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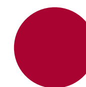

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OXIDATION OF REDUCING EQUIVALENTS DURING ETHANOL METABOLISM. STUDIES WITH 1-^{3}H-ETHANOL

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The rate of transfer of reducing equivalents from cytosolic NADH to the mitochondrial cell compartment has been estimated in isolated hepatocytes from fasted rats. Hepatocytes were incubated with 6 mM R-ethanol-1-^{3}H or 6 mM S-ethanol-1-^{3}H. Measurements of the incorporation of tritium into water and β-hydroxybutyrate allowed calculations of the rate of NAD-dependent shuttles (probably the malate-aspartate shuttle) as well as the rate of FAD-dependent shuttles (probably the α-glycerophosphate shuttle).

Incorporation of tritium from R- and S-ethanol-1-^{3}H into lactate and glucose showed that less than 20% of the acetaldehyde oxidation takes place in the cytosolic compartment of the liver cell.

With an ethanol oxidation rate of 2.5 μmoles x min^{-1} x ml of cells^{-1}, the activity of the malate-aspartate shuttle was estimated to be about 1.5 μmoles x min^{-1} c ml of cells $^{-1}$ and that of the α-glycerophosphate shuttle to be about 0.6 μmoles x min^{-1} x ml of cells $^{-1}$.

I. INTRODUCTION

Oxidation of ethanol to acetaldehyde by alcohol dehydrogenase (ADH) results in the formation of NADH in the cytosolic compartment of the liver cell (1). In the fasted liver, most of this cytosolic NADH probably is oxidized via the mitochondrial respiratory chain, since the capacity of cytosolic synthetic pathways is too low to reoxidize the reducing equivalents formed by ethanol oxidation. Oxidation of cytosolic NADH by the respiratory chain requires the transfer of the reducing equivalents of NADH to the mitochondria by a hydrogen shuttle mechanism. These hydrogen shuttles may be NAD-linked (e.g. the malate-

aspartate shuttle) or FAD-linked (e.g. the α-glycerophosphate shuttle). The participation of the malate-aspartate shuttle in ethanol metabolism has been demonstrated by the use of the inhibitors of the aspartate aminotransferase (2,3). Ethanol metabolism can be inhibited only 50-60% by these agents, indicating that more than one shuttle-mechanism is involved in ethanol oxidation. Attempts to evaluate the importance of the α-glycerophosphate shuttle involve experiments with rotenone-inhibition of ethanol oxidation (4,5), and studies of the metabolism of 1-^{3}H-ethanol (6). In the present study, 1-^{3}H-ethanol is used to estimate the rate of transfer of reducing equivalents from the cytosol to the mitochondria *via* NAD-dependent and FAD-dependent shuttles, respectively, during ethanol metabolism.

II. MATERIALS AND METHODS

Cells from 24 h fasted Wistar female rats weighing 150-220 g were prepared as described previously (7). About 100 mg wet wt of cells were incubated at 37°C in Krebs-Henseleit bicarbonate buffer containing 1% purified albumin, 25 mM N-(2-hydroxyethyl) -N'2-ethanesulfonic acid (HEPES), 8 mM glucose, 2 mM L-lactate and 0.2 mM pyruvate in a total volume of about 2.3 ml. The gas phase was 95% O_2 + 5% CO_2. The reaction was stopped after 1, 3 5 and 15 min by addition of $HClO_4$. The neutralized $HClO_4$ supernatant was used for isolation of water, glucose, lactate and β-hydroxybutyrate. Radioactivity in water was obtained as the difference between radioactivity in water plus dry matter and the radioactivity in dry matter. Glucose was isolated as the neutral fraction from ion-exchange chromatography and lactate and β-hydroxybutyrate by the gas-chromatographic procedure previously described (7).

R-ethanol-1-^{3}H and S-ethanol-1-^{3}H were prepared from R,S-ethanol-1-^{3}H (New England Nuclear, Boston, Ma., USA), R,S-ethanol-1-^{3}H was incubated with NAD^{+}, semicarbazide and ADH. When the reaction was complete, NAD^{3}H and ^{3}H-acetaldehyde-semicarbazone were separated on a DEAE-sephadex A-25 column (7). Acetaldehyde was reduced by NAD^{3}H (plus ADH) giving R-ethanol-1-^{3}H. Acetaldehyde from the ^{3}H-acetaldehyde-semicarbazone was isolated by distillation after acidification, and 1-^{3}H-acetaldehyde was reduced by NADH (plus ADH) giving S-ethanol-1-^{3}H. Both R- and S-ethanol-1-^{3}H were purified by distillation.

III. THEORY

1. Rate of NAD-Dependent Shuttles

Oxidation of ethanol to acetaldehyde catalyzed by ADH, transfers the pro-R-hydrogen of ethanol to NAD^{+}, while the pro-S

hydrogen is retained on acetaldehyde. The oxidation of acetaldehyde to acetate catalyzed by aldehyde dehydrogenase results in formation of NADH containing the pro-S- hydrogen of ethanol (Fig. 1).

