The reversibility of cytosolic dehydrogenase reactions in hepatocytes from starved and fed rats

Effect of fructose

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The metabolism of [2-³H]lactate was studied in isolated hepatocytes from fed and starved rats metabolizing ethanol and lactate in the absence and presence of fructose. The yields of ³H in ethanol, water, glucose and glycerol were determined. The rate of ethanol oxidation (3 μmol/min per g wet wt.) was the same for fed and starved rats with and without fructose. From the detritiation of labelled lactate and the labelling pattern of ethanol and glucose, we calculated the rate of reoxidation of NADH catalysed by lactate dehydrogenase, alcohol dehydrogenase and triosephosphate dehydrogenase. The calculated flux of reducing equivalents from NADH to pyruvate was of the same order of magnitude as previously found with [³H]ethanol or [³H]-xylitol as the labelled substrate [Vind & Grunnet (1982) *Biochim. Biophys. Acta* 720, 295–302]. The results suggest that the cytoplasm can be regarded as a single compartment with respect to NAD(H). The rate of reduction of acetaldehyde and pyruvate was correlated with the concentration of these metabolites and NADH, and was highest in fed rats and during fructose metabolism. The rate of reoxidation of NADH catalysed by lactate dehydrogenase was only a few per cent of the maximal activity of the enzymes, but the rate of reoxidation of NADH catalysed by alcohol dehydrogenase was equal to or higher than the maximal activity as measured *in vitro*, suggesting that the dissociation of enzyme-bound NAD⁺ as well as NADH may be rate-limiting steps in the alcohol dehydrogenase reaction.

Isolated cytoplasmic dehydrogenases have been studied intensively, but the details of their behaviour and interaction in the intact cell are still not available. Lactate and ethanol are major NADH-producing substrates in the liver cytoplasm. The rate of ethanol oxidation *in vivo* corresponds to the rate expected from measured or calculated concentrations of substrates and products and the kinetic constants of alcohol dehydrogenase *in vitro* (Crabb *et al.*, 1983; Cornell, 1983b). At low concentrations of ethanol and acetaldehyde (below approx. 20 mM and 5 μM, respectively), inhibition by NADH appears to be the major rate-determining factor, whereas at high concentrations of ethanol or acetaldehyde significant inhibition of alcohol dehydrogenase may be exerted by these compounds. It is generally assumed that little or none of the acetaldehyde produced is reduced to ethanol, but when the [acetaldehyde]/[ethanol] ratio increases while the [NAD⁺]/[NADH] ratio decreases

acetaldehyde can be reduced to ethanol (Lindros *et al.*, 1972). It was found in mice that the rate of acetaldehyde reduction under physiological conditions was at least 2% of the rate of ethanol oxidation (Gershman, 1975).

The high activity of lactate dehydrogenase (approx. 200 μmol/min per g wet wt.) in rat liver (Weber, 1963) and the assumption that the lactate dehydrogenase system is close to thermodynamic equilibrium is used in numerous investigations, where the [lactate]/[pyruvate] ratio has been utilized as an indicator for the cytosolic [NADH]/[NAD⁺] ratio. In such calculations, a single cytoplasmic NAD(H) pool is assumed. Studies (Berry, 1980; Cronholm *et al.*, 1974) have suggested, however, that metabolites and NAD(H) may be compartmented in the cytoplasm. However, we found (Vind & Grunnet, 1982) that at least alcohol dehydrogenase and xylitol dehydrogenase share a common pool of NAD(H).

Fructose is rapidly metabolized by the liver, gives rise to a large increase in the concentration of pyruvate (Mapungwana & Davies, 1982) and enhances the rate of the glucose/glucose 6-phosphate and fructose 6-phosphate/fructose 1,6-bisphosphate substrate cycles (Clark *et al.*, 1979). Furthermore, fructose stimulates ethanol metabolism in hepatocytes with a sub-optimal supply of precursors of the malate-aspartate hydrogen shuttle (Scholz *et al.*, 1975; Cornell, 1983a). The effect of fructose addition was therefore examined in the present experiments. In the present work some of the above-mentioned aspects of cytoplasmic metabolism have been elucidated by studies of the metabolism of ^3H from $[2\text{-}^3\text{H}]\text{lactate}$ in isolated hepatocytes from fed and starved rats incubated with ethanol in the presence or absence of fructose.

