Rate-Limiting Factors in the Oxidation of Ethanol by Isolated Rat Liver Cells

By ALFRED J. MEIJER,* GEORGE M. VAN WOERKOM,* JOHN R. WILLIAMSON†
and JOSEPH M. TAGER*

*Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam,
Plantage Muidergracht 12, Amsterdam, The Netherlands, and

†The Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. 19174, U.S.A.

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The oxidation of ethanol by isolated liver cells from starved rats is limited by the rate of removal of reducing equivalents generated in the cytosol by alcohol dehydrogenase. Evidence is presented suggesting that, in these cells, transfer of reducing equivalents from the cytosol to the mitochondria is regulated by the intracellular concentrations of the intermediates of the malate–aspartate and glycerol 3-phosphate cycles, as well as by flux through the respiratory chain. In liver cells isolated from fed rats, the availability of substrate increased the cell content of intermediates of the hydrogen-transfer cycles, and enhanced ethanol uptake. Under these conditions, ethanol consumption is limited by the availability of ADP for oxidative phosphorylation.

It is generally considered that ethanol oxidation in liver is limited by the rate of reoxidation of cytosolic NADH (see, e.g., Videla & Israel, 1970; Lindros *et al.*, 1972; Williamson *et al.*, 1974c). It is unlikely that alcohol dehydrogenase (EC 1.1.1.1) itself is rate-limiting (Videla & Israel, 1970). In the absence of a cytosolic hydrogen acceptor, the reducing equivalents generated in the alcohol dehydrogenase reaction must be transported to the mitochondria via one of the hydrogen-translocating shuttles in order to react with O₂. Since hydrogen transfer to the mitochondria via NAD-linked shuttles is likely to be regulated by the mitochondrial NAD oxidation–reduction state (Williamson *et al.*, 1971a), uncertainty arises as to whether the rate-determining factor during ethanol oxidation is the activity of the shuttles, or flux through the phosphorylating electron-transport chain.

In a survey of the literature, we concluded that ethanol oxidation in the liver of the starved rat is probably limited by the activity of the shuttles, in contrast with the liver of the fed rat, where the mitochondrial reoxidation of NADH is limiting (Meijer & van Dam, 1974). The most important arguments in favour of this conclusion were (a) ethanol oxidation in the liver of the fed rat is faster than that in the liver of the starved rat (Smith & Newman, 1959; Ylikahri & Mäenpää, 1968), unless the latter is supplemented with exogenous substrates (Williamson *et al.*, 1974c) (b) thyroxine treatment of the animals, which increases the activity of mitochondrial glycerol 3-phosphate dehydrogenase (EC 1.1.99.5) and thereby the activity of the glycerol 3-phosphate cycle (Lee & Lardy, 1965), increases the rate of ethanol utilization in livers from starved rats considerably (Ylikahri & Mäenpää, 1968), but has less effect in

livers from fed rats (Ylikahri & Mäenpää, 1968; Rawat & Lundquist, 1968); and (c) addition of each of the components of the malate–aspartate cycle stimulates ethanol oxidation in isolated liver cells from starved rats (Williamson *et al.*, 1974b). Additional experimental evidence in support of this conclusion is described in the present paper.

Experimental

Rat liver cells were prepared from livers of 24h-starved male rats (Sprague–Dawley) or from rats fed *ad libitum*, by the procedure of Berry & Friend (1969), by using 0.04% collagenase and 0.01% hyaluronidase. The perfusion technique was that of Williamson *et al.* (1969). The cells were used immediately after isolation. Cells (5–10mg dry wt./ml) were incubated for 60min at 37°C in Krebs bicarbonate buffer, pH 7.4 (Krebs & Henseleit, 1932), supplemented with 4% (w/v) dialysed serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo., U.S.A.), and gassed with O₂+CO₂ (95:5) as described previously (Meijer & Williamson, 1974). Samples were removed for assays at 20min intervals and were deproteinized by the addition of HClO₄ to a final concentration of 3.5% (w/v). After removal of the denatured protein by centrifugation in the cold, the pH of the supernatant was adjusted to about 6.5 with 2M-KOH plus 0.3M-Mops [3-(N-morpholino)propanesulphonic acid]. Metabolic rates were linear with time unless otherwise noted. Metabolites were measured in cells plus medium. The amount of extracellular ATP was always very low (less than 10% of the total ATP present in the suspension) and therefore the total amount of ATP is approximately equal to that of