ADH is confined to the cytosol of the liver cell (1), while it is generally agreed upon that acetaldehyde oxidation takes place exclusively or predominantly in the mitochondrial matrix compartment (8,9) (Fig. 1.). Therefore, if R-ethanol-1-^{3}H is the substrate for liver cells, NAD^{3}H will be formed exclusively in the cytosol, and if S-ethanol-1-^{3}H is the substrate, NAD3 will be formed almost exclusively in the mitochondria.

Cytosolic NAD^{3}H may be oxidized in several reactions and the reducing equivalents can be retained in 'cytosolic' end-products like glucose or lactate or they can be transferred to the mitochondrial NADH-pool. Mitochondrial NAD^{3}H may react with intramitochondrial dehydrogenases, and if oxidized by the respiratory chain, they will give rise to tritium-labelled water.

With S-ethanol-1-3H as a substrate, all NAD^{3}H is formed in the mitochondria (see above) and the rate of NAD^{3}H formation equals the rate of ethanol oxidation, since no accumulation of acetaldehyde takes place. With R-ethanol-1-^{3}H as the substrate, the rate of mitochondrial NAD^{3}H formation equals the rate of transfer of reducing equivalents from the cytosol to the mitochondria via NAD-dependent shuttles.

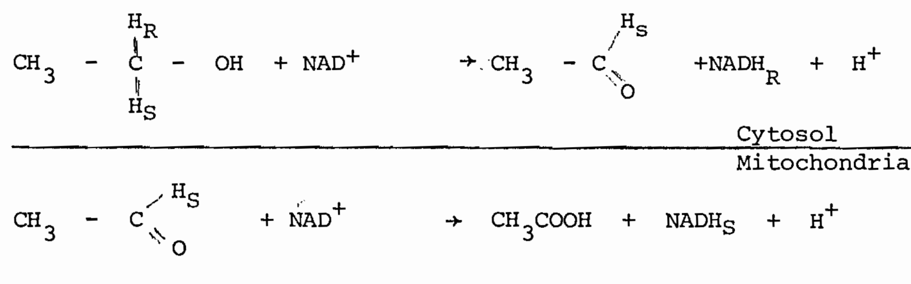

Fig.1. Pathway of the pro-R and pro-S Hydrogen of Ethanol.

β-hydroxybutyrate (β-HBA) dehydrogenase is located exclusively in the mitochondrial compartment and is generally assumed to equilibrate only with the mitochondrial NADH-pool. For a given experimental condition (cell concentration, incubation time), the incorporation of tritium into βhydroxybutyrate will be proportional to the formation of NAD^{3}H in the mitochondria. The ratio between tritium in β-hydroxybutyrate from R- and S-ethanol-1-^{3}H thus equals that fraction of the total cytosolic NAD^{3}H which is transferred to the mitochondria via NAD-dependent shuttles:

Fraction of cytosolic NAD^3H transferred via NAD-shuttles =
[^3H in β-HBA from R-ethanol-1-^3H}/ {^3H in β-HBA from S-ethanol-1-^3H}.

Incubation of liver cells with R- and S-ethanol-1-^3H and determination of tritium incorporation into β-hydroxybutyrate therefore allows calculation of the rate of NAD-linked hydrogen shuttles. The two isotopes should be applied to the same batch of liver cells to account for differences between individual animals regarding e.g. ethanol oxidation and ketogenesis. It has been assumed that the isotope effect on ethanol oxidation is close to 1, and it has been assumed that tritium from ytosolic NAD^3H is transferred to the mitochondrial NADH-pool only via shuttle mechanisms. The first assumption seems justified as the isotope effect on ethanol oxidation at low concentrations of ethanol is 1-1.2 (Table 1) (10.11). The second assumption may be more difficult to validate. A rapid exchange of malate across the mitochondrial membrane may result in the transfer of tritium from cytosolic NAD^3H to mitochondrial NAD^+ without any net transport of reducing equivalents. Evidence has been obtained for a slow transport of dicarboxylate anions across the mitochondrial membrane in liver cells from fasted rats (12). Other experiments, however, indicate a relatively fast exchange of malate between the cytosol and the mitochondria (13). We, therefore, consider our estimates of the rate of NAD-dependent (^3H) shuttles as maximum rates.

2. Rates of Other Shuttles

Cytosolic reducing equivalents in NADH may be transferred to the mitochondria via NAD-linked shuttles or via FAD-linked shuttles. The total transfer of cytosolic NAD^3H to the mitochondria may be estimated from tritium incorporation from etha-ol-1-^3H into water, and as the transfer via NAD-linked shuttles s known from the tritium incorporation into β-hydroxybutyrate, the transfer via FAD-linked shuttles can be obtained by difference.

