting hepatic ethanol metabolism, did not inhibit testicular ethanol metabolism enough to prevent some mechanism other than acetaldehyde [such as increased NADH/NAD⁺ ratio (Ellingboe & Varanelli, 1979)] from inhibiting testosterone biosynthesis. This possibility is, however, not supported by the efficient inhibition of endogenous ethanol-dependent NADH formation in testis preparations from the 4-methylpyrazole-treated animals (Table 4). It is not known, however, to what extent, if at all, the measured alcohol dehydrogenase activity represented the functional enzyme activity responsible for possible ethanol metabolism in the Leydig cells. In this connection it should be noted that pyrazole-sensitive alcohol dehydrogenase activity has been previously observed in testis (Erwin & Deitrich, 1972) and that in a

preliminary communication it has been reported that testicular alcohol dehydrogenase activity may be confined to the interstitial cells (Messiha *et al.*, 1980). That 4-methylpyrazole treatment blocked the ethanol-induced increase in testicular lactate/pyruvate ratio also argues against the role of an increased NADH/NAD⁺ ratio being responsible for the inhibition of the steroidogenesis. On the other hand, there is no evidence that the whole-testis lactate/pyruvate ratio really represents the functional free NADH/free NAD⁺ ratio at the site of the steroidogenesis, rather than merely being a reflection of the corresponding hepatic redox change (as supported by the correlation between hepatic and testicular lactate/pyruvate ratio during ethanol intoxication).

The shift to a lower testicular 3-hydroxybutyrate/acetoacetate ratio during ethanol intoxication is difficult to understand. Nothing is known about the intratesticular localization and functional activity of 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30). Neither is it known whether testicular ketone bodies may serve as precursors for steroidogenesis. In theory, it could be possible for testicular acetoacetate to become activated and enter the cholesterol pathway leading to pregnenolone and testosterone synthesis in testis. If this were true, the decreased total ketone-body concentration in testis during ethanol intoxication (with and without 4-methylpyrazole) may have contributed to decreased testosterone production. This argument is supported by the positive correlation (significant in the first series of experiments) between testicular acetoacetate and plasma testosterone concentration during ethanol oxidation. Whether there is a meaningful relationship between decreased testosterone biosynthesis and the ketone-body changes during ethanol intoxication is not clear from the present investigation, but may prove to be a challenging target for further investigations.