intracellular ATP. Control experiments have shown that, under our experimental conditions, more than 60% of the total amounts of metabolites such as malate, aspartate and glycerol 3-phosphate were located intracellularly. Therefore with these metabolites, the true intracellular contents will be lower by 40%, or less than indicated by the amounts in the whole-cell suspension. However, since the metabolite changes reported in the present paper are very large, we have confined ourselves to measurements in cells plus medium. Metabolites were determined by standard enzymic procedures (Williamson & Corkey, 1969; Bergmeyer, 1970). Values for ethanol consumption were corrected for evaporation by carrying out parallel incubations in the absence of cells. Extracellular lactate dehydrogenase (EC 1.1.1.27) was measured in some cases at the end of the experiment, after centrifuging the cells for 2 min at 50g. Collagenase was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and hyaluronidase was from Sigma. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine was a gift from Dr. Peter Heytler of E.I. DuPont de Nemours and Co., Wilmington, Del., U.S.A.

Results and Discussion

Ethanol oxidation in liver cells from starved rats

Since ethanol oxidation in liver cells from starved rats is partly inhibited by the transaminase inhibitors amino-oxyacetate and DL-cycloserine (Scholz & Zimmer, 1973; Williamson *et al.*, 1974c), it may be concluded that the malate-aspartate cycle participates in the reoxidation of cytosolic NADH under these conditions. The results in Table 1 show that the malate-aspartate cycle does not operate at full capacity, as indicated by the fact that the addition of malate to the cells results in a marked cycloserine-

sensitive stimulation of ethanol oxidation. A similar increase in ethanol consumption, which is sensitive to inhibition by transaminase inhibitors, can be obtained by adding any one of the other components of the malate-aspartate cycle (Williamson *et al.*, 1974a,b,c). It is important to note that notwithstanding the low permeability of the plasma membrane for malate and other dicarboxylic acids (Hems *et al.*, 1968), sufficient malate is transported into the cells to augment the concentration of the shuttle intermediates. The small increase in glucose production on addition of malate (Table 1) shows that the added malate is indeed slowly metabolized, and indicates that the effect of malate is catalytic.

The fact that ethanol consumption, but not oxygen uptake (results not shown), is increased by augmenting the intracellular concentration of components of the malate-aspartate shuttle, suggests that in liver cells from starved-rats, hydrogen transfer from cytosol to mitochondria, and not the mitochondrial oxidation of NADH, limits the oxidation of ethanol. This conclusion is supported by the observation that the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine to liver cells from starved rats did not increase ethanol consumption (Table 2, Expt. a), unless the cells were pre-incubated with lactate and quinolinate (Fig. 1). Quinolinate inhibits the conversion of oxaloacetate into phosphoenolpyruvate, and results in the accumulation of malate and aspartate in the liver (Veneziale *et al.*, 1967). At concentrations of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine above 6.6 μM , an inhibition of ethanol consumption was observed with liver cells from starved rats, which was paralleled by a decreased respiratory rate and increased leakage of lactate dehydrogenase into the medium (not shown).

We have previously reported that ethanol oxidation in liver cells from starved rats is stimulated by addition of lactate (Williamson *et al.*, 1974c). Table 3 shows that this stimulatory effect has two components.

(a) *Stimulation by an increased energy demand, owing to glucose synthesis.* This component is sensitive to quinolinate. Assuming a P/O ratio of 3, six molecules of ATP can be synthesized for each molecule of ethanol oxidized to acetate. [Operation of the glycerol 3-phosphate cycle will decrease the ATP/acetate ratio from 6 to 5, since acetaldehyde is mainly oxidized in the mitochondria (Marjanen, 1972; Grunnet, 1973; Parilla *et al.*, 1974; Rognstad & Clark, 1974)]. Thus if no recycling of carbon occurs, the increase in ethanol oxidation should be equal to the amount of glucose formed from lactate, since 6 mol of ATP are required for the synthesis of 1 mol of glucose from lactate. Actually, changes of ethanol consumption caused by the addition of lactate or quinolinate were larger than the changes of gluconeogenesis, indicating a greater-than-theoretical energy cost for

Table 1. *Effect of malate and DL-cycloserine on ethanol uptake in liver cells of starved rats*

For details see the Experimental section. The substrate was 7 mM-ethanol. The results shown are those obtained by using a single batch of cells. Similar results were obtained in several experiments of this type.

