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Interaction of Cytoplasmic Dehydrogenases: Quantitation of Pathways of Ethanol Metabolism

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VIND, C. AND N. GRUNNET. *Interaction of cytoplasmic dehydrogenases: Quantitation of pathways of ethanol metabolism.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 209–213, 1983.—The interaction between xylitol, alcohol and lactate dehydrogenase has been studied in hepatocytes from rats by applying specifically tritiated substrates. A simple model, describing the metabolic fate of tritium from [2-^{3}H] xylitol and (1R) [1-^{3}H]ethanol is presented. The model allows calculation of the specific radioactivity of free, cytosolic NADH, based on transfer of tritium to lactate, glucose and water. From the initial labelling rate of lactate and the specific radioactivity of cytosolic NADH, we have determined the reversible flow through the lactate dehydrogenase catalyzed reaction to 1–5 μmol/min·g wet wt. The results suggest that xylitol, alcohol and lactate dehydrogenase share the same pool of NAD(H) in the cytoplasma. This finding allows estimation of the ethanol oxidation rate by the non-alcohol dehydrogenase pathways from the relative yield of tritium in water and glucose. The calculations are based on a comparison of the fate of the 1-pro-R hydrogen of ethanol and the hydrogen bound to carbon 2 of xylitol or carbon 2 of lactate under identical conditions.

Xylitol metabolism Ethanol metabolism Cytosolic dehydrogenases Compartmentation
Non-ADH pathways MEOS

IT is generally accepted that the major pathway of ethanol oxidation occurs via the NAD^+-linked alcohol dehydrogenase with transfer of the pro-1-R hydrogen of ethanol to cytoplasmic NAD^+ [3]. It is also reported that a significant part of the oxidation of ethanol occurs via an alcohol dehydrogenase (ADH) independent pathway, which might be either a microsomal ethanol oxidizing system (MEOS) or catalase [9]. Xylitol is oxidized in hepatocytes exclusively by a cytosolic NAD^+-linked dehydrogenase (Xylitol: NAD^+ 2-oxidoreductase (D-xylulose-forming), E.C.1.1.1.9) [7]. The product, D-xylulose, is converted via the pentose phosphate cycle to fructose-6-phosphate and triosephosphate. It is assumed that lactate oxidation not involving the cytosolic lactate dehydrogenase is negligible. Alcohol, xylitol and lactate dehydrogenase have the same (A) specificity for the coenzyme NAD^+.

In the present project we have estimated the contribution to ethanol oxidation by non-alcohol dehydrogenase pathways by comparing the fate of the 1-pro-R hydrogen of ethanol and the hydrogen bound to carbon-2 of xylitol or to carbon-2 of lactate under identical conditions. A presumption for the approach is that alcohol dehydrogenase, xylitol dehydrogenase and lactate dehydrogenase use the same pool of NAD(H). The interaction between reducing equivalents by these cytosolic dehydrogenases was studied by estimation of the flux of reducing equivalents from NADH to pyruvate, when labelled ethanol or labelled xylitol was metabolized. We conclude that the three dehydrogenases share a single cytosolic NAD(H) pool.

By comparing the relative yield of tritium in water and glucose when xylitol and ethanol are metabolized together (either with xylitol or ethanol as the labelled substrate), we can calculate the contribution to ethanol oxidation by non-alcohol dehydrogenase pathways.

This paper describes our methods and our calculations. Preliminary results indicate that about 30% of the oxidation of ethanol occurs via non-ADH pathways, when xylitol and ethanol are metabolized together and somewhat less, when xylitol is omitted and labelling from lactate and ethanol are compared.

METHOD

Hepatocytes from female Wistar rats weighing 150–200 g were prepared by collagenase perfusion. Cells (0.1 ml) were incuabed at 37°C in Krebs-Henseleit bicarbonate buffer containing 1% serum albumin and 7.5 mM glucose in a total volume of 2.25 ml. The gas phase was 95% O_2/5% CO_2. The cells were incubated with 2.5 mM lactate and 0.25 mM pyruvate for 5 or 10 min prior to the ethanol and xylitol addition (zero time). After incubation with 6 mM[2-^{3}H]xylitol or 12 mM (1R) [1-^{3}H]ethanol with or without unlabelled 12 mM ethanol or 6 mM xylitol, respectively, the reaction was stopped by addition of 0.7 M perchloric acid [14].

