Lactate-Stimulated Ethanol Oxidation in Isolated Rat Hepatocytes

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1. Hepatocytes isolated from starved rats and incubated without other substrates oxidized ethanol at a rate of 0.8–0.9 $\mu\text{mol}/\text{min}$ per g wet wt. of cells. Addition of 10 mM-lactate increased this rate 2-fold. 2. Quinolate (5 mM) or tryptophan (1 mM) decreased the rate of gluconeogenesis with 10 mM-lactate and 8 mM-ethanol from 0.39 to 0.04–0.08 $\mu\text{mol}/\text{min}$ per g wet wt. of cells, but rates of ethanol oxidation were not decreased. From these results it appears that acceleration of ethanol oxidation by lactate is not dependent upon the stimulation of gluconeogenesis and the consequent increased demand for ATP. 3. As another test of the relationship between ethanol oxidation and gluconeogenesis, the initial lactate concentration was varied from 0.5 mM to 10 mM and pyruvate was added to give an initial [lactate]/[pyruvate] ratio of 10. This substrate combination gave a large stimulation of ethanol oxidation (from 0.8 to 2.6 $\mu\text{mol}/\text{min}$ per g wet wt. of cells) at low lactate concentrations (0.5–2.0 mM), but rates remained nearly constant (2.6–3.0 $\mu\text{mol}/\text{min}$ per g wet wt. of cells) at higher lactate concentrations (2.0–10 mM). 4. In contrast, owing to the presence of ethanol, the rate of glucose synthesis was only slightly increased (from 0.08 to 0.12 $\mu\text{mol}/\text{min}$ per g wet wt. of cells) between 0.5 mM- and 2.0 mM-lactate and continued to increase (from 0.12 to 0.65 $\mu\text{mol}/\text{min}$ per g wet wt. of cells) with lactate concentrations between 2 and 10 mM. 5. In the presence of ethanol, O_2 uptake increased with increasing substrate concentration over the entire range. 6. Changes in concentrations of glutamate and 2-oxoglutarate closely paralleled changes in the rate of ethanol oxidation. 7. In isolated hepatocytes, rates of ethanol oxidation are lower than those *in vivo* apparently because of depletion of malate–aspartate shuttle intermediates during cell preparation. Rates are returned to those observed *in vivo* by substrates that increase the intracellular concentration of shuttle metabolites.

Rates of ethanol metabolism in rats *in vivo* range from 2.4 to 4.0 $\mu\text{mol}/\text{min}$ per g wet wt. of liver (Crow *et al.*, 1977a) and it has been suggested (Plapp, 1975; Crow *et al.*, 1977a) that the activity of alcohol dehydrogenase (EC 1.1.1.1) may be an important factor in determining that rate. With hepatocytes isolated from rats, however, the rates are much lower (0.7–0.9 $\mu\text{mol}/\text{min}$ per g wet wt. of cells; Krebs & Stubbs, 1975; Meijer *et al.*, 1975; Crow *et al.*, 1977a,b) when these cells are incubated with ethanol and no other added substrate. Addition of 5 mM-pyruvate increases the rate of ethanol metabolism to 2.6–3.1 $\mu\text{mol}/\text{min}$ per g wet wt. of cells (Crow *et al.*, 1977a,b), which shows that the isolated cells do have the capacity to oxidize ethanol at rates comparable with those *in vivo* but that, in the absence of substrates, something other than the amount of alcohol dehydrogenase becomes the major rate-determining

factor. The rate of reoxidation of NADH has been suggested to be the major factor limiting the rate of ethanol oxidation in isolated hepatocytes (Grunnet *et al.*, 1973). An apparent explanation for pyruvate-induced acceleration in ethanol metabolism by isolated liver cells is that rapid reoxidation of cytosolic NADH in the lactate dehydrogenase reaction provides conditions more favourable for ethanol oxidation by alcohol dehydrogenase. By a similar reasoning, it might be expected that lactate added to hepatocyte suspensions would inhibit ethanol metabolism, since lactate oxidation produces NADH and competes with alcohol dehydrogenase for free cytosolic NAD^+ . However, as is now well documented (Meijer *et al.*, 1975; Krebs & Stubbs, 1975; Crow *et al.*, 1977b) and also illustrated by data in Table 1 of the present paper, lactate increases the rate of ethanol oxidation in hepatocytes.

