Metabolism of 1-³H-Ethanol by Isolated Liver Cells. Time-course of the Transfer of Tritium from *R,S*-1-³H-Ethanol to Lactate and β -Hydroxybutyrate

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Parenchymal cells isolated from the liver of 24 h fasted rats were incubated with 65 mM *R,S*-1-³H-ethanol plus 3 mM pyruvate as substrates in the absence and presence of 1.7 mM 4-methylpyrazole. Metabolite levels and the time-course of the transfer of tritium from ethanol to lactate and β -hydroxybutyrate was measured during the first 15 min of ethanol metabolism.

The time-course of the loss of tritium from 2-³H-L-lactate and 3-³H- β -D-hydroxybutyrate in experiments identical to the above-mentioned was estimated.

A GLC method for the isolation of lactate and β -hydroxybutyrate and the preparation of 2-³H-L-lactate and 3-³H- β -D-hydroxybutyrate is described.

The incorporation rate of tritium from ethanol into lactate and β -hydroxybutyrate decreased with time. Addition of 4-methylpyrazole decreased the incorporation rate roughly proportional to the decrease in ethanol and acetaldehyde metabolism. The observed incorporation rates of tritium to lactate were corrected for the detritiation rates measured in experiments with 2-³H-L-lactate and 3-³H- β -D-hydroxybutyrate as substrates.

The rate of extramitochondrial acetaldehyde oxidation was calculated from the corrected initial rates of incorporation of tritium into lactate to 0–0.4 $\mu\text{mol min}^{-1}$ (ml of cells)⁻¹.

Acetaldehyde is the first product of the oxidation of ethanol by liver tissue. Ethanol may be oxidized in the cytosol by alcohol dehydrogenase,^{1*} and when present in high concentra-

tions² also in the peroxisomes by catalase³ or in the endoplasmatic reticulum by MEOS.⁴ The intracellular production of acetaldehyde must therefore be localized to these subcellular structures.

The oxidation of acetaldehyde in the liver apparently proceeds quite efficiently as only a minor fraction of the ethanol metabolized is released from the liver as acetaldehyde and as no accumulation of acetaldehyde occurs during ethanol metabolism⁵. It may be inferred from distribution studies and kinetic properties of aldehyde dehydrogenases in liver tissue^{6–9} that the intracellular localization of acetaldehyde oxidation might be microsomal, cytoplasmic, or mitochondrial.

The intracellular localization of acetaldehyde oxidation was suggested as mainly mitochondrial by Lindroos *et al.*^{10,11}

The evidence for this suggestion was, however, rather indirect and moreover the liver was exposed to unphysiologically high concentration of acetaldehyde. Recently, reports have appeared concerning the intracellular localization of acetaldehyde oxidation during ethanol metabolism at low concentrations (< 10 mM).^{12–14} These reports indicate a mainly mitochondrial oxidation of acetaldehyde.

The present work was undertaken in an attempt to estimate the amount of acetaldehyde oxidized in the extramitochondrial cell compartment at physiological concentrations of acetaldehyde, *i.e.* during ethanol metabolism.

R,S-1-³H-Ethanol was used as a substrate for ethanol oxidation by isolated rat liver

* *Enzymes*: Alcohol dehydrogenase (EC 1.1.1.1); aldehyde dehydrogenase (EC 1.2.1.3); catalase (EC 1.11.1.6); glutamate dehydrogenase (EC 1.4.1.3); β -hydroxybutyrate dehydrogenase (EC 1.1.1.30); lactate dehydrogenase (EC 1.1.1.27).

parenchymal cells. The method applied allows measurement of the time-course of transfer of tritium from ethanol *via* NAD⁺ to lactate and β -hydroxybutyrate: The *S*-tritium atom of *R,S*-1-³H-ethanol is retained at C-1 in acetaldehyde formed by ethanol oxidation, catalyzed by any of the three enzymes mentioned above, whereas the *R*-tritium atom is transferred to NAD⁺ in the reaction catalyzed by alcohol dehydrogenase only. Further oxidation of acetaldehyde to acetate results in the transfer of tritium to the 4-A position of NAD⁺. As alcohol dehydrogenase can be inhibited competitively with ethanol by 4-methylpyrazole,¹⁵ it is possible to avoid the formation of 4-³H-NADH in the reaction catalyzed by this enzyme, the only source of 4-³H-NADH being acetaldehyde formed by oxidation of ethanol, catalyzed by enzymes other than alcohol dehydrogenase. The intracellular localization of 4-³H-NADH formation is then identical to the localization of the acetaldehyde oxidation.