The neutralized $HClO_4$ supernatant was used for isolation of water, xylitol, glucose and lactate. Radioactivity in water was obtained as the difference between total radioactivity and the radioactivity in dry matter plus ethanol. The

radioactivity in ethanol was obtained as the radioactivity in lactate (dry matter) after separation of ethanol and water from dry matter by freeze-drying in a Thurnberg tube and incubating the fraction of ethanol and water with pyruvate, NAD^+, lactate dehydrogenase and alcohol dehydrogenase in Tris-HCl buffer, pH=7.5.

Glucose and xylitol were isolated as the neutral fraction from ion exchange chromatography. Lactate was eluted with 4 N acetic acid. Glucose and xylitol were separated by phosphorylation of glucose and subsequent separation by ion exchange chromatography.

[2-^{3}H]Xylitol and (1R) [1-^{3}H]ethanol were prepared from [4A-^{3}H]-NADH and D-xylulose plus sorbitol dehydrogenase or acetaldehyde plus alcohol dehydrogenase, respectively [14].

The hyperthyroid state was induced by 3 intraperitoneal injections of 50 μg L-3,3',5'triiodothyronine/100 g body weight every second day.

RESULTS

Metabolic Changes

Rates of xylitol and ethanol uptake by cells were linear with time in the 60 min incubation. The rate of xylitol oxidation (1.6±0.2, (n=4) μmol/min g wet wt.) as well as the rate of ethanol oxidation (2.6±0.4, (n=4) μmol/min g wet wt.) decreased 30% when ethanol and xylitol were metabolized together in cells from fed rats, but in cells from T_3-treated rats, we observed no mutual inhibition [14]. The results are similar to those reported by others [6,16]. Lactate was taken up by cells from all groups except in those from fed rats metabolizing xylitol in which lactate accumulates.

Interaction Between Xylitol Dehydrogenase, Alcohol Dehydrogenase and Lactate Dehydrogenase

We estimate the contribution to ethanol oxidation by non-alcohol dehydrogenase pathways by comparing the fate of the R hydrogen of ethanol with the fate of the hydrogen bound to carbon-2 of xylitol under identical conditions. A presumption for this comparison is that alcohol dehydrogenase and xylitol dehydrogenase use the same pool of NAD(H). Our approach to elucidate the interaction between reducing equivalents produced by these cytosolic dehydrogenases is based on the measurement of transfer of tritium from [2-^{3}H]xylitol or (1R) [1-^{3}H]ethanol to water, lactate and glucose.

From these results and the model of Fig. 1, we calculate the lactate dehydrogenase catalyzed oxidation rate of NADH labelled with tritium either from oxidation of [2-^{3}H]xylitol or (1R) [1-^{3}H]ethanol. If comparable calculated rates are obtained, it will indicate a single pool of NAD^+ for the two dehydrogenases. The lactate dehydrogenase catalyzed oxidation rate of NADH was calculated from the initial labelling rate of lactate and the steady-state specific radioactivity of cytoplasmic NADH [14]. These parameters were determined as described below.

Specific radioactivity of NADH. To calculate the specific radioactivity of NADH, we consider the model depicted in Fig. 1, assume isotopic steady state, and set input of tritium into NADH equal to output of tritium from NADH. Thus, with xylitol as the labelled substrate (subscript X) and the specific activity set to one:

