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Metabolic Alterations Produced in the Liver by Chronic Ethanol Administration

INCREASED OXIDATIVE CAPACITY

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1. Administration of ethanol (14g/day per kg) for 21–26 days to rats increases the ability of the animals to metabolize ethanol, without concomitant changes in the activities of liver alcohol dehydrogenase or catalase. 2. Liver slices from rats chronically treated with ethanol showed a significant increase (40–60%) in the rate of O_2 consumption over that of slices from control animals. The effect of uncoupling agents such as dinitrophenol and arsenate was completely lost after chronic treatment with ethanol. 3. Isolated mitochondria prepared from animals chronically treated with ethanol showed no changes in state 3 or state 4 respiration, ADP/O ratio, respiratory control ratio or in the dinitrophenol effect when succinate was used as substrate. With β-hydroxybutyrate as substrate a small but statistically significant decrease was found in the ADP/O ratio but not in the other parameters or in the dinitrophenol effect. Further, no changes in mitochondrial Mg^{2+}-activated adenosine triphosphatase, dinitrophenol-activated adenosine triphosphatase or in the dinitrophenol-activated adenosine triphosphatase/Mg^{2+}-activated adenosine triphosphatase ratio were found as a result of the chronic ethanol treatment. 4. Liver microsomal NADPH oxidase activity, a H_2O_2-producing system, was increased by 80–100% by chronic ethanol treatment. Oxidation of formate to CO_2 *in vivo* was also increased in these animals. The increase in formate metabolism could theoretically be accounted for by an increased production of H_2O_2 by the NADPH oxidase system plus formate peroxidation by catalase. However, an increased production of H_2O_2 and oxidation of ethanol by the catalase system could not account for more than 10–20% of the increased ethanol metabolism in the animals chronically treated with ethanol. 5. Results presented indicate that chronic ethanol ingestion results in a faster mitochondrial O_2 consumption *in situ* suggesting a faster NADH reoxidation. Although only a minor change in mitochondrial coupling was observed with isolated mitochondria, the possibility of an uncoupling in the intact cell cannot be completely discarded. Regardless of the mechanism, these changes could lead to an increased metabolism of ethanol and of other endogenous substrates.

Previous studies by our group (Videla & Israel, 1970) indicate that the rate of ethanol metabolism by the liver depends on the rate of mitochondrial reoxidation of the cytoplasmic NADH produced in the oxidation of ethanol. Uncoupling agents such as 2,4-dinitrophenol or arsenate were shown to increase markedly the rate of ethanol metabolism by normal rat liver slices. Dinitrophenol was also shown to be effective in increasing the rate of ethanol metabolism *in vivo* (Israel *et al.*, 1970). However, although dinitrophenol increased the ethanol metabolism in liver slices from control animals, it was not effective in those from animals chronically treated with ethanol, in which the rate of ethanol metabolism had already been increased. This suggested that the rate of ethanol metabolism in these animals was no longer limited by the ability of the mitochondria to oxidize NADH. The possibility exists, however, that in these livers dinitrophenol fails to increase the mitochondrial O_2 consumption because mitochondria are already working at a maximal rate. This could occur if mitochondria are uncoupled or if they are in state 3 of phosphorylation rather than in state 4 (Chance & Williams, 1956). This would lead to an increased O_2 consumption and to a faster reoxidation of NADH and would also explain the more rapid metabolism of ethanol following chronic ethanol treatment. The possibilities of an increased O_2 consumption and mitochondrial uncoupling were investigated and the results are presented in the present paper. The subsequent paper (Bernstein *et al.*, 1973) deals with the cellular changes leading towards a state 3.

