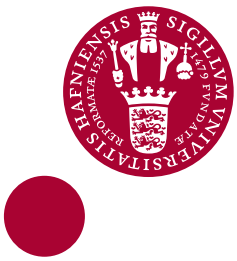

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Rate-Limiting Factors in Ethanol Oxidation by Isolated Rat-Liver Parenchymal Cells

Effect of Ethanol Concentration, Fructose, Pyruvate and Pyrazole

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1. Isolated rat-liver parenchymal cells oxidized ethanol at a rate of 1.4, 1.7, 1.9 and 2.5 μmol/min per ml packed cells at 4, 20, 40 and 65 mM ethanol, respectively. Between 40 and 65 mM ethanol an abrupt 30% increase in the ethanol oxidation rate was observed.

2. The activity of the NAD-dependent alcohol dehydrogenase in a homogenate of isolated cells corresponded to 1.25–5 U/ml packed cells, depending on the assay method used.

3. Fructose or pyruvate enhanced the oxidation rate of ethanol by 1.4–2.0 μmol/min per ml packed cells independent of the ethanol concentration applied. No additive effect of the two compounds upon ethanol oxidation was observed.

4. Pyrazole inhibited the NAD dependent alcohol dehydrogenase activity in a homogenate of isolated cells as well as the unstimulated and the fructose-stimulated ethanol oxidation with a K_i-value of 9–13 μM.

The "fructose effect" was inhibited 100% by pyrazole concentrations, which inhibited the unstimulated ethanol oxidation only 30%, indicating that the "fructose effect" is mediated *via* alcohol dehydrogenase.

4 mM pyrazole only slightly inhibited that part of the ethanol oxidation which was not catalyzed by alcohol dehydrogenase, whereas 18 mM pyrazole inhibited also this pathway significantly.

5. 50 μM pyrazole had no effect at all upon the basal ethanol oxidation rate whereas the fructose-stimulated ethanol oxidation was 30% inhibited.

6. The results concerning the effect of fructose and pyruvate upon ethanol oxidation are interpreted in terms of the mechanism for the reaction catalyzed by alcohol dehydrogenase. At low concentrations of ethanol and in the absence of fructose, the rate-limiting step in ethanol oxidation appears to be dissociation of the enzyme-NADH complex, whereas, in the presence of fructose, the maximal activity of ethanol dehydrogenase may be rate-limiting for the oxidation of ethanol.

7. The results also suggest that enzyme systems other than alcohol dehydrogenase participate in ethanol oxidation at high concentrations of ethanol.

Three different reaction mechanisms for oxidation of ethanol to acetaldehyde have been described in liver tissue. These are the pathway catalyzed by the NAD-dependent alcohol dehydrogenase [1], the pathway involving hydrogen peroxide and catalase [2–5] and the microsomal ethanol-oxidizing system involving NADPH and cytochrome P-450 [6–8]. The pathway catalyzed by alcohol dehydrogenase is localized to the cytoplasmic compartment of the cell, while the system dependent on cytochrome P-450 is microsome-bound.

Enzymes. Alcohol dehydrogenase (EC 1.1.1.1); catalase (EC 1.11.1.6).

The oxidation of ethanol catalyzed by alcohol dehydrogenase, which prevails at low (below approx. 10 mM) ethanol concentrations [9], probably is limited by the rate of NADH oxidation [10, 11]. The enhancement of ethanol oxidation by fructose (the "fructose effect") or by pyruvate [12, 13] is thought to be mediated *via* the NAD-dependent alcohol dehydrogenase. If the increase in ethanol oxidation rate caused by fructose or pyruvate is limited by the maximal activity of alcohol dehydrogenase, this effect should be inhibited by pyrazole or pyrazole derivatives, which are potent inhibitors of alcohol dehydrogenase [14].

Ethanol oxidation catalyzed by enzymes other than alcohol dehydrogenase has a K_m-value for ethanol of about 10 mM [2,3]. This alternative pathway is thus expected to contribute significantly to the ethanol oxidation rate only at high (above approx. 10 mM) ethanol concentrations.

