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Shuttles for Translocation of NADH in Isolated Liver Cells from Fed Rats during Oxidation of Xylitol

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Translocation of xylitol-derived NADH *via* malate-aspartate and α-glycerophosphate shuttles was studied in liver cells isolated from fed rats.

In bicarbonate medium amino-oxyacetate, rotenone and antimycin A, were equally efficient in depressing the xylitol removal. Incubation of cells in nonbicarbonate medium did not affect the rate of xylitol removal. In this medium amino-oxyacetate and antimycin A, but not rotenone, inhibited xylitol removal.

Xylitol inhibited the lactate accumulation found when the cells were incubated without any exogenous substrates. Glucose was the main end product of xylitol oxidation.

In nonbicarbonate medium ketogenesis was high, whereas in bicarbonate medium a low rate of ketone body formation was found. Xylitol had no effect on the rate of ketone body formation in either medium tested.

Xylitol markedly decreased the ATP and P_i contents of the cells, but no change in the ATP/ADP × P_i ratio or the rate of oxygen consumption was found.

The results suggest that NADH formed during xylitol oxidation is translocated to the mitochondria mainly through the malate-aspartate shuttle and only when this shuttle is inhibited does the α-glycerophosphate shuttle transfer NADH. Intramitochondrial reactions which form NADH and $FADH_2$ are also suggested to be important regulators of the activity of the α-glycerophosphate shuttle.

The role of the malate-aspartate shuttle in the translocation of NADH between cytoplasm and mitochondria has been studied widely with inhibitors such as fluoromalate, difluoro-oxaloacetate, cycloserine, and amino-oxyacetate.[1] *In vitro* studies have frequently shown that the inhibitory effect of these compounds varies according to the incubation medium. Translocation of NADH during oxidation of ethanol, glycerol, or sorbitol is efficiently inhibited in bicarbonate-buffered medium but not in phosphate of HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) buffered media.[2–4] The difference has been attributed to inhibition of the pyruvate carboxylase-catalyzed reaction which has a K_m value of 1 mM for the bicarbonate ion.[5] Inhibition of this reaction either by shortage of bicarbonate[6] or other causes[7] leads to diminuation of the cell content of oxaloacetate and thus inhibition of the malate-aspartate shuttle.[3]

The malate-aspartate shuttle has been shown to be the main shuttle for NADH formed from ethanol, glycerol, and sorbitol,[3,8,9] but the α-glycerophosphate shuttle has been suggested to transfer NADH, too, especially when α-glycerophosphate is accumulated,[1,10] or when the malate-aspartate shuttle is inhibited.[4,9,11–13] In the present study a possible activation of the α-glycerophosphate shuttle was studied in isolated rat liver cells supplied with xylitol.

Xylitol was chosen for two main reasons. First it forms NADH only in the cytoplasm[10] and thus the respiratory chain inhibitors rotenone and antimycin A can be used to determine how much NADH is translocated through the malate-aspartate (a rotenone- and antimycin A-sensitive) shuttle and how much through the α-glycerophosphate (a rotenone-insensitive, antimycin A-sensitive) shuttle. Similar experiments have been done with ethanol,[4,12] but these are more complicated since the acetaldehyde formed from ethanol is mainly oxidized intramitochondrially and both rotenone and antimycin A inhibit the

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acetaldehyde oxidation; the accumulation of acetaldehyde may therefore affect the rate of ethanol elimination. The second reason for the use of xylitol is the apparent accumulation of α-glycerophosphate during xylitol elimination;[10,14,15] this compound has been suggested to affect the activity of the α-glycerophosphate shuttle.[1,16]

During isolation of liver cells, metabolites of the malate-aspartate shuttle are depleted,[17,18] and thus the capacity of this shuttle is diminished.[3] To prevent limitations in the NADH translocation,[18,19] cells were accordingly isolated from fed rats. The malate-aspartate shuttle was inhibited with amino-oxyacetate and by incubation of the cells in nonbicarbonate medium. The participation of the α-glycerophosphate shuttle in the NADH translocation was estimated with the respiratory chain inhibitors, rotenone and antimycin A.