It has been proposed that lactate stimulation of ethanol oxidation in hepatocytes isolated from starved rats is due in part to increases in malate and aspartate concentrations (Meijer *et al.*, 1975) and

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in part to ATP utilization for glucose synthesis (Meijer *et al.*, 1975; Krebs & Stubbs, 1975). Increased malate and aspartate concentrations would increase reoxidation of cytosolic NADH via the malate-aspartate shuttle (Borst, 1963; Lardy *et al.*, 1965; Haynes, 1965), whereas ATP utilization for glucose synthesis has been assumed to increase the rate of reoxidation of mitochondrial NADH. The proposed explanations for lactate stimulation of ethanol oxidation have been evaluated in the present experiments. Our results indicate that the ATP demand for glucose synthesis plays no role in the lactate stimulation of ethanol oxidation by isolated hepatocytes.

Materials and Methods

Rats

Male Wistar rats (170–220 g), obtained from Carworth (Wilmington, MA, U.S.A.), were maintained on NIH standard rat chow (National Institutes of Health, Bethesda, MD, U.S.A.) and were starved for 48 h before use.

Reagents

Except where stated otherwise, enzymes and co-factors were obtained from Boehringer (Mannheim) Corp. (Indianapolis, IN, U.S.A.). Other chemicals were reagent-grade products.

Preparation and incubation of hepatocytes

Isolated hepatocytes were prepared by the procedure of Berry & Friend (1969), modified as described by Cornell *et al.* (1973). All hepatocyte preparations were washed and incubated as described by Cornell *et al.* (1973) and Krebs *et al.* (1974). Each incubation mixture contained 70–100 mg wet wt. of cells in a final volume of 4 ml. Reactions were stopped by addition of 0.2 ml of 60% (v/v) HClO₄.

O₂ uptake

Uptake of O₂ was measured with a Clark oxygen electrode with a YSI model 53 oxygen monitor (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.). To obtain results that were comparable with those observed with manometric methods, the following procedure was used. A fresh suspension containing approx. 20 mg wet wt. of cells/ml was gassed with O₂/CO₂ (19:1), stoppered and shaken at 37°C for 30 min. After this, 2 ml of the cell suspension was added to 2 ml of medium and substrates in a 50 ml Erlenmeyer flask. The mixture was gassed with O₂/CO₂, stoppered, shaken at 37°C for 12 min and then transferred to a chamber of the O₂ monitor. The electrode, which was at 37°C, was quickly placed in the chamber and O₂ uptake was measured for 4 min. Calibration of the monitor was carried out as described by Billiar *et al.* (1970).

Metabolite measurements

As described by Krebs & Stubbs (1975), accurate measurement of ethanol oxidation by isolated hepatocytes requires special precautions to minimize evaporative losses of ethanol. We found the following procedure gave reproducible results and complete recovery of ethanol. At the end of the incubation period, the 25 ml Erlenmeyer flasks were unstoppered only long enough to add HClO₄. The flasks were then immersed in ice for 20 min, after which the samples were centrifuged at 4°C. A portion (20 µl) of the acidic supernatant was taken immediately for the determination of ethanol (Dickinson & Dalziel, 1967). High-purity Tris obtained from Sigma (Trizma base, reagent grade; Sigma Chemical Co., St. Louis, MO, U.S.A.) was used in this assay and did not require recrystallization. NAD⁺ (grade III) was also from Sigma.