Supposing that the extra- and intramitochondrial 4-³H-NADH is equilibrated isotopically with lactate and with β -hydroxybutyrate *via* the lactate dehydrogenase and the β -hydroxybutyrate dehydrogenase reaction, respectively, information about the intracellular localization of acetaldehyde oxidation should arise from a time-course study of the transfer of tritium from ethanol to lactate and β -hydroxybutyrate. However, such time-course studies can be used quantitatively only if all tritium formed by ethanol and acetaldehyde oxidation is transferred to the two substrates mentioned, and as this is not the case, a correction for the 'tritium loss' should be applied. In the present study the time-course for the tritium loss from added 2-³H-L-lactate and 3-³H- β -D-hydroxybutyrate have been used to correct the labelling pattern obtained.

EXPERIMENTAL

Materials.

[¹⁴C]Lactate was obtained from NEN Chemicals GmbH (Frankfurt/Main, Germany) and [¹⁴C] β -hydroxybutyrate from the Radiochemical Center (Amersham, England). The purity of these compounds was determined by the GLC procedure described in 'Methods'.

R,S-1-³H-Ethanol (specific activity 100 mCi/mmol) was purchased from NEN Chemicals

GmbH (Frankfurt/Main, Germany) or from ICN Pharmaceuticals, Inc. (Cleveland, Ohio, U.S.A.). The content of water-exchangeable tritium was determined by extraction with chloroform.¹⁶ The *R,S*-1-³H-ethanol used in the experiments contained less than 5 % of water-exchangeable radioactivity. One sample obtained from NEN Chemicals contained 80 % of water-exchangeable radioactivity.

Enzymes and coenzymes were from Boehringer Mannheim GmbH (Mannheim, Germany) except that collagenase II from Worthington Biochemical Corporation (Freehold, New Jersey, U.S.A.) was used in some experiments. Hexamethyldisilazane and trichloromethylsilane were from Pierce (Rockford, Illinois, U.S.A.) and DEAE-Sephadex A 25 from Pharmacia Fine Chemicals (Uppsala, Sweden). Serum albumin from Armour Chemical Co. (England) was treated with charcoal to remove fatty acids.¹⁷ 4-Methylpyrazole was synthesized and kindly provided by Dr. S. E. Hansen (this laboratory). The purity of this compound was checked by GLC and IR spectrometry. Other chemicals used were of analytical grade.

Methods.

Isolated liver parenchymal cells were prepared from 24 h fasted female Wistar rats of 200 g weight as previously described,¹⁸ except for the omission of hyaluronidase in the perfusion fluid.

Cells were incubated at 37 °C in 15 ml Erlenmeyer flasks with atmospheric air as the gas phase. CO₂ was absorbed in KOH placed in a center well. The incubation buffer was Hanks' buffer¹⁹ containing 25 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES), 10 mM phosphate, 8 mM glucose, 1.2 mM CaCl₂ and 1 % serum albumin. The cell concentration was 80–120 μ l of tightly packed cells (corresponding to 130–200 mg of liver tissue² in a total volume of about 2.3 ml. The cells were incubated 5 min with 3 mM pyruvate and 1.7 mM 4-methylpyrazole (when added) prior to the ethanol addition. The experiments were terminated at the times indicated by perchloric acid precipitation of a sample (2 ml) of the incubation mixture. The final concentration of perchloric acid was 0.7 M.

Results are expressed per ml of cells. The volume of the cells was measured after centrifugation to constant volume.

Metabolite levels. Lactate,²⁰ pyruvate,²¹ β -hydroxybutyrate,²² and acetoacetate²³ were measured by the enzymatic procedures indicated. Acetaldehyde was determined with yeast alcohol dehydrogenase.²⁴ The concentration of these metabolites was determined in separate experiments run parallel to the experiments with tritium-labelled ethanol as a substrate.