Materials and methods

Reagents

Enzymes and coenzymes were from Boehringer, Mannheim, Germany. NaB^3H_4 was purchased from Amersham International, Amersham, Bucks, U.K.

Chemicals were of analytical grade. Serum albumin from Armour Pharmaceutical Co. (Eastbourne, U.K.) was treated with charcoal to remove fatty acids (Chen, 1967). Dowex 1 was from Fluka A.G. (Buchs, Switzerland), Sephadex G-50 from Pharmacia Fine Chemicals (Uppsala, Sweden) and Instafluor from Packard (Groningen, The Netherlands).

Preparation and incubation of hepatocytes

Hepatocytes from female Wistar rats (150–200g), either fed or starved for 24h, were prepared as described by Kondrup *et al.* (1980). More than 90% of the cells excluded Trypan Blue initially and more than 85% after 120min incubation.

Packed cells (0.8–1.1ml; approx. 0.9–1.2g wet wt.) were incubated in a 300ml conical flask at 37°C in Krebs–Henseleit (1932) bicarbonate buffer containing 1% serum albumin and 7.5mM-glucose in a total volume of 22ml. The gas phase was O_2/CO_2 (19:1).

To the incubations were added 2.5mM-lactate (giving a final concentration of about 3mM), 0.25mM-pyruvate, 20mM-ethanol, and in some of the experiments 10mM-fructose 10min before addition (at zero time) of a trace amount of $[2\text{-}^3\text{H}]\text{lactate}$ to an initial specific radioactivity of lactate of approx. 500c.p.m./nmol. Samples (2ml) were taken from the incubation at –10, 0, 10, 20, 30, 40, 50 and 70min and added to HClO_4 (final concn. 0.7M). The supernatant was neutralized by KOH containing triethanolamine.

Determination of the distribution of ^3H from $[2\text{-}^3\text{H}]\text{lactate}$

The neutralized HClO_4 supernatant was used for determination of ^3H in water, ethanol, glucose, glycerol and lactate. Radioactivity in water was obtained as the difference between total radioactivity and that in dry matter plus that in ethanol.

^3H in ethanol was determined by incubation of 250 μl of a freeze-dried preparation with 36 μmol of pyruvate, 2 μmol of NAD^+ , 300nkat of lactate dehydrogenase and 300nkat of alcohol dehydrogenase in 0.2M-Tris/HCl buffer, pH 7.5, for 10h at room temperature (Rognstad & Clark, 1974). After freeze-drying, the $[^3\text{H}]\text{lactate}$ formed from (1R)- $[1\text{-}^3\text{H}]\text{ethanol}$ was counted for radioactivity.

Glucose, lactate and 'other anions' were separated by Dowex 1 (acetate form) ion-exchange chromatography (Vind & Grunnet, 1981). From incubations with fructose, glucose and glycerol were separated by phosphorylation of glucose with ATP and hexokinase and subsequent separation by Dowex 1 ion-exchange chromatography. Samples were counted for radioactivity in a Packard 2425 scintillation spectrometer. Instafluor/Triton X-114 (2:1, v/v) was used as the scintillation liquid, with a sample volume of 23%.

The recovery of ^3H removed from $[2\text{-}^3\text{H}]\text{lactate}$ in water, ethanol, glucose, glycerol and the fraction of 'other anions' was 90–110%. The relative yield found in the fraction of 'other anions' was less than 2% and has been neglected in our calculations. We only found ^3H in glycerol when $[2\text{-}^3\text{H}]\text{lactate}$ was metabolized together with fructose.

