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ETHANOL OXIDATION BY ISOLATED HEPATOCYTES FROM ETHANOL-TREATED AND CONTROL RATS; FACTORS CONTRIBUTING TO THE METABOLIC ADAPTATION AFTER CHRONIC ETHANOL CONSUMPTION*

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Abstract—Chronic consumption of ethanol by rats produced a fatty liver and resulted in a pronounced increase in the rate of ethanol oxidation by isolated hepatocytes. Despite the increase in ethanol oxidation, oxygen consumption with several substrates was not enhanced after chronic ethanol treatment. Ouabain, an inhibitor of the $(Na^+ + K^+)$-ATPase activity, did not abolish the increase in the rate of ethanol oxidation. About 40–50 per cent of the increase in ethanol oxidation persisted after inhibition of alcohol dehydrogenase, mitochondrial oxygen consumption or the malate–aspartate shuttle. The addition of substrates for the malate–aspartate shuttle slightly increased the rate of ethanol oxidation in hepatocytes from control and ethanol-treated animals. The increased rate of ethanol oxidation was not abolished by the uncoupling agent dinitrophenol, which by itself had little effect on ethanol oxidation. In the presence of aspartate or α-glycerophosphate, dinitrophenol augmented the rate of ethanol oxidation; in the presence of glutamate, the rate of ethanol oxidation was doubled by dinitrophenol. However, the higher rate of ethanol oxidation after ethanol consumption was still found in the presence of various combinations of substrate shuttles, with or without dinitrophenol. Pathways independent of alcohol dehydrogenase may contribute, at least in part, to the increase in ethanol oxidation found after chronic ethanol consumption. It is concluded that ethanol oxidation may be enhanced after chronic ethanol consumption without the estabishment of a hypermetabolic state of the liver.

Chronic administration of ethanol to rats results in an increase in the rate of ethanol oxidation [1–4]. The mechanisms underlying this metabolic adaptation to ethanol are not clear. Alcohol dehydrogenase is the major enzyme responsible for the oxidation of ethanol in the normal liver. The reaction of ethanol with alcohol dehydrogenase generates NADH in the cytoplasm; reoxidation of the alcohol dehydrogenase-NADH complex is believed to represent the rate-limiting step in the overall oxidation of ethanol by the alcohol dehydrogenase-mediated pathway [5, 6]. Since cytoplasmic processes do not appear to be capable of oxidizing all the NADH generated during the metabolism of ethanol, the reducing equivalents must enter the mitochondria for their eventual oxidation by the respiratory chain. In view of the impermeability of intact mitochondria to NADH [7], it is necessary for this transport to be mediated via substrate shuttles. The malate–aspartate [8, 9] and the α-glycerophosphate [10, 11] shuttles have been proposed as the major pathways for the transport of reducing equivalents into the mitochondria. In particular the importance of the malate–aspartate shuttle in ethanol oxidation has been demonstrated [12, 13]. The capacity of the mitochondria to oxidize reducing equivalents may also be a controlling factor in ethanol metabolism. Dinitrophenol, an uncoupler of oxidative phosphorylation, has been reported to increase the rate of ethanol oxidation *in vitro* and *in vivo* [3, 14, 15].

Hepatic microsomes are also capable of oxidizing ethanol [1, 16, 17]. The microsomal ethanol oxidizing system shares certain characteristics with the microsomal mixed function oxidase system, and has been shown to increase after chronic ethanol consumption [1, 2, 18]. The peroxidatic activity of catalase may play some role in microsomal acetaldehyde production [19, 20]. NADPH oxidase, the microsomal enzyme system which produces hydrogen peroxide, increases in activity after chronic ethanol consumption [21, 22]. The residual ethanol oxidation, which persists in the presence of pyrazole, a potent inhibitor of alcohol dehydrogenase, is believed to represent ethanol metabolism by hepatic microsomes [1, 18, 23–25].

The rate of ethanol metabolism may therefore be affected by several factors, including the activity of alcohol dehydrogenase, the activity of substrate shuttles, the capacity of mitochondria to oxidize reducing equivalents and the activity of the microsomal pathways which oxidize ethanol to acetaldehyde. Of particular importance are the reports that chronic ad-

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ministration of ethanol results in a hypermetabolic state of the liver [14, 26, 27], thus stimulating mitochondrial oxidation of NADH, and consequently ethanol oxidation.

To study factors which may contribute to the metabolic adaptation found after chronic ethanol consumption, various aspects of ethanol oxidation by hepatocytes isolated from control rats and those fed ethanol chronically were measured.

MATERIALS AND METHODS

Ethanol-treatment. Male Sprague–Dawley rats, initially weighing about 150 g, were fed for 4 weeks a nutritionally adequate liquid diet [28], in which ethanol provided 36 per cent of total calories, protein 18 per cent, fat 35 per cent and the remainder, carbohydrate. Pair-fed litter-mates consumed the same diet, except that ethanol was isocalorically replaced by carbohydrate. The hepatic triglyceride content increased about 6-fold [28].

