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ETHANOL OXIDATION BY ISOLATED HEPATOCYTES FROM FED AND STARVED RATS AND FROM RATS EXPOSED TO ETHANOL, PHENOBARBITONE OR 3-AMINO-TRIAZOLE

NO EVIDENCE FOR A PHYSIOLOGICAL ROLE OF A MICROSOMAL ETHANOL OXIDATION SYSTEM

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Abstract—In isolated hepatocytes from fed and starved rats, basal rates of ethanol oxidation were 1.15 and 0.71 μmoles/g wet wt, respectively, and were unchanged over the ethanol concentration range 8–96 mM. The addition of 4-methyl pyrazole (4 mM), a competitive inhibitor of alcohol dehydrogenase, largely abolished ethanol oxidation from 8 mM ethanol, while at an ethanol concentration of 96 mM, the oxidation rate was inhibited by 87 per cent. Pyrazole was a less effective inhibitor of alcohol oxidation than 4-methyl pyrazole. In hepatocytes isolated from rats treated with ethanol, phenobarbitone or 3-amino-triazole, basal rates of ethanol oxidation were the same at ethanol concentrations of 8–96 mM and the rates were similar to, and never exceeded, the rate found in hepatocytes from normal fed rats. 4-Methyl pyrazole inhibited ethanol oxidation to the same extent in all liver cell preparations, regardless of the treatment the donor animal had received. Pyruvate stimulated cellular ethanol oxidation irrespective of the prior treatment of the donor animal. This stimulation, together with the ethanol-induced accumulation of lactate, was abolished by 4-methyl pyrazole. This suggests that the capacity for alcohol oxidation in isolated liver cells is generally limited by the lack of suitable acceptors for the hydrogen generated in the cytoplasm by the alcohol dehydrogenase-catalysed oxidation of ethanol to acetaldehyde. Methylene blue, phenazine methosulphate and menadione stimulated both ethanol oxidation and respiration, irrespective of the prior treatment of the donor animal. This enhancement of ethanol oxidation and respiration was prevented by 4-methyl pyrazole. These artificial electron acceptors appear to act by circumventing normal pathways for the oxidation of cytoplasmic NADH generated in the conversion of ethanol to acetaldehyde. In cells from each treatment group, antimycin was more effective than rotenone as an inhibitor of ethanol oxidation; inhibition of ATP formation by oligomycin had least effect on alcohol oxidation. Ethanol oxidation by cells from alcohol-treated rats was most affected by these inhibitors of mitochondrial respiration. These results indicate that under a wide variety of experimental conditions the contribution of the postulated microsomal ethanol oxidizing system to ethanol oxidation in isolated, intact liver cells appears minimal. Thus they cast doubt on a physiological role for this system *in vivo*.

Since the description of an hepatic microsomal ethanol oxidizing system termed 'MEOS' by Lieber and De Carli [1], there has been continuing debate concerning the physiological role, if any, that it plays in ethanol oxidation by the liver *in vivo*. MEOS has been suggested to contribute significantly to ethanol oxidation at high ethanol concentrations, because of its high K_m for ethanol of 7–10 mM [2], in contrast to a K_m of 0.5–2 mM ethanol for the cytoplasmic alcohol dehydrogenase [2]. Reports of higher rates of ethanol oxidation at high ethanol concentrations [3–6] have tended to support the idea of a second pathway of ethanol oxidation in addition to alcohol dehydrogenase, as have observations of increased rates of ethanol oxidation in rats fed an ethanol diet [7]. However, other studies have suggested that the rate of ethanol oxidation does not increase with increased ethanol concentrations [8] and that MEOS has little [9] or no role [10] in ethanol oxidation *in vivo*. In addition, a role for catalase in ethanol oxidation has also been suggested [5].

In view of these apparent discrepancies and inconsistencies, and because some of these studies have been done in liver slices [8, 9] and some in isolated enzyme systems [2, 5], with relatively few in intact liver preparations [7, 11], it seemed desirable to try to assess the possible physiological roles of alcohol dehydrogenase, MEOS and catalase, by a detailed examination of ethanol oxidation in isolated hepatocytes. Accordingly, rates of ethanol oxidation at low and high ethanol concentrations have been determined in liver cells isolated from normal fed and starved rats, and from rats chronically fed ethanol or treated with phenobarbitone or 3-amino-triazole. The effects of these two drugs were studied because phenobarbitone is known to cause prolifer-

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ation of the endoplasmic reticulum [12] and to enhance drug metabolism, while 3-amino-triazole is a known inhibitor of catalase [5].