Preparation of $[2\text{-}^3\text{H}]\text{lactate}$

$[2\text{-}^3\text{H}]\text{Lactate}$ was prepared from $[1,1\text{-}^3\text{H}]\text{ethanol}$ by incubation with 75mM-pyruvate, 4mM- NAD^+ , 300nkat of lactate dehydrogenase and 300nkat of alcohol dehydrogenase in 0.2M-Tris/HCl buffer, pH 7.5. After freeze-drying, $[2\text{-}^3\text{H}]\text{lactate}$ was separated from the dehydrogenases on a Sephadex G-50 column.

$[1,1\text{-}^3\text{H}]\text{Ethanol}$ was prepared from freshly distilled acetaldehyde and excess of NaB^3H_4 in a Thunberg tube, with subsequent freeze-drying.

Metabolite concentrations

Metabolites were determined in the neutralized HClO_4 supernatant. Lactate, pyruvate, glucose, fructose, acetaldehyde and ethanol were measured by standard enzymic procedures (Bergmeyer, 1970). Before measurement of acetaldehyde, it was separated from glyceraldehyde by freeze-drying in a Thunberg tube. In some cases acetaldehyde was estimated by g.l.c. To 1 vol. of the cell suspension was added 1 vol. of 1M- HClO_4 (containing 50mM-thiourea). Acetaldehyde was determined in the gas

phase after incubation for 15 min at 65°C of the protein-free supernatant.

The NADH concentration was calculated from the [lactate]/[pyruvate] ratio, assuming a free cytoplasmic NAD⁺ concentration of 0.5 mM (Bücher, 1970) and 1.11×10^{-4} as the equilibrium constant [pyruvate][NADH]/[lactate][NAD⁺] (Williamson *et al.*, 1967).

Results

Metabolic changes

Rates of ethanol and fructose removal and of glucose formation were linear with time in the 80 min incubation period. Rates of lactate oxidation or formation in the absence or presence of fructose were linear with time for 80 min.

As shown in Table 1, the rate of ethanol oxidation by hepatocytes from starved rats was 3.1 $\mu\text{mol/min}$ per ml of packed cells and from fed rats 2.5 $\mu\text{mol/min}$ per ml. The lower rate in cells from fed rats is explainable by the larger volume of these cells (Declercq *et al.*, 1982).

A stimulatory effect of fructose on ethanol oxidation has been reported by many authors (for review, see Scholz *et al.*, 1975). We found no activation by fructose of the rate of ethanol oxidation in hepatocytes from either fed or starved rats (when incubated with lactate, pyruvate and glucose as in the present experiments). This is in agreement with the values reported by Cornell (1983a), when the hepatocytes from starved rats have enough substrate to restore the malate-aspartate-shuttle intermediates lost during cell preparation (Cornell, 1983b).

Glucose was the major end product of fructose metabolism in hepatocytes from both fed and starved rats. Glucose and lactate production accounted for the fructose metabolized, leaving little room for the production of glycerol, in agreement with results reported by others (Scholz *et al.*, 1975).

Fructose caused an increase in the concentration of pyruvate and acetaldehyde in both fed and starved rats (Table 3). The increased pyruvate concentration in the presence of fructose is well known and has been explained by an activation of pyruvate kinase by fructose 1,6-bisphosphate (Taylor & Bailey, 1967) and fructose 1-phosphate (Eggleston & Woods, 1970). The increase in the acetaldehyde concentration is in accordance with results obtained by others (H. L. Iversen, unpublished work), but in disagreement with those by Andersen *et al.* (1977), who found that fructose stimulated the acetaldehyde uptake in perfused rat liver. It should be pointed out that the capacity of aldehyde dehydrogenase in rat liver is just sufficient to oxidize the amount of acetaldehyde formed by oxidation of ethanol, and thus a slight inhibition of aldehyde dehydrogenase is sufficient to cause a large increase in the concentration of acetaldehyde.

The mean rate of glucose formation (Table 1) in the absence of fructose (0.78 $\mu\text{mol/min}$ per ml of cells for fed rats and 0.36 $\mu\text{mol/min}$ per ml for starved rats) was similar to previous reported values [0.78 $\mu\text{mol/min}$ per g wet wt. for fed rats (Cornell, 1983a) and 0.39 $\mu\text{mol/min}$ per g wet wt. for starved rats (Crow *et al.*, 1978)].