All other metabolites were assayed enzymically, after adjusting the cell extract to pH 6.5 with 3 M-KOH. Pyruvate was assayed by the method of Czok & Lamprecht (1974), and 2-oxoglutarate was determined in the same cuvette by addition of 12 units (µmol/min) of glutamate dehydrogenase (EC 1.4.1.2), after completion of the pyruvate assay. When 2-oxoglutarate was to be measured, 0.75 mM-ADP, which did not interfere with the pyruvate assay, was included in the assay mixture (Lowry & Passonneau, 1972a). Glucose was assayed by the method of Bergmeyer *et al.* (1974a), lactate and malate by those of Lowry & Passonneau (1972b) and ATP by that of Lamprecht & Trautshold (1974), with the glucose concentration decreased as suggested by Lund *et al.* (1975). Glutamate was assayed as described by Cornell *et al.* (1974). The aspartate assay of Bergmeyer *et al.* (1974b) was modified to obtain a more rapid reaction and a more well-defined stable end point. The major modifications were a 4-fold increase in aspartate aminotransferase (EC 2.6.1.1) and a 3-fold decrease in malate dehydrogenase. The final assay mixture contained, in a total volume of 2 ml, 0.5 ml of 0.2 M-potassium phosphate buffer, pH 7.6, 10 µmol of 2-oxoglutarate, 0.33 µmol of NADH, 12 units of malate dehydrogenase (pig heart, 10 mg/ml) and was complete within 8 min with standard solutions of aspartate or with extracts of freeze-clamped liver or isolated cells. This modified method gave 100% recovery of aspartate from standard solutions, 89–95% recovery of aspartate added to powdered freeze-clamped liver and 86–93% recovery of aspartate added to HClO₄-treated hepatocyte suspensions.

In the present paper, metabolite contents (µmol/g) and metabolic rates (µmol/min per g) are expressed on the basis of cell or tissue wet wt. In our experiments, cell wet wt. was determined as described by Krebs *et al.* (1974). In all cases where results are

given as means $\pm$ S.E.M., they represent determinations made on the indicated numbers of different cell preparations.

Results

As shown in Table 1, the rate of ethanol oxidation by hepatocytes isolated from starved rats was increased 2.5-fold in the presence of 10mM-lactate; this observation agrees with other reports (Meijer *et al.*, 1975; Krebs & Stubbs, 1975). The physiological quality of hepatocytes used in the present study was evaluated by measuring adenine nucleotide content and rates of gluconeogenesis (Krebs *et al.*, 1974). In experiments with 10mM-lactate as substrate (Table 1), the mean rate of gluconeogenesis in the absence of ethanol ($0.59 \mu\text{mol/min per g}$) was similar to previously reported values ($0.65\text{--}0.8 \mu\text{mol/min per g wet wt. of cells}$; Krebs *et al.*, 1974; Cornell *et al.*, 1974). ATP concentrations were measured in all experiments and ranged from 2.3 to $2.5 \mu\text{mol/g}$ in cells incubated with substrates. These values are close to those reported for freeze-clamped liver ($2.5 \mu\text{mol/g}$, Krebs *et al.*, 1974; $2.1\text{--}3.0 \mu\text{mol/g}$, Williamson & Brosnan, 1974).

Effects of quinolinate and tryptophan on ethanol oxidation and glucose synthesis

Addition of quinolinate (pyridine-2,3-dicarboxylate) or tryptophan to incubation mixtures containing lactate caused a marked inhibition of gluconeogenesis both in the absence (64–73% inhibition) and in the presence (80–90% inhibition) of ethanol. In contrast with the results of Meijer *et al.* (1975), however, neither inhibitor had any effect on the rate of ethanol oxidation in the presence of lactate (Table

1). When 5mM-pyruvate was the substrate, the rate of ethanol oxidation ($2.8 \mu\text{mol/min per g wet wt.}$) was similar to the average rate in rats *in vivo* ($3.3 \mu\text{mol/min per g}$; Crow *et al.*, 1977a). Ethanol oxidation in incubations containing pyruvate was not significantly inhibited by either quinolinate or tryptophan (Table 1), which is to be expected if pyruvate accelerates ethanol oxidation by causing reoxidation of cytosolic NADH in the lactate dehydrogenase reaction. As predicted by the proposed modes of action of quinolinate and tryptophan (Ray *et al.*, 1966; Veneziale *et al.*, 1967), both compounds inhibited glucose synthesis from pyruvate, although the inhibition with pyruvate as substrate (43–51%) was less than that with lactate. One possible explanation for this difference is that pyruvate may cause a higher oxaloacetate concentration. Increased oxaloacetate concentrations would decrease the inhibitory effect of quinolinate, since this compound inhibits phosphoenolpyruvate carboxykinase by competing with oxaloacetate (McDaniel *et al.*, 1972).