Preparation of hepatocytes. Hepatocytes were prepared by a slight modification of the method of Berry and Friend [29]. Rats were placed under light ether anaesthesia and the portal vein cannulated. Perfusion of the liver at a rate of 20–30 ml/min with Ca^{2+}- and $-Mg^{2+}$-free Hanks buffer, was initiated at the time of cannulation and continued until the liver blanched. The entire liver was excised and placed in a plastic support in the perfusion apparatus. The perfusion rate was increased to about 40 ml per min, and collagenase and hyaluronidase (both to a final concentration of 0.05%) were added. The buffer was continuously equilibrated at pH 7.4 with 95% O_2-5% CO_2 in a disc oxygenator. The recirculating medium was maintained at 37° and passed through a nylon filter before entering the liver. After 10–15 min of perfusion, the liver was transferred to a plastic beaker containing Mg^{2+} + Ca^{2+} Hanks buffer, and dispersed by gentle stirring. The resulting cell suspension was filtered through nylon mesh. The cells were collected by centrifugation, washed twice and resuspended in the complete Hanks buffer supplemented with 10 mM phosphate buffer.

Incubation. The oxidation of ethanol was carried out, under air, in closed 25 ml plastic Erlenmeyer flasks at 37° in a Dubnoff metabolic shaker, using a reaction mixture consisting of Hank's buffer, about 20–30 mg liver cell protein and ethanol at a final concentration of 12.5 mM, in a total reaction volume of 3.0 ml. Cells were incubated with the various additives for 5 min before the reaction was initiated by the addition of ethanol. Although the usual reaction period was 60 min, ethanol oxidation with both preparations was linear up to at least 90 min. After 60 min the reaction was terminated by the addition of trichloracetic acid (final concentration of 4.5%) and the remaining ethanol was determined on aliquots of the supernatant obtained after centrifugation as previously described [30]. Oxygen consumption was assayed at 30° using a Clark oxygen electrode and Yellow Springs Oxygen Monitor.

Source of materials. Collagenase (CLS Type II) was from Worthington Biochemicals, Freehold, NJ. Hyaluronidase was either from Worthington or Sigma Chemical Co., St. Louis, MO. Antimycin, rotenone, oligomycin, hydrazine, NAD^+, amino-oxyacetate, atractyloside and ouabain were from Sigma Chemical Co., Iodobenzylmalonic acid was obtained from K & K Laboratories Inc., Plainview, NJ. Avenaciolide was a generous gift of Dr. W. B. Turner and Dr. D. C. Aldridge, Imperial Chemical Industries Ltd., Macclesfield, Cheshire, U.K. Methylpyrazole was a generous gift of Dr. R. Pietruszko, Center of Alcohol Studies, Rutgers University, New Brunswick, NJ.

Statistics. All values refer to mean ± S.E. of the mean. Statistical analysis was performed by the paired *t* test. The number of pairs is given in the legends.

RESULTS

The viability of the isolated liver cells was routinely determined by Trypan Blue exclusion, and by assaying for the leakage of lactic dehydrogenase into the suspension medium. Liver cells prepared from ethanol-fed rats and controls were comparable, both preparations showing 75–90 per cent cells which excluded the dye. After incubation for 1 hr at 37° the number of cells which excluded trypan blue decreased by about 10 per cent in both preparations, whereas about 90 per cent of the lactic dehydrogenase activity was still associated with the pellets.

Effect of chronic ethanol administration and methylpyrazole on the rate of ethanol metabolism. The mean rate of ethanol oxidation in liver cells isolated from controls was 56 μmoles/hr/g liver, wet weight (Fig. 1). The mean rate of ethanol oxidation in cells from ethanol-fed rats was 78 μmoles/hr/g liver, wet weight (Fig. 1). The increase of 39 per cent in the rate of ethanol oxidation found with isolated liver cells is comparable to that found *in vivo* (44–45% [1, 18]). In the presence of 10 mM pyruvate, the rates of ethanol oxidation were 159 ± 14.9 and 171 ± 13.7 μmoles/hr/g liver, wet weight for control and chronic ethanol

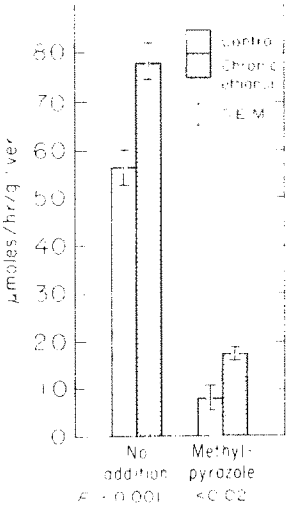

Fig. 1. Effect of chronic ethanol consumption and methylpyrazole on the rate of ethanol oxidation by isolated hepatocytes. Ethanol oxidation (12.5 mM ethanol) was assayed as described in Methods, in the absence, or presence, of 2 mM methylpyrazole. Results are from nine pairs of animals.