Lactate was taken up by the hepatocytes from both starved and fed rats in the absence of fructose, but accumulated in the presence of fructose.

Fate of labelling from [2-³H]lactate

Our approach to calculate the flux of reducing equivalents from NADH to pyruvate (*x*), acetaldehyde (*y*) and 1,3-bisphosphoglycerate (*f*) is based on the model shown in Fig. 1 and the measurement of transfer of ³H from [2-³H]lactate to water, ethanol, glucose and glycerol. The relative yields of ³H in the different fractions were for both starved and fed rats at the end of the experiment (110 min after addition of [2-³H]lactate) 75% in water, 5% in ethanol, 16% in glucose and 4% in glycerol, if the

Table 1. *Metabolic changes*

Hepatocytes were incubated for 120 min and metabolite concentrations estimated during the incubation as described in the Materials and methods section. Initial concentration of substrates were 2.5 mM-lactate, 0.25 mM-pyruvate and 20 mM-ethanol in the presence or absence of 10 mM-fructose. Rates are means $\pm$ S.E.M. for five different cell preparations.

	Fructose (mM)	Change in metabolite ($\mu\text{mol/min}$ per ml of packed cells)			
		Lactate	Ethanol	Glucose	Fructose
Starved rats	0	-0.68 ± 0.13	-3.09 ± 0.19	0.36 ± 0.04	
	10	0.39 ± 0.09	-2.81 ± 0.22	2.33 ± 0.10	-2.30 ± 0.28
Fed rats	0	-0.21 ± 0.12	-2.52 ± 0.12	0.78 ± 0.09	
	10	0.74 ± 0.05	-2.30 ± 0.13	2.10 ± 0.13	-2.47 ± 0.13

Fig. 1. Model for flux of reducing equivalents from $[2-^3\text{H}]\text{lactate}$

a and b represent the oxidation rates of lactate and ethanol respectively; $a+x$ and $b+y$ represent the rates of formation of NADH from $[2-^3\text{H}]\text{lactate}$ and from ethanol respectively, and x , y and f are the oxidation rates of NADH catalysed by lactate dehydrogenase, alcohol dehydrogenase and triose-phosphate dehydrogenase respectively.

hepatocytes were incubated in the presence of fructose. At the beginning of the experiment (10 min after addition of $[2-^3\text{H}]\text{lactate}$) the relative yield in water and glucose was lower and that in ethanol and glycerol higher than at later time points. This can be explained by removal of ethanol and glycerol and synthesis of glucose and water during the experiment. If the hepatocytes were incubated without fructose, no ^3H was found in glycerol and the relative yield of ^3H in glucose remained constant around 7% throughout the experiment for both starved and fed rats. The relative yield in water became higher (approx. 90% at the end of the experiment) than with fructose.

The labelling patterns of lactate, ethanol and glucose shown in Figs. 2 and 3 were used to calculate the specific radioactivity of NADH and the oxidation rate of NADH catalysed by lactate dehydrogenase, alcohol dehydrogenase and triose-phosphate dehydrogenase.

Calculation of the flux of reducing equivalents from NADH to pyruvate, acetaldehyde and 1,3-bisphosphoglycerate

The decrease in specific radioactivity (c.p.m./nmol) of lactate (L) with time and the increase in specific radioactivity of ethanol (E) and glucose (T) with time (Figs. 2 and 3) are used to calculate the reoxidation rates of NADH ($\mu\text{mol}/\text{min}$ per ml of packed cells) catalysed by lactate dehydrogenase (x), alcohol dehydrogenase (y) and triosephosphate dehydrogenase (f). The specific radioactivity of NADH (N) is also obtained by the calculations.