The present work was undertaken to investigate the ability of isolated rat-liver parenchymal cells to metabolize ethanol and to examine the effect of ethanol concentration, fructose, pyruvate and pyrazole upon ethanol oxidation. The work was carried out with isolated parenchymal cells to eliminate diffusion problems with oxygen and substrates and to exclude the possibility that non-parenchymal cells might contribute to the phenomenae studied [16].

The results allow conclusions about the rate-limiting steps in unstimulated and in fructose-stimulated ethanol oxidation and show that an ethanol-oxidizing pathway, alternative to that catalyzed by alcohol dehydrogenase, is present in the parenchymal cells of rat liver.

MATERIALS AND METHODS

Materials

Enzymes and coenzymes were obtained from Boehringer Mannheim GmbH (Mannheim, Germany), and 1-^{14}C-ethanol and Aquasol from NEN Chemicals GmbH (Frankfurt/Main, Germany). Chemicals were of analytical grade. Serum albumin from Armour Chemical Co. (England) was treated with charcoal to remove fatty acids [15].

Preparation of Rat-Liver Parenchymal Cells

Cells were prepared from 24-h-fasted 250-g female Wistar rats as described [16].

Incubation

In all experiments cells were incubated in Hanks' solution [17] containing 1.2 mM $CaCl_2$, 1% serum albumin, 8 mM glucose, 10 mM phosphate and 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid. pH was adjusted to 7.4. Incubations were carried out at 37 °C with atmospheric air as the gas phase. Carbon dioxide was absorbed in KOH in the center well of the incubation flasks. The cell concentration was 60—120 µl tightly packed cells in a total volume of approx. 2 ml (corresponding to 5—10 mg protein/ml). The cells were incubated for 5 min with glucose, fructose or pyruvate prior to ethanol addition. Incubation time was 20 min and no decline in ethanol oxidation rate was observed during incubation. If not otherwise stated the experiments were terminated by perchloric acid precipitation of a sample of the incubation mixture. The final concentration of perchloric acid was 0.7 M.

Ethanol Determination

At concentrations of ethanol higher than 20 mM the relative amount of ethanol which disappeared during incubation was too small to be measured accurately as a difference and the ethanol oxidation rate was measured as non-volatile products formed from ethanol [18]: 1-^{14}C-ethanol was added to the incubation mixture to a specific activity of 0.16 nCi/µmol. Incubations were terminated by addition of 20 µl 2 N NaOH to 200 µl of the incubation mixture. Samples were evaporated overnight at room temperature in an exsiccator with P_2O_5 at reduced pressure (approx. 5 mm Hg). The P_2O_5 was renewed and the samples were kept in the exsiccator for another 24 h at reduced pressure. The whole procedure was carried out in scintillation counting vials. After the last evaporation acetic acid (3 ml 30 mM) and scintillation liquid (11 ml Aquasol, see Materials) was added to suspend the residue. Samples were counted in a Packard 2002 Tri-Carb scintillation spectrometer. Blank experiments contained no cells.

$^{14}CO_2$ production was measured after acidification and shaking of the incubation mixture for 30 min at 37 °C. The filter papers, impregnated with KOH, from the center well of the incubation flasks were placed in scintillation liquid (acetic acid plus Aquasol as described above) and counted. In no experiments did the CO_2 production exceed 5% of the ethanol oxidation rate. The CO_2 production therefore is not included in the results.

Results obtained by the procedure were identical to those measured enzymatically [19], when the two methods were compared on incubation mixtures containing 4 mM ethanol.

In some experiments (Fig. 3) the ethanol disappearance was determined by gas-chromatographic measurement of the ethanol concentration. The measurements were carried out on a Hewlett-Packard gaschromatograph (model 7620 A). The column was 16.5% Carbowax on Diatoport S, nitrogen flow-rate 25 ml/min, air flow-rate 250 ml/min, hydrogen flow-rate 40 ml/min, oven temperature 95 °C, injection port temperature 130 °C and detector (FID) temperature 250 °C. 2 µl samples were injected.

NAD-dependent alcohol dehydrogenase activity was measured spectrophotometrically [20] under incubation conditions as specified in Table 1.