preparations, respectively. These rates approach the rates measured *in vivo* [31–36]. At a concentration of 2 mM, 4-methylpyrazole strikingly decreased the rate of ethanol oxidation in cells from controls (86 per cent) as well as ethanol-fed rats (77 per cent) (Fig. 1). However, the rate of ethanol oxidation by cells from ethanol-fed rats (17.5 μmoles/hr/g) was still higher than the rate from control cells (7.7 μmoles/hr/g, $P < 0.02$). About 40 per cent of the increase in the rate of ethanol oxidation found after chronic ethanol administration persisted in the presence of concentrations of methylpyrazole which totally blocked the activity of alcohol dehydrogenase in liver supernatant fractions.

Effect of inhibitors of mitochondrial respiration on the rate of ethanol metabolism. Antimycin, which inhibits NAD^+-dependent and flavin-linked oxygen uptake, blocks both the malate-aspartate and α-glycerophosphate shuttles. Rotenone, which inhibits only NAD^+-dependent oxygen uptake, inhibits the malate–aspartate, but not the α-glycerophosphate shuttle. Antimycin decreased the rate of ethanol oxidation by 70–80 per cent with both cell preparations, whereas rotenone decreased the rate less than 50 per cent (Table 1). This suggests that although a system which transports reducing equivalents into the mitochondria and is sensitive to rotenone, plays an important role in ethanol metabolism, a second system which is rotenone-insensitive, but antimycin-sensitive (presumably the α-glycerophosphate shuttle) also contributes to the transport of reducing equivalents into hepatic mitochondria. In the presence of either of these inhibitors of mitochondrial oxygen consumption, the rate of ethanol oxidation was greater in cells from ethanol-fed rats than controls (Table 1). About 43 per cent (antimycin) and 50 per cent (rotenone) of the increase in ethanol oxidation persisted in the presence of these inhibitors. Cyanide, which inhibits both mitochondrial oxygen consumption and cytochrome P-450 mediated oxidation of ethanol [1], strikingly decreased the rate of ethanol oxidation with both preparations (Table 1). In the presence of this inhibitor, the rate of ethanol oxidation in cells from ethanol-fed rats was not increased (Table 1). Oligomycin, which is an inhibitor of ADP-dependent oxygen consumption by mitochondria, decreased the rate of ethanol oxidation by about 50 per cent. However, the rate of ethanol oxidation still remained higher in cells from ethanol-fed rats (Table 1). Similar results were obtained with atractyloside, an inhibitor of the adenine nucleotide translocase, and hence of ADP entry into the mitochondria. (Table 1). In the presence of these inhibitors of ADP-dependent oxygen consumption, 40–50 per cent of the increase in ethanol oxidation found after chronic ethanol consumption persisted.

Effect of inhibitors of the malate-aspartate shuttle on the rate of ethanol metabolism. In view of the importance of the malate–aspartate shuttle in transporting reducing equivalents produced by the oxidation of ethanol into the mitochondria [12, 13], the effects of inhibitors of this shuttle were studied. Hydrazine sulfate and amino-oxyacetate were used to inhibit aspartate aminotransferase. Previous results indicated that the activities of the mitochondrial or cytosolic enzyme, as well as the sensitivity to inhibitors or an activator were not changed by ethanol consumption [30]. For operation of the malate–aspartate shuttle, glutamate and malate must enter the mitochondria, while α-ketoglutarate and aspartate exit. Avenaciolide is a specific inhibitor of glutamate transport [37], whereas iodobenzylmalonate blocks the transport of malate and α-ketoglutarate across the mitochondrial membrane [38]. These two inhibitors decreased the activity of the malate–aspartate shuttle reconstituted *in vitro* with mitochondria from ethanol-fed rats and controls [30, 39]. Chronic ethanol consumption did not alter the response to these transport inhibitors [30]. The rate of ethanol oxidation was decreased by about 50 per cent in the presence of these inhibitors of the malate–aspartate shuttle (Fig. 2). However, part of the increase (50 per cent) in the rate of ethanol oxidation found after chronic ethanol consumption persisted in the presence of these inhibitors of the malate–aspartate shuttle, in agreement with the increase which remained in the presence of rotenone.