$$a + L(x + d) = N_X (a + (b-c) + (h-d) + (x + d)) \quad (1)$$

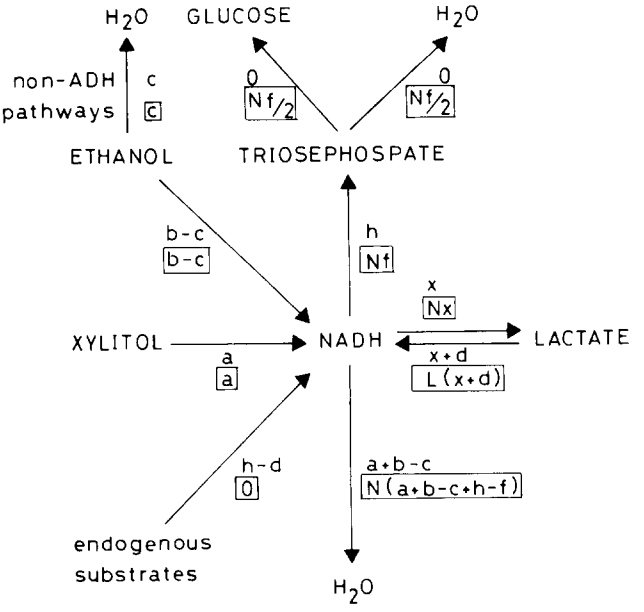

FIG. 1. Model for flux of reducing equivalents and tritium from [2-^{3}H]xylitol or (1R) [1-^{3}H]ethanol. Small letters, a, b−c, x+d and h−d represent the formation of NADH from xylitol, ethanol, lactate and endogenous substrates, respectively; a+b−c represents consumption of NADH in the microsomal and mitochondrial electron transport chain and h in the triosephosphate dehydrogenase reaction, respectively; c represents the amount of ethanol oxidized by non-alcohol dehydrogenase pathways. Framed letters represent the flux of tritium. N and L are the specific radioactivity of NADH and lactate, respectively. For explanation of f see Results section.

L represents the average specific radioactivity of lactate. N_X represents the average specific radioactivity of NADH.

The yield of tritium in lactate equals input minus output (still with xylitol as the labelled substrate):

$$aM_X = N_X x - L(x + d) \quad (2)$$

M_X represents the relative yield of tritium in lactate after the oxidation of [2-^{3}H]xylitol.

From equations (1) and (2) we eliminate x and L and obtain the equation:

$$N_X = \frac{a\,(1-M_X)}{a + (b-c) + h}$$

With ethanol as the labelled species, b−c substitutes for a in left side of equation (1) and b for a in equation (2), and the specific radioactivity of NADH is

$$N_E = \frac{b\,(1-M_E)-c}{a + (b-c) + h}$$

In the equations for N_X and N_E only h, the flux of reducing equivalents through the triosephosphate dehydrogenase reaction is unknown.

In cells from starved rats, h can be calculated from measurements of glucose formation, and xylitol and lactate uptake [13]. The value of h must be at least large enough to

account for the reducing equivalents from the carbon necessary for the formation of glucose from other substrates than xylitol. f is an operational value of the flux of tritium labelled reducing equivalents to glyceraldehyde-3-phosphate and is different from h because the specific radioactivity of the 4B-H of NADH is less than that of the 4A-H [11] (triosephosphate dehydrogenase is a B-specific dehydrogenase, whereas alcohol and xylitol dehydrogenase are A-specific enzymes [1]) and because more than half of the tritium may be lost to water in the aldolase reaction due to cycling between fructose 1,6-diphosphate and the triosephosphates. In cells from starved rats, we determined a value of f~0.5 h [13]. This value has been applied to the present experiments with cells from fed rats, thus assuming the same degree of equilibration between the 4A and 4B hydrogen of NADH and the same rate of cycling between fructose 1,6-diphosphate and the triosephosphates as in cells from starved rats. It should be pointed out, however, that the estimation of ethanol metabolism via non-ADH pathways (see below) is independent of the value of h (cf. equation (I), below).

Lactate dehydrogenase catalyzed oxidation rate of NADH. The labelling pattern of lactate (Fig. 2) follows the exponential equation $L=K(1-e^{-t/\tau})$. L represents the specific radioactivity of lactate and K is the steady state value of the specific radioactivity of lactate and K/τ the initial labelling rate of lactate. We have determined the values τ and K from a straight-line plot of the equation, which when differentiated with respect to t gives

$$\frac{dL}{dt} = \frac{K}{\tau} \times e^{-t/\tau};$$

taking the logarithm of both sides, the equation for a straight-line is obtained:

$$\ln \frac{\Delta L}{\Delta t} = -\frac{1}{\tau} t + \ln K/\tau$$

K and τ were obtained [14] from the regression line and used to calculate the flux through lactate dehydrogenase.