An increased metabolism of ethanol after chronic ethanol ingestion could conceivably also be produced by mechanisms that are not operational, or that are of minor quantitative importance, in naive animals. Catalase is known to metabolize ethanol *in vitro* (Keilin & Hartree, 1945; Lundquist *et al.*, 1963) if provided with enough H_2O_2. However, this reaction seems to be of little importance in untreated animals

(Kinard *et al.*, 1956) even though the amount of catalase present in the liver cell could more than account for the total metabolism of ethanol. It appears, therefore, that *in vivo* the production of H_2O_2 is rate-limiting (Aebi, 1960; Portwich & Abei, 1960). Lieber & De Carli (1970*a*) and Carter & Isselbacher (1971) have reported that a microsomal NADPH oxidase, an enzyme system that can produce H_2O_2 (Gillette *et al.*, 1957; Thurman *et al.*, 1972), is increased in rats treated chronically with ethanol. Thus an increased H_2O_2 production could conceivably cause an increased metabolism of ethanol by catalase. We have also explored this possibility.

Materials and Methods

Chronic ethanol treatment

Male Wistar rats (Canadian Breeding Laboratories, Montreal, P.Q., Canada or High Oak Ranch Ltd., Toronto, Ont., Canada) weighing 150–180g were separated in two groups and housed in individual wire-bottomed cages. An ethanol-containing liquid diet of the type used by Lieber *et al.* (1963) was given in Richter tubes as the only source of food and water. The composition of the diet expressed as percentage of the total energy (cal) was as follows: 28% protein, 10% fat, 35% ethanol and 27% carbohydrate. Ethanol was replaced by an energetically equivalent amount of carbohydrate in the sucrose-control diet, giving a total of 62% of energy from carbohydrate. The diets were prepared to provide 4.2kJ/ml (1kcal/ml). The composition of the ethanol diet in g/100ml was: sucrose, 6.75; cod liver oil, 0.088; corn oil, 0.293; olive oil, 0.075; enzymic hydrolysate of casein (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), 8.75; ethanol, 5.0; salt mixture (General Biochemical Inc., Chagrin Falls, Ohio, U.S.A.), 1.31; L-methionine, 0.035; choline chloride, 0.10; sodium carrageenate (Viscarin, Marine Colloids Inc., New York, U.S.A.), 0.4; vitamin mixture (General Biochemical Inc.), 0.197; and water to 100ml. The total choline content of the diets was 176mg/100ml and the content of methionine added as such was 35mg/100ml, values that correspond to a combined lipotropic value of 188mg of choline/420kJ (100kcal).

Each control rat was pair-fed with one in the ethanol group for 21–26 days. The average daily energy intake of both groups of rats in kJ/day per kg (kcal/day per kg) and ethanol consumption of the ethanol-treated group in g/day per kg calculated from the pair-feeding results are presented in Table 1. After an initial 3-day period of adaptation to the liquid diet both groups continued to grow at virtually identical rates (3.8g/day in control rats and 4.1g/day in the ethanol-treated group) so that the final weights of the two groups were not significantly different. This insured that any of the changes produced by ethanol were not due to dietary deficiencies. At the end of the treatment, liver weights were measured and total proteins determined by the biuret method (Gornall *et al.*, 1949) and triglycerides by the method of Carlson (1963). Liver weights and protein content were not significantly different. However, hepatic triglycerides were increased by 80% in the ethanol-treated group ($P<0.001$) (Table 1). It should be noted that this increase in triglycerides does not constitute a fatty liver, since the control value with these low-fat high-protein diets was quite low [see the discussion by Lieber (1971*a*) on this matter]. To insure an equal nutritional postprandial state in both groups and complete ethanol disappearance, the day before the experiments ethanol-treated and control animals were given equal amounts of sucrose diet for 4h (00:14–00:18h) and were starved overnight (16–18h). Water was given during the period of starvation.

Table 1. *Parameters indicating nutritional dietary adequacy in the chronic ethanol treatment*

Animals kept on semi-synthetic liquid diets for 21–26 days and starved for 18h were killed by decapitation and different parameters were determined. Energy intake and ethanol consumption are averages obtained between 0–21 days of treatment. The values represent the means ± S.E.M. with the numbers of animals in parentheses. The *P* values refer to differences between ethanol-treated and sucrose-control rats as determined by Student's *t* test for paired results. N.S., not significant.