MATERIALS AND METHODS

Collagenase was a product of Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). [1-^{14}C]Ethanol was obtained from the Radiochemical Centre Ltd., Amersham, U.K. Any contaminating [1-^{14}C]acetaldehyde was removed by distillation. The redistilled ethanol remained stable for at least one month. Methylene blue, menadione and mitochondrial inhibitors were purchased from Sigma (St. Louis, MO, U.S.A.). Pyrazole and phenazine methosulphate were obtained from K and K Laboratories Inc., Hollywood, CA, U.S.A. and 4-methyl pyrazole was a gift from Dr. N. Grunnet, Copenhagen, Denmark.

Male hooded Wistar rats weighing between 250 and 300 g were used either in the fed state or after being starved for 18 hr to deplete hepatic glycogen. Phenobarbitone treatment consisted of intraperitoneal (i.p.) injections (0.1 mg/g body wt) on five consecutive days. Animals were used for liver cell preparation on the seventh day. 3-Amino-triazole (2.4 mg/g body wt) was injected i.p. 24 hr prior to use. Two liquid ethanol diets were employed, one equivalent to the high-fat, ethanol diet of Lieber *et al.* [13], in which fat contributed 40 per cent of total calories, ethanol 36 per cent, protein 18 per cent and carbohydrate 6 per cent, and the other a low-fat ethanol diet in which fat supplied 5 per cent of calories, ethanol 36 per cent, protein 18 per cent and carbohydrate 41 per cent. The diets were as described in detail by Savolainen *et al.* [14]. The ethanol content of the diet was gradually increased to the maximum by the end of the first week. The rats were kept on the liquid diet for 21–28 days prior to use. A control group of rats was kept on a liquid diet with sucrose replacing ethanol. This control group had the same rates of ethanol utilization as the normal chow-fed rats.

Suspensions of isolated cells were prepared as described elsewhere [15] by a simplified version of the method of Berry and Friend [16]. The standard procedure did not work satisfactorily for the chronic alcohol-fed rats, especially those on the high-fat diet. The liver was perfused and digested with collagenase as usual, but the resultant isolated cell population had a very low percentage of intact cells. It was necessary to shake the cell suspension for 2 min with DNase (2 mg) and trypsin (3 mg) [17] to digest the damaged cells and to allow harvesting of the intact cells. This procedure did not affect rates of ethanol oxidation in cell suspensions prepared from normal liver (M. N. Berry, unpublished observations). Yields of liver cells from rats on the high-fat ethanol diet were still very low (< 1 g wet wt per liver) but over 90 per cent excluded trypan blue and there was no excessive loss of lactate dehydrogenase.

[1-^{14}C]Ethanol oxidation and oxygen uptake were estimated as described previously [18]. All results are expressed on the basis of liver wet wt [18].

RESULTS

Ethanol oxidation by isolated cells from fed and starved rats. Ethanol oxidation by isolated hepatocytes from fed rats was linear with time and proceeded at the same rate (1.15 μmoles/min/g) at concentrations of 8, 32, 48 and 96 mM ethanol. The competitive inhibitors of alcohol dehydrogenase, pyrazole and 4-methyl pyrazole, were used to evaluate the contribution of this enzyme to ethanol oxidation. At an ethanol concentration of 8 mM, pyrazole (4 mM) inhibited ethanol oxidation by 90 per cent and 4-methyl pyrazole (4 mM) by 95 per cent

Table 1. Rates of ethanol utilization by isolated liver cells from fed and starved rats. Effects of 4-methyl pyrazole, pyrazole and pyruvate*