The lactate dehydrogenase catalyzed oxidation rate of NADH (x μmol NADH oxidized by LDH/min·g wet wt., Table 1) can be calculated by using the labelling rate K/τ (μmol ^{3}H/μmol lactate·min) multiplied by the lactate content (μmol lactate) and divided by the steady-state specific radioactivity of cytoplasmic NADH (N) in μmol ^{3}H/μmol NADH.

The specific radioactivity of NADH (N) in Table 1 has been calculated as the mean of the N values for all four time intervals used in the incubations, as no significant change in N with time could be detected.

Contribution to Ethanol Oxidation by Non-Alcohol Dehydrogenase Pathways

As alcohol dehydrogenase and xylitol dehydrogenase use the same pool of cytoplasmic NAD^+, we can estimate the extent of non-ADH pathways. This approach involves a comparison of the relative amount of labelling of glucose (G_E and G_X) and water (W_E and W_X) from (1R)-[1-^{3}H]ethanol and [2-^{3}H]xylitol when labelled ethanol or labelled xylitol were metabolized under identical conditions.

From the specific radioactivity of NADH, we can calculate the relative yield of tritium in glucose (G) and water (W)

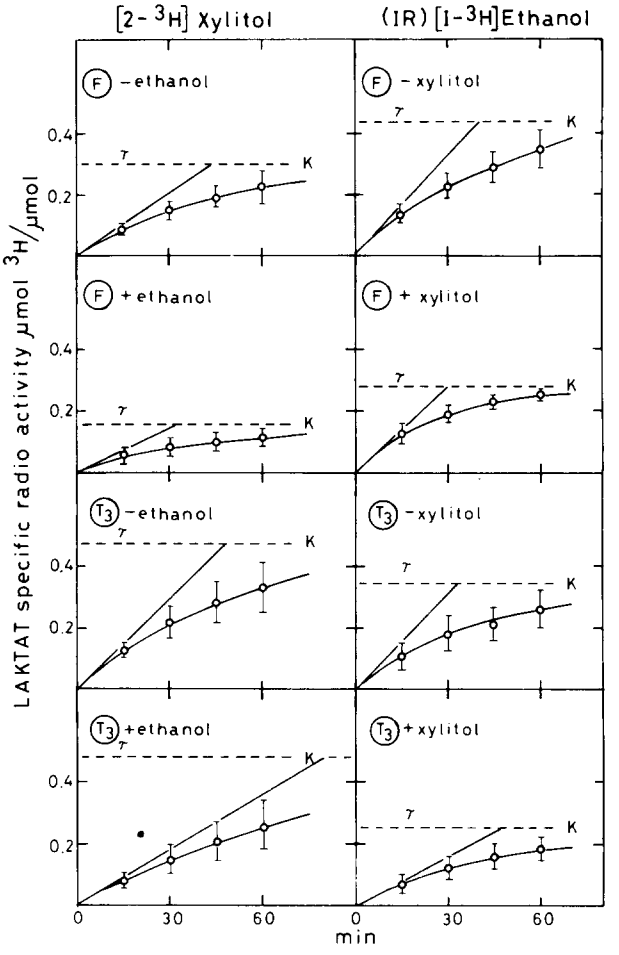

FIG. 2. Specific radioactivity of lactate L (in μmol ^{3}H/μmol) as a function of time. The values of K and τ were calculated from the equation $L=K(1-e^{-t/\tau})$ from a straight line plot of the equation

$$\ln \frac{\Delta L}{\Delta t} = -\frac{1}{\tau} t + \ln K/\tau$$

(see Results section) and used to estimate the initial labelling rate of lactate. Results with labelled xylitol (left column) or labelled ethanol (right column) are shown. The presence or absence of unlabelled ethanol or xylitol is indicated on each curve and the treatment of the rats is indicated in circles, F for fed and T_3 for triiodothyronine. Results are mean values of 4 cell preparations. Bars indicate SD.