Additions	Metabolic changes ($\mu\text{mol/h}$ per g dry wt. of cells)	
	$-\Delta\text{Ethanol}$	$\Delta\text{Glucose}$
None	255	2.5
Malate (10 mM)	338	8.0
DL-Cycloserine (10 mM)	173	1.2
Malate + DL-cycloserine	202	2.1

Table 2. *Effect of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone on ethanol uptake by liver cells from starved and fed rats*

For details see the Experimental section. The substrate was 7 mM-ethanol. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was dissolved in 4% (w/v) albumin. One batch of cells was used in each of the two experiments shown. Similar results were obtained in several analogous experiments.

Expt.	Condition of animal	FCCP (μM)	$-\Delta\text{Ethanol}$ ($\mu\text{mol/h}$ per g dry wt. of cells)	ATP ($\mu\text{mol/g}$ dry wt. of cells after 1 h)
<i>a</i>	Starved	0	210	6.1
		3.3	201	4.0
		6.6	187	4.0
		13.2	85	0.7
<i>b</i>	Fed	0	432	8.3
		8	612	6.7
		16	776	5.9
		24	739	4.8

Fig. 1. *Effect of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone on ethanol uptake in liver cells from starved rats*

The initial ethanol concentration was 7 mM. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 5 μM) was added as indicated. In Fig. 1(b), 10 mM-lactate and 5 mM-quinolinate were also present. At 20 min, 0.6 and 19.4 μmol of malate/g dry wt. of cells were found in the cell suspensions of Figs. 1(a) and 1(b) respectively. The same cell preparation was used in Figs. 1(a) and 1(b).

gluconeogenesis, owing to the activity of energy-consuming carbon-recycling reactions (Williamson *et al.*, 1971b).

(b) *Stimulation of ethanol oxidation by increased activity of the malate-aspartate cycle because of increased malate and aspartate concentrations.* This component is insensitive to quinolinate.

The effect of inhibiting electron transport in liver cells from starved rats by addition of Amytal is illustrated in Table 4. Addition of dihydroxyacetone

stimulated ethanol uptake by 45% and caused a threefold increase in glycerol 3-phosphate content. Since ethanol uptake under these conditions is insensitive to inhibition by cycloserine (Williamson *et al.*, 1974c), it may be assumed that hydrogen transport to the mitochondria occurs by the glycerol 3-phosphate cycle. The increased energy demand for gluconeogenesis (2 mol of ATP/mol of glucose from dihydroxyacetone) was about equivalent to the energy yield from the oxidation of the extra ethanol

Table 3. *Ethanol oxidation in liver cells from starved and fed rats*

The substrate was 7 mM-ethanol. Concentrations: lactate, 10 mM; quinolinate, 5 mM; malate, 10 mM. There was a lag period in the synthesis of glucose from lactate in the liver cells of starved rats (cf. Cornell *et al.*, 1974). The values for glucose production represent the amount of glucose formed at 60 min. All values represent the means $\pm$ s.e.m. All observations were paired, so that exactly the same experiment, with the five different additions mentioned, was repeated with different cell preparations, the number of which is given in parentheses. The concentrations of malate, aspartate and glycerol 3-phosphate were not measured in all these experiments. In such cases the values belonging to one particular cell preparation have been listed in the same column.