Additions		Rate of ethanol oxidation (μmoles/min/g)			
		Fed		Starved	
		8 mM Ethanol	96 mM Ethanol	8 mM Ethanol	96 mM Ethanol
None		1.15 ± 0.03	1.16 ± 0.04	0.71 ± 0.06	0.71 ± 0.04
4-Methyl pyrazole	1 mM	0.07 ± 0.01	0.17 ± 0.02		
	4 mM	0.06 ± 0.01	0.15 ± 0.01	0.07 ± 0.01	0.17 ± 0.02
Pyrazole	4 mM	0.12 ± 0.01	0.61 ± 0.05	0.12 ± 0.02	0.56 ± 0.11
	16 mM	0.10 ± 0.01	0.29 ± 0.03		
Pyruvate	10 mM	2.87 ± 0.32	2.81 ± 0.13	2.95 ± 0.17	2.37 ± 0.22
Pyruvate 10 mM + 4-methyl pyrazole	4 mM	0.08 ± 0.01	0.14 ± 0.01	0.09 ± 0.01	0.18 ± 0.02

* Liver cells from fed or starved rats were incubated in the presence of 8 or 96 mM [1-^{14}C]ethanol in a medium containing 0.115 M NaCl, 5.4 mM KCl, 0.8 mM $MgSo_4$, 2 mM $CaCl_2$, 10 mM sodium phosphate and 25 mM $NaHCO_3$. The medium was maintained at pH 7.4 by gassing with O_2 + CO_2 (95:5 v/v). Pyrazole and 4-methyl pyrazole were added at the concentrations indicated. Pyruvate was added at an initial concentration of 10 mM. The final volume was 2 ml containing 60–80 mg wet wt cells, and incubations were carried out for 30 min at 37°. Results are given as means ± S.E. Data represent results from at least 5 experiments.

(Table 1). At an ethanol concentration of 96 mM, 4 and 16 mM pyrazole inhibited ethanol oxidation 47 and 75 per cent respectively. The corresponding inhibitions with 4-methyl pyrazole, which is a more potent inhibitor of alcohol dehydrogenase, were 87 and 93 per cent respectively.

In isolated hepatocytes from starved rats ethanol oxidation proceeded at the rate of 0.71 μmoles/min/g (Table 1). This rate was appreciably lower than the rate in cells from fed rats but again was essentially constant over the range 8–96 mM ethanol. Ethanol oxidation was largely abolished by 4-methyl pyrazole (4 mM), although pyrazole (4 mM) was less effective, especially in the presence of 96 mM ethanol. These inhibitor studies suggest that most of the ethanol oxidation occurring, even at very high ethanol concentrations, can be attributed to a pathway involving alcohol dehydrogenase.

These findings are not conclusive since these rates of ethanol oxidation by isolated hepatocytes incubated in an unsupplemented saline medium are substantially less than that observed *in vivo* [19]. It appears that a number of substances which serve as carriers of reducing equivalents from the cytosol to the mitochondrion are eluted from the cells during the preparative procedure. In consequence, intercompartmental hydrogen transfer can become rate-limiting for ethanol oxidation. This difficulty can be overcome by supplementing the incubation medium with pyruvate which acts as a cytoplasmic sink for ethanol hydrogen.

The presence of pyruvate stimulated ethanol oxidation by cells from both fed and starved animals to a rate of about 2.9 μmoles/min/g, again irrespective of whether the initial ethanol concentration was 8 or 96 mM (Table 1). Lactate accumulated in the medium under these conditions at a rate of 3.28 ± 0.12 μmoles/min/g (N = 5). This enhanced oxidation of ethanol was virtually abolished by the addition of 4-methyl pyrazole (4 mM). Moreover, the rate of lactate accumulation was reduced to that observed in the absence of added ethanol. These findings imply that the pyruvate-induced stimulation of ethanol oxidation was mediated entirely by alcohol dehydrogenase, since lactate accumulation should have been unaffected or perhaps enhanced if MEOS was the target of 4-methyl pyrazole inhibition. Thus cytoplasmic alcohol dehydrogenase is capable of catalysing a rate of ethanol oxidation equal to that observed *in vivo*, when a suitable sink is available for the hydrogen generated in the cytoplasm by the oxidation of ethanol to acetaldehyde.