(Fig. 1). The relative yield of tritium during the metabolism of [2-^{3}H]xylitol is

$$\text{in glucose } G_X = \frac{N_X f}{2a} \text{ and} \quad (3)$$

$$\text{in water } W_X = \frac{N_X (a + (b-c) + h-f + {}^1/_2 f)}{a} \quad (4)$$

The relative yield of tritium during the metabolism of (1R)[1-^{3}H]-ethanol is

$$\text{in glucose } G_E = \frac{N_E f}{2b} \text{ and} \quad (5)$$

TABLE 1

CALCULATED SPECIFIC RADIOACTIVITY OF NADH (N) AND THE LACTATE DEHYDROGENASE FLUX (x)

Treatment	^{3}H-Substrate	Unlabelled Substrate	N μmol ^{3}H/μmol	μmol/min g wet wt.
Fed	[2-^{3}H]xylitol	−ethanol	0.388 ± 0.066 (5)	1.8 ± 0.1 (5)
		+ethanol	0.257 ± 0.047 (5)	2.0 ± 1.1 (5)
	(1 R) [1-^{3}H]ethanol	−xylitol	0.450 ± 0.112 (5)	3.0 ± 1.9 (5)
		+xylitol	0.193 ± 0.072 (5)	5.5 ± 2.0 (5)
T_3-treated	[2-^{3}H]xylitol	−ethanol	0.393 ± 0.113 (5)	1.9 ± 0.7 (4)
		+ethanol	0.170 ± 0.047 (5)	2.4 ± 0.5 (4)
	(1 R) [1-^{3}H]ethanol	−xylitol	0.472 ± 0.130 (5)	1.3 ± 0.5 (4)
		+xylitol	0.190 ± 0.072 (5)	2.3 ± 0.4 (4)

Values are mean ± S.D. with the number of cell preparations in parentheses.

$$\text{in water } W_E = \frac{N_E\,(a+(b-c)+h-f+{}^1/_2\,f)}{b} + \frac{c}{b} \quad (6)$$

where the last term, $\frac{c}{b}$, represents the part of the total ethanol oxidation (b) which is oxidized in non-ADH pathways with the transport of tritium directly to oxygen without use of NAD^+.

From equations (3), (4), (5) and (6) we eliminate N_X and N_E

$$\frac{N_X}{N_E} = \frac{a \times G_X}{b \times G_E} = \frac{a \times W_X}{b \times W_E - c} \quad \text{or}$$

$$\frac{\text{ethanol oxidation via non-ADH-pathways}}{\text{total ethanol oxidation}} =$$

$$\frac{c}{b} = W_E - \frac{G_E}{G_X} \times W_X \quad (I)$$

If the calculation of the contribution to ethanol oxidation by non-alcohol-dehydrogenase pathways is done by comparing the labelling of glucose and water from (1R)[1-^{3}H]ethanol and L-[2-^{3}H]lactate, we can also use equation (I),

$$\frac{c}{b} = W_E - \frac{G_E}{G_L} W_L ,$$

because lactate and xylitol both donate reducing equivalents exclusively to cytosolic NAD^+.

DISCUSSION

As can be seen from Fig. 2 the labelling pattern of lactate differs widely with the labelled substrate, treatment of the animal and the substrates metabolized. But even so, the flux of reducing equivalents from NADH to pyruvate (Table 1) is in the same order of magnitude when it is calculated by means of the initial labelling rate, the specific radioactivity of NADH and the lactate content and whether calculated from results with labelled xylitol or labelled ethanol. These observations strongly indicate that the two enzymes, xylitol dehydrogenase and alcohol dehydrogenase, equilibrate with the same pool of cytoplasmic NAD(H) [14]. The rate of pyruvate reduction of about 2 μmol/min · g wet wt. (Table 1) is identical to the rate obtained in pulse-labelling experiments with the perfused rat liver [12] and with the rate calculated from the kinetic parameters of lactate dehydrogenase and cytosolic concentrations of pyruvate, lactate, NAD^+ and NADH [12], which strongly support the concept of a single pool of NAD(H) for xylitol, alcohol and lactate dehydrogenase. These results thus justify the estimation of the extent of the non-ADH pathways by comparison of the relative yield of tritium in glucose and water from [2-^{3}H]xylitol or L-[2-^{3}H]lactate and (1R)[1-^{3}H]ethanol. Our approach requires parallel incubations with the same batch of liver cells with everything identical except for the radioactive labelling. In the calculations, possible isotope effects should be taken into account. We find no significant isotope effect on the metabolism of [2-^{3}H]xylitol by isolated hepatocytes, and it has been found that the isotope effect on ethanol oxidation in liver cells is close to 1 [4]. It therefore appears justifiable to neglect isotope effects in the calculations.