Influence of initial lactate concentration on glucose synthesis and ethanol oxidation

Since the inhibition of gluconeogenesis from lactate by quinolinate or tryptophan had no effect on the rate of ethanol oxidation, a second means of varying the rate of glucose synthesis was sought to evaluate the influence of that process on ethanol oxidation. A series of experiments was carried out in the presence of ethanol and initial lactate concentrations of 0.5, 1.0, 2.0, 5.0 and 10mM. All incubations were stopped after 20min to prevent depletion of lactate at lower concentrations. Because of the short incubation time, pyruvate was added at one-tenth of the lactate concentration, to give an initial [lactate]/[pyruvate] ratio similar to that found

Table 1. *Effects of quinolinate and tryptophan on rates of glucose synthesis and ethanol oxidation in hepatocytes*
Cells from rats starved for 48 h were incubated for 60min. Initial concentrations of substrates and inhibitors were: lactate, 10mM; pyruvate, 5mM; tryptophan, 1mM; quinolinate, 5mM; ethanol, 8mM, where rates of ethanol oxidation or glucose synthesis in the presence of ethanol were determined. Rates are means $\pm$ S.E.M. for three to six cell preparations.

Additions	Rates ($\mu\text{mol/min per g wet wt. of cells}$)		
	Ethanol oxidation	Glucose synthesis	
		–Ethanol	+Ethanol
None	0.75 ± 0.09	0.07 ± 0.004	0.02 ± 0.002
Lactate	1.86 ± 0.07	0.59 ± 0.03	0.39 ± 0.03
Lactate, quinolinate	1.83 ± 0.09	0.16 ± 0.02	0.04 ± 0.003
Lactate, tryptophan	1.88 ± 0.07	0.21 ± 0.03	0.08 ± 0.02
Quinolinate	0.93 ± 0.07	—	0.02 ± 0.002
Tryptophan	1.01 ± 0.10	—	0.02 ± 0.005
Pyruvate	2.78 ± 0.14	0.61 ± 0.03	0.83 ± 0.03
Pyruvate, quinolinate	2.76 ± 0.08	0.35 ± 0.02	0.41 ± 0.01
Pyruvate, tryptophan	2.52 ± 0.18	0.34 ± 0.03	0.47 ± 0.03

in freeze-clamped liver (Veech *et al.*, 1972). With this substrate combination, maximal rates of ethanol oxidation ($2.6\text{--}3.0\mu\text{mol/min per g}$) were similar to rates observed *in vivo* (average rate, $3.3\mu\text{mol/min per g}$; Crow *et al.*, 1977a).

Fig. (1a) shows that the rate of gluconeogenesis increased only slightly between 0.5 mM- and 2.0 mM-lactate (from 0.08 to $0.12\mu\text{mol/min per g}$). Larger increases in the rate of gluconeogenesis (from 0.12 to $0.65\mu\text{mol/min per g}$) were observed when the lactate concentration was increased from 2 to 10 mM. In contrast, the lactate-stimulated increase in ethanol oxidation (Fig. 1b) occurred at the lower lactate concentrations (0.5–2.0 mM) and no significant increase was observed between 2 mM- and 10 mM-lactate. The present results thus show that the dependence of ethanol oxidation on substrate concentration is completely different from that for glucose synthesis.

Parallel experiments with 5 mM-quinolinate gave an even more pronounced separation of the two

processes. Gluconeogenesis was inhibited by 5 mM-quinolinate at 2 mM-, 5 mM- and 10 mM-lactate (Fig. 1a) but not at 0.5 or 1.0 mM, where rates in the absence of quinolinate were not significantly higher than in the control without lactate. Rates of ethanol oxidation were not significantly affected by quinolinate throughout the entire range of substrate concentrations (Fig. 1b).

The inhibition of glucose synthesis by quinolinate was 50% in experiments with 10 mM-lactate and 1 mM-pyruvate, compared with the 90% inhibition observed with 10 mM-lactate and no added pyruvate (Table 1). As suggested earlier, the decreased effect of quinolinate in incubation mixtures containing pyruvate may be due to an increased oxaloacetate concentration.

O₂ uptake in hepatocytes with varied [lactate]

Although the rate of glucose synthesis increased over the entire range of lactate concentrations (Fig. 1a), this did not necessarily mean that the rate of mitochondrial reoxidation of NADH was increasing. As a more direct indication, rates of O₂ uptake were measured in cells incubated with various concentrations of lactate.