Estimation of the total rate of transfer of reducing equivalents to the mitochondria from cytosolic NAD^3H may be obtained from incorporation of tritium into water from R- and S-ethanol 1-^3H. It is assumed that tritium from these labeled substrates is incorporated into water only by mitochondrial reactions. By analogy to the incorporation of tritium into β-hydroxybutyrate (see above) the fraction of cytosolic NAD^3H which is transferred to the mitochondria, equals the ratio between tritium in water from R- and from S-ethanol-1-^3H:

Fraction of cytosolic NAD^3H transferred to mitochondria =
{^3H in water from R-ethanol-1-^3H}/ {^3H in water from S-ethanol-1-^3H}

Clearly, the assumption that tritiated water is formed exclusively in the mitochondria is incorrect, as e.g. non-ADH oxidation of R-ethanol-1-^{3}H also gives rise to the formation of tritiated water. Our estimates of the total rate of (^{3}H) shuttle activity also represents maximum values.

IV. RESULTS AND DISCUSSION

The distribution of tritium from R-and S-ethanol-1-^{3}H in various metabolites is shown in Table 1. With S-ethanol-1-^{3}H as the substrate, most of the tritium is found in water as expected, and relatively little is incorporated into glucose and lactate. These results, therefore, support the concept that acetaldehyde is oxidized in the mitochondrial compartment of the liver cell. Incorporation of tritium in glucose and lactate from S-ethanol-1-^{3}H may be due to cytosolic oxidation of acetaldehyde or to transfer of mitochondrial NAD^{3}H to the cytosol. If no transfer of reducing equivalents from the mitochondria to the cytosol took place, it may be seen, by comparing tritium labeling of glucose and lactate from R- and from S-ethanol-1-^{3}H, that less than 20% of the acetaldehyde is oxidized extramitochondrially.

TABLE 1
Distribution of Tritium from R-and S-Ethanol-1-^{3}H,

Isotope	% of Tritium found in Water	Lactate	Glucose	β-HBA	Δ^{3}H-Ethanol / Δ^{14}C-Ethanol
R-Ethanol-1-^{3}H	73	28	6.5	0.7	1.08
S-Ethanol-1-^{3}H	92	5.9	1.4	1.1	1.03

Liver cells were incubated for 15 min as described in METHODS with 6 mM R- or 6 mM S-ethanol-1-^{3}H. Ethanol oxidation was measured with ^{14}C-ethanol as described previously (14).

The ratio between tritium incorporation into β-hydroxybutyrate and water from R- and S-ethanol-1-^{3}H is shown in Table 2. These results show that about 80% of the NAD^{3}H formed by oxidation of ethanol to acetaldehyde is transferred to the mitochondria. Of these 80% about 55-60% is transferred via NAD-dependent shuttles and the difference - about 20-25% - is transferred via other shuttles. About 20% of the NAD^{3}H from the ADH-reaction is detected in lactate, glucose and other metabolites. Calculation of the activity of the hydrogen shuttles from the tritium-data presented above is made uncertain by the largely unknown isotope effects on the reactions of the hydrogen-shuttles.

TABLE 2

Tritium Incorporation into Water and β-Hydroxybutyrate from R-and S-Ethanol-1-^3H

$$\frac{^3H \text{ in water from R-ethanol-1-}^3H}{^3H \text{ in water from S-ethanol-1-}^3H} = 0.79 \quad (n = 3)$$

$$\frac{^3H \text{ in } \beta\text{-HBA from R-ethanol-1-}^3H}{^3H \text{ in } \beta\text{-HBA from S-ethanol-1-}^3H} = 0.57 \quad (n = 4)$$

Liver cells were incubated for 15 min as described in METHODS with 6 mM R- or 6 mM S-ethanol-1-^3H. n is the number of cell preparations. β-HBA = β-hydroxybutyrate.

Such isotope effects would cause an underestimation of the rate of the hydrogen shuttles. On the other hand, a transfer of tritium from the cytosol to the mitochondria by a malate-exchange and ^3H_2O formation in the cytosol from NAD^{3}H tend to overestimate these rates (see above). Since the rate of ethanol oxidation in the present experiments was about 2.5 μmoles x min $^{-1}$ x ml of cell^{-1}, our best estimate of the activity of the malate-aspartate shuttle is about 1.5 μmoles x min^{-1} x ml of cells $^{-1}$ and that that of the α-glycerophosphate shuttle is about 0.6 μmoles x min^{-1} x ml of cells $^{-1}$.

The figure calculated for the transfer of cytosolic NADH via the malate-aspartate shuttle is in good agreement with results from experiments with inhibitors of the aspartate aminitransferase (2,3), indicating that 50-60% of NADH from the oxidation of ethanol to acetaldehyde is transferred to the mitochondria via the malate-aspartate shuttle.

V. ACKNOWLEDGEMENTS

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