Ethanol oxidation by liver cells from rats treated with alcohol, phenobarbitone or 3-amino-triazole. The rates of ethanol oxidation at 8 and 96 mM ethanol in hepatocytes from rats chronically fed ethanol are shown in Table 2. In all instances the rate of ethanol oxidation was independent of the concentration of ethanol presented to the cell (8 or 96 mM). The rate in cells from rats on the low-fat ethanol diet was somewhat higher than in cells from rats on the high-fat ethanol diet, but neither was higher than the rates in cells from normal fed rats. The alcohol dehydrogenase inhibitors pyrazole and 4-methyl pyrazole inhibited ethanol oxidation to the

Table 2. Rates of ethanol utilization by isolated liver cells from fed rats treated with ethanol, phenobarbitone or 3-amino-triazole. Effects of 4-methyl pyrazole, pyrazole and pyruvate*

Additions	Rate of ethanol oxidation (μmoles/min/g)							
	Ethanol-fed (high-fat)		Ethanol-fed (low-fat)		Phenobarbitone		3-Amino-triazole	
	8 mM Ethanol	96 mM Ethanol	8 mM Ethanol	96 mM Ethanol	8 mM Ethanol	96 mM Ethanol	8 mM Ethanol	96 mM Ethanol
None	0.96 ± 0.05	0.99 ± 0.04	1.15 ± 0.08	1.03 ± 0.06	1.03 ± 0.01	1.04 ± 0.10	0.99 ± 0.02	1.04 ± 0.10
4-Methyl pyrazole (4 mM)	0.04 ± 0.01	0.16 ± 0.01	0.03 ± 0.01	0.17 ± 0.01	0.05 ± 0.01	0.13 ± 0.04	0.03 ± 0.01	0.13 ± 0.02
Pyrazole (4 mM)	0.10	0.58	0.08	0.47	0.12 ± 0.01	0.44	0.10 ± 0.02	0.58 ± 0.07
Pyruvate (10 mM)	2.09 ± 0.14	2.28 ± 0.27	1.87 ± 0.19	2.12 ± 0.13	2.55 ± 0.21	2.23 ± 0.22	2.84 ± 0.15	2.62 ± 0.33
Pyruvate (10 mM) + 4-methyl pyrazole (4 mM)	0.04 ± 0.01	0.14 ± 0.04	0.04 ± 0.01	0.15 ± 0.01	0.05 ± 0.01	0.14 ±0.04	0.04 ± 0.01	0.14 ± 0.03

* Liver cells from rats chronically fed a high-fat ethanol diet or a low-fat ethanol diet and from rats treated with phenobarbitone or 3-amino-triazole were incubated as described for Table 1. For details of diets and treatments see Materials and Methods.

same extent in cells from the ethanol-fed rats as in cells from normal fed rats, implying that alcohol dehydrogenase was responsible for ethanol oxidation in both groups. Ethanol oxidation by cells from alcohol-fed rats was increased by the addition of pyruvate and this stimulated activity was abolished by the addition of 4-methyl pyrazole (Table 2). Thus, alcohol dehydrogenase activity in liver cells from alcohol-fed rats is also limited by the rate of removal of cytoplasmic hydrogen.

The rate of ethanol oxidation in cells from phenobarbitone-treated rats was the same whether 8 or 96 mM ethanol was present in the incubation medium (Table 2). Phenobarbitone treatment, despite its known capacity to cause proliferation of the endoplasmic reticulum membrane system [12], did not increase the rate of ethanol oxidation. Ethanol oxidation in cells from phenobarbitone-treated rats showed the same response to inhibition by 4-methyl pyrazole and pyrazole and to stimulation by pyruvate. This stimulated activity remained totally sensitive to inhibition by 4-methyl pyrazole (Table 2).

In order to assess the role of catalase in ethanol oxidation *in vivo*, rats were injected with the catalase inhibitor 3-amino-triazole. The rate of ethanol oxidation in isolated liver cells from these rats was the same whether 8 or 96 mM ethanol was in the incubation medium (Table 2), and was similar to the rate in cells from normal fed rats. Again, pyrazole and 4-methyl pyrazole inhibited to the same extent as in cells from fed animals. Ethanol oxidation was stimulated by pyruvate and the total activity was largely abolished by 4-methyl pyrazole (4 mM). These results imply that in liver cells from 3-amino-triazole-treated rats, ethanol was oxidized almost entirely via a pathway catalysed by alcohol dehydrogenase. Moreover, inhibition of catalase activity had no effect on the rate of ethanol oxidation.