Our O₂ uptake measurements, carried out with a Clark oxygen electrode as described in the Materials and Methods section, gave results comparable with those from manometric measurements. With the latter method, a 52% increase in O₂ uptake was observed with 10 mM-lactate (Krebs *et al.*, 1974). We observed a 47% stimulation by 10 mM-lactate (Table 2b). Also in agreement with previous studies (Berry, 1974), the results of Table 2(b) show that O₂ uptake was not increased when ethanol was added to cell suspensions containing 10 mM-lactate.

Table 2(a) contains the results obtained when substrate concentrations were varied. In all of these experiments, lactate and pyruvate were added in the ratio 10:1. In the absence of ethanol, the rate of O₂ uptake was maximal at 1 mM-lactate and did not change between 1 mM- and 10 mM-lactate. However, when ethanol was also present the rate of O₂ uptake increased with increasing lactate concentration over the entire concentration range. Thus it appears that increased glucose synthesis was accompanied by an increased mitochondrial rate of NADH reoxidation, even at lactate concentrations of 2–10 mM, where the rate of ethanol oxidation was nearly constant.

Concentrations of aspartate-shuttle components

It has been shown previously that amino acids and other metabolites are depleted in freshly prepared hepatocytes, but that these can be restored during incubation with substrates (Krebs *et al.*, 1974). Similar results were found in this study when malate, aspartate, glutamate and 2-oxoglutarate were measured in fresh cells and at the end of incubations for

Fig. 1. Effect of varying [lactate] on rates of glucose synthesis and ethanol oxidation

Cells from rats starved for 48 h were incubated for 20 min. Controls (○) contained 8 mM-ethanol, lactate at the concentrations indicated and pyruvate at one-tenth of the lactate concentration. Other incubations contained, in addition, 5 mM-quinolinate (□). Each point represents the mean of determinations on at least three different cell preparations. (a) Rates of glucose synthesis; (b) rates of ethanol oxidation.

Table 2. *Effect of ethanol on relative rates of O₂ uptake by hepatocytes*

Uptake of O₂ was measured with a YSI model 53 oxygen monitor and the rate in the absence of added substrates was assigned a value of 1.0. Addition of 8 mM-ethanol alone had no effect on the rate. Subsequent calibration of the monitor indicated that the absolute rate in the absence of substrates was 2.3–2.5 μmol of O₂/min per g wet wt. of cells. For the experiments in (a) ($n = 4$), pyruvate was also added to give an initial [lactate]/[pyruvate] value of 10. For the single experiment shown in (b), no pyruvate was added.

[Lactate] (mM)	Relative rates of O ₂ uptake	
	–Ethanol	+8 mM-Ethanol
(a) Incubations with lactate and pyruvate		
0	1.00	1.00
0.5	1.34 $\pm$ 0.05	1.18 $\pm$ 0.05
1.0	1.43 $\pm$ 0.07	1.31 $\pm$ 0.02
2.0	1.44 $\pm$ 0.07	1.41 $\pm$ 0.03
5.0	1.42 $\pm$ 0.07	1.58 $\pm$ 0.03
10.0	1.42 $\pm$ 0.07	1.71 $\pm$ 0.03
(b) Incubations with lactate alone		
2.0	1.47	1.44
10.0	1.47	1.49

Table 3. *Effects of lactate concentration on concentrations of malate and aspartate*

Cells were incubated for 20 min. All incubations contained 8 mM-ethanol and pyruvate to give an initial [lactate]/[pyruvate] value of 10. The quinolinate concentration was 5 mM. Values are means $\pm$ S.E.M. for three cell preparations, with one exception, where results for two preparations are shown.

Added lactate (mM)	Malate ($\mu\text{mol/g}$ wet wt. of cells)		Aspartate ($\mu\text{mol/g}$ wet wt. of cells)	
	–Quinolinate	+Quinolinate	–Quinolinate	+Quinolinate
0.0	<0.2	—	<0.2	—
0.5	<0.2	0.40 $\pm$ 0.16	<0.2	0.35 $\pm$ 0.05
1.0	0.22 $\pm$ 0.03	0.81 $\pm$ 0.21	<0.2	0.23 $\pm$ 0.05
2.0	0.57 $\pm$ 0.06	1.75 $\pm$ 0.14	<0.2	0.39 $\pm$ 0.04
5.0	1.21 $\pm$ 0.10	3.54 $\pm$ 0.22	<0.2	0.37 $\pm$ 0.05
10.0	1.62 $\pm$ 0.16	4.93 $\pm$ 0.45	<0.2	0.33, 0.46