RESULTS

Effect of Ethanol Concentration

The ethanol oxidation rate as a function of the ethanol concentration is shown in Fig. 1. At

Table 1. *NAD-dependent alcohol-dehydrogenase activity in homogenates of isolated rat-liver parenchymal cells*
Cells were homogenized in the buffer indicated and alcohol dehydrogenase activity measured in the supernatant after centrifugation at 40000 × *g* for 15 min [20]. The assay buffer was, at pH 7.4: 0.1 M triethanolamine plus 0.08 M semicarbazide, at pH 9.0: 0.02 M glycine plus 0.08 M pyrophosphate plus 0.08 M semicarbazide and at pH 10.0: 0.1 M glycine. Figures are μmoles NAD^+ reduced/min per ml packed cells at 37 °C ± S.E., with the number of cell preparations in parentheses

Homogenization buffer	Activity with assay buffer		
	pH 7.4	pH 9.0	pH 10.0
	$\mu mol \times min^{-1} \times ml^{-1}$		
0.1 M triethanolamine, pH 7.2	1.25 ± 0.06 (6)	1.86 ± 0.11 (6)	3.03—3.43 (2)
0.05 M Tris, pH 8.4	—	2.52—2.81 (2)	4.09—5.05 (2)

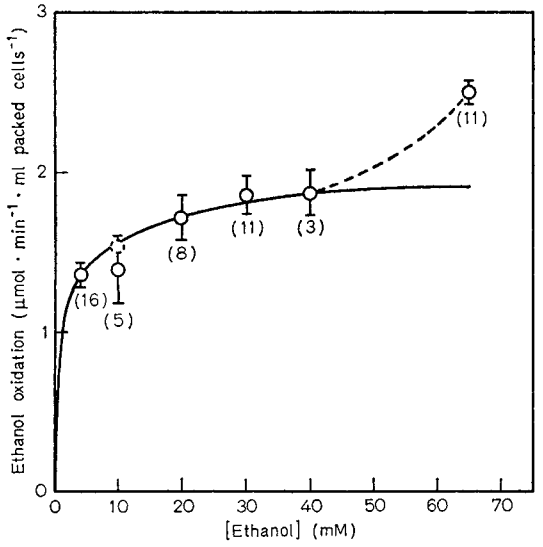


Fig. 1. *Effect of ethanol concentration upon ethanol oxidation by isolated rat-liver parenchymal cells.* Experiments were performed as described in Methods. Bars indicate S.E. and figures below the bars the number of cell preparations. The point marked with a stippled circle at 10 mM ethanol was obtained by omission of a single, very low, value. The solid graph was calculated as described in the Discussion section ("Effect of ethanol concentration")

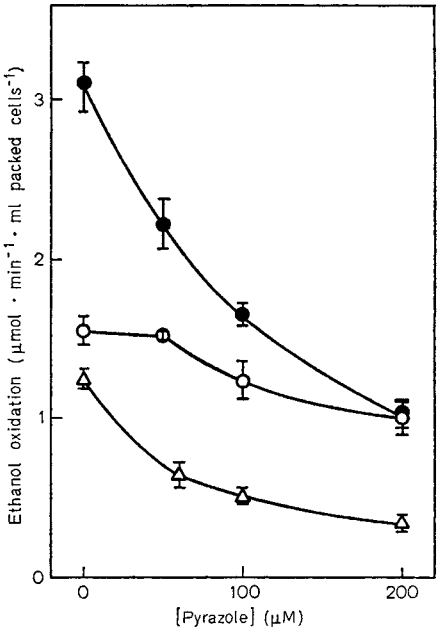


Fig. 2. *Inhibition by pyrazole of ethanol oxidation (○), fructose-stimulated ethanol oxidation (●) and NAD-dependent alcohol dehydrogenase activity (△).* Experiments were performed as described in "Methods" with 4 mM ethanol. NAD dependent alcohol dehydrogenase activity was measured at pH 7.4 (*cf.* Table 1). Results are mean values of four cell preparations and bars indicate S.E.