Specific activity of lactate and β -hydroxybutyrate. The neutralized supernatant from the

perchloric acid precipitation was lyophilized and redissolved in dry pyridine (0.50 ml). Solutions of [^{14}C]lactate and of [^{14}C] β -hydroxybutyrate were added as internal standards and the trimethylsilyl esters of lactate and β -hydroxybutyrate prepared by addition of hexamethyldisilazane (100 μl) and trimethylchlorosilane (50 μl). A 25 μl sample was injected into a gas chromatograph (Hewlett-Packard 7620A) equipped with a beam-splitting device. The conditions for the chromatography were: 1.6 m $\times$ 6 mm o.d. glass column packed with 2 % E. 350 diatomite M-AW-DMCS, 90–100 mesh (JJ's Chromatography Ltd., Norfold, England), oven temperature 90 $^{\circ}\text{C}$, injection port temperature 200 $^{\circ}\text{C}$, detector temperature 260 $^{\circ}\text{C}$, and nitrogen flow rate 30 ml/min. Retention times for lactate and β -hydroxybutyrate were 200 and 370 s, respectively.

The eluates corresponding to the lactate and the β -hydroxybutyrate peaks were collected over 60 and 90 s, respectively, in either small cold traps or in water. The recovery by the chromatography was about 50 %. Samples were counted in a Packard 2425 scintillation spectrometer with Instagel as a scintillation liquid.

Preparation of specifically labelled substrates. 2- ^3H -Lactate (specific activity 10 $\mu\text{Ci}/\mu\text{mol}$) was prepared by incubation of *R,S*-1- ^3H -ethanol, NAD^+ , and pyruvate together with alcohol dehydrogenase and lactate dehydrogenase. The incubation mixture was applied to a column of DEAE-Sephadex A 25 equilibrated with 25 mM ammonium acetate. The column was washed with 25 mM ammonium acetate and lactate eluted with 100 mM ammonium acetate. The radiochemical purity of the product was determined to larger than 85 % by the GLC-procedure described above.

3- ^3H -D- β -Hydroxybutyrate (specific activity 40 $\mu\text{Ci}/\mu\text{mol}$) was prepared by reduction of acetoacetate by 4-A,B- ^3H -NADH in the presence of β -hydroxybutyrate dehydrogenase, and was purified by thin layer chromatography on cellulose plates with butan-2-ol; (2 N NH_3 , H_2O) (80:20, v/v) as a solvent. The radiochemical purity was determined by the GLC procedure described above as larger than 80 %.

4-A,B- ^3H -NADH was prepared by reduction of 4- ^3H - NAD^+ by *R,S*-1- ^3H -ethanol in the presence of alcohol dehydrogenase and semicarbazide.²⁵ Purification was achieved on a DEAE-Sephadex A 25 column as described for the preparation of 2- ^3H -lactate. NADH was eluted with 500 mM ammonium acetate.

4- ^3H - NAD^+ was prepared by oxidation of 4-A- ^3H -NADH with α -oxoglutarate and NH_4^+ in the presence of glutamate dehydrogenase. The reaction mixture was ultrafiltrated and lyophilized before conversion of the 4- ^3H - NAD^+ to 4-A,B- ^3H -NADH.

4-A- ^3H -NADH was prepared by reduction of NAD^+ with *R,S*-1- ^3H -ethanol in the presence of alcohol dehydrogenase and semicarba-

zide. NADH was purified as described above for 4-A,B- ^3H -NADH.

RESULTS

Metabolite levels. All results on metabolite concentrations appear in Table 1. The lactate production, the metabolism of pyruvate to metabolites other than lactate, and the ketone body production have been calculated from the measurements of metabolite levels.

The 15 min values in the absence and presence of 4-methylpyrazole as regards the lactate to pyruvate and β -hydroxybutyrate to acetoacetate ratios and the level of acetaldehyde, agree with previously reported results.^{5,27,28}

Lactate and pyruvate. As judged from the lactate to pyruvate ratios the results of Table 1 show that the cells do not attain a steady-state with regard to the redox state of the cytoplasmic NAD-couple within the first 15 min after addition of ethanol. This may seem at variance with previously reported results^{13,26} and is probably due to the high initial concentration of pyruvate in the present experiments. The production of lactate is high within the first 5 min after ethanol addition and declines with the level of pyruvate in the 5–15 min period after ethanol addition (at the end of the 15 min incubation period, the pyruvate added was nearly used up by the cells). Concomitant with the decrease in the lactate production from 5–15 min after ethanol addition, a very striking increase in the lactate to pyruvate ratio is observed (Table 1). The presence of 4-methylpyrazole does not affect the metabolism of pyruvate by pathways other than that catalyzed by lactate dehydrogenase.