Effect of artificial electron acceptors on the rate of ethanol oxidation. A number of artificial electron acceptors were tested for their effects on ethanol oxidation. Since these agents promote NAD(P)H oxidation they would be expected to stimulate alcohol dehydrogenase-mediated ethanol oxidation but impair any NADPH-dependent (microsomal) ethanol oxidation. The results are shown in Table 3. Methylene blue, phenazine methosulphate and menadione each stimulated both ethanol oxidation and respiration in cells from fed rats. This supports the contention that in these experimental circumstances the rate of ethanol oxidation by the cells was limited by the rate of oxidation of cytoplasmic NADH. Again, the stimulated activity was abolished by the addition of 4-methyl pyrazole (4 mM), indicating that the increased ethanol oxidation was accounted for by alcohol dehydrogenase activity.

The addition of artificial electron acceptors increased the rate of ethanol oxidation in cells from starved rats and from fed rats treated with phenobarbitone or 3-amino-triazole to about the same level as in cells from normal fed rats (Table 3). However, the artificial electron acceptors were less effective in stimulating ethanol oxidation in cells from alcohol-treated rats. Cells from phenobarbitone-treated rats showed a very high rate of respiration in the presence of phenazine methosulphate and menadione, presumably reflecting increased extra-mitochondrial respiration associated with the proliferation of the endoplasmic reticulum membranes. In cells from all treatment groups ethanol oxidation in the presence of the electron acceptors was reduced by the addition of 4-methyl pyrazole (4 mM) to 0.04 μmoles/min/g at 8 mM ethanol and to 0.16 μmoles/min/g at 96 mM ethanol, indicating that alcohol dehydrogenase activity was responsible for the increased ethanol oxidation. Moreover, in the presence of the electron acceptors, 4-methyl pyrazole reduced the respiratory rate by about 1.5 μmoles/min/g.

Effect of mitochondrial inhibitors on ethanol oxidation. The effects of rotenone, antimycin and oligomycin on the basal rates of ethanol oxidation in cells from fed or fasted rats and from rats treated with ethanol, phenobarbitone or 3-amino-triazole are shown in Table 4. Antimycin, which inhibits both NAD^+-dependent and flavin-linked oxygen uptake, was slightly more inhibitory in cells from both fed and fasted rats than was rotenone, which inhibits only NAD^+-dependent oxygen uptake. Oligomycin, which inhibits ATP formation during oxidative phosphorylation, was much less inhibitory than either antimycin or rotenone, indicating that some of the respiration associated with ethanol oxidation may not be coupled to phosphorylation.

In cells from alcohol-treated rats, ethanol oxidation was much more sensitive to inhibition by these mitochondrial inhibitors (Table 4). Ethanol oxidation in cells from phenobarbitone-treated rats was less sensitive to rotenone inhibition than cells from fed and fasted rats, but antimycin and oligomycin inhibited to the same extent as in normal cells. Cells from 3-amino-triazole treated rats were more sensitive to antimycin inhibition than were cells from untreated rats, but were affected by rotenone and oligomycin to the same degree as in normal cells.

DISCUSSION

Pathways of ethanol oxidation in cells from normal rats. The activity of alcohol dehydrogenase in the liver of normal rats is sufficient to account for the rate of ethanol oxidation *in vivo* [19]. Hence in order to demonstrate a contribution by other pathways it is necessary to identify features of ethanol oxidation which are clearly not attributable to alcohol dehydrogenase. For example, use can be made of the differences in K_m for ethanol between alcohol dehydrogenase (0.5–2 mM) and MEOS or catalase (~8 mM). If the rate of ethanol oxidation is shown to increase at ethanol concentrations above 10 mM, this implies the involvement of a pathway other than alcohol dehydrogenase. Another approach is to use inhibitors of alcohol dehydrogenase such as pyrazole [20] or the more potent 4-methyl pyrazole [21]. It can be assumed that in the presence of these inhibitors ethanol oxidation is proceeding by pathways not involving alcohol dehydrogenase. However, the inhibition is of a competitive nature [21] and may not be complete particularly at high ethanol concentrations, since it is possible that the pyrazole derivative may not readily enter the liver cell.