65 mM ethanol the oxidation rate was 1.85 times the oxidation rate at 4 mM ethanol. At ethanol concentrations above 10 mM a significant increase in the ethanol oxidation rate was observed compared to the oxidation rate at 4 mM (P values < 0.025). Between 40 and 65 mM ethanol an abrupt 30% increase in the ethanol oxidation rate was observed (Fig. 1).

The alcohol dehydrogenase activity in homogenates of isolated cells varied with the homogenization and assay conditions between 1.25 and 5 U/ml of packed cells (Table 1).

In the "pyrazole titration" experiments (Fig. 2) the activity of the NAD-dependent alcohol dehydrogenase, measured in a homogenate of isolated cells at pH 7.4 and at 37 °C, corresponded to the ethanol oxidation rate in the absence of fructose (Fig. 2), as ethanol oxidation catalyzed by enzymes other than alcohol dehydrogenase at 4 mM ethanol amounts to approx. 0.2 μmol/min per ml packed cells (see Discussion).

The alcohol dehydrogenase activity, measured in a homogenate of isolated cells at pH 7.4 and 37 °C, probably is lower than the maximal activity of the NAD-dependent alcohol dehydrogenase, as indicated by the rate of ethanol oxidation catalyzed by alcohol dehydrogenase in the presence of fructose (corresponding to approx. 2.9 U/ml packed cells) (Fig. 2).

Effect of Fructose and Pyruvate

Fructose or pyruvate in concentrations of 8 and 4 mM, respectively, increased the oxidation rate of ethanol in isolated rat-liver parenchymal cells to 195—240% at 4 mM ethanol and to 130—160% at higher concentrations of ethanol (Tables 2 and 3). The absolute increase in ethanol oxidation rate due to fructose appeared independent of the ethanol concentration. The effect of pyruvate was quantitatively identical to the effect of fructose and no additive effects of the two compounds was observed (Table 2).

The effect of fructose upon ethanol oxidation varied in the two experimental series reported (*cf.* Tables 2 and 3). The reason for this variation is not clear. In a single experiment, which is not included in the present results, fructose caused an increase in the ethanol oxidation rate from 2.70 to 6.41 μmol/min per ml packed cells at 4 mM ethanol.

Effect of Pyrazole

4 and 18 mM pyrazole inhibited the oxidation of 4 mM ethanol almost completely, whereas the oxidation of ethanol at high concentrations was inhibited only partly (Table 3). The effect of fructose upon ethanol oxidation was abolished 100% by 4 or 18 mM pyrazole independent of the concentration of ethanol (Table 3).

At 4 mM ethanol a titration of the ethanol oxidation rate with pyrazole was carried out (Fig. 2). 50 μM pyrazole did not decrease the basal ethanol oxidation rate, whereas the effect of fructose was inhibited about 30%. At pyrazole concentrations above 100 μM the effect of fructose was completely abolished, whereas the ethanol oxidation rate was inhibited 20—30% only. The inhibition of the NAD-dependent alcohol dehydrogenase, measured in a homogenate of isolated cells, closely parallels the inhibition of the fructose-stimulated ethanol oxidation (Fig. 2).

Table 2. *Effect of fructose and pyruvate upon ethanol oxidation in isolated rat-liver parenchymal cells*
The experiments were performed as described in Methods. Final concentration of fructose and pyruvate was 8 and 4 mM, respectively. Figures represent μmol ethanol oxidized/min per ml packed cells ± S.E. with the number of cell preparations in parentheses

Fructose	Pyruvate	Oxidation with ethanol concentration	
		4 mM	65 mM
		μmol × min^{-1} × ml^{-1}	
−	−	1.53 ± 0.13 (4)	3.06 ± 0.27 (4)
+	−	3.65 ± 0.33 (4)	
−	+	3.86 ± 0.25 (4)	5.07 ± 0.54 (4)
+	+	3.71 ± 0.32 (4)	