20 min containing 8 mM-ethanol and 0.5, 1.0, 2.0, 5.0 and 10 mM-lactate. With 2 mM-lactate, glutamate (Fig. 2) and malate (Table 3) reached concentrations similar to those seen in freeze-clamped liver (Williamson & Brosnan, 1974). As [lactate] was increased from 2 to 10 mM, [malate] continued to increase, but [glutamate] remained constant. Aspartate content was less than 0.2 $\mu\text{mol/g}$ at all lactate concentrations, and was considerably lower than in freeze-clamped liver (0.7 $\mu\text{mol/g}$, Williamson & Brosnan, 1974). In contrast with the other shuttle components, 2-oxoglutarate was higher in hepatocytes at all substrate concentrations than in freeze-clamped liver, and it increased with increasing lactate up to 5 mM.

The changes in [glutamate], in particular, closely paralleled changes in the rate of ethanol oxidation with increasing [lactate]. Changes in [2-oxoglutarate] also correlated fairly closely with changes in ethanol oxidation (Figs. 1b and 2), whereas malate concentrations showed a closer correlation with rates of glucose synthesis (Table 3 and Fig. 1a).

As in previous experiments with perfused rat liver (Veneziale *et al.*, 1967; Alvares & Ray, 1974), the addition of quinolinate to hepatocyte incubation mixtures caused an increase in aspartate and a marked increase in malate (Table 3). Quinolinate caused only slight increases in 2-oxoglutarate and glutamate concentrations, and a close correlation between

Fig. 2. Effects of varying [lactate] on glutamate and 2-oxoglutarate content

Cells from rats starved for 48 h were incubated for 20 min. Glutamate (○, □) and 2-oxoglutarate (●, ■) concentrations were measured as described in the Materials and Methods section. Controls (○, ●) contained, initially, 8 mM-ethanol, lactate at the concentrations indicated and pyruvate at one-tenth of the lactate concentration. Other incubations (□, ■) contained, in addition, 5 mM-quinolinate. Each point represents the mean of determinations on three different cell preparations.

changes in ethanol oxidation and glutamate and 2-oxoglutarate concentrations was still evident (Figs. 1b and 2).

Discussion

Since quinolinate or tryptophan prevents utilization of two-thirds of the ATP required for glucose synthesis from lactate, either inhibitor should decrease the lactate-stimulated rate of ethanol oxidation if the latter depends on an increased ATP demand for glucose synthesis. However, the results in Table 1 show that these inhibitors decreased the rate of glucose synthesis from 10 mM-lactate by 80–90% in cells from starved rats, but the rate of ethanol oxidation was not decreased. The increment in ethanol oxidation in the presence of lactate and quinolinate, relative to incubations with no added substrates, was 54 times greater than the increment in glucose synthesis. Under all conditions, we find that acetate accumulation in hepatocyte incubations accounts for at least 90% of the ethanol disappearance (K. E. Crow, unpublished work). Conversion of ethanol into acetate via the alcohol dehydrogenase and aldehyde dehydrogenase reactions yields 2 molecules of NADH/molecule of ethanol, and reoxidation of 2 molecules of NADH via the mitochondrial respiratory chain generates 6 molecules of ATP, the amount required in the

synthesis of 1 glucose molecule from lactate. Though the design of our experiments does not permit a strict comparison, it should be noted that, in every case (Table 1 and Fig. 1), the increase in glucose synthesis due to substrates is less than the increase in ethanol oxidation. In the experiments with lactate and quinolinate (Table 1), the ATP required for glucose synthesis would account for reoxidation of only a small percentage of the NADH produced in the lactate-stimulated portion of ethanol metabolism. The present results indicate that the accelerating effect of lactate on ethanol oxidation is not primarily due to an increased ATP demand for glucose synthesis.