Initial studies on the effect of ethanol concentra-

Table 3. Effect of artificial electron acceptors on the rate of ethanol utilization and oxygen uptake by isolated liver cells from fed and starved rats, and from rats treated with ethanol, phenobarbitone or 3-amino-triazole*

Pre-treatment of animal	Initial ethanol concentration	Artificial electron acceptor: None — Ethanol removal	None — O_2 uptake	Methylene blue — Ethanol removal	Methylene blue — O_2 uptake	Phenazine methosulphate — Ethanol removal	Phenazine methosulphate — O_2 uptake	Menadione — Ethanol removal	Menadione — O_2 uptake
Fed	8 mM	1.15 ± 0.03	2.01 ± 0.04	2.14 ± 0.22	4.46 ± 0.47	1.82 ± 0.17	5.47 ± 0.26	1.78 ± 0.08	5.15 ± 0.27
	96 mM	1.16 ± 0.04	1.92 ± 0.06	1.84 ± 0.20	4.44 ± 0.30	1.79 ± 0.17	4.80 ± 0.03	2.32 ± 0.20	5.97 ± 0.47
Starved	8 mM	0.71 ± 0.06	1.84 ± 0.06	2.36 ± 0.11	4.50 ± 0.44	2.27 ± 0.10	3.34 ± 0.73	1.79 ± 0.13	2.79 ± 0.57
	96 mM	0.71 ± 0.04	1.67 ± 0.05	2.02 ± 0.06	3.97 ± 0.36	2.24 ± 0.09	3.20 ± 0.48	2.08 ± 0.09	3.59 ± 0.71
Ethanol-fed (low fat)	8 mM	0.96 ± 0.05	1.77 ± 0.10	1.56 ± 0.06	4.04 ± 0.42	1.55 ± 0.29	3.43 ± 0.36	1.42 ± 0.02	3.15 ± 0.60
	96 mM	0.99 ± 0.04	1.44 ± 0.12	1.91 ± 0.06	3.80 ± 0.41	1.66 ± 0.29	3.56 ± 0.53	1.83 ± 0.04	3.72 ± 0.42
Phenobarbitone	8 mM	1.03 ± 0.01	2.14 ± 0.01	2.48 ± 0.07	4.09 ± 0.31	2.13 ± 0.08	7.06 ± 0.17	1.84 ± 0.10	6.09 ± 0.15
	96 mM	1.04 ± 0.10	1.93 ± 0.08	2.61 ± 0.11	3.70 ± 0.48	2.54 ± 0.12	7.25 ± 0.17	2.63 ± 0.08	7.16 ± 0.16
3-Amino-triazole	8 mM	0.99 ± 0.02	1.97 ± 0.10	2.24 ± 0.09	5.63 ± 0.21	2.07 ± 0.12	5.17 ± 0.72	1.63 ± 0.11	3.67 ± 1.00
	96 mM	1.04 ± 0.10	1.82 ± 0.12	2.20 ± 0.10	4.90 ± 0.34	2.26 ± 0.23	4.62 ± 0.42	2.07 ± 0.03	4.29 ± 0.70

* Liver cells from fed and starved rats, from rats chronically fed a low-fat ethanol diet, and from rats treated with phenobarbitone or 3-amino-triazole were incubated in the medium described in Table 1. Initial concentrations of methylene blue (70 μM), phenazine methosulphate (0.16 mM) and menadione (0.3 mM, added in 5 μl acetone) were added as indicated. For measurement of oxygen uptake, cells were incubated in bicarbonate-free medium [18]. Rates are expressed as μmoles/min/g.

Table 4. Effect of inhibitors of mitochondrial oxygen consumption on the rate of ethanol oxidation by isolated liver cells from fed and starved rats, and from rats treated with ethanol, phenobarbitone or 3-amino-triazole*