β -Hydroxybutyrate and acetoacetate. The β -hydroxybutyrate to acetoacetate ratio shows a significant change within the first 5 min after ethanol addition if compared with the ratio in the presence of 4-methylpyrazole. A very abrupt increase in ketone body production is observed from 3 to 5 min after ethanol addition.

Acetaldehyde. The concentration of acetaldehyde in the incubation medium is almost constant within 15 min of incubation and about 100 μM (Table 1). The fact that the concentration of acetaldehyde is unaffected by the rate of acetaldehyde production as re-

Table 1. Metabolic variables as a function of incubation time. Figures are means $\pm$ S.E. of six experiments, except where noted. Comparison of results in the absence and presence of 4-methylpyrazole was carried out by the paired-data *t*-test and * indicates *P* values < 0.05 . In the last three lines of the table, average rates of lactate production, pyruvate metabolism minus lactate production and ketone body formation are calculated in $\mu\text{mol min}^{-1}$ (ml of cells) $^{-1}$ in the time intervals indicated (i.e. 1–3, 3–5 and 5–15 min).

Incubation time, min	– 4-Methylpyrazole			+ 4-Methylpyrazole		
	1	3	5	1	3	5
[Acetaldehyde] μM	100 $\pm$ 13	105 $\pm$ 20	115 $\pm$ 17	81 $\pm$ 10	117 $\pm$ 14	114 $\pm$ 16
[β -Hydroxybutyrate] μM	196 $\pm$ 37	232 $\pm$ 58	304 $\pm$ 60	599 $\pm$ 87	190 $\pm$ 26	240 $\pm$ 51
[Acetoacetate] μM	280 $\pm$ 42	327 $\pm$ 50	502 $\pm$ 53	395 $\pm$ 30	367 $\pm$ 65	547 $\pm$ 80
[β -Hydroxybutyrate]/[Acetoacetate]	0.75 $\pm$ 0.11	0.73 $\pm$ 0.11	0.63 $\pm$ 0.11	1.49 $\pm$ 0.13	0.58 $\pm$ 0.09*	0.44 $\pm$ 0.05*
[Lactate] mM	0.59 $\pm$ 0.04	0.95 $\pm$ 0.05	1.29 $\pm$ 0.09	1.49 $\pm$ 0.08	0.57 $\pm$ 0.05*	0.60 $\pm$ 0.06*
[Pyruvate] mM	1.61 $\pm$ 0.19	1.03 $\pm$ 0.22	0.53 $\pm$ 0.21	0.04 $\pm$ 0.01 ^a	1.32 $\pm$ 0.21*	1.06 $\pm$ 0.26*
[Lactate]/[Pyruvate]	0.39 $\pm$ 0.06	1.22 $\pm$ 0.32	8.10 $\pm$ 3.44	47.5 $\pm$ 6.5 ^a	0.51 $\pm$ 0.11*	0.77 $\pm$ 0.20*
Δ Lactate		4.76 $\pm$ 0.37	4.29 $\pm$ 0.78	0.57 $\pm$ 0.41	0.36 $\pm$ 0.42*	0.80 $\pm$ 0.36*
Δ Pyruvate – Δ Lactate		– 2.54 $\pm$ 0.80	– 2.13 $\pm$ 0.59	– 0.95 $\pm$ 0.19	– 3.18 $\pm$ 0.41	– 2.18 $\pm$ 1.15
Δ Ketone bodies		1.01 $\pm$ 0.38	3.18 $\pm$ 0.59	0.40 $\pm$ 0.14	1.04 $\pm$ 0.43	3.32 $\pm$ 0.47

^a In two experiments the concentration of pyruvate was below 5 μM . These experiments have been omitted in this value.