	Sucrose-control	Ethanol-treated	*P*
Energy intake (kJ/day per kg of rat)	1249.5 ± 15.1 (67)	1232.70 ± 13.9 (67)	N.S.
(kcal/day per kg of rat)	297.5 ± 3.6 (67)	293.5 ± 3.3 (67)	N.S.
Ethanol consumption (g/day per kg of rat)	—	14.3 ± 0.2 (67)	—
Body weight (g)			
Initial	164 ± 15 (67)	164 ± 15 (67)	N.S.
Final	234 ± 13 (67)	242 ± 15 (67)	N.S.
Liver weight (g/100g of rat)	4.09 ± 0.05 (20)	4.29 ± 0.09 (20)	N.S.
Hepatic protein content (mg/g of liver)	128.8 ± 11.2 (6)	152.1 ± 9.6 (6)	N.S.
Hepatic triglycerides (mg/g of liver)	2.1 ± 0.2 (38)	3.8 ± 0.4 (38)	<0.001

Blood ethanol disappearance

The test dose of ethanol (2.5g/kg) was given intraperitoneally as a 25% (w/v) solution in 0.9% NaCl. Blood samples were taken from the cut tails of the animals with heparinized micro-sampling pipettes at 3, 4, 5 and 6h after the administration of the test dose. Ethanol was determined enzymically as described by Hawkins *et al.* (1966). The disappearance rate of blood ethanol in mg/h per 100ml and the rate of ethanol metabolism in mg/h per kg were calculated by the method of Widmark as described by Kalant (1971).

Formate oxidation in vivo

The oxidation of formate was measured by determining $^{14}CO_2$ production after intraperitoneal administration of [^{14}C]formate (New England Nuclear Corp., Boston, Mass., U.S.A.) diluted to a final specific radioactivity 0.5μCi/mmol (Weinhouse & Friedmann, 1952). Immediately after the injection the rat was placed in a metabolic chamber. A vacuum pump, operating at a constant pressure of 8.31kPa (62.5mmHg), drew air into a drying column of $CaCl_2$ then through the chamber and finally through two gas-washing cylinders connected in series, which each contained 100ml of 0.1M-NaOH to collect the $^{14}CO_2$ produced (Makar *et al.*, 1968). Samples were taken from the cylinders at 30, 60, 90 and 120min after the injection and mixed with Bray's (1960) solution, for counting of radioactivity in a Packard Tri-Carb scintillation counter. The efficiency of counting was 75% and quenching was corrected by external standardization. Since more than one-half of the injected formate is excreted in the urine in 3–6h (Weinhouse & Friedmann, 1952), preliminary experiments were conducted to determine the dose needed to reach a saturating concentration in the liver and thus a constant rate of metabolism. The minimal dose to obtain linearity was 10mmol/kg. A standard dose of 13.3mmol/kg was adopted for subsequent experiments.

O_2 consumption by liver slices

O_2 consumption by rat liver slices was measured polarographically (Estabrook, 1967) with a Clark O_2 electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio, U.S.A.). Liver slices were prepared as described by Videla & Israel (1970) and preincubated for 30min in Ringer bicarbonate buffer, pH7.4, containing 10mM-glucose (Umbreit *et al.*, 1959) and oxygenated with O_2+CO_2 (95:5) in a Dubnoff bath at 37°C and shaken at 80 strokes/min with continuous bubbling of the gas mixture into the medium. When the effect of different substances was studied a 15min preincubation in the buffer described above was followed by another 15min preincubation in Ringer bicarbonate buffer containing the agents studied. The slices were then transferred to the O_2-electrode chamber containing 3ml of Ringer solution with or without the substances under study, equilibrated with $O_2+CO_2+N_2$ (18:5:77), and the O_2 consumption was recorded for 2–4min at 37°C. Under these conditions O_2 consumption was linear with time. Initial studies revealed that the amount of O_2 consumed/min was linear with the amount of tissue in the range of 5–30mg. In the experiments in which the effect of ethanol on Q_{O_2} was studied, a basal Q_{O_2} was recorded after which ethanol was added to a final concentration of 20mM in the electrode chamber containing the slices.