Effect of ouabain on ethanol oxidation. Ouabain is classically used as the inhibitor of the $(Na^+ + K^+)$-activated ATPase [40]. Since ADP derived from this

Table 1. Effect of inhibitors of mitochondrial oxygen consumption on the rate of ethanol oxidation*

Inhibitor	Ethanol oxidation		Increase in ethanol oxidation	P
	Control	Ethanol-treated		
	μmoles/hr/g wet wt			
None	56.53 ± 3.58	78.37 ± 3.93	21.84	<0.001
Antimycin	14.40 ± 3.48	23.78 ± 2.26	9.38	<0.05
Rotenone	32.73 ± 3.84	44.04 ± 7.25	11.31	<0.05
Cyanide	6.95 ± 2.50	10.15 ± 5.32	3.20	N.S.
Oligomycin	30.46 ± 3.59	38.52 ± 2.81	8.06	<0.05
Atractyloside	32.96 ± 4.76	45.55 ± 4.05	12.59	<0.02

* Ethanol oxidation was assayed as described in Methods in the presence or absence of the indicated inhibitors. Final concentrations of the inhibitors were; antimycin, 4 μM; rotenone, 4.3 μM; cyanide 2 mM; oligomycin, 4 μM; atractyloside, 0.1 mM. Results are from nine pairs of rats except for the experiments with atractyloside, in which case results are from 5 pairs. All values are means ± S.E.M.

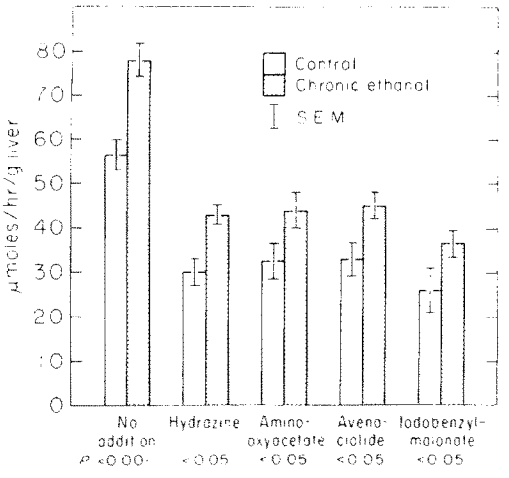

Fig. 2. Effect of inhibitors of the malate–aspartate shuttle on ethanol oxidation by isolated hepatocytes. Ethanol oxidation was assayed as described in Methods in the absence or presence of the indicated inhibitors. Final inhibitor concentrations were: hydrazine sulfate, 10 mM; amino oxyacetate, 0.2 mM; avenaciolide, 50 μM; iodobenzylmalonate, 5 mM. Results are from 8–9 pairs of animals.

enzyme activity could stimulate mitochondrial respiration, and hence the reoxidation of reducing equivalents generated during ethanol oxidation, the effect of ouabain on ethanol oxidation was studied. The addition of 1 or 3.3 mM ouabain produced about a 20 per cent decrease in the rate of ethanol oxidation by both liver cell preparations (Fig. 3). This is consistent with the estimate that the $(Na^+ + K^+)$-activated ATPase system utilizes about 7–10 per cent of the total ATP formed in the liver [14, 41, 42]. In perfusion studies, 2 mM ouabain produced a 19 per cent decrease in the control rate of ethanol oxidation [43]. After chronic ethanol consumption, the net increase in the rate of ethanol oxidation in the presence of ouabain was as great as that found in the absence of ouabain (Fig. 3). Ouabain did not prevent the adaptive increase in ethanol oxidation produced by the chronic consumption of ethanol.

Effect of shuttle substrates on ethanol oxidation. The rate of ethanol oxidation is greater in fed rats than fasted rats [44, 45]. The concept that the level of substrates participating in shuttles, especially the malate–aspartate shuttle, plays an important role in ethanol oxidation has been recently emphasized [13, 46, 47]. It was concluded that in the liver of the starved rat, ethanol oxidation is limited by the activity of the shuttles since the levels of substrates for the shuttles is low. This contrasts with the liver of the fed rat, in which the mitochondrial reoxidation of NADH may be limiting [46]. The addition of the components of the malate–aspartate shuttle stimulated ethanol oxidation in liver cells isolated from starved animals [46, 47]. We have confirmed that the rate of ethanol oxidation was greater in isolated liver cells prepared from fed animals than fasted.* The addition of malate, glutamate of α-glycerophosphate stimulated ethanol oxidation considerably more in cells prepared from fasted rats than in those from fed rats, a finding which is consistent with the suggestion that shuttle metabolites are especially limiting in the fasted state. Dinitrophenol stimulated ethanol oxidation in the presence of substrates for the malate–aspartate shuttle to a greater extent than the stimulation produced by these substrates alone.* This suggests that when the shuttles are fully functional, the capacity of the respiratory chain is rate-limiting for ethanol oxidation. The possibility that the higher rate of ethanol oxidation in cells prepared from rats chronically fed ethanol may reflect varying levels of endogenous metabolites was tested by assaying ethanol oxidation in the presence of shuttle substrates. The addition of malate, aspartate or glutamate produced a 10–15 per cent increase in the rate of ethanol oxidation with both liver cell preparations, whereas α-glycerophosphate had no effect (Fig. 4). The absence of significant effects by these shuttle substrates indicates that the animals were in the fed state. The increase in the rate of ethanol oxidation in cells from ethanol-fed rats was the same in the presence of the shuttle substrates, as in the absence of these components (Fig. 4).