Condition of animal	Additions	Metabolite changes (μ mol/h per g dry wt. of cells)		Metabolite concentrations (μ mol/g dry wt. of cells)		
		$-\Delta$ Ethanol	Δ Glucose	Malate	Aspartate	Glycerol 3-phosphate
Starved	None	215 $\pm$ 22 (4)	3.4 $\pm$ 0.4 (4)	0.6 $\pm$ 0.15 (3)	0.3; 0.9 (2)	1.9 $\pm$ 0.3 (3)
	Lactate	416 $\pm$ 43 (4)	66 $\pm$ 7 (4)	9.9 $\pm$ 1.5 (3)	1.2; 1.2 (2)	3.3 $\pm$ 0.5 (3)
	Quinolinate	214 $\pm$ 37 (4)	1.9 $\pm$ 0.4 (4)	0.9 $\pm$ 0.1 (3)	—	1.3 $\pm$ 0.4 (3)
	Quinolinate + lactate	317 $\pm$ 37 (4)	7.5 $\pm$ 0.8 (4)	24.2 $\pm$ 1.1 (3)	—	2.2 $\pm$ 0.2 (3)
	Malate	303 $\pm$ 18 (3)	9.4 $\pm$ 0.8 (3)	—	—	1.9; 3.0 (2)
Fed	None	433 $\pm$ 12 (3)	369 $\pm$ 86 (3)	1.9; 2.1 (2)	1.7; 2.5 (2)	7.8; 22.6 (2)
	Lactate	575 $\pm$ 89 (3)	483 $\pm$ 64 (3)	13.3; 17.9 (2)	5.5; 5.9 (2)	3.9; 6.5 (2)
	Quinolinate	443 $\pm$ 29 (3)	358 $\pm$ 80 (3)	1.1; 2.5 (2)	2.4; 3.8 (2)	5.3; 19.3 (2)
	Quinolinate + lactate	490 $\pm$ 50 (3)	394 $\pm$ 84 (3)	18.4; 41.4 (2)	11.4; 6.4 (2)	4.3; 4.5 (2)
	Malate	470 $\pm$ 28 (3)	346 $\pm$ 75 (3)	—	—; 7.2 (1)	—

Table 4. *Effect of Amytal on ethanol uptake by liver cells from starved rats*

For details see the text. The concentrations of the substrates were: ethanol, 2.5 mM; dihydroxyacetone, 10 mM.

Substrate	Amytal (mM)	Metabolite changes (μ mol/h per g dry wt. of cells)			Metabolite concentrations (μ mol/g dry wt. of cells)	
		$-\Delta$ Ethanol	Δ Glucose	Δ Lactate	ATP	Glycerol 3-phosphate
None	0		20		10.3	0.6
Ethanol	0	350	25	-30	9.3	4.4
Ethanol + dihydroxyacetone	0	510	475	62	7.5	13.8
	1	526	400	225	6.2	13.0
	2	434	180	382	4.4	11.7
	4	248	15	485	3.6	11.0

consumption. Amytal (1–4 mM) inhibited ethanol uptake by 50%, and caused a progressive switch of dihydroxyacetone metabolism from glucose to lactate formation. Since the oxidation of ethanol via the flavin-linked glycerol 3-phosphate cycle should be insensitive to inhibition by Amytal, this experiment illustrates that ethanol uptake is also regulated by the rate of oxidation of acetaldehyde in the mitochondria, which, being NAD-linked, is directly inhibited by Amytal (Parilla *et al.*, 1974).

Ethanol oxidation in liver cells from fed rats

The rate of ethanol consumption in cells prepared from the livers of fed rats was about twice that observed in those from starved rats (Table 3). This is in agreement with earlier observations in perfused liver (Smith & Newman, 1959; Ylikahri & Mäenpää, 1968). Since the concentrations of malate, aspartate and particularly glycerol 3-phosphate in liver cells from fed rats were much higher than in the cells from

starved rats, it seemed likely that the higher rate of ethanol oxidation in the liver cells of fed rats is caused by increased activities of the hydrogen-transport cycles.

As in liver cells of starved rats, lactate stimulated ethanol consumption. However, in the liver cells of fed rats the effect of lactate was largely inhibited by quinolinate, indicating that the stimulation by lactate was due solely to an increased requirement for ATP in gluconeogenesis. At the concentration of quinolinate used, there was still some stimulation by lactate of ethanol consumption, which was statistically significant when paired controls were compared. This was presumably caused by the incomplete inhibition of phosphoenolpyruvate carboxykinase (EC 4.1.1.32), as indicated by the slight increase in glucose production caused by lactate in the presence of quinolinate.

Malate was rather ineffective in stimulating ethanol oxidation in the liver cells of fed rats, in contrast with the situation in starved rats (Table 3),

indicating that the hydrogen-transport cycles were not rate-limiting under these conditions.

Contrary to the situation in liver cells from starved rats, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine caused a large stimulation of ethanol oxidation in cells from fed animals, which were also less sensitive to the uncoupling agent (Table 2, Expt. *b*). Apparently, in liver cells from fed rats the mitochondrial reoxidation of NADH, whether transported from the cytosol or generated directly in the mitochondria by acetaldehyde dehydrogenase (EC 1.2.1.3), is the rate-limiting step in ethanol oxidation.

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