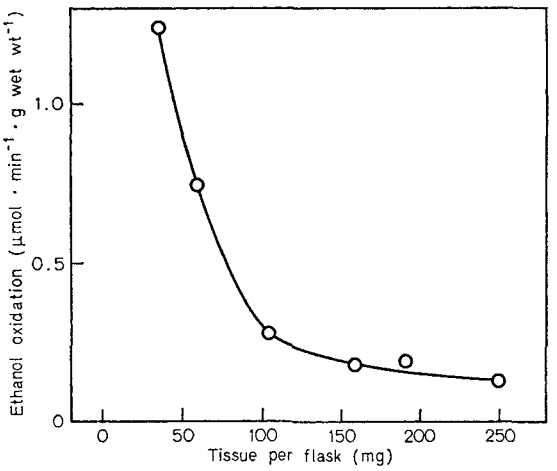


Fig. 3. *Ethanol oxidation by rat-liver slices as a function of tissue amount per incubation flask.* 0.3-mm liver slices were cut with a McIlwain tissue slicer and incubated with 4 mM ethanol in 2 ml of the same buffer as used for incubation of isolated cells. Every second minute a sample of the incubation medium was taken for ethanol determination. Ethanol concentration was plotted *versus* time and the rate of ethanol oxidation calculated from the slope of the linear part of the curve

Table 3. *Effect of 4 and 18 mM pyrazole upon ethanol oxidation in isolated rat-liver parenchymal cells*
The experiments were performed as described in Methods. Figures represent μmol ethanol oxidized/min per ml packed cells ± S.E. with the number of cell preparations in parentheses

Additions	Concentration of ethanol	Ethanol oxidation with concentration of pyrazole		
		0 mM	4 mM	18 mM
	mM	μmol × min^{-1} × ml^{-1}		
None	4	1.41 ± 0.08 (4)	0.17 ± 0.04 (4)	0.09 ± 0.02 (4)
	30	1.90 ± 0.17 (4)	0.93 ± 0.06 (4)	0.50 ± 0.07 (4)
	65	2.48 ± 0.12 (4)	2.01 ± 0.08 (4)	1.22 ± 0.13 (4)
Fructose 8 mM	4	2.75 ± 0.16 (4)	0.15 ± 0.02 (4)	0.09 ± 0.02 (4)
	30	2.88 ± 0.16 (4)	0.92 ± 0.06 (4)	0.54 ± 0.07 (4)
	65	3.93 ± 0.25 (4)	2.22 ± 0.08 (4)	1.12 ± 0.01 (4)

Addition of 200 μM pyrazole decreased the stimulation of the ethanol oxidation rate by pyruvate (4 mM) from 152% to 31% (4 mM ethanol, 4 experiments).

Ethanol Oxidation by Liver Slices

For the purpose of comparison, the ethanol oxidation rate in rat liver slices was measured. In Fig. 3 a typical experiment with varying amounts of tissue per flask is shown.

DISCUSSION

The present results demonstrate in accordance with the work of Berry [20a] that isolated parenchymal cells from rat liver oxidize ethanol at a rate comparable to that of the perfused rat liver [21—23] and that the cells may be suitable for studies of ethanol metabolism.

At 4 mM ethanol the oxidation rate is more than twice the oxidation rate reported for rat liver slices, (see Results and [18]), assuming that 0.6 ml packed cells correspond to 1 g wet wt liver [24]. This difference may be due to insufficient oxygen supply to the liver slices, depending partly on the amount of tissue (Fig. 3) and partly on the oxygen tension [24a].

Effect of Ethanol Concentration

The increase in ethanol oxidation rate observed with increasing concentrations of ethanol (Fig. 1) indicates the presence in the isolated parenchymal cells of an ethanol-metabolizing enzyme system different from that of the NAD-dependent alcohol dehydrogenase. Rat-liver alcohol dehydrogenase has a K_m-value for ethanol of 0.5—2 mM [14, 20]. A constant rate of ethanol oxidation therefore is to be expected at ethanol concentrations above 10 mM, provided that the oxidation rate of cytoplasmic NADH remains unchanged (see below).

Further evidence for an ethanol oxidation pathway alternative to that catalyzed by the NAD-dependent alcohol dehydrogenase is obtained by the inhibition pattern caused by pyrazole. Pyrazole concentrations, which inhibit the NAD-dependent alcohol dehydrogenase by more than 93% (assuming a K_m-value for ethanol of 0.5 mM and a K_i-value for pyrazole of 9 μM) still leave 50% of the ethanol-metabolizing capacity of the isolated cells (Table 3, line 3).