The data in Table 1 are in contrast with those of Meijer *et al.* (1975), who reported that quinolinate decreased the lactate-stimulated rate of ethanol oxidation. We are unable to explain this discrepancy. However, the conclusion drawn from our quinolinate and tryptophan data is supported by results of experiments in which ethanol oxidation and glucose synthesis were measured at various lactate concentrations (Fig. 1). The major stimulation in ethanol oxidation occurred at low lactate concentrations (0.5, 1.0 mM) where, because of the presence of ethanol, glucose synthesis was not stimulated at all. At higher lactate concentrations (2.0–10.0 mM), where rates of glucose synthesis increased with increasing lactate concentration, ethanol oxidation rates remained almost constant. As with glucose synthesis, O_2 uptake continued to increase at higher lactate concentrations, indicating that an increased mitochondrial rate of reoxidation of NADH did accompany the increasing ATP utilization for gluconeogenesis. There was no concomitant increase in rate of ethanol oxidation, which again indicates that NADH reoxidation via the mitochondrial electron-transport chain is not a limiting factor for ethanol metabolism in isolated hepatocytes.

The results in Figs. 1 and 2 and Table 3 show that changes in concentrations of malate–aspartate shuttle intermediates, especially glutamate, parallel changes in the rate of ethanol oxidation. These data suggest that lactate accelerates ethanol oxidation in isolated hepatocytes by increasing the rate at which reducing equivalents are transported into the mitochondria via the malate–aspartate shuttle. Exchange of cytosolic glutamate for mitochondrial aspartate is an essential process in the operation of this shuttle (Azzi *et al.*, 1967; Tischler *et al.*, 1976). The exchange is catalysed by a specific carrier, for which a K_m for glutamate of approx. 6 mM has been determined with isolated mitochondria in the presence of low external aspartate concentrations (Tischler *et al.*, 1976). In the present experiments, total cellular concentrations of glutamate ranged from 1.5 to 3.9 $\mu\text{mol/g}$ wet wt. (Fig. 2). Assuming uniform distribution of glutamate throughout the cell, these concentrations of gluta-

mate would correspond to cytosolic concentrations of 2–5 mM. Since these concentrations are below the glutamate K_m for the glutamate–aspartate carrier, it is possible that the rate of operation of the malate–aspartate shuttle and the resultant concentration of free cytosolic NADH are dependent on the glutamate concentration. This would explain the particularly close correlation between glutamate concentration and rates of ethanol oxidation with various lactate concentrations.

To relate the results of the present or other studies with isolated cells to ethanol metabolism *in vivo*, a major difference between isolated hepatocytes and the liver *in vivo* must be considered. The concentrations of shuttle intermediates (malate, glutamate and aspartate) seen in freshly prepared cells (Krebs *et al.*, 1974, and the present paper) are much lower than those in freeze-clamped liver, even after starvation for 48 h (Krebs *et al.*, 1974; Williamson & Brosnan, 1974). Because of these metabolite losses during cell preparation, the metabolism of isolated hepatocytes is altered. However, the shuttle intermediates are restored and the metabolism readily reverts to that of the intact liver after addition of appropriate substrates to hepatocyte suspensions (see e.g., Cornell *et al.*, 1974). When that occurs, it becomes possible to draw inferences about the cellular factors that are rate-determining in the normal intact liver. In the presence of appropriate substrates (Fig. 1), ethanol is metabolized by isolated hepatocytes at rates similar to those observed *in vivo*, but we know of no substrate combination or experimental condition that will cause the rate to be greater than *in vivo*. Thus, though the malate–aspartate shuttle appears to be a rate-limiting factor for ethanol oxidation by isolated hepatocytes in some instances, this is probably not the case *in vivo* where extensive depletion of shuttle metabolites does not normally occur, and where rates of ethanol metabolism are as fast as the activity of alcohol dehydrogenase will allow with the prevailing substrate concentrations (Crow *et al.*, 1977a). The reversible effects of hepatocyte preparation are useful in that they provide an opportunity to study systems that participate in ethanol metabolism but which are not rate-limiting and, therefore, more difficult to study in living animals.