The addition of 50 μM dinitrophenol had no significant effect on ethanol oxidation with both cell preparations (Fig. 5). The adaptive increase in ethanol oxidation was the same in the presence and absence of dinitrophenol. The addition of aspartate or α-glycerophosphate in the presence of dinitrophenol produced about a 30 per cent increase in the rate of ethanol oxidation with both preparations, whereas the combination of dinitrophenol plus glutamate increased ethanol oxidation by 80–90 per cent (Fig. 5). Almost the entire increase in ethanol oxidation persisted in the presence of the various combinations of dinitrophenol with shuttle substrates. Furthermore, the adaptive increase also persisted in the presence of various combinations of shuttle substrates, with or without dinitrophenol (Table 2).

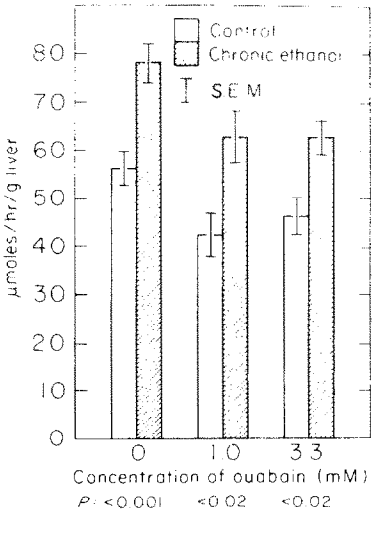

Fig. 3. Effect of ouabain on ethanol oxidation by isolated hepatocytes. Ethanol oxidation was assayed in the absence or presence of either 1.0 or 3.3 mM ouabain. Results are from 9 (no addition and 1 mM ouabain) or 7 (3.3 mM ouabain) pairs of animals.

* A. I. Cederbaum, E. Dicker and E. Rubin, *Archs Biochem. Biophys.* (in press).

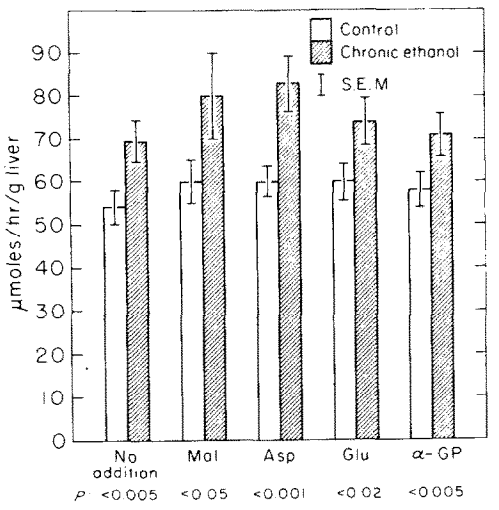

Fig. 4. Effect of components of the malate–aspartate and α-glycerophosphate shuttles on ethanol oxidation by isolated hepatocytes. Ethanol oxidation was assayed in the absence or presence of the indicated shuttle components. All substrates were added to a final concentration of 13.3 mM. Results are from 7 pairs of animals.

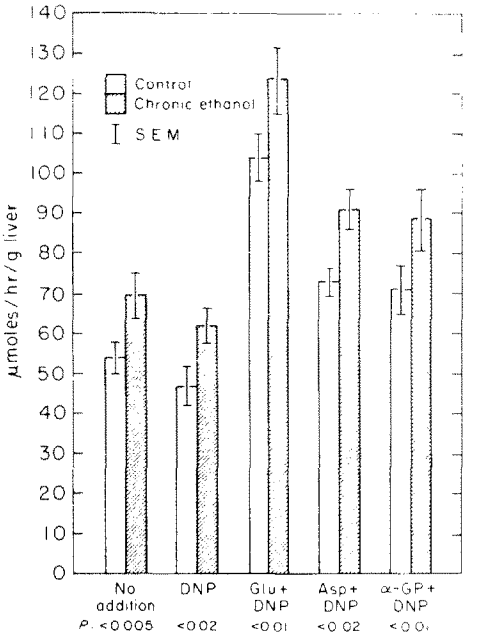

Fig. 5. Effect of shuttle components plus dinitrophenol on ethanol metabolism by isolated hepatocytes. Ethanol oxidation was assayed in the absence or presence of the indicated additions. The final concentration of all the substrates was 13.3 mM, and of dinitrophenol, 50 μM. Results are from 6 to 7 pairs of animals.