Rognstad [10] and Havre *et al.* [5] also estimated the contribution to ethanol oxidation by non-ADH pathways by comparing the fate of the 1R-hydrogen of ethanol with the fate of hydrogen from a substrate oxidized only via a cytosolic NAD^+-dependent dehydrogenase. Rognstad [10] compared the incorporation of tritium from (1R)[1-^{3}H]ethanol and [2-^{3}H]lactate into glucose and water and obtained a value of 33–37% for ethanol metabolism via non-ADH pathways. Rognstad, however, neglects incorporation of tritium from (1R)[1-^{3}H]ethanol into lactate, which in our experiments is about 30%, and therefore underestimates the contribution of non-ADH pathways. (In our notation, Rognstad [10] calculates the non-ADH pathway.

$$\frac{c}{b}, \text{ as } \left(W_E - \frac{G_E}{G_L} W_L\right)/(G_E + W_E) ,$$

cf. our equation (I). The underestimation is therefore $1/(G_E + W_E)$ or about 1.4). Also, the experimental conditions as re-

TABLE 2

CONTRIBUTION OF NON-ADH PATHWAYS TO ETHANOL OXIDATION

Experiment	Labelled substrate	Tritium yields % of used ^3H Water	Lactate	Glucose	% non-ADH pathways
1	^3H-Ethanol	67	26	7	49
	^3H-Xylitol	48	32	19	
2	^3H-Ethanol	54	35	10	36
	^3H-Xylitol	49	23	27	
3	^3H-Ethanol	60	28	11	28
	^3H-Xylitol	46	39	16	
4	^3H-Ethanol	57	39	4	31
	^3H-Xylitol	53	39	8	
5a	^3H-Ethanol	65	31	5	24
	^3H-Xylitol	66	26	8	
5b	^3H-Ethanol	44	52	4	17
	^3H-Lactate	87	—	13	

Hepatocytes from fed rats were incubated with 7.5 mM glucose + 2.5 mM lactate + 12 mM ethanol + 6 mM xylitol 10 min prior to addition of either [2-^3H]-xylitol or (1R) [1-^3H]ethanol except for experiment 5b where xylitol was omitted and L [2-^3H]lactate was used as the labelled substrate. Incubations were carried out for 60 min.

gards substrate concentrations appear different in experiments with tritiated lactate and tritiated ethanol (Table 1 in [10]), which may also lead to incorrect estimates of the contribution of non-ADH pathways.

Havre *et al.* [5] have compared the incorporation of tritium from (1R)[1-^3H]ethanol and [2-^3H]sorbitol into lactate and water in liver slices, and conclude that ethanol oxidation via non-ADH pathways is negligible. They consider the incorporation of tritium into lactate as irreversible. If we calculate the contribution of non-ADH pathways from our results and assume an irreversible incorporation of tritium into lactate we find values between −21% and 28% with a mean value of only 5%. Obviously, the metabolism of ethanol via the non-ADH pathways is seriously underestimated when the reversibility of the lactate dehydrogenase reaction is neglected.

The preliminary results (Table 2) reported on the contribution of the non-ADH pathways is different in the presence of xylitol (30%) and in the absence of xylitol (20%). This might suggest that the simultaneous oxidation of xylitol and ethanol have activated the hydroxylation of ethanol by the microsomal ethanol oxidizing system (MEOS) by creating a massive supply of NADH to cytochrome b_5 in the microsomal electron transport system. Cytochrome b_5 reductase will preferentially use NADH instead of NADPH [2] and the reduction is dependent on the ratio NADPH/NADH [15]. The apparent increase in MEOS activity caused by xylitol might also be due to a loss of adenine nucleotides [16] thereby deinhibiting NADPH cytochrome c reductase and one or more NADPH-generating enzymes [8].

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