MATERIALS AND METHODS

Animals. Male Spraque-Dawley rats had free access to pelleted laboratory diet (Orion, Espoo, Finland) and tap water. Rats 3 to 4 months in age were used in the experiment. No fasting period preceded the cell isolation.

Isolation and incubation of liver cells. Liver cells were isolated from fed rats by the method of Berry and Friend [20] as described and modified by Seglen.[21] Viability of the cells was tested with the toluidine blue exclusion test and varied between 85 and 95 %. A factor of 3.7 was used to calculate the liver cell wet weight.[17] After isolation liver cells were suspended into either Krebs-Henseleit bicarbonate buffer [22] which was 2 % with respect to bovine plasma albumin (fraction V), or into the HEPES, TES (*N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid), and Tricine (*N*-tris(hydroxymethyl)-methylglycine) buffered medium described by Seglen.[21] The latter buffer contains no bicarbonate and will be referred to in the text as nonbicarbonate medium even though it contains the bicarbonate produced by metabolism during incubation of liver cells.

Cell suspensions (1.5 ml; containing about 80 – 100 mg of liver cells, wet weight) were incubated in 1.5 ml of either bicarbonate or nonbicarbonate buffer which contained substrate and inhibitors. Rotenone and antimycin A were dissolved in acetone and administered to the incubation vessels immediately before the experiment according to Grundin.[23] Concentrations at the beginning of the incubation period were as follows: xylitol 10 mM, ethanol 10 mM, amino-oxyacetate 0.5 mM, rotenone 20 μM, and antimycin A 20 μM. Cells were incubated in a shaking water bath for 60 min at 37 °C with air as the gas phase. Incubation was stopped with ice-cold perchloric acid to produce an acid concentration of 0.3 M. The protein precipitate was centrifuged down, the supernatant was neutralized, and the concentrations of the metabolities were determined within a few hours.

Analytical. Oxygen consumption was measured with the conventional Warburg apparatus [24] in nonbicarbonate medium with air as the gas phase. Xylitol was determined according to Bässler *et al.*,[25] except that L-iditol dehydrogenase was used instead of xylitol dehydrogenase. The rate of xylitol elimination was determined from the difference in the xylitol concentration before and after the incubation. Glucose was determined as described by Werner *et al.*[26] Lactate, pyruvate, β-hydroxybutyrate, acetoacetate, ATP, ADP and AMP were determined by previously described enzymatic methods.[27] Inorganic phosphate was determined by the method of Fiske and Subba-Row as modified by Bartlett.[28] Glucose and lactate formation during the incubation were calculated by subtracting the concentrations at the beginning of the incubation from the final concentrations of glucose and lactate. The rates of ketone body formation were calculated from the final concentrations after the incubation. Ethanol and acetaldehyde were determined gas chromatographically (Perkin-Elmer F 40) by head-space techniques. The temperature of the water bath was 65 °C.

Biochemicals. Collagenase for the isolation of liver cells was purchased from Worthington Biochemical Corp. (Freehold, New Jersey, U.S.A.). Rotenone, antimycin A, amino-oxyacetate, and bovine plasma albumin were from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). Lactate dehydrogenase was from Miles-Seravac LTD (Maidenhead, Berkshire, England). L-Iditol dehydrogenase, β-hydroxybutyrate dehydrogenase, and the test kits for ATP, ADP/AMP, and glucose from Boehringer GmbH (Mannheim, W. Germany).

Statistics. The different groups were compared with a control group by the paired *t*-test so that samples from the same liver are compared.

RESULTS

Xylitol elimination in isolated rat liver cells. Xylitol is oxidized in the liver cell cytoplasm by NAD-dependent dehydrogenase (Xylitol:NAD oxidoreductase, E.C. 1.1.1.9) to D-xylulose, phosphorylated to xylulose 5-phosphate and then converted to either glucose, lactate, pyruvate, or α-glycerophosphate.[10]

In contrast to the results obtained during oxidation of ethanol, glycerol, or sorbitol,[3–4] the rate of xylitol elimination appeared to be

independent of the bicarbonate content of the incubation buffer (Table 1). However, both rotenone and amino-oxyacetate strongly inhibited xylitol elimination, indicating that in bicarbonate medium NADH from xylitol is transported from cytoplasm mainly *via* the malate-aspartate shuttle. Also in nonbicarbonate buffer amino-oxyacetate inhibited xylitol elimination which probably indicates that there is some activity of the malate–aspartate shuttle left in the nonbicarbonate medium.