Mitochondrial respiration and oxidative phosphorylation

Mitochondria were isolated as described by Wainio (1970) by using the following isolation solution: 70mM-sucrose (calcium-free) (Schwarz/Mann, Dickinson and Co., Orangeburg, N.Y., U.S.A.), 210mM-mannitol, 1mM-Tris–HCl buffer, pH7.4, and 1mM-EDTA. The washed mitochondrial pellet obtained was resuspended in an incubation medium containing 62.5mM-sucrose (calcium-free), 185.5mM-mannitol, 10mM-KCl, 10mM-Tris–HCl buffer, pH7.4, 5mM-K_2HPO_4, 5mM-$MgCl_2$ and 0.2mM-EDTA (Hagihara, 1961). Mitochondrial respiration and oxidative phosphorylation were studied by the polarographic technique in a Clark O_2 electrode at 25°C (Estabrook, 1967). The substrates were added to give a final concentration of 7mM (succinate) and 10mM (β-hydroxybutyrate). Dinitrophenol was added to a final concentration of 70μM. ADP/O ratios, state 3 and 4 respiration, respiratory control ratio and respiration in the uncoupled state were determined according to Chance & Williams (1956).

Alcohol dehydrogenase activity

Homogenates of liver (10%, w/v) in 0.25M-sucrose containing 1% Triton X-100 (Räihä & Koskinen, 1964) were centrifuged at 5000g for 30min and the supernatants were used for the determination of alcohol dehydrogenase activity as described by Bonnischsen & Brink (1955).

Catalase activity

Catalase activity was determined in a medium containing 0.067M-potassium phosphate buffer, pH7.4, and 12.5mM-H_2O_2 by measuring the decrease in u.v. absorption of H_2O_2 at 240nm (Lück, 1965). The determinations were done in extracts of homogenates (10%, w/v) prepared in 0.25M-sucrose containing 0.1% ethanol (Leighton *et al.*, 1968). The addition of 0.1% ethanol has been reported to prevent the

formation of the inactive catalase compound II (Chance, 1950*a*). Microsomal fractions were prepared by the method of Gillette *et al.* (1957) except that the high-speed centrifugation was done at 105000*g* for 1 h and were assayed for catalase activity as before.

NADPH oxidase activity

NADPH oxidase activity was measured spectrophotometrically in microsomal preparations by determining the rate of disappearance of NADPH at 340nm as described by Gillette *et al.* (1957).

Mg^{2+} and dinitrophenol-stimulated mitochondrial ATPase activities*

To compare the ATPase activities with the polarographic results the mitochondrial Mg^{2+}- and dinitrophenol-stimulated ATPases were determined by measuring the amount of P_i released after a 3min incubation of mitochondrial suspensions (0.5–1 mg of protein) at 25°C. The incubation medium was that described by Hagihara (1961) except that phosphate was omitted and 1 μmol of ATP/ml was added; duplicate sets were tested in the absence and in the presence of 1 mM-dinitrophenol. P_i was measured as described by Martin & Doty (1949).

* Abbreviation: ATPase, adenosine triphosphatase.

Results

The rate of ethanol metabolism *in vivo* was increased by chronic ethanol administration under our experimental conditions (Table 2). This is consistent with the findings of numerous other investigators, both in the rat (Hawkins *et al.*, 1966; Lieber & De Carli, 1970*b*; Tobon & Mezey, 1971; Mezey, 1972) and in man (Iber *et al.*, 1969; Ugarte & Valenzuela, 1971; Mezey & Tobon, 1971). However, the livers of the same animals showed no significant increases in alcohol dehydrogenase activity [controls ±S.E.M. (numbers of animals), 4.5±0.8 (6) nmol/min per mg of protein; treated, 4.3±1.3 (6)].

