OXIDATION OF EXTRAMITOCHONDRIAL NADH BY RAT LIVER MITOCHONDRIA. POSSIBLE ROLE OF ACYL-SCoA ELONGATION ENZYMES.

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SUMMARY. Oxidation of extramitochondrial NADH by rat liver mitochondria was studied in vitro with a new model for the inward transport of NADH as basis. The model involves chain-elongation of acyl-SCoA compounds.

Extramitochondrial NADH was generated during the experiment by ethanol, NAD^+ and ADH and the ethanol disappearance rate was used as a measure of the transport of NADH. Addition to the incubation medium of the extramitochondrial components of the transport model resulted in a fiftyfold increased oxidation of ethanol and gave rise to changes of the intramitochondrial redox-level and of the oxidation of palmitate similar to the changes observed during ethanol oxidation in vivo. The capacity of the transport mechanism compares well with the oxidation rates observed in rat liver slices.

INTRODUCTION. Since the discovery by Lehninger (1) of the impermeability of the mitochondrial membrane to NADH, several systems designed to transfer reducing equivalents in form of NADH from the cytosol to the mitochondria have been proposed (2, 3, 4). The most widely accepted mechanism for the transport of reducing equivalents across membranes of rat liver mitochondria is the MDH[x)] and GOT catalyzed system as proposed by Borst (4).

The system for transport of NADH, recently proposed by Whereat et al. (5), which is catalyzed by the mitochondrial fatty acid elongation enzymes and the enzymes catalyzing β-oxidation, is shown in fig. 1. It should be pointed out that this mechanism, in contrast to the MDH

x)Abbreviations: ADH, Alcohol Dehydrogenase (EC 1.1.1.1); CCCP, Carbonyl cyanide m-chlorophenylhydrazone; DNP, Dinitrophenol; GOT, Glutamate-oxaloacetate Transaminase (EC 2.6.1.1); HSCoA, Coenzyme A; MDH, Malate Dehydrogenase (EC 1.1.1.37); TMPD, N,N, N', N'-tetramethyl-p-phenylenediamine.

and GOT catalyzed mechanism, furnishes the energy needed for the transport of NADH against a concentration gradient. In the model of fig.1, one molecule of ATP is expended per two reducing equivalents transfered. The fatty acid elongation enzymes of rat liver mitochondria are well known (6, 7, 8) and are located to the outer and/or inner membrane. No evidence for or against the hypothetical acyl-SCoA shuttle is available. This communication reports some preliminary experiments concerning the model of Whereat et al. (fig. 1). See also (9).

METHODS AND MATERIALS. In the experiments reported, extramitochondrial NADH was produced by addition of ethanol, NAD^+ and ADH to the mitochondrial suspension. The equilibrium of the ADH catalyzed reaction is in favour of ethanol + NAD^+ and the rate limiting step in the reaction is, at least in case of the liver enzyme, dissociation and reoxidation of NADH bound to the enzyme (10). This implies that the ethanol disappearance rate in a system composed of mitochondria, ethanol, NAD^+ and ADH plus a NADH transferring system, may reflect the rate of transfer of reducing equivalents. Mitochondria were prepared from two livers of 200 g fed female Wistar rats as described in (11), except that the mitochondria were washed three times and the preparation medium, adjusted to pH 6.8 before use, contained 225 mM mannitol, 75 mM sucorse, 1 mM TRIS plus 0.1 mM EDTA. The yield of mitochondria by this procedure was 28 % as determined by the yield of succinate dehydrogenase (12). On this basis ethanol and oxygen disappearance rates obtained as µmoles/mg of mitochondrial protein can be converted to nmoles/g fresh liver, thus enabling a comparison with results obtained with more intact liver preparations. The mitochondria contained virtually no pyruvate kinase or lactate dehydrogenase activity, showed control ratios above 3.5 with succinate as substrate and were impermeable to NADH. The standard deviation of -Δethanol values, calculated from experiments with five different preparations of mitochondria, was 10-15 %.

The oxygen consumption was determined in a Gilson respirometer at 30.0°C. The incubation medium contained 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 5 mM $MgCl_2$, 5 mM phosphate and 0.5 mM EDTA and was adjusted to pH 7.35. The total incubation volume was 2.3 - 2.5 ml and the amount of mitochondria corresponded to 10-15 mg of protein. After a five min. equilibration period the endogenous respiration was measured for 10 min. Phosphate acceptor was then added and the oxygen consumption measured in a 20 min. period. Manometer readings were taken every second minute and the slope of the oxygen consumption versus time curve was calculated by the method of least squares. The carbon dioxide produced was absorbed by KOH on filter paper in the center well and determination of the production of $^{14}CO_2$ from radioactive substrates was carried out by counting of the filter paper after acidification and 10 min. shaking of the reaction mixture.