The difference in the inhibitory capacity of rotenone and antimycin A has been used to distinguish the roles of the malate–aspartate and the α-glycerophosphate shuttles.[4,12] The results in Table 1 show that in bicarbonate medium rotenone and antimycin A were equally efficient in depressing the elimination of xylitol. This result indicates that other factors than merely the accumulation of α-glycerophosphate regulate the functioning of the α-glycerophosphate shuttle. However, when rotenone or antimycin A together with amino-oxyacetate was used in nonbicarbonate medium or in bicarbonate medium, antimycin A was found to be 40 and 20 %, respectively, more efficient in its inhibitory capacity that rotenone. This confirms previous suggestions [9,11,13] that if the malate-aspartate shuttle is inhibited the α-glycerophosphate shuttle transfers NADH.

In nonbicarbonate medium where the capacity of the malate-aspartate shuttle is lowered,[3,4] rotenone did not inhibit xylitol elimination (Table 1). To test whether rotenone was ever inhibitory in nonbicarbonate medium the rate of ethanol elimination was measured. It was found that under similar conditions rotenone inhibited ethanol elimination by 40 % without amino-oxyacetate and only 10 % when amino-oxyacetate was present. When rotenone was used, acetaldehyde accumulation in the medium was increased from 28 nmol/(g liver cells/min) to 106 nmol/(g liver cells/min). It would seem, therefore, that the activity of the α-glycerophosphate shuttle is influenced not only by inhibition of the malate-aspartate shuttle, but by inhibition of NADH oxidation in the respiratory chain, for example with rotenone, as well.

Glucose and lactate formation. Both lactate and glucose accumulated in the incubation medium when the cells were incubated without exogenous substrates (Table 2). Addition of xylitol to the incubation medium inhibited lactate accumulation. Since the lactate-to-pyruvate ratio increases during oxidation of xylitol [10] (Table 3), the inhibition is probably due to the inhibition of glycolysis at the level of glyceraldehydephosphate dehydrogenase, as in the oxidation of ethanol [29] (Table 2). Thus in accordance with previous findings in liver perfusion,[10] glucose was the main end product of xylitol oxidation (Table 2). Effects of xylitol on lactate and glucose formation were equal in both buffers tested.

Table 1. The rate of xylitol elimination in isolated rat liver cells. Rat liver cells (80–100 mg of wet weight) were incubated in either Krebs–Henseleit bicarbonate buffer or in nonbicarbonate buffer for 60 min at 37 °C. The concentrations at the beginning of the incubation were as follows: xylitol 10 mM, amino-oxyacetate (AOA) 0.5 mM, rotenone (R) 20 μM and antimycin A (A) 20 μM. The results are means ± S.D. of four experiments.

Inhibitor	Bicarbonate buffer		Nonbicarbonate buffer	
	Xylitol elimination μmol g^{-1} min^{-1}	% of control	Xylitol elimination μmol g^{-1} min^{-1}	% of control
– (Control)	1.19 ± 0.19	100	1.24 ± 0.44	100
AOA	0.36 ± 0.07 [c]	30	0.65 ± 0.23 [a]	52
R	0.62 ± 0.22 [a]	52	1.13 ± 0.35	93
A	0.53 ± 0.13 [c]	45	0.73 ± 0.38 [b]	59
AOA + R	0.73 ± 0.20 [b]	61	1.11 ± 0.38	90
AOA + A	0.49 ± 0.17 [c]	41	0.74 ± 0.32 [a]	60

[a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ for statistical difference from the control.

Table 2. Glucose formation and change in the lactate concentration during incubation. Liver cells (80–100 mg of wet weight) were incubated in Krebs-Henseleit bicarbonate buffer for 60 min at 37 °C. Concentrations at the beginning of the incubation were: ethanol 10 mM, xylitol 10 mM, and amino-oxyacetate (AOA) 0.5 mM. The results are means ± S.D. of four experiments.