Pre-treatment of animal	Initial ethanol concentration	Inhibitor: Rotenone — Ethanol removal (μmoles/min/g)	Rotenone — Inhibition (%)	Antimycin — Ethanol removal (μmoles/min/g)	Antimycin — Inhibition (%)	Oligomycin — Ethanol removal (μmoles/min/g)	Oligomycin — Inhibition (%)
Fed	8 mM	0.62 ± 0.06	42	0.61 ± 0.04	43	0.77 ± 0.01	28
	96 mM	0.71 ± 0.02	38	0.65 ± 0.03	43	0.84 ± 0.07	23
Starved	8 mM	0.41 ± 0.05	42	0.39 ± 0.05	45	0.52 ± 0.03	27
	96 mM	0.45 ± 0.03	37	0.37 ± 0.05	48	0.55 ± 0.02	23
Ethanol-fed	8 mM	0.32 ± 0.03	72	0.17 ± 0.02	85	0.38 ± 0.03	67
(low fat)	96 mM	0.42 ± 0.03	59	0.26 ± 0.02	75	0.45 ± 0.05	56
Phenobarbitone	8 mM	0.76 ± 0.04	26	0.55 ± 0.08	47	0.67 ± 0.08	35
	96 mM	0.86 ± 0.07	17	0.55 ± 0.07	47	0.77 ± 0.09	26
3-Amino-triazole	8 mM	0.61 ± 0.01	38	0.38 ± 0.05	62	0.64 ± 0.01	35
	96 mM	0.57 ± 0.01	45	0.41 ± 0.06	61	0.75 ± 0.04	28

* Liver cells were incubated as described in Table 3. Initial concentrations of rotenone (15 μM, added in 5 μl acetone), antimycin (11 μM, added in 5 μl acetone) and oligomycin (5 μg in 5 μl acetone) were added as indicated. Control values without inhibitors are shown in Table 3.

tion on ethanol oxidation revealed that in the first 10 min of the incubation period, there was an apparent enhancement of ethanol oxidation that was directly proportional to the ethanol concentration. This phenomenon proved to be due to the contamination of [1-^{14}C]ethanol with another volatile, labelled material which was rapidly metabolized to a form not volatile in alkali. This substance was most likely [1-^{14}C]acetaldehyde which is formed during storage of [1-^{14}C]ethanol (H. E. Hargraves, Radiochemical Centre, Amersham, U.K., personal communication). When this was removed by distillation, the redistilled ethanol remained stable for at least one month.

After purification of the [1-^{14}C]ethanol it was found that the rate of ethanol oxidation was linear with time and constant over a range of ethanol concentrations of 8–96 mM. This was the case in the absence of added substrate or in the presence of pyruvate for cells from fed or fasted rats. Hence in these experiments no evidence was obtained for a pathway for ethanol oxidation catalysed by an enzyme with a lower affinity for ethanol than alcohol dehydrogenase. Our studies with pyrazole and 4-methyl pyrazole likewise revealed no evidence for a significant contribution to ethanol oxidation of pathways other than alcohol dehydrogenase.

Since the hydrogen arising in ethanol oxidation catalysed by alcohol dehydrogenase is transferred in large part to the mitochondria [22], it would seem feasible to employ inhibitors of mitochondrial metabolism to discriminate between alternate pathways of ethanol oxidation. The hydrogen arising in catalase or MEOS-catalysed ethanol oxidation reacts in the cytosol and it can be anticipated that mitochondrial inhibitors would be less effective in reducing the activity of these enzymes. However, as shown in Table 4, the oxidation of ethanol by cells from normal rats was blocked to the same extent by mitochondrial inhibitors in the presence of 8 or 96 mM ethanol.

An alternative approach is to examine the effects on ethanol oxidation of agents which might be expected to inhibit a microsomal ethanol-oxidizing system. Since this system requires NADPH for activity [2], substances which deplete the hepatic cell of NADPH should reduce the rate of ethanol oxidation. On this premise the actions of menadione, phenazine methosulphate and methylene blue were tested. These artificial electron carriers all strongly increased oxygen uptake and stimulated ethanol oxidation over the range 8–96 mM, although gluconeogenesis was substantially reduced (M. N. Berry, unpublished observations). It seems probable that the stimulatory effect of these agents was mediated through the regeneration of cytoplasmic NAD, the rate-limiting step for alcohol dehydrogenase-dependent ethanol oxidation under these circumstances [23]. The absence of any inhibitory effect on ethanol oxidation even in the presence of 96 mM ethanol suggests that availability of NADPH was not a requirement for ethanol oxidation and that MEOS was not functional in this system.

Pathways of ethanol oxidation in alcohol-fed rats. It is difficult to maintain in rats an alcohol intake high enough to mimic the intake of alcoholics. Such high levels of alcohol ingestion have been achieved by providing a total liquid diet. Whereas Lieber *et al.* [13] chose to supply the residual calories largely as fat, others [14] have used a low-fat diet where fat provided only 5 per cent of the calories.