Ethanol was determined by the method of Bonnichsen (13) and protein by the method of Groves et al. (14) using bovine serum albumine as a standard. Enzymes and coenzymes were supplied by Boehringer & Soehne, Mannheim, Germany, and 1-^{14}C-palmitate and 1-^{14}C-acetate by The Radiochemical Center, Amersham, U.K.

RESULTS AND DISCUSSION. The maximal NADH oxidizing capacity of the mitochondria was determined by measurements of the ethanol oxidation rate in the presence of TMPD, which oxidizes extramitochondrial NADH, penetrates the mitochondrial membrane and is reoxidized via the respiratory chain (table 1). Proportionality between ethanol oxidation and time was maintained throughout the 20 min. period after addition of phosphate acceptor.

Addition of ethanol, NAD^+ and ADH to mitochondria plus ADP resulted in only a small oxidation of ethanol. Further addition of oleic acid, ATP and coenzyme A caused a fifty-fold increase in the ethanol oxidation rate (table 1). The amount of ethanol disappearing was more than

Table 1. Oxidation of ethanol by rat liver mitochondria. The incubation mixture contained buffer and mitochondria as described in 'Materials and Methods', 10 mM ethanol, 0.4 mM NAD^+ and 3 units of dialyzed yeast ADH. Final concentrations of added substances: fatty acids, 0.2 mM; HSCoA, 0.2 mM; ATP, 2 mM; ADP, 2 mM; CCCP, 2 µM; DNP, 0.1 mM; TMPD, 50 µM; succinate, 10 mM. All values are corrected for the ethanol disappearance in the absence of mitochondria and are expressed as nmoles x min^{-1} (mg of mitochondrial protein)$^{-1}$. The values are averages of experiments with at least three different preparations of mitochondria.

Additions	-Δethanol	-Δoxygen
ADP	0.3	5.4
TMPD + ADP	29.2	21.1
Oleate + HSCoA + ATP + ADP	16.9	21.3
Oleate + HSCoA + ATP	11.5	12.0
Oleate + HSCoA	1.7	4.8
HSCoA + ATP	0.3	2.1
Oleate + ATP	7.7	10.o
Oleate + HSCoA + ATP + CCCP or DNP	2.5	9.9
Palmitate + HSCoA + ATP + ADP	8.5	7.0
Palmitate + HSCoA + ATP	4.6	3.1
Laurate + + HSCoA + ATP + ADP	11.2	5.9
Laurate + HSCoA + ATP	11.0	6.9
Octanoate + HSCoA + ATP + ADP	2.4	9.1
Octanoate + HSCoA + ATP	4.1	3.3
Succinate + ADP	0.4	6.9

five times the amount of oleic acid added, which indicates a cyclic transfer mechanism for NADH. The oxygen consumption listed in table 1 is the increase in oxygen consumption on addition of ethanol, NAD^+

and phosphate acceptor. This means that a possible stimulation or inhibition of the endogenous (fatty acid) respiration is not taken into account. The change in oxygen consumption associated with the ethanol oxidation may therefore be about 1 mole of oxygen per mole of ethanol corresponding to oxidation of ethanol to acetate, which is in accordance with the capability of rat liver mitochondria to oxidize acetaldehyde (15, 16, 17).

If the model for the transfer of reducing equivalents outlined in fig. 1 is correct, the ethanol oxidation by mitochondria should be strictly dependent upon addition of fatty acids, ATP and coenzyme A. This is the case only for fatty acids and ATP (table 1) whereas omission of coenzyme A caused only a partial reduction of the ethanol oxidation. This may be

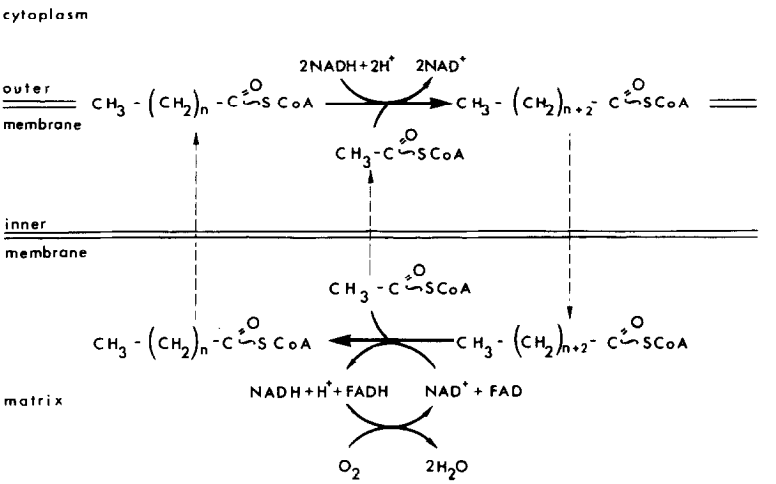

Fig. 1. Mechanism for the transport of reducing equivalents as NADH from the cytoplasm to the mitochondria involving fatty acid elongation and β-oxidation (5). The model does not take into account impermeability of the inner mitochondrial membrane to acyl-SCoA esters. In the experiments reported, the transfer of the acyl-SCoA esters, which are synthesized in the outer membrane (20, 33) and elongated in the outer compartment of the mitochondria (6, 7), might have been mediated by carnitine, present in the mitochondrial suspension (34). Another possibility would be that the elongation mechanism involves a transfer of the elongated acyl-SCoA ester to the matrix compartment.