Substrate	AOA	Glucose formation μmol g^{-1} min^{-1}	% of control	△ Lactate concentration μmol g^{-1} 60 min^{-1}	% of control
– (Control)	–	0.42 ± 0.22	100	16.6 ± 3.8	100
–	+	0.42 ± 0.16	100	19.2 ± 8.0	116
Ethanol	–	0.55 ± 0.28	131	3.1 ± 2.6 [a]	19
Ethanol	+	0.49 ± 0.23	117	2.3 ± 1.8 [a]	14
Xylitol	–	0.84 ± 0.28 [a]	212	2.4 ± 2.4 [a]	14
Xylitol	+	0.37 ± 0.22	88	0.5 ± 0.9 [a]	3

[a] $p < 0.001$ for statistical difference from the control.

Table 3. Effect of xylitol on oxygen consumption, adenine nucleotides, and lactate/pyruvate. Liver cells were incubated in Krebs-Henseleit bicarbonate buffer for 60 min at 37 °C. Concentrations at the beginning of the incubation were: xylitol 10 mM, amino-oxyacetate (AOA) 0.5 mM, rotenone (R) 20 μM, and antimycin A (A) 20 μM. Oxygen consumption was measured in nonbicarbonate medium, where the effects of xylitol were similar to those in bicarbonate medium. The results are means ± S.D. of four experiments.

Substrate	–	–	Xylitol	Xylitol	Xylitol	Xylitol
Inhibitor	(Control) –	AOA	–	AOA	R	A
O_2 (μg-atom O_2 g^{-1} min^{-1})	2.86 ± 1.22	2.88 ± 0.72	2.90 ± 0.54	2.64 ± 0.62	1.84 ± 0.82 [b]	1.16 ± 0.44 [c]
ATP (μmol g^{-1})	3.06 ± 0.68	3.42 ± 0.70	1.38 ± 0.33 [c]	1.19 ± 0.27 [c]	1.12 ± 0.24 [c]	0.51 ± 0.12 [c]
ADP (μmol g^{-1})	0.60 ± 0.16	0.66 ± 0.14	0.59 ± 0.13	0.63 ± 0.15	0.67 ± 0.16	0.54 ± 0.14
AMP (μmol g^{-1})	0.89 ± 0.61	0.92 ± 0.56	1.01 ± 0.56 [b]	0.95 ± 0.59	1.07 ± 0.60 [c]	1.38 ± 0.67 [c]
P_i (μmol g^{-1})	8.6 ± 1.3	7.5 ± 0.8 [a]	3.3 ± 0.9 [c]	2.4 ± 0.4 [c]	6.2 ± 3.0	6.9 ± 0.8 [b]
ATP/ADPxHPO_4^{-2} (M^{-1})	1060 ± 380	1180 ± 160	1210 ± 120	1320 ± 210	600 ± 370 [a]	240 ± 60 [b]
L/P	9.1 ± 1.8	37 ± 7 [c]	62 ± 24 [b]	38 ± 18 [a]	280 ± 140 [b]	370 ± 130 [b]

[a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ for statistical difference from the control.

Amino-oxyacetate decreased the amount of glucose formation during xylitol oxidation to the level of controls. Since amino-oxyacetate without exogenous substrates or in the presence of ethanol did not affect glucose formation, *i.e.* amino-oxyacetate seems not to affect the glucose formation itself, the malate-aspartate shuttle must have a central role in the transport of NADH formed during the elimination of xylitol.

Phosphorylation state during oxidation of xylitol. Oxidation of xylitol depends on ATP which is needed to phosphorylate the D-xylulose formed from xylitol.[10] If xylitol inhibits glycolysis, as suggested above, and thus formation of ATP in the cytoplasm, the ATP necessary for xylitol oxidation must be generated through oxidative phosphorylation in the mitochondria. Xylitol decreased the ATP and P_i contents of the cells, but did not alter the phosphorylation state which is defined as the ratio of ATP/ADP × HPO_4^{2-} (Table 3). The effect of xylitol on adenine nucleotides in isolated liver cells is thus similar to that found in human liver *in vivo*.[15] Oxygen consumption (Table 3) also remained unaltered (oxygen

consumption was measured in nonbicarbonate medium, where the effects of xylitol on the phosphorylation state and adenine nucleotides were similar to the effects in bicarbonate buffer). This result is in accordance with the results obtained during glycerol elimination.[30] Erecińska *et al.*[30] found that although glycerol decreased the ATP/ADP ratio and the P_i content in isolated liver cells it had no effect on the phosphorylation state or on the rate of oxygen consumption.