Using the model depicted in Fig. 1, assuming that the decrease in ^3H content of lactate equals input minus output (in c.p.m./min per ml of

packed cells), we obtain

$$\frac{dS_t L}{dt} = xN - (x+a)L$$

with S_t being the lactate concentration at time t ($\mu\text{mol}/\text{ml}$ of packed cells). Since the change in lactate concentration is constant with time (in the 70 min period considered), S_t can be substituted by $S_0 - at$, where S_0 is the lactate concentration at zero time and a is the oxidation rate of lactate ($\mu\text{mol}/\text{min}$ of ml packed cells).

$$\frac{d(S_0 - at)L}{dt} = xN - (x+a)L \quad (1)$$

Analogously, the increase in ^3H content in ethanol equals input minus output (in c.p.m./min per ml of packed cells) (Fig. 1):

$$\frac{d(A_0 - bt)E}{dt} = yN - (y+b)E \quad (2)$$

where A_0 is the ethanol concentration at zero time and b is the oxidation rate of ethanol ($\mu\text{mol}/\text{min}$ per ml of packed cells).

The ^3H content of glucose will depend on the flux of reducing equivalents from NADH to 1,3-bisphosphoglycerate (f) (Fig. 1). Since half of the ^3H incorporated into glyceraldehyde 3-phosphate is lost in the aldolase reaction, the flux of reducing equivalents through triosephosphate dehydrogenase must be multiplied by a factor α , which is 0.5 or less (see below), to obtain the ^3H incorporated in glucose:

$$\frac{d(G_0 + \Delta G)T}{dt} = \alpha fN \quad (3)$$

where G_0 is the glucose concentration at zero time and ΔG the rate of glucose formation. In this equation the output of ^3H from glucose is considered negligible.

Assuming one pool of NAD^+ (Vind & Grunnet, 1982), inflow of ^3H to NAD^+ equals outflow of ^3H from NADH (in c.p.m./min per ml of packed cells). Assuming the concentrations of NADH (Q) to be constant with time, the model shown in Fig. 1 gives:

$$\frac{QdN}{dt} = (x+a)L + (y+b)E - (x+a+y+b+f)N \quad (4)$$

with the output of ^3H from NADH being the total amount of reducing equivalents that goes through NADH multiplied by the specific radioactivity of NADH (N).

Fig. 2. Specific radioactivity (c.p.m./nmol) of lactate, ethanol and glucose as a function of time (fed rats) Hepatocytes were incubated for 10 min with 2.5 mM-lactate, 0.25 mM-pyruvate and 20 mM-ethanol in the presence or absence of 10 mM-fructose before addition of $[2\text{-}^3\text{H}]\text{lactate}$. The curves are given as an example and represent values from a cell preparation pooled from two fed rats. The open circles represent the measured data. The lines A, B and C represent the computed least-square fits to the measured data: line A represents the fit for $\alpha = 0.50$, B the fit for $\alpha = 0.25$ and C the fit for $\alpha = 0.10$. From the curve fitting with $\alpha = 0.10$, we find the oxidation rate of NADH by lactate dehydrogenase, alcohol dehydrogenase and triosephosphate dehydrogenase to be 3.8, 10.7 and $14.3 \mu\text{mol/min}$ per ml of packed cells with fructose and 2.6, 10.2 and $15.1 \mu\text{mol/min}$ per ml of packed cells without fructose, respectively.

Fig. 3. Specific radioactivity (c.p.m./nmol) of lactate, ethanol and glucose as a function of time (starved rats) Hepatocytes were incubated for 10 min with 2.5 mM-lactate, 0.25 mM-pyruvate and 20 mM-ethanol in the presence or absence of 10 mM-fructose before addition of $[2\text{-}^3\text{H}]\text{lactate}$. The curves are given as an example and represent values from a cell preparation pooled from two starved rats. The open circles represent the measured data. The lines A and B represent the computed least-square fit to the measured data: line A represents the fit for $\alpha = 0.50$ and B the fit for $\alpha = 0.25$. From the curve fitting with $\alpha = 0.25$, we find the oxidation rate of NADH by lactate dehydrogenase, alcohol dehydrogenase and triosephosphate dehydrogenase to be 1.9, 14.1 and 19.8 $\mu\text{mol}/\text{min}$ per ml of packed cells with fructose and 0.6, 0.5 and 1.5 $\mu\text{mol}/\text{min}$ per ml of packed cells without fructose, respectively.