Oxygen consumption. Oxygen uptake was assayed in the presence of 12.5 mM ethanol, the same concentration used in the studies of ethanol metabolism. The endogenous rate of oxygen uptake by isolated hepatocytes was slightly decreased (−11 per cent) after chronic ethanol consumption (Table 3). Similar results were obtained in the presence of succinate, glutamate, α-glycerophosphate and pyruvate (Table 3). Dinitrophenol stimulated endogenous oxygen uptake by about 35 per cent with both preparations. In the presence of dinitrophenol, the rate of oxygen uptake by cells from ethanol-treated rats was 87 per cent that of controls (Table 3). These data suggest that chronic ethanol consumption in a diet which leads to a fatty liver does not produce an increase in oxygen consumption per g liver, wet weight.

DISCUSSION

Chronic administration of ethanol in a diet which leads to a fatty liver [28], was previously shown to result in a 45 per cent increase in the rate of blood ethanol clearance [1, 18]. The increase in ethanol oxidation did not correlate with the activity of alcohol dehydrogenase, the activity of which was slightly decreased after ethanol consumption [1, 18]. The reconstituted malate–aspartate, fatty acid, and α-glycerophosphate shuttles were equally effective in transporting reducing equivalents into the mitochondria from both ethanol-fed and control rats [30]. The activities

Table 2. Effect of shuttle components and dinitrophenol on ethanol oxidation*

Addition	Ethanol oxidation: Control	Ethanol oxidation: Ethanol-treated	Increase in ethanol oxidation	P
	μmoles/hr/g/wet wt			
None	54.01 ± 3.70	69.57 ± 5.41	15.56	<0.005
Aspartate + glutamate	62.13 ± 4.82	79.17 ± 6.73	17.04	<0.02
Malate + aspartate + glutamate	66.56 ± 3.01	83.65 ± 5.81	17.09	<0.02
Malate + aspartate + glutamate + α-glycerophosphate	70.63 ± 3.60	88.86 ± 5.40	18.23	<0.002
Malate + aspartate + glutamate + α-glycerophosphate + dinitrophenol	121.39 ± 8.43	147.06 ± 16.61	25.67	<0.02

* Ethanol oxidation was assayed as described in the Legends to Figs 4 and 5. Results are from 7 pairs of animals. All values are means ± S.E.M.

of enzymes involved in the shuttles, such as cytoplasmic and mitochondrial α-glycerophosphate dehydrogenase and glutamic oxalacetic transaminase, were either decreased or unchanged by chronic ethanol consumption [30]. The ability of isolated mitochondria from ethanol-treated rats to oxidize numerous substrates, including NAD^+-dependent substrates [48], flavin-linked substrates [30, 48], fatty acids [49] and ascorbate [48] was depressed. Furthermore the amount of mitochondrial protein per g liver, wet weight, was not changed by ethanol-treatment [30]. Studies by others have also suggested that oxygen consumption is impaired after chronic ethanol ingestion [50–52]. Of interest is the recent observation by Thurman *et al.* [43] that in a model which does not produce a fatty liver, chronic consumption of ethanol resulted in a decrease in state 3 succinate oxidation, without affecting the respiratory control. These results are identical with those reported for the fatty liver model with this flavin-linked substrate [48]. These data indicate that total mitochondrial activity, as reflected by studies *in vitro* was decreased after chronic ethanol consumption and could therefore not account for the increased rate of ethanol oxidation. Since experiments with isolated mitochondria need not necessarily reflect conditions which exist in the cell, especially with regard to factors regulating oxygen consumption, the studies reported here with isolated hepatocytes were carried out to determine factors which may contribute to the increased rate of ethanol oxidation.

Isolated rat liver cells from ethanol-treated rats show a 30–40 per cent increase in the rate of ethanol oxidation compared to controls. This increase is comparable to the increase in blood ethanol clearance [1, 18], and indicates that most of the metabolic adaptation occurs in the liver. There is considerable evidence that in addition to the alcohol dehydrogenase pathway, ethanol is metabolized by alcohol dehydrogenase-independent pathways [1, 18, 23–25]. Chronic ethanol treatment results in adaptive changes, which include proliferation of the smooth endoplasmic reticulum, increased activities of the microsomal drug metabolizing enzymes and augmented activity of the microsomal ethanol oxidizing system [53–56]. It has been suggested that when corrected for microsomal losses, $\frac{1}{2}$–$\frac{2}{3}$ of the increase in the rate of ethanol oxidation after chronic ethanol administration can be accounted for by the microsomal ethanol oxidizing pathway [18]. Methylpyrazole, an inhibitor of alcohol dehydrogenase, strikingly lowered the rate of ethanol oxidation in liver cells from controls and ethanol-treated rats, confirming the dominant role of alcohol dehydrogenase. However, the rate of ethanol oxidation by cells from ethanol-treated rats was still higher than the rate shown by control cells. Of the 22 μmole/hr/g increase in the rate of ethanol oxidation found after chronic ethanol consumption, about 40–45 per cent of this increase (10 μmoles/hr/g) persisted in the presence of methylpyrazole. Thus whereas at 12.5 mM ethanol, only 10–15 per cent of the ethanol-oxidizing capacity of the normal liver cell seems to be independent of alcohol dehydrogenase, this proportion is increased to about 25 per cent in hepatocytes from animals given ethanol. Even these values may be somewhat of an underestimation since methylpyrazole can slightly inhibit the activity of the microsomal pathways of ethanol oxidation. Of considerable interest are the observations that after chronic ethanol consumption by male rats the activity of the microsomal pathways of ethanol oxidation increase by about 0.25 μmoles/min/g liver (15 μmoles/hr/g) [1, 2, 57, 58], which can account for the increase of about 10 μmoles/hr/g in the rate of pyrazole insensitive ethanol oxidation by liver cells from ethanol-treated rats. It is possible that the non-alcohol dehydrogenase pathways may account for an even greater extent of the increase in the rate of ethanol oxidation found at higher ethanol concentrations [24].