The quantitatively identical effect of fructose or pyruvate upon the ethanol elimination rate at all ethanol concentrations applied (Table 3) and the inhibition of these effects by pyrazole (Table 2 and 3), demonstrate that these effects are different from the effect of high ethanol concentrations.

Fig. 1 illustrates the relation between the ethanol oxidation rate and the concentration of ethanol. Assuming that the oxidation rate observed at each concentration of ethanol is the sum of the rate of the reaction catalyzed by alcohol dehydrogenase (with a K_m-value for ethanol of 0.5 mM) and the rate of the reaction catalyzed by an enzyme system with a K_m-value for ethanol of 10 mM, the V for the reaction catalyzed by alcohol dehydrogenase and for the alternative ethanol oxidation pathway can be calculated to 1.50 and 0.67 μmol/min per ml packed cells respectively (the experiments with 65 mM ethanol have been omitted in the calculations). The fully drawn graph of Fig. 1 represents the expected rate of ethanol oxidation assuming the rates measured at 4, 10, 20, 30 and 40 mM ethanol to reflect the combined action of two enzymes with the K_m and V values mentioned above.

The V-values calculated above correspond to 0.9 and 0.4 μmol/min per g wet wt liver for the alcohol dehydrogenase pathway and for the alternative ethanol oxidation pathway respectively [24]. The perfused rat liver oxidizes ethanol at a rate of about 2 μmol/min per g wet wt [21—23], a figure corresponding to the maximally obtainable rate in the isolated cells. The activity *in vitro* of the microsomal ethanol-oxidizing system has been reported to be 0.5 μmol/min per g wet wt [25].

One explanation of the high ethanol-oxidation rate at 65 mM ethanol (Fig. 1), could be an increased rate of hydrogen peroxide formation when the ethanol concentration is raised from 40 to 65 mM. It has been reported that the apparent K_m-value for ethanol and the rate of ethanol oxidation, catalyzed by catalase, varies with the rate of hydrogen peroxide production [26], which again may depend on the amount of pyruvate or acetate [27].

The abrupt increase in the ethanol oxidation rate between 40 and 65 mM might also be a consequence of a permeability effect upon the mitochondrial membrane. High concentrations of ethanol might increase the respiratory-chain-linked oxidation of cytoplasmic NADH ([28], *cf.* however [29]), which is believed to be the rate-limiting step in ethanol oxidation. This mechanism should imply a smaller effect of fructose upon ethanol oxidation at 65 mM than at 40 mM ethanol, which is however not observed (Tables 2 and 3).

Effect of Fructose and Pyruvate

The absolute increase in ethanol oxidation rate caused by fructose or pyruvate (Fig. 2, Tables 2 and 3) is independent of the concentration of ethanol in the range studied. Low concentrations of pyrazole (100—200 μM) inhibits the acceleration of ethanol metabolism almost completely, whereas the un-

stimulated ethanol oxidation is only slightly affected (Fig. 2). The concentrations of pyrazole applied are so low that inhibition of enzymes other than the NAD-dependent alcohol dehydrogenase is improbable [14]. The results of Fig. 2 may therefore be taken as evidence for the suggestion [30] that the NAD-dependent alcohol dehydrogenase is involved in the "fructose effect".

The inhibition of the "fructose effect" by concentrations of pyrazole which cause little or no inhibition of the unstimulated ethanol oxidation (Fig. 2) also suggest that fructose or fructose metabolites affect the rate-limiting step in ethanol oxidation, so that the oxidation of ethanol in the presence of fructose or pyruvate proceeds at a rate corresponding to the maximal activity of the NAD-dependent alcohol dehydrogenase (see below).