The O_2 consumption by liver slices from treated animals was significantly elevated by 56% (Table 3). Table 3 also shows that the effect of dinitrophenol and arsenate on respiration was completely lost after

Table 2. *Effect of chronic ethanol treatment on the rate of ethanol metabolism in vivo*

Experimental animals were as described in Table 1. For the determination of the rate of ethanol metabolism blood ethanol was at 3, 4, 5 and 6h after intraperitoneal administration of ethanol (2.5g/kg). The values represent the means ±S.E.M. with the numbers of animals in parentheses. The *P* values were determined by the Student's *t* test for paired results.

	Sucrose-control	Ethanol-treated	*P*
Blood ethanol disappearance (mg/h per 100ml of blood)	33.7±4.1 (6)	51.3±3.2 (6)	<0.01
Rate of ethanol metabolism (mg/h per kg of rat)	345±30 (6)	471±24 (6)	<0.02

Table 3. *Effect of uncouplers on O_2 consumption of liver slices from control and ethanol-treated rats*

Experimental animals were as described in Table 1. Liver slices were preincubated with the uncouplers for 15min before the polarographic determination of O_2 consumption. The values represent the means ±S.E.M. with the numbers of animals in parentheses. Significance studies were done by the Student's *t* test for unpaired results: (a) versus (b), $P<0.05$; (a) versus (c), $P<0.001$; (d) versus (*f*), $P<0.01$; (a) versus (d), $P<0.02$; (d) versus (e), (b) versus (e), (c) versus (f), (b) versus (d) and (c) versus (d) were not statistically different. Dinitrophenol (0.1 mM) and arsenate (1 mM) were used.

		Additions		O_2 consumption
		Dinitrophenol	Arsenate	(μmol of O_2/min per g of liver)
Sucrose-control	(a)	−	−	0.74±0.06 (7)
	(b)	+	−	1.09±0.14 (7)
	(c)	−	+	1.03±0.08 (7)
Ethanol-treated	(d)	−	−	1.16±0.10 (7)
	(e)	+	−	1.01±0.08 (7)
	(f)	−	+	0.98±0.09 (7)

Table 4. *Mitochondrial respiration and oxidative phosphorylation of isolated liver mitochondria from rats chronically treated with ethanol and sucrose-control rats*

Experimental animals were as described in Table 1. Liver mitochondria (1.5–2.5 mg of protein) were incubated at 25°C in a medium containing 62.5 mM-sucrose, 185.5 mM-mannitol, 10 mM-KCl, 10 mM-Tris–HCl buffer, pH 7.4, 5 mM-K_2HPO_4, 5 mM-$MgCl_2$ and 0.2 mM-EDTA. O_2 consumption was determined polarographically. Uncoupled respiration and state 3 respiration were elicited by addition of dinitrophenol and ADP to final concentrations of 70 and 83.3 μM respectively. The values represent the means ± S.E.M. with the numbers of animals in parentheses. Significance was determined by using Student's *t* test for paired results. N.S., not significant.