Fig. 1. Incorporation of tritium into lactate from *R,S*-1-³H-ethanol. Triangles are the values actually measured and circles these values as corrected for the detritiation of 2-³H-L-lactate (see 'Discussion'). Open and closed symbols represent values in the absence and presence of 4-methylpyrazole (1.7 mM), respectively. Ethanol was added at time zero. Average values of three experiments.

flected in the independency of 4-methylpyrazole addition is at present not understood. The acetaldehyde concentration in the presence of ethanol agrees closely with the levels obtained in the intact rat liver during ethanol metabolism.⁵

Labelling pattern of lactate and β -hydroxybutyrate. The incorporation of tritium from ethanol into lactate (Fig. 1) and the specific activity of lactate increases with time. The specific activity of lactate relative to that of ethanol after 15 min of incubation is about 0.13, in accordance with previously reported values.^{18,20} The labelling rate of lactate apparently declines with the fall in lactate production and the concomitant decrease in the concentration of pyruvate (cf. Table 1). In the presence of 4-methylpyrazole, the transfer of label from ethanol to lactate is about 6 times smaller than in the absence of inhibitor, which corresponds roughly to the decrease in the [³H]NADH production due to the lower rate of ethanol and acetaldehyde oxidation.

Fig. 2. Incorporation of tritium into β -hydroxybutyrate from R,S -1- ^3H -ethanol. Triangles are the values actually measured and circles these values as corrected for the detritiation of $3\text{-}^3\text{H}$ - β -D-hydroxybutyrate (see 'Discussion'). Open and closed symbols represent values in the absence and presence of 4-methylpyrazole (1.7 mM), respectively. Ethanol was added at time zero. Average values of three experiments.

The incorporation of tritium from ethanol into β -hydroxybutyrate is shown in Fig. 2.

The relative specific activity of β -hydroxybutyrate of 0.015 obtained in the present work is in accordance with values reported previously.³⁰ The extensive labelling of β -hydroxybutyrate reported by us³¹ probably was due to insufficient methods for the isolation of β -hydroxybutyrate.

The detritiation of lactate and of β -hydroxybutyrate was measured with $2\text{-}^3\text{H}$ -lactate and $3\text{-}^3\text{H}$ - β -D-hydroxybutyrate plus ethanol as substrates in experiments identical to those applying R,S -1- ^3H -ethanol as a substrate (Figs. 3 and 4). The results have been used to correct the results of Figs. 1 and 2 for the detritiation of lactate and β -hydroxybutyrate, respectively, to obtain a measure of the true $[^3\text{H}]$ -NADH production in the cytosol (see 'Discussion').

The correction was carried out by assuming a linear detritiation of both the initial amount of labelled substrate present and of the tritium incorporation in each of the three time intervals measured, e.g. for the labelling of lactate: The fractional detritiation of lactate in the

Fig. 3. Detritiation of $2\text{-}^3\text{H}$ -L-lactate by isolated liver cells. Ethanol (65 mM) and $2\text{-}^3\text{H}$ -L-lactate (27 000 cpm) were added at time zero. O, -4-methylpyrazole; ●, +1.7 mM 4-methylpyrazole. Average values of two experiments.

Fig. 4. Detritiation of $3\text{-}^3\text{H}$ - β -D-hydroxybutyrate by isolated liver cells. Ethanol (65 mM) and $3\text{-}^3\text{H}$ - β -D-hydroxybutyrate (46 000 cpm) were added at zero time. O, -4-methylpyrazole; ●, +1.7 mM 4-methylpyrazole. One experiment.

time interval from t_1 to t_2 is denoted x , the observed labelling at t_1 is denoted α , the observed labelling at t_2 is denoted $\alpha + \Delta\alpha$ and the amount of label incorporated into lactate in the absence of any detritiation in the time interval from t_1 to t_2 is denoted β . The detritiation from t_1 to t_2 of α is αx and of the increase in labelling from t_1 to t_2 it is $0.5\beta x$, resulting in a total detritiation of $\alpha x + 0.5\beta x$. The observed increase in labelling from t_1 to t_2 equals the true increase in labelling from t_1 to t_2 minus the detritiation in this time interval: $\Delta\alpha = \beta - (\alpha x + 0.5\beta x)$ or $\beta = (\Delta\alpha + \alpha x) / (1 - 0.5x)$.