due to coenzyme A independent oxidation of the fatty acids in the presence of phosphate (18, 19) or to leakage of coenzyme A from the mitochondria to the incubation medium (20). Uncoupling of the respiratory chain by CCCP or DNP caused an inhibition of the ethanol oxidation. The cause of this inhibition could be a depletion of ATP or a diversion of the metabolism of fatty acids from elongation to oxidation. The experiment with addition of succinate shows that the requirement for fatty acids, ATP and coenzyme A is not a simple substrate requirement.

Oxidation of ethanol in the described system increased the intramitochondrial β-hydroxybutyrate/acetoacetate ratio (table 2) and decreased the rate of oxidation of both acetate and palmitate (table 3), which agrees well with known effects of ethanol metabolism upon these parameters (21 and 22; 23 and 24 and 25, 26 and 27 respectively).

Table 2. Effect of ethanol oxidation upon the intramitochondrial redox level of rat liver mitochondria. Experimental conditions as described in the text. After 15 min of incubation 1.6 mM acetoacetate was added. Equilibrium of the β-hydroxybutyrate dehydrogenase catalyzed reaction was obtained after five minutes and acetoacetate and β-hydroxybutyrate was determined according to (32). The incubation mixture contained buffer, mitochondria, 0.2 mM palmitate, 0.2 mM HSCoA and 2 mM ATP. Final concentration of added substances: Ethanol, 10 mM; NAD^+, 0.4 mM; dialyzed yeast ADH, 3 units.

Additions	$\frac{\text{β-hydroxybutyrate}}{\text{acetoacetate}}$	β-hydroxybutyrate + acetoacetate, mM
none	0.056	1.97
Ethanol + NAD^+ + ADH	0.156	2.10

The efficiency of the fatty acids to catalyze ethanol oxidation decrease in the order oleate, laurate, palmitate and octanoate (table 1). Although the fatty acids were added in amounts too small

Table 3. Effect of ethanol oxidation upon 1-^{14}C-palmitate and 1-^{14}C-acetate oxidation by rat liver mitochondria. The radioactive substrates were added together with ATP. The incubation medium contained buffer, mitochondria, 0.2 mM palmitate, 0.2 mM HSCoA, 2 mM ATP and 25 nC of 1-^{14}C-palmitate (55.2 mC/mM) or 10 nC of 1-^{14}C-acetate (0.12 mC/mM). Final concentration of added substances: Ethanol, 10 mM; NAD^+, 0.4 mM; dialyzed yeast ADH, 3 units. The $^{14}CO_2$ production is corrected for $^{14}CO_2$ production in parallel experiments without mitochondria present. In experiments with 1-^{14}C-acetate possible isotope dilution by acetate formed from ethanol is not taken into consideration.

Addition	$^{14}CO_2$ (c.p.m.)	%
1-^{14}C-palmitate	690	100
1-^{14}C-palmitate + ethanol + NAD^+ + ADH	60	9
1-^{14}C-acetate	5840	100
1-^{14}C-acetate + ethanol + NAD^+ + ADH	280	5

to cause uncoupling (28) this order reflects the uncoupling efficiency of the fatty acids (28) and the oxidation rate of the acids when no ADP is added (15). The low ethanol oxidation rate in experiments with octanoate may reflect that octanoate by the liver is oxidized to C_2-units preferential to esterification (29, 30).

The values for the ethanol disappearance rate expressed as nmoles/mg of mitochondrial protein may be converted to ethanol oxidation rates per gram fresh liver (see section Methods and Materials). The maximal value obtained correspond to 0.70 μmol/g liver·min.$^{-1}$ (experiments with TMPD added). This figure compares well with ethanol oxidation rate of liver slices at 37°C (0.75 μmol/g min^{-1} (31). The best figure obtained for the fatty acid dependent ethanol oxidation at 30° is 0.40 μmol x g^{-1} min^{-1}.

The results presented here thus indicate that the reactions of fatty acid elongation and β-oxidation may constitute a mechanism where-

by reducing equivalents produced in the cytoplasma of the cell can be oxidized via the respiratory chain. This mechanism is more attractive from an energetical point of view than the mechanism involving MDH and GOT. Quantitatively the capacity of the fatty acid stimulated NADH transport system may be 2-3 times as high as that of the MDH and GOT catalyzed system (22). In vivo both systems may be operating simultaneously.

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