We found considerable difficulty in obtaining preparations of intact cells in good yield from the very fatty livers of rats fed an ethanol high-fat diet.

For this reason the low-fat diet of Savolainen *et al.* [14] was preferred for most experiments. Even so, it was found that neither diet caused any increase in the oxidation of ethanol by isolated cells and again no discrimination was observed between the effects of 8 and 96 mM ethanol. Recently, Kondrup *et al.* [24] have reported a lack of stimulation of ethanol oxidation by perfused livers from rats fed an ethanol high-fat diet.

Using perfused livers from normal and chronic ethanol-fed rats, Damgaard *et al.* [4] found no differences in the rates of ethanol oxidation at high and low concentrations. On the other hand, in a recent paper Cederbaum *et al.* [7] reported an increased rate of ethanol oxidation in cells from ethanol-fed rats when incubated with 12.5 mM ethanol. This phenomenon was observed only when ethanol was being oxidized at about 45 per cent of maximal rate in each preparation due to the lack of a hydrogen acceptor such as pyruvate. When pyruvate was added to the cells the rates of ethanol oxidation were similar whether or not the cells were obtained from normal or ethanol-treated rats and approached the level seen *in vivo*. Thus, the data of Cederbaum *et al.* [7] agree essentially with our own in that maximal rates of ethanol oxidation were similar in cells from normal and from ethanol-treated rats.

Pathways of ethanol oxidation in rats fed phenobarbitone. The effectiveness of phenobarbitone treatment in stimulating proliferation of the hepatic endoplasmic reticulum was confirmed by the substantial residual respiration in the presence of rotenone (1.95 μmoles/min/g for cells from phenobarbitone-treated rats compared with 1.23 μmoles/min/g for cells from fed rats), and the extremely high respiration observed in the presence of phenazine methosulphate or menadione (Table 3). Nevertheless, phenobarbitone treatment failed to bring about a stimulation of ethanol oxidation. In addition, the residual ethanol oxidation in cells poisoned with antimycin or oligomycin was not greater than that of normal cells incubated with either 8 or 96 mM ethanol. Thus, these data provide no evidence that phenobarbitone administration brought about the induction of an ethanol-oxidizing system in the proliferating endoplasmic reticulum.

Role of catalase in ethanol oxidation. Treatment of donor fed or fasted rats with 3-amino-triazole 24 hr before cell preparation had no effect on endogenous rates of ethanol oxidation by hepatocytes. Likewise, pyruvate-stimulated ethanol oxidation was similar in cells from normal and 3-amino-triazole-treated rats. These studies, which were carried out with both 8 and 96 mM ethanol, imply that catalase is of little or no importance under these conditions. However, glycollate-stimulated ethanol oxidation was substantially impaired by pre-treatment of rats with 3-amino-triazole (M. N. Berry, unpublished observations). Hence, it is evident that under certain circumstances catalase can be responsible for a significant proportion of ethanol oxidation.

Physiological role of hepatic ethanol-oxidizing systems. In the present studies pyruvate and artificial electron acceptors greatly stimulated the rate of ethanol oxidation in cells from all treatment groups, although cells from ethanol-treated rats showed least stimulation. In all instances this stimulated activity was totally abolished by 4-methyl pyrazole, demonstrating that the pathway of ethanol oxidation consistently involved alcohol dehydrogenase. Rates of ethanol oxidation obtained in the presence of stimulating agents were close to those observed *in vivo*. Hence, alcohol dehydrogenase clearly has sufficient capacity to account for total ethanol oxidation, provided that hydrogen generated in the cytoplasm can be transferred to oxygen. Although this circumstance will not always pertain in isolated cells which may have been depleted of key metabolites by washing, there is no evidence that a deficiency of carrier or acceptor molecules for the hydrogen arising in ethanol oxidation exists *in vivo*.

In the present studies no evidence has been obtained either for an increased rate of ethanol oxidation at high ethanol concentrations or for an increased rate of ethanol oxidation in hepatocytes from ethanol-fed or phenobarbitone-treated rats. These studies therefore provide no support for the view that a 'microsomal ethanol-oxidizing system' is likely to have a significant role in the hepatic oxidation of ethanol *in vivo*.

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