Lactate/pyruvate ratio during oxidation of xylitol. Xylitol increased the lactate-to-pyruvate ratio significantly (Table 3) as found previously in perfused livers.[10] Inhibition of the NADH reoxidation with rotenone caused a marked increase in the lactate-to-pyruvate ratio in the cytoplasm. Inhibition of the malate-aspartate shuttle with amino-oxyacetate decreased the effect of rotenone and antimycin A significantly ($p < 0.05$ and $p < 0.01$, respectively) on the cytoplasmic lactate-to-pyruvate ratio. The result is similar to results obtained with ethanol under similar conditions.[4]

In nonbicarbonate medium where xylitol elimination was not inhibited after rotenone administration, increase in the lactate/pyruvate ratio was greater than it could be as the effect of xylitol alone (Table 3). This suggests that the lactate/pyruvate ratio is not the only regulative factor in the removal of xylitol.

Ketone body formation. In the absence of exogenous substrates liver oxidizes mainly fatty acids.[31] Depletion of malate and oxaloacetate during liver cell isolation [17,18] leads to inhibition of the citric acid cycle [32,33] and thus a higher rate of ketogenesis is found in isolated liver cells than in perfused livers.[3] In the present study ketogenesis was further increased when the liver cells isolated from fed rats were incubated in nonbicarbonate medium (Table 4). The lack of bicarbonate ion has a similar effect on ketone body formation as fasting.[34] Thus the cells isolated from fed rats but incubated in nonbicarbonate medium are more like the cells from fasted rats.

In nonbicarbonate medium neither ethanol nor xylitol had any effect on the rate of ketogenesis (Table 4) and also in bicarbonate medium xylitol had no antiketogenic effect. Therefore it can be assumed that in isolated liver cells xylitol oxidation produces only a part of the NADH which is needed for the respiratory chain. Ethanol strongly depressed the ketone body formation in bicarbonate medium. The difference in the antiketogenic effects of ethanol and xylitol may be due to the difference in the NADH supply in the mitochondria since amino-oxyacetate increased ketone body formation during both ethanol and xylitol oxidation. Amino-oxyacetate alone had no effect on ketogenesis in either buffer

Table 4. Ketone body formation. Liver cells were incubated in either bicarbonate or nonbicarbonate medium for 60 min at 37 °C. Concentrations at the beginning of the incubation were: xylitol 10 mM, ethanol 10 mM, amino-oxyacetate (AOA) 0.5 mM, rotenone (R) 20 μM. The results are means ± S.D. of four experiments.

Substrate	Inhibitor	Bicarbonate medium		Nonbicarbonate medium	
		Ketone body formation nmol g^{-1} min^{-1}	% of control	Ketone body formation nmol g^{-1} min^{-1}	% of control
–	–	63 ± 25	100	154 ± 84	100
–	AOA	58 ± 16	92	146 ± 75	95
Xylitol	–	62 ± 23	98	127 ± 59	83
Xylitol	AOA	76 ± 20	121	118 ± 42	77
Xylitol	R	31 ± 14 [a]	49	66 ± 45 [a]	43
Xylitol	AOA + R	59 ± 15	94	82 ± 59	53
Ethanol	–	25 ± 9 [b]	40	161 ± 36	105
Ethanol	AOA	67 ± 27	106	173 ± 51	112
Ethanol	R	13 ± 4 [c]	21	93 ± 21 [a]	60
Ethanol	AOA + R	44 ± 4	70	82 ± 30 [a]	53

[a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ for statistical difference from the control.

tested. Inhibition of NADH reoxidation also inhibited ketone body formation, indicating that both NADH and $FADH_2$ are important regulators of fatty acid oxidation, as demonstrated previously by Lumeng *et al.*[33]

DISCUSSION

The effects of xylitol metabolism on adenine nucleotides, the phosphorylation state, and the rate of oxygen consumption were found to resemble those observed during glycerol elimination.[30] In both cases ATP concentration, and ATP/ADP ratio are lowered but no change is observed in the phosphorylation state or the rate of oxygen consumption. The results of the present study thus support the view [30,35] that not only the energy consumption, but also the phosphorylation state is an important regulator of the respiratory chain.