By using the standard form of differentiation $d(Lt) = Ldt + t dL$, we can rearrange eqns. (1), (2) and (3), and obtain the following four simultaneous differential equations:

$$(S_0 - at) \frac{dL}{dt} = xN - xL \quad (I)$$

$$(A_0 - bt) \frac{dE}{dt} = yN - yE \quad (II)$$

$$(G_0 + \Delta Gt) \frac{dT}{dt} = \alpha fN - \Delta GT \quad (III)$$

$$\frac{QdN}{dt} = (x + a)L + (y + b)E - (x + a + y + b + f)N \quad (IV)$$

In these equations the concentrations of lactate (S_0), ethanol (A_0) and glucose (G_0) at zero time, the rates of metabolism of lactate (a), ethanol (b) and glucose (ΔG), and the changes with time of the specific radioactivities of lactate (L), ethanol (E) and glucose (T) have been determined by analysis (Figs. 2 and 3). The concentration of NADH was calculated from the [lactate]/[pyruvate] ratio (see the Materials and methods section).

To estimate the unknowns x , y and f , we have used a weighted least-mean-squares fit to the measured data $L(t)$, $E(t)$ and $T(t)$.

The solution to the differential equations, computed for each trial value of x , y and f , was accomplished by the Gear (1971) code operated in its stiff mode. Optimization of the resulting weighted sum of squared deviations was achieved by a quasi-Newton method with automatic estimation of the gradient of F (Stewart's method) (Stewart, 1967) and automatic updating of the Hessian (Broyden's method), as described by Dixon (1972). Both codes are found in commercially available software packages, such as the NAG library (available from Numerical Algorithms Group, Mayfield House, 256 Banbury Road, Oxford OX2 7DE, U.K.).

The given choices of x , y and f to start the computer calculations were estimated from the equations and values of N at $0.8 L_0$. The calculated values of N varied from 15 to 20% of L during the 10–70 min of incubation in cells from fed rats ($\alpha = 0.10$, see below) and was around 30% of L during the experiments with cells from starved rats ($\alpha = 0.25$).

Influence of the concentration of NADH and the value of α on the calculations

The concentration of NADH (Q) cannot be measured directly and was calculated from the [lactate]/[pyruvate] ratio and a free cytosolic

NAD⁺ concentration of 0.5 mM (see the Materials and methods section). We have therefore tested the influence of a change in the value of Q , and found that even a 10-fold change did not alter either the calculated values of x , y and f or the fitting of the curves $L(t)$, $E(t)$ and $T(t)$ (Figs. 2 and 3).

The values of x , y and f will depend on the value of α in eqn. (3). Lactate dehydrogenase is, like alcohol dehydrogenase, an *A*-specific dehydrogenase, and triosephosphate dehydrogenase is *B*-specific. The value of α will be 0.5, if the specific radioactivity of the 4*B*-hydrogen equals that of the 4*A*-hydrogen of NADH and the only ³H lost on the way from triose phosphate to glucose is that lost in the aldolase reaction. If, however, the exchange of ³H in the reactions catalysed by aldolase and triosephosphate isomerase is extensive because of cycling, the value of α will be less than 0.5. Furthermore, lack of equilibrium between the 4*B*- and 4*A*-hydrogens of NADH will give a value of α smaller than 0.5.

In a previous study (Vind & Grunnet, 1981) we found the value of α to be 0.25 in hepatocytes from starved rats metabolizing xylitol in the presence or absence of ethanol. In the present study, we have chosen the value of α giving the best curve fitting (Figs. 2 and 3), i.e. 0.25 for hepatocytes from starved rats metabolizing ethanol in the presence or absence of fructose, and 0.10 in hepatocytes from fed rats metabolizing ethanol in the presence or absence of fructose.

Our choice of $\alpha = 0.25$ for starved rats and $\alpha = 0.10$ for fed rats is not only appropriate because the computer calculations give the best curve fitting with those values (Figs. 2 and 3), but is also in agreement with other observations.