Pyruvate has an effect upon ethanol oxidation quantitatively identical to that of fructose (Table 2). The enhancement in ethanol oxidation caused by pyruvate is inhibited to the same degree by pyrazole as the "fructose effect" and no additive effect of the two compounds was observed (Table 2). These results indicate that the "fructose effect" could be an effect of pyruvate, formed by the metabolism of fructose. The results may very well be interpreted in terms of the "malic enzyme shuttle" mechanism for the effect of fructose or pyruvate upon ethanol metabolism [13].

Effect of Pyrazole

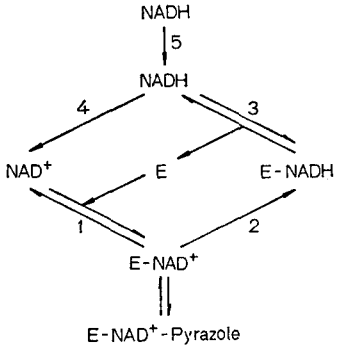
Pyrazole is reported to inhibit purified NAD-dependent alcohol dehydrogenase from rat liver competitively with respect to ethanol with a K_i-value of 4 μM [14].

The apparent K_i-values for the inhibition by pyrazole of the ethanol oxidation, catalyzed by alcohol dehydrogenase, can be calculated from the data of Fig. 2, assuming that the ethanol oxidation rate in the presence of fructose represents the maximal activity of the NAD-dependent alcohol dehydrogenase in isolated cells: In the presence and absence of fructose, respectively, the K_i-values for pyrazole were 9.8—12.8 μM and 9.1—9.3 μM (the actual oxidation rates were corrected for ethanol oxidation not catalyzed by alcohol dehydrogenase (approx. 0.2 μmol/min per ml packed cells at 4 mM ethanol) and the experiments with 50 μM pyrazole in the absence of fructose were omitted). The K_i-values for the pyrazole inhibition of alcohol dehydrogenase in a homogenate of isolated cells were calculated to be 9.2—9.9 μM (from Fig. 2, △).

The K_i-values obtained above and the K_m and V values for the two ethanol-metabolizing enzyme systems (see Results, "Effect of Ethanol Concentration") may be applied in calculations of the expected rates of ethanol oxidation at high concentrations of pyrazole. Assuming no inhibition of ethanol oxidation catalyzed by enzymes other than alcohol dehydrogenase the rate of ethanol oxidation in the presence of 4 mM pyrazole was calculated to 0.26 and 0.90 μmol/min per ml packed cells at 4 and 30 mM ethanol, respectively. These figures coincide with the actually observed rates of ethanol oxidation (Table 3). In the presence of 18 mM pyrazole, the calculated oxidation rates at 4 and 30 mM ethanol are 0.21 and 0.63 μmol/min per ml packed cells, respectively, indicating some inhibition of ethanol oxidation catalyzed by enzymes other than alcohol dehydrogenase by 18 mM pyrazole (*cf.* Table 3). This effect of high concentrations of pyrazole has been reported earlier [31].

Rate-Limiting Steps in Ethanol Oxidation and the "Fructose Effect"

Ethanol oxidation catalyzed by isolated (horse) liver alcohol dehydrogenase obeys the Theorell-Chance mechanism (Scheme I) and is limited by dissociation of the enzyme-NADH complex (Scheme I, reaction 3) [10]. The dissociation rate of this complex depends upon the concentrations of free NADH [10] and of free NAD^+ (by competition with NADH [32]).



Scheme I. *Possible pathways.* Mechanism for the NAD-dependent alcohol-dehydrogenase-catalyzed oxidation of ethanol according to Theorell and Chance [10] (reaction 1—3). Reaction 4 symbolizes oxidation of cytosolic NADH and reaction 5 formation of cytoplasmic NADH by reactions besides that catalyzed by alcohol dehydrogenase. For further explanation see the text

The basal ethanol-oxidation rate in isolated parenchymal cells is independent of the total amount of enzyme present, as no inhibition was observed by 50 μM pyrazole (Fig. 2). The rate of unstimulated ethanol oxidation does not reflect the maximal rate of interconversion of the ternary complex (Scheme I, reaction 2), as the overall rate of the reaction can be increased almost three-fold by fructose (Fig. 2). Considering the reaction catalyzed by alcohol dehydrogenase the rate-limiting step in

unstimulated ethanol oxidation therefore must be either formation of the enzyme-NAD^+ complex (Scheme I, reaction 1) or dissociation of the enzyme-NADH complex.