Accumulation of α-glycerophosphate during oxidation of xylitol,[10,14,15] glycerol,[36,37] sorbitol,[36] and to a minor extent also ethanol,[9,38] has led to the suggestion that when the content of α-glycerophosphate is increased NADH can be transfered to mitochondria for reoxidation *via* either the α-glycerophosphate or the malate–aspartate shuttle.[1] In earlier studies with glycerol,[3] sorbitol,[3] and ethanol,[3,4,8,9,12,13,19] the malate-aspartate shuttle has been shown to be the most active NADH translocation shuttle in rat liver, and in the present study (Table 1) it was found that also during xylitol elimination the malate-aspartate shuttle plays the major role. Moreover, the respiratory chain inhibitors, rotenone which inhibits only NADH-mediated shuttles, and antimycin A which inhibits both NADH- and flavoprotein-mediated shuttles, were observed to be equally efficient in inhibiting the elimination of xylitol. Thus it seems that the accumulation of α-glycerophosphate during the elimination of xylitol [10,14,15] merely indicates the low capacity of the α-glycerophosphate shuttle to transfer NADH and, also, that when α-glycerophosphate is accumulated the transfer of NADH *via* the α-glycerophosphate shuttle is regulated mainly by other factors, *e.g.* the activity of glycerophosphate oxidase, as earlier suggested by both *in vivo* [13] and *in vitro* [39] studies.

The metabolic situation is quite different if the cells are incubated in nonbicarbonate medium. Metabolites of the malate–aspartate shuttle are already depleted [17,18] during isolation of the liver cells. If the cells are incubated in bicarbonate-containing medium, these metabolites are synthesized at the beginning of the incubation; [3,40] but no synthesis takes place in nonbicarbonate medium where the flux through the pyruvate carboxylase reaction is decreased,[3,6] since CO_2 production from the metabolism seems not to produce a high enough bicarbonate level in the cell. Decrease in the concentrations of both malate and oxaloacetate is also seen as an inhibition of the citric acid cycle [32,33] and leads to increased formation of ketone bodies [33] (Table 4).

Rotenone inhibited xylitol elimination in bicarbonate buffer, but not in nonbicarbonate medium, suggesting differences in the activity of the α-glycerophosphate shuttle. Two basic differences exist in the experimental conditions: one is the activity of the malate–aspartate shuttle and the other is the production of mitochondrial NADH by β-oxidation and the citric acid cycle. Direct inhibition of the malate-aspartate shuttle by amino-oxyacetate inhibited xylitol elimination in nonbicarbonate medium. It is, therefore, concluded that other factors besides the inhibition of the malate–aspartate shuttle affect the activity of the α-glycerophosphate shuttle. Furthermore, in bicarbonate medium a combination of rotenone and amino-oxyacetate produced a smaller inhibition than either rotenone or amino-oxyacetate alone, suggesting that the inhibition of NADH oxidation with NADH dehydrogenase in the respiratory chain participates in the regulation of the activity of the α-glycerophosphate shuttle. Inhibition of the malate–aspartate shuttle and the intramitochondrial formation of NADH both decrease the flux of hydrogen *via* NADH dehydrogenase in the respiratory chain, which might in turn increase the flux *via* other flavoproteins like glycerophosphate oxidase, and thus activate the α-glycerophosphate shuttle. Since inhibition of both NADH dehydrogenase and malate–aspartate shuttle affects the activity of the α-glycerophosphate shuttle it must be emphasized that the exact role of the α-glycerophosphate shuttle in the translocation of NADH can be determined only with direct measurements but not by using

inhibitors of the malate-aspartate shuttle or the respiratory chain.

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