Rognstad & Clark (1974) found that only when the rates of the reversible dehydrogenases are greatly in excess of the net fluxes in the system can the difference in the specific radioactivity of 4*A*- and 4*B*-hydrogen in NADH become negligible. In starved rats this is not the case, and α is around 0.25.

The most obvious explanation for $\alpha = 0.10$ in cells from fed rats with or without fructose is a more extensive cycling of hydrogen in the reactions catalysed by aldolase and triosephosphate isomerase, because of the increased concentration of substrate for these reactions as compared with cells from starved rats (Scholz *et al.*, 1975).

Discussion

Rate of NADH oxidation catalysed by lactate dehydrogenase

The calculated flux of reducing equivalents from NADH to pyruvate is 1–4 μ mol/min per ml of packed cells (Table 2), which is in agreement with

Table 2. *Effects of fructose on the oxidation rate of NADH catalysed by cytoplasmic dehydrogenases*

Hepatocytes were incubated for 10 min with 2.5 mM-lactate, 0.25 mM-pyruvate and 20 mM-ethanol in the presence or absence of 10 mM-fructose before addition of [2-³H]lactate. Samples were taken every 10 min and used to estimate the labelling pattern of lactate, ethanol and glucose, from which the oxidation rate of NADH catalysed by cytoplasmic dehydrogenases were calculated. Values are expressed as means $\pm$ S.E.M. for five different cell preparations. * $P < 0.01$, ** $P < 0.02$, *** $P < 0.05$, **** $P \leq 0.10$ compared with experiments without fructose addition.

	Fructose (mM)	NADH oxidized (μ mol/min per ml of packed cells) catalysed by:		
		Lactate dehydrogenase (x)	Alcohol dehydrogenase (y)	Triosephosphate dehydrogenase (f)
Starved	0	1.2 $\pm$ 0.2	1.2 $\pm$ 0.4	1.8 $\pm$ 0.3
	10	2.8 $\pm$ 0.5**	12.0 $\pm$ 5.1****	14.3 $\pm$ 4.5***
Fed	0	1.5 $\pm$ 0.2	13.3 $\pm$ 2.1	15.0 $\pm$ 2.4
	10	3.4 $\pm$ 0.4*	9.0 $\pm$ 1.8	17.7 $\pm$ 2.9

Table 3. *Initial concentrations of pyruvate, acetaldehyde and NADH*

Hepatocytes were incubated for 10 min with 2.5 mM-lactate, 0.25 mM-pyruvate and 20 mM-ethanol in the presence or absence of 10 mM-fructose before addition of [2-³H]lactate at zero time. The initial concentrations are mean values for 0 and 10 min incubations. For calculation of NADH concentrations see the Materials and methods section. Values are expressed as means $\pm$ S.E.M. for five different cell preparations, except for starved rats without fructose ($n = 3$). * $P < 0.01$ and ** $P < 0.10$, compared with incubations in the absence of fructose.

		Pyruvate (μ M)	Acetaldehyde (μ M)	NADH (μ M)
Starved	– fructose	19 $\pm$ 5	23 $\pm$ 14	11.2 $\pm$ 3.5
	+ fructose	52 $\pm$ 7*	171 $\pm$ 19	4.1 $\pm$ 0.5**
Fed	– fructose	30 $\pm$ 6	117 $\pm$ 28	8.4 $\pm$ 2.1
	+ fructose	44 $\pm$ 10	169 $\pm$ 20	3.6 $\pm$ 1.1**

values reported by others and obtained by a different method (Schwab, 1982). These values are only a few per cent of the maximal enzyme activity (200 μ mol/min per g wet wt.; Weber, 1963).

In a previous study (Vind & Grunnet, 1982), we calculated the rates of NADH oxidation by lactate dehydrogenase by following the incorporation of ³H into lactate from [2-³H]xylitol or (1R)-[1-³H]-ethanol and obtained values similar to those obtained in the present work by using [2-³H]-lactate. These results indicate that the three enzymes xylitol dehydrogenase, alcohol dehydrogenase and lactate dehydrogenase do equilibrate with the same pool of cytoplasmic NAD(H).