	+Succinate (7 mM)			+β-Hydroxybutyrate (10 mM)		
	Sucrose-control	Ethanol-treated	*P*	Sucrose-control	Ethanol-treated	*P*
ADP/O ratio	1.65 ± 0.07 (9)	1.60 ± 0.09 (9)	N.S.	2.60 ± 0.09 (9)	2.29 ± 0.09 (9)	<0.001
State 3 respiration (ng-atoms of O/min per mg)	164.0 ± 13.5 (9)	147.9 ± 9.1 (9)	N.S.	61.2 ± 7.3 (9)	59.3 ± 2.6 (9)	NS.
State 4 respiration (ng-atoms of O/min per mg)	31.9 ± 3.4 (9)	29.7 ± 1.8 (9)	N.S.	14.9 ± 2.3 (9)	14.9 ± 1.1 (9)	N.S.
Respiratory control ratio	5.14 ± 0.36 (9)	5.00 ± 0.50 (9)	N.S.	4.11 ± 0.45 (9)	4.00 ± 0.24 (9)	N.S.
Dinitrophenol-uncoupled respiration (ng-atoms of O/min per mg)	280.2 ± 31.0 (9)	286.5 ± 16.7 (9)	N.S.	33.2 ± 3.7 (9)	30.4 ± 1.9 (9)	N.S.
O_2 utilization in state 3 (ng-atoms of O)	158.2 ± 6.9 (9)	162.3 ± 7.9 (9)	N.S.	98.4 ± 3.2 (9)	113.2 ± 4.4 (9)	<0.001

the chronic ethanol treatment. The increased O_2 consumption by liver slices from ethanol-treated animals was also observed in the presence of ethanol *in vitro*; sucrose controls ± S.E.M. (number of animals), 0.82±0.07 (11) μmol/min per g of liver; chronic ethanol-treated, 1.12±0.11 (11); sucrose controls plus 20mM-ethanol *in vitro*, 0.82±0.07 (11); chronic ethanol-treated plus 20mM-ethanol *in vitro*, 1.11±0.11 (11).

Various parameters of respiratory and phosphorylation activity and dinitrophenol effect were also studied in mitochondria isolated from livers of control and ethanol-treated animals with succinate and β-hydroxybutyrate as substrates (Table 4). No differences in dinitrophenol-uncoupling ability were found in isolated mitochondria. The only significant effect of chronic ethanol treatment was a small decrease of the ADP/O ratio from 2.60 to 2.29 ($P<0.001$) in the presence of β-hydroxybutyrate (Table 4). These results would suggest partial uncoupling in site 1 of phosphorylation of the order of 30%. The type of uncoupling is unusual, since the respiratory control ratio was not significantly altered. No changes were found after chronic ethanol treatment in the ATP-hydrolysing ability of mitochondria in the presence or in the absence of 1mM-dinitrophenol [Mg^{2+}-stimulated ATPase: controls ± S.E.M. (numbers of animals), 13.7±2.5 (5)nmol of P_i/min per mg of protein; chronically ethanol-treated, 12.9±1.7 (5). Dinitrophenol-activated ATPase: controls, 88.8 ±14.6 (5); chronically ethanol-treated, 90.2±9.8 (5)].

Lieber & de Carli (1970*a*) and Carter & Isselbacher (1971) have reported that the liver microsomal NADPH oxidase, an enzyme system which produces H_2O_2 (Gillette, *et al.*, 1957; Thurman *et al.*, 1972), is increased after chronic ethanol treatment. An increased rate of H_2O_2 production might be in part responsible through the catalase mechanism for the greater rate of ethanol metabolism after chronic ethanol treatment. Therefore we have examined whether the changes in the microsomal NADPH oxidase also occur in our animals and whether an increased H_2O_2 formation is associated with increased peroxidative activity by the catalase system *in vivo*. For this latter purpose we have studied the effect of chronic ethanol treatment, on the rate of [^{14}C]formate metabolism to $^{14}CO_2$ *in vivo*. Formate is not oxidized by the alcohol dehydrogenase system but like ethanol it can be peroxidized by the catalase–H_2O_2 complex (Chance, 1950*b*; Friedmann *et al.*, 1954; Oro & Rappoport, 1959). The NADPH oxidase activity of liver microsomal fractions was significantly increased by about 80–100% after chronic ethanol treatment (Table 5). Similar increases were obtained if the activity is expressed as units/mg of microsomal protein or as units/g of liver or units/100g of animal. The rate of formate oxidation to CO_2 *in vivo* (±S.E.M.) was 57.5±5.3μmol/h per 100g of rat in the controls and 86.4±5.1 in the chronically ethanol-treated group (eight animals/group, $P<0.001$). Nevertheless, no increases were found in the liver catalase activity after chronic ethanol treatment either in the whole homogenates or in microsomal preparations (Table 6).