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References

- Badr, F. M. & Bartke, A. (1974) *Steroids* **23**, 921–928
- Badr, F. M., Bartke, A., Dalterio, S. & Bulger, W. (1977) *Steroids* **30**, 647–655
- Badr, F. M., Smith, M. S., Dalterio, S. L. & Bartke, A. (1979) *Steroids* **34**, 477–482
- Cicero, T. J. & Badger, T. M. (1977) *J. Pharmacol. Exp. Ther.* **201**, 427–433
- Cicero, T. J. & Bell, R. D. (1980) *Biochem. Biophys. Res. Commun.* **94**, 814–819
- Cicero, T. J., Bernstein, D. & Badger, T. M. (1978) *Alcoholism* **2**, 249–254
- Cicero, T. J., Meyer, E. R. & Bell, R. D. (1979) *J. Pharmacol. Exp. Ther.* **208**, 210–215
- Cicero, T. J., Bell, R. D., Meyer, E. R. & Badger, T. M. (1980) *J. Pharmacol. Exp. Ther.* **213**, 228–233
- Cicero, T. J., Newman, K. S. & Meyer, E. R. (1981) *Life Sci.* **28**, 871–877
- Cobb, C. F., Van Thiel, D. H., Ennis, M. F., Gavalier, J. S. & Lester, R. (1978) *Gastroenterology* **75**, 958
- Cobb, C. F., Ennis, M. F., Van Thiel, D. H., Gavalier, J. S. & Lester, R. (1980) *Metab. Clin. Exp.* **29**, 71–79
- Cobb, C. F., Gavalier, J. S., Van Thiel, D. H. (1981) *Clin. Toxicol.* **18**, 149–154
- Deitrich, R. A., Troxell, P. A. & Worth, W. S. (1976) *Biochem. Pharmacol.* **25**, 2733–2737
- Dotson, L. E., Robertson, L. S. & Tuchfeld, B. (1975) *J. Studies Alcohol* **36**, 578–586
- Ellingboe, J. & Varanelli, C. C. (1979) *Res. Commun. Chem. Pathol. Pharmacol.* **24**, 87–102
- Eriksson, C. J. P. (1972) *Anal. Biochem.* **47**, 235–242
- Eriksson, C. J. P., Sippel, H. W. & Forsander, O. A. (1977) *Anal. Biochem.* **80**, 116–124
- Eriksson, K. & Rusi, M. (1981) in *Development of Animal Models as Pharmacogenetic Tools: Proc. Workshop, Boulder, CO* (McClern, G. E., Deitrich, R. A. & Erwin, V. G., eds.), pp. 87–117, NIAA Research Monograph-6, US Government Printing Office, Washington, DC
- Erwin, V. G. & Deitrich, R. A. (1972) *Biochem. Pharmacol.* **21**, 2915–2924
- Hammond, G. L., Ahonen, V. & Vihko, R. (1978) *Int. J. Androl. Suppl.* **2**, 391–399
- Hohorst, H. J., Kreutz, F. H. & Bücher, T. (1959) *Biochem. Z.* **332**, 18–46
- Ismail, A. A., Niswender, G. D. & Midgley, A. R. (1972) *J. Clin. Endocrinol. Metab.* **34**, 177–184
- Jänne, O., Apter, D. & Vihko, R. (1974) *J. Steroid Biochem.* **5**, 155–162
- Linnoila, M., Prinz, P. N., Wonsowicz, C. J. & Lepäluoto, J. (1980) *Br. J. Addiction* **75**, 207–212
- Mendelson, J. H., Mello, N. K. & Ellingboe, J. (1977) *J. Pharmacol. Exp. Ther.* **202**, 676–682
- Messiha, F. S., Hutson, J., Webb, J. & Wilkinson, T. (1980) *Pharmacol. Biochem. Behav.* **12**, 325
- Murono, E. P., Lin, T., Osterman, J. & Nankin, H. R. (1980) *Steroids* **36**, 619–631
- Reynier, M. (1969) *Acta Chem. Scand.* **23**, 1119–1129
- Rowe, P. H., Racey, P. A., Shenton, J. C., Ellwood, M. & Lehane, J. (1974) *J. Endocrinol.* **63**, 50p
- Senter, R. J. (1969) *Analysis of Data*, p. 403, Scott, Foresman and Company, Glenview
- Seto, A., Tricomi, S., Goodwin, D. W., Kolodney, R. & Sullivan, T. (1978) *J. Studies Alcohol* **39**, 1–11
- Snedecor, G. W. (1961) *Statistical Methods*, 5th edn., p. 35, Iowa State University Press, Ames
- Toro, G., Kolodny, R. C., Jacobs, L. S., Masters, W. H. & Daughaday, W. H. (1973) *Clin. Res.* **21**, 505
- Van Thiel, D. H., Gavalier, J. & Lester, R. (1974) *Science* **186**, 941–942
- Ylikahri, R., Huttunen, M., Härkönen, M., Seuderling, U., Onikki, S., Karonen, S.-L. & Adlercreutz, H. (1974) *J. Steroid Biochem.* **5**, 655–658
- Ylikahri, R. H., Huttunen, M. O., Härkönen, M., Leino, T., Helenius, T., Liewendahl, K. & Karonen, S.-L. (1978) *J. Clin. Endocrinol. Metab.* **46**, 715–720
- Yu, J. Y.-L., Roan, S.-Y., Hsu, Z.-S. & Wan, W. C.-M. (1981) *Bull. Inst. Zool. Acad. Sin.* **20**, 67–74