In agreement with the data obtained using methylpyrazole, about 40–50 per cent of the increase in ethanol oxidation found after chronic ethanol consumption was not abolished in the presence of inhibitors of the mitochondrial respiratory chain, inhibitors of ADP-dependent oxygen uptake, inhibitors of aspartate amino-transferase and inhibitors of glutamate and malate transport across the mitochondrial membrane. Most of the increase was abolished in the presence of cyanide. However, this compound can inhibit both the mitochondrial and microsomal-dependent pathways of ethanol oxidation. Rognstad presented the same explanation for his data concerning the absence of an accelerated rate of ethanol oxidation

Table 3. Effect of chronic ethanol consumption on oxygen consumption by isolated hepatocytes*

Addition	Oxygen consumption (μmoles/min/mg protein)	
	Control	Ethanol-treated
None	2.45 ± 0.13	2.22 ± 0.21
Glutamate	2.79 ± 0.17	2.53 ± 0.23
α-Glycerophosphate	2.88 ± 0.18	2.66 ± 0.22
Pyruvate	2.49 ± 0.09	2.19 ± 0.41
Succinate	4.35 ± 0.37	4.02 ± 0.44
Dinitrophenol	3.36 ± 0.28	3.01 ± 0.39

* Oxygen uptake was assayed as described in Methods, in a reaction mixture consisting of Hanks buffer, 12.5 mM ethanol, the indicated additions and about 4–8 mg cell protein in a final volume of 3.0 ml. Substrate concentrations were 13.3 mM and the concentration of dinitrophenol was 50 μM. Results are from 5 to 9 pairs of animals.

in the presence of cyanide [23]. It is therefore possible that part of the increase in ethanol oxidation is contributed by pathways that are independent of alcohol dehydrogenase and the transfer and oxidation of reducing equivalents by the mitochondrial respiratory chain. However, when comparing the percentage inhibition produced by the various inhibitors, in most cases each inhibitor produces almost the same degree of inhibition in control and chronic ethanol preparations, e.g. antimycin produced 75 per cent (controls) and 70 per cent (chronic ethanol) inhibition; oligomycin produced 46 per cent (controls) and 51 per cent (chronic ethanol) inhibition; iodobenzylmalonate produced 54 per cent (controls) and 53 per cent (chronic ethanol) inhibition, etc. Since these inhibitors are affecting both preparations in the same way, the differences in ethanol oxidation between the two preparations can also be attributable to different degrees of activity of the same enzyme system, i.e. the alcohol dehydrogenase pathway. It is possible that the metabolic adaptation may involve alcohol dehydrogenase-dependent and -independent pathways. It has been suggested that acceleration of the NADPH-dependent microsomal oxidation of ethanol pathway by chronic ethanol treatment could also accelerate the NAD^+-dependent alcohol dehydrogenase pathway [55]. The microsomal pathways utilize NADPH, thereby generating $NADP^+$. Transhydrogenation between $NADP^+$ and NADH (generated from the alcohol dehydrogenase reaction) could regenerate the NAD^+ required for the alcohol dehydrogenase reaction. Cytoplasmic pathways linking diphospho- and triphospho-pyridine nucleotides, e.g., the malic enzyme reaction which can produce pyruvate, have been suggested [59]. Under these conditions, since alcohol dehydrogenase is involved, part of the increase in ethanol oxidation would be blocked by methylpyrazole. However, it would not be sensitive to inhibitors of mitochondrial oxygen consumption or the malate–aspartate shuttle, because reoxidation of the NADH would not require transport and oxidation of reducing equivalents by the mitochondrial respiratory chain. The fact that methylpyrazole did not abolish the increment in ethanol oxidation produced by the chronic consumption of ethanol to a greater extent than did inhibitors of mitochondrial oxygen consumption or the malate aspartate shuttle might suggest that cytoplasmic processes linking $NADP^+$ and NADH do not play a major role in the metabolic adaptation. However, the inhibition of part of the increase in ethanol oxidation produced by antimycin, rotenone or oligomycin may reflect non-specific secondary effects; the inhibition of oxygen uptake and energy transduction produced by these compounds may interfere with functions of the liver cell itself. Moreover, methylpyrazole causes some inhibition of the microsomal oxidation of ethanol, whereas antimycin and oligomycin do not (unpublished observations). Thus the effects of methylpyrazole may reflect action on microsomal pathways in addition to pathways mediated by alcohol dehydrogenase. Further studies are required to determine if these "linking" pathways contribute to the metabolic adaptation.