Discussion

Chronic ethanol treatment increased the capacity of rats to metabolize ethanol *in vivo*, in agreement with previous observations in man (Iber *et al.*, 1969; Ugarte & Valenzuela, 1971; Mezey & Tobon, 1971) and in animals (Hawkins *et al.*, 1966; Lieber & De Carli, 1970*b*; Tobon & Mezey, 1971; Mezey, 1972) and consistent with our previous observations *in vitro* in liver slices prepared from chronically ethanol-treated animals (Videla & Israel, 1970). No effect was found in the liver alcohol dehydrogenase activity after the chronic ethanol treatment, which agrees with observations by other workers indicating that the rate of ethanol metabolism is not directly related to the amount of this enzyme (Hawkins *et al.*, 1966; Lieber & De Carli, 1970*b*; Tobon & Mezey, 1971).

Table 5. *NADPH oxidase activity in liver microsomal preparations from control rats and rats chronically treated with ethanol*

Experimental animals were as described in Table 1. Liver microsomal preparations (1–1.5mg of protein) were added to a medium (3.0ml) containing 100μmol of phosphate buffer, pH 7.4, 100μmol of nicotinamide and 150nmol of NADPH for spectrophotometric measurements at 340nm. The values represent the means ± S.E.M. with the numbers of animals in parentheses. The P values were calculated by Student's t test for paired results. Values for total activity were calculated assuming total recovery of the microsomal fractions present in the crude homogenate.

	Sucrose-control	Ethanol-treated	P
Specific activity (nmol/min per mg of protein)	6.0±0.7 (7)	11.8±1.1 (7)	<0.01
Total activity			
(nmol/min per g of liver)	115.5±22.5 (7)	201.8±24.7 (7)	<0.001
(μmol/h per 100g of rat)	28.6±5.1 (7)	49.4±5.6 (7)	<0.001

Table 6. *Catalase activity in liver homogenates and microsomal fractions from control rats and rats chronically treated with ethanol*

Experimental animals were as described in Table 1. Liver homogenates and microsomal preparations were added to a medium containing 66.7mM-phosphate buffer, pH 7.4, and 12.5mM-H_2O_2 for spectrophotometric measurements at 240nm. The values represent the means ± S.E.M. with the numbers of animals in parentheses. Significance studies were done by Student's *t* test for paired results. One international unit (i.u.) is the number of μmol of H_2O_2 converted/min at 25°C (Lück, 1965). Values per g of liver or per 100g of rat were calculated assuming total recovery of the microsomal fractions originally present in the crude homogenate. N.S., not significant.

	Sucrose-control	Ethanol-treated	*P*
Liver homogenates (10^{-4} × i.u./g of liver)	6.1 ± 0.9 (8)	5.9 ± 0.9 (8)	N.S.
Liver microsomal preparations			
(i.u./mg of protein)	794 ± 162 (7)	755 ± 104 (7)	N.S.
(10^{-4} × i.u./g of liver)	1.32 ± 0.35 (7)	1.42 ± 0.39 (7)	N.S.
(10^{-4} × i.u./100g of rat)	5.60 ± 1.54 (7)	5.79 ± 1.15 (7)	N.S.