The oxidation rate of NADH in the lactate dehydrogenase reaction may be determined by the concentration of pyruvate, as we observed a positive correlation ($r = 0.88$) between the concentration of pyruvate (Table 3) and the rate of NADH oxidation catalysed by lactate dehydrogenase (Table 2). In accordance with this conclusion, the pyruvate concentrations were well below the K_m value for pyruvate (0.14–1.0 mM; Winer & Schwert, 1958; Pesce *et al.*, 1964). In contrast, the

rate of NADH oxidation catalysed by lactate dehydrogenase was inversely proportional to the concentration of NADH (Tables 2 and 3) ($r = -0.96$).

Rate of NADH oxidation catalysed by alcohol dehydrogenase

The rate of NADH oxidation catalysed by alcohol dehydrogenase is 1 μ mol/min per ml of packed cells in starved rats. The addition of fructose accelerates the rate to the same rate as in cells from fed rats with or without fructose (9–13 μ mol/min per ml of packed cells) (Table 2). These results are in agreement with values for acetaldehyde reduction (9–15 μ mol/min per ml of cells) obtained by measuring the isotope effect in ethanol metabolism (Lundquist & Iversen, 1983).

When the rate of acetaldehyde reduction is about 10 μ mol/min per ml of packed cells (Table 3) and the net oxidation of ethanol is 2.5–2.8 μ mol/min per ml of packed cells (Table 1), the rate of ethanol oxidation must be about 13 μ mol/min per ml of packed cells. This is more than twice the maximal capacity of alcohol dehydrogenase to

oxidize ethanol as measured *in vitro* (Crow *et al.*, 1977). The reaction catalysed by alcohol dehydrogenase occurs in a series of five reversible steps (Wratten & Cleland, 1965):

catalysed by alcohol dehydrogenase seems to correlate with the NADH concentration, following simple Michaelis–Menten kinetics at high concentrations of acetaldehyde.

where E = enzyme catalytic site.

Reaction 1 is the only step that is not involved in the labelling of ethanol via NADH (labelled from [2-³H]lactate), but is involved in measurements of alcohol dehydrogenase activity *in vitro*. Our results therefore suggest that the dissociation of NAD⁺ as well as of NADH can be rate-limiting in the alcohol dehydrogenase reaction.

When the concentration of acetaldehyde is greater than $100\text{ }\mu\text{M}$ in cells from fed rats and in cells from starved rats in the presence of fructose (Table 3), the reduction of acetaldehyde appears independent of the concentration of acetaldehyde (Table 2). This is an agreement with a K_m value for acetaldehyde around $40\text{ }\mu\text{M}$ (Cornell, 1983b). At acetaldehyde concentrations greater than $100\text{ }\mu\text{M}$, the rate of acetaldehyde reduction appears positively correlated with the NADH concentration (Tables 2 and 3). The apparent K_m and V_{max} values can be calculated as $4.7\text{ }\mu\text{M}$ and $21\text{ }\mu\text{mol/min per ml}$ of packed cells respectively, in agreement with values reported for the isolated enzymes (Cornell, 1983b; Crabb *et al.*, 1983; Katavama *et al.*, 1983).

Rate of NADH oxidation catalysed by triose-phosphate dehydrogenase

The calculated flux of reducing equivalents from NADH to 1,3-bisphosphoglycerate is $2\mu\text{mol/min}$ per ml of packed cells in cells from starved rats without fructose. The addition of fructose accelerates the rate to the same value as in cells from fed rats with or without fructose ($14\text{--}18\mu\text{mol/min}$ per ml of packed cells) (Table 2). The most obvious explanation for this acceleration of the rate of NADH oxidation is an increase in the concentration of 1,3-bisphosphoglycerate.

Conclusions

The present results support the concept of a single cytoplasmic NAD(H) pool, at least for the dehydrogenases considered. Exchange rates in the reactions catalysed by alcohol dehydrogenase and lactate dehydrogenase are substantial as compared with the net oxidation rate in these reactions. The rate of NADH oxidation catalysed by lactate dehydrogenase is correlated with the concentration of pyruvate, and the rate of NADH oxidation

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