In Eqn (1) the notation and φ-values of Dalziel [33] have been used. The equation is derived from [10] and [33]. φ is the reciprocal rate constant, x_1 is the concentration of NAD^+, x_2 that of NADH and a_1 the concentration of ethanol (4 mM). K_{m2} is the K_m-value for NADH (0.1 μM [10]) and e the amount of enzyme present.

If Eqn (1) is used to calculate the velocity (V/e) of the alcohol-dehydrogenase-catalyzed reaction it appears that V/e is nearly independent of the concentration of free NAD^+ in the range 200 to 500 μM, whereas NADH inhibits the reaction considerably (*e.g.* 50% inhibition by 5 μM NADH at 400 μM NAD^+).

$$\frac{e}{V} = \varphi_0 + \frac{\varphi_1}{x_1} + \frac{\varphi_2}{a_1} + \frac{\varphi_{12}}{a_1 \cdot x_1} + \frac{x_2}{K_{m2}}\left(\frac{\varphi_1}{a_1} + \frac{\varphi_{12}}{a_1 \cdot x_1}\right). \quad (1)$$

The cytosolic concentration of free NAD^+ has been estimated to about 0.5 mM [24] and that of free NADH to about 0.5 μM [24]. During ethanol oxidation the cytosolic NADH/NAD^+ ratio is increased about 10 times [9], probably reflecting an increase in the concentration of free NADH. The above-mentioned calculations therefore strongly indicate that the rate-limiting step in the alcohol-dehydrogenase-catalyzed reaction during unstimulated ethanol metabolism is the dissociation of the enzyme-NADH complex.

The "fructose effect" must then be a consequence of a decreased cytosolic concentration of free NADH due to an increased rate of oxidation of cytosolic NADH during fructose metabolism (Scheme I, reaction 4). In accordance with this view, the cytosolic NADH/NAD^+ ratio is decreased in ethanol-metabolizing liver slices after administration of fructose [30]. In the perfused rat liver, the unstimulated ethanol oxidation rate is about twice that in the isolated rat-liver parenchymal cells (see above). Furthermore, no "fructose effect" and an increase in the cytosolic NADH/NAD^+ ratio after administration of fructose to an ethanol-metabolizing perfused liver preparation is observed [22, 23].

These differences between isolated cells and perfused liver indicate that the oxidation rate of cytosolic NADH is higher in the perfused liver than in unstimulated parenchymal cells or in liver slices, although the maximal capacity to oxidize cytosolic NADH appears identical in isolated parenchymal cells and in perfused liver.

The activity of alcohol dehydrogenase *in vitro* as measured in a homogenate of isolated cells corresponds to the unstimulated ethanol-oxidation rate (see above and Fig. 2), but much lower concentrations of pyrazole is required to inhibit the activity *in vitro* (Fig. 2). This difference in pyrazole inhibition pattern may be due to the very different ratio between enzyme concentration (e) and K_i for pyrazole under the two experimental conditions [34]. In the isolated cells the concentration of alcohol dehydrogenase is approx. 4 μM [14] and $e/K_i \cong 0.5$. Under the assay conditions used for determination of the activity *in vitro* e/K_i is approx. 1.5×10^{-4}.

The difference in pyrazole inhibition pattern between a homogenate of isolated cells and intact cells (Fig. 2, △ and ○) probably is not due to slow diffusion of pyrazole into the cells or to destruction of pyrazole by the intact cells, as concentrations of pyrazole which do not affect the unstimulated ethanol oxidation inhibit the fructose-stimulated oxidation of ethanol in intact cells (Fig. 2).

In conclusion, the basal (unstimulated) ethanol oxidation and measurements of the activity of alcohol dehydrogenase *in vitro* are limited by the rate of dissociation of the alcohol dehydrogenase-NADH complex. Fructose (or pyruvate) metabolism indirectly activates this dissociation by an increased rate of NADH oxidation, probably mediated *via* the "malic enzyme shuttle" [13].

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