Mitochondrial uncouplers were shown previously to increase the rate of ethanol metabolism in normal animals but not in those chronically treated with ethanol (Videla & Israel, 1970; Israel *et al.*, 1970). The present work shows parallel changes in the consumption of O_2 by liver slices of chronically ethanol-treated animals. O_2 utilization was increased about 50% over that from the controls, but could not be further activated by dinitrophenol or arsenate. These results can be interpreted as an indication that the mitochondria are either uncoupled or in state 3 in the tissue. Nevertheless, when liver mitochondria from the animals chronically treated with ethanol were prepared and studied in an isolated system no changes in the effect of dinitrophenol occurred with either succinate or β-hydroxybutyrate as substrates. Only a slight degree of uncoupling in site 1 of phosphorylation measured as ADP/O ratio could be observed. It should be noted, however, that no changes occurred in the respiratory control ratio, a parameter also considered an indication of mitochondrial uncoupling (Estabrook, 1967; Klingenberg, 1968). The changes observed in ADP/O ratio in the presence of β-hydroxybutyrate were due only to a prolongation of state 3, since the absolute respiratory rates in state 3 and state 4 were not altered. This would suggest that some of the ATP produced in site 1 is utilized or hydrolysed. However, no changes in Mg^{2+}- or dinitrophenol-stimulated ATPase activities were observed after chronic ethanol treatment.

Results given by other workers in this field are contradictory. Sardesai & Walt (1969) observed no changes in the P/O ratio nor in mitochondrial Q_{O_2} (possibly state 3) after chronic ethanol treatment when either succinate or β-hydroxybutyrate were used as substrates. Rubin *et al.* (1970), with succinate, found no changes in ADP/O or in the respiratory control ratio but reported decreases in both state 3 and 4 respiration. Kiessling & Tilander (1961) and Walker & Gordon (1970) reported an increase in the mitochondrial Mg^{2+}-stimulated ATPase activity. These differences could conceivably be caused by differences in the preparation of mitochondria or by differences in the amount of fat infiltration in the livers produced by different diets, which could contaminate the mitochondrial preparations. It is well known that some fatty acids are effective mitochondrial uncouplers (Borst *et al.*, 1962; Lardy *et al.*, 1965). Despite these differences it is clear that a mitochondrial uncoupling as measured *in vitro* is not a constant concomitant of chronic ethanol ingestion. Although we cannot discard the possibility of a mitochondrial uncoupling *in situ* in the intact cell, results in the subsequent paper indicate that there appears to be a change from phosphorylation state 4 towards state 3 in the cell, which could explain the increased O_2 consumption and the loss of dinitrophenol effect produced after chronic ethanol ingestion (Bernstein *et al.*, 1973).

Role of the catalase–H_2O_2 system

We have confirmed the observations by Lieber & De Carli (1970*a*) and Carter & Isselbacher (1971) that chronic ethanol treatment increases the microsomal NADPH oxidase activity, a H_2O_2-producing system (Gillette *et al.*, 1957; Thurman *et al.*, 1972). Further, we have found that this increase can account for the increased oxidation of formate *in vivo*, a compound known to be oxidized by the catalase–H_2O_2 complex (Chance 1950*b*; Oro & Rappoport, 1959). NADPH oxidase activity was significantly increased by the chronic ethanol treatment from 28.6 to 49.4μmol/h per 100g of rat. Considering that microsomal recovery is not 100% but rather about 40% (Lieber, 1971*b*) these values correspond to 71.4 and 122.4μmol/h per 100 g of rat respectively, a change of 51.0μmol/h per 100g of rat. Formate metabolism *in vivo* was, on the other hand, significantly increased from 57.5 to 86.4μmol/h per 100g of rat (change = 28.9μmol/h per 100g of rat) thus indicating that

all of the increase in formate metabolism could be accounted for by the increased microsomal NADPH oxidase activity, even if *in vivo* not all NADPH oxidized yields H_2O_2.

Since the reactivity (k_4) of catalase–H_2O_2 complex is almost identical for formate and for ethanol (Chance, 1950*b*), this system could conceivably also contribute to oxidation of ethanol. The increase in the metabolism of ethanol after the chronic treatment however is 12.6mg/h per 100g of rat (274μmol/h per 100g of rat) (Table 2), of which only 10–20% could be accounted for by the catalase–H_2O_2 mechanism.

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