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References

- Alvares, F. L. & Ray, P. D. (1974) *J. Biol. Chem.* **249**, 2058–2062
- Azzi, A., Chappell, J. B. & Robinson, B. H. (1967) *Biochem. Biophys. Res. Commun.* **29**, 148–152
- Bergmeyer, H. U., Bernt, E., Schmidt, F. & Stork, H. (1974a) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U. & Gawehn, K., eds.), vol. 3, pp. 1196–1201, Academic Press, New York and London
- Bergmeyer, H. U., Bernt, E., Möllering, H. & Pfeleiderer, G. (1974b) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U. & Gawehn, K., eds.), vol. 4, pp. 1696–1700, Academic Press, New York and London
- Berry, M. N. (1974) in *Regulation of Hepatic Metabolism* (Lundquist, F. & Tygstrup, N., eds.), pp. 568–578, Academic Press, New York
- Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520
- Billiar, R. B., Knappenberger, M. & Little, B. (1970) *Anal. Biochem.* **36**, 101–104
- Borst, P. (1963) in *Funktionelle und Morphologische Organisation der Zelle* (Karlson, P., ed.), pp. 137–158, Springer-Verlag, New York
- Cornell, N. W., Lund, P., Hems, R. & Krebs, H. A. (1973) *Biochem. J.* **134**, 671–672
- Cornell, N. W., Lund, P. & Krebs, H. A. (1974) *Biochem. J.* **142**, 327–337
- Crow, K. E., Cornell, N. W. & Veech, R. L. (1977a) in *Alcohol and Aldehyde Metabolising Systems* (Thurman, R. G., Williamson, J. R., Drott, H. & Chance, B., eds.), vol. 3, pp. 335–342, Academic Press, New York
- Crow, K. E., Cornell, N. W. & Veech, R. L. (1977b) *Alcoholism: Clinical and Experimental Research* **1**, 43–47
- Czok, R. & Lamprecht, W. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U. & Gawehn, K., eds.), vol. 3, pp. 1446–1451, Academic Press, New York and London
- Dickinson, F. M. & Dalziel, K. (1967) *Biochem. J.* **104**, 165–172
- Grunnet, N., Quistorff, B. & Thieden, H. I. D. (1973) *Eur. J. Biochem.* **40**, 275–282
- Haynes, R. C., Jr. (1965) *J. Biol. Chem.* **240**, 4103–4106
- Krebs, H. A. & Stubbs, M. (1975) in *Alcohol Intoxication and Withdrawal II* (Gross, M. M., ed.), pp. 149–161, Plenum Press, New York
- Krebs, H. A., Cornell, N. W., Lund, P. & Hems, R. (1974) in *Regulation of Hepatic Metabolism* (Lundquist, F. & Tygstrup, N., eds.), pp. 726–750, Academic Press, New York
- Lamprecht, W. & Trautschold, I. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U. & Gawehn, K., eds.), vol. 4, pp. 2101–2110, Academic Press, New York and London
- Lardy, H. A., Paetkau, V. & Walter, P. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **53**, 1410–1415
- Lowry, O. H. & Passonneau, J. V. (1972a) *A Flexible System of Enzymatic Analysis*, pp. 78–82, Academic Press, New York and London
- Lowry, O. H. & Passonneau, J. V. (1972b) *A Flexible System of Enzymatic Analysis*, pp. 194–201, Academic Press, New York and London
- Lund, P., Cornell, N. W. & Krebs, H. A. (1975) *Biochem. J.* **152**, 593–599
- McDaniel, H. G., Reddy, W. J. & Boshell, B. R. (1972) *Biochim. Biophys. Acta* **276**, 543–550
- Meijer, A. J., Van Woerkom, G. M., Williamson, J. R. & Tager, J. M. (1975) *Biochem. J.* **150**, 205–209

- Plapp, B. V. (1975) in *Biochemical Pharmacology of Ethanol* (Majchrowicz, E., ed.), pp. 77-109, Plenum Press, New York and London
- Ray, P. D., Foster, D. O. & Lardy, H. A. (1966) *J. Biol. Chem.* **241**, 3904-3908
- Tischler, M. E., Pachence, J., Williamson, J. R. & La Noue, K. F. (1976) *Arch. Biochem. Biophys.* **173**, 448-462
- Veech, R. L., Guynn, R. & Veloso, D. (1972) *Biochem. J.* **127**, 387-397
- Veneziale, C. M., Walter, P., Kneer, N. & Lardy, H. A. (1967) *Biochemistry* **6**, 2129-2138
- Williamson, D. H. & Brosnan, J. T. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U. & Gawehn, K., eds.), vol. 4, p. 2293, Academic Press, New York and London