As discussed above, no changes in the mitochondria themselves seem to explain any adaptive mechanism in ethanol oxidation. The mechanism underlying the alcohol dehydrogenase-dependent contribution to the metabolic adaptation does not appear to involve levels of shuttle substrates, since the increase in ethanol oxidation was the same in the presence or absence of malate, aspartate, glutamate or α-glycerophosphate. The increase in ethanol oxidation was also observed when dinitrophenol was added together with the shuttle substrates. The addition of the shuttle substrates produced only a slight increase in the rate of ethanol oxidation, indicating that substrate levels were not rate-limiting in these experiments, i.e., the rats were not in a starved state. In the presence of the substrates, especially with glutamate, dinitrophenol strikingly increased the rate of ethanol oxidation with liver cells from both control and ethanol-fed animals. Thus when the shuttles appear to be operating near maximum capacity, i.e. substrate levels are not limiting, ethanol oxidation may be limited by the capacity of the respiratory chain. Under these conditions, dinitrophenol, which stimulates oxygen uptake (Table 3) could therefore stimulate the rate of ethanol oxidation. However, the possibility that dinitrophenol promotes the entry of the shuttle substrates cannot be discarded at this time. The liver cell membrane shows restricted permeability to dicarboxylate anions [60]. It is possible that dinitrophenol increases this permeability. Massive entry of substrate anions is not required to promote ethanol oxidation; the entry of a few μmoles is sufficient to initiate cyclic shuttle processes. This is more readily seen in starved rats, in which case the addition of shuttle substrates led to a significant increase in the rate of ethanol oxidation [46, 47].

Israel and his collaborators [14, 26, 27] suggest that chronic ethanol consumption results in the production of a hypermetabolic state of the liver, thereby accelerating the reoxidation of NADH produced in the alcohol dehydrogenase reaction. They postulate that an increase in $(Na^+ + K^+)$-activated ATPase generates more ADP, and brings the hepatocyte closer to "state 3", i.e. the phosphorylation potential which regulates the rate of oxygen consumption would be lowered [14, 26, 27]. Ouabain, an inhibitor of the $(Na^+ + K^+)$-activated ATPase was reported to abolish the hypermetabolic state as well as the increase in ethanol oxidation found after chronic ethanol consumption [14, 26, 27]. However, in hepatocytes isolated from a fatty liver, oxygen consumption in the presence of ethanol and several substrates was not increased in cells from ethanol-treated rats, and ouabain did not abolish the increase in ethanol oxidation (Fig. 3). The fact that after chronic ethanol treatment oxygen consumption was depressed 20–35 per cent with mitochondria [48] whereas a nonsignificant decrease of about 10 per cent was observed with liver cells of ethanol-treated rats suggests the possibility that in the intact liver cell, the decrease in mitochondrial oxygen uptake may be coupled to an increase in microsomal oxygen uptake, thereby resulting in no significant change in total oxygen uptake by the whole cell. Some increase in microsomal oxygen uptake would be consistent with the proliferation of the endoplasmic reticulum and the adaptive increase in microsomal oxidation of ethanol and drugs found after chronic ethanol consump-

tion [53–56]. Preliminary experiments with the fatty liver model reveal no change in the activity of the sodium plus potassium-activated ATPase in liver homogenates and plasma membranes of ethanol-treated rats.* Gordon [61] has recently reported that chronic ethanol treatment did not produce a hypermetabolic state of the liver and that the $(Na^+ + K^+)$-activated ATPase was not significantly affected. Christensen *et al.* [62] did not find a hypermetabolic state of the liver after chronic ethanol ingestion as hepatic oxygen uptake was slightly decreased. Of major consideration is the fact that the model system used in this report (and that by Gordon [61]) results in the production of a fatty liver, whereas the regimen used by Israel produces no significant elevation in triglyceride levels [26, 27].

The studies reported here employed a diet containing an amount of fat comparable to that in the human diet. The differences in ATPase activities, ouabain sensitivity and "hypermetabolic state" of the liver may consequently reflect the different diets employed, and may also be related to other differences in the design of the experiments such as the use of liver slices versus hepatocytes, use of Wistar versus Sprague–Dawley rats, differences in the protein content of the diet (28 per cent versus 18 per cent of total calories) and fasting or not-fasting the rats for 16–18 hr prior to sacrifice. Nevertheless, under our conditions, chronic ethanol consumption leads to an accelerated rate of ethanol metabolism which is not associated with a hypermetabolic state of the liver.

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