The corrected labelling pattern of lactate is shown in Fig. 1 (○,●) and that of β -hydroxybutyrate in Fig. 2 (○,●). In the experiments with 2-³H-lactate as a substrate, no labelling of β -hydroxybutyrate could be detected. Similarly, no labelling of lactate could be detected with 3-³H- β -D-hydroxybutyrate as a substrate.

The tritium content of both lactate and β -hydroxybutyrate after 1 min of ethanol oxidation appeared independent of the presence of 4-methylpyrazole (Figs. 1 and 2). However, in parallel experiments it has been shown that ethanol oxidation, catalyzed by alcohol dehydrogenase becomes maximally inhibited by 1.7 mM 4-methylpyrazole already 15 s after addition of the inhibitor to a cell suspension (unpublished results).

DISCUSSION

Metabolite levels. The production of ketone bodies shows a very abrupt increase 3 to 5 min after addition of ethanol (Table 1) and corresponds in this time interval to 6.5 (μ mol C-2 units) $\text{min}^{-1} \text{ml}^{-1}$ and is independent of the presence of 4-methylpyrazole. Ketone bodies may be formed from pyruvate, acetaldehyde⁶ or fatty acids. In the absence of 4-methylpyrazole, the ketone bodies produced from 3 to 5 min after ethanol addition, might be derived from acetaldehyde and pyruvate (*cf.* Table 1), whereas in the presence of 4-methylpyrazole, about 3 μ mol min^{-1} of ketone bodies must be formed from other substrates, *e.g.* by an increase in the β -oxidation pathway. An inhibition of the utilization of ketone bodies is not a likely explanation of the increase in the level of ketone bodies, as the capacity of liver tissue to metabolize ketone bodies is very limited.³²

Labelling pattern. The specific activity of lactate after 15 min was about 0.13 (with 4-methylpyrazole only 0.06) relative to the specific activity of ethanol. The deviation from the theoretical value of 0.5 may be due to a cytosolic NADH production from substrates other than ethanol and/or to a rapid exchange reaction of the cytosolic NADH-pool with water, *e.g.* caused by a rapid exchange of malate between the cytosolic and the mitochondrial cell compartment.³³ A possible compartmentation between NADH formed in the

reaction catalyzed by alcohol dehydrogenase and the cytosolic NADH-pool^{29,30,34} might also contribute to the low specific activity of lactate in the steady-state situation.

The specific activity of β -hydroxybutyrate is only about 0.02 relative to the specific activity of ethanol. This very low labelling of β -hydroxybutyrate may be due to an extensive transfer of tritium from mitochondria [³H]NADH to water^{35,36} and/or to a lack of isotopic equilibration between the mitochondrial NADH-pool and β -hydroxybutyrate.

The intracellular localization of acetaldehyde oxidation. The results of the present experiments allow calculation of the cytosolic oxidation of acetaldehyde from the measurements of the initial incorporation of tritium into lactate.

Cytosolic [³H]NADH is formed from *R,S*-1-³H-ethanol in the reaction catalyzed by alcohol dehydrogenase (reaction 1 of Scheme 1) and by the oxidation of that part of the acetaldehyde, which is oxidized extramitochondrially (reaction 2 of Scheme 1).

The total amount of [³H]NADH formed by the cytosolic oxidation of ethanol and acetaldehyde equals the total amount of tritium actually measured in lactate plus the amount of tritium, which is deposited in substrates other than lactate (*cf.* Scheme 1). The significance of the last mentioned pathway is assumed to be measured by the rate of detritiation of 2-³H-L-lactate. This assumption is valid only if lactate/pyruvate equilibrates rapidly with the cytoplasmic NADH/NAD⁺ pool as compared to other redox couples sharing the same NADH/NAD⁺ pool, and if no significant recirculation of tritium to ethanol takes place. Recirculation of tritium to ethanol probably will be insignificant during the 15 min of incubation, as the major labelled products of reaction 4 (Scheme 1) will be water and glucose.^{12,16}

The total radioactivity measured in lactate plus the radioactivity removed by reaction 4 (Scheme 1) (as determined by the detritiation of 2-³H-lactate) is then a measure of the production of cytosolic [³H]NADH by reaction 1 and 2 (Scheme 1). The rate of reaction 1 is known from measurements of the rate of ethanol oxidation and the rate of reaction 2 can therefore be calculated.

Scheme 1. Schematic flow-diagram for the C-1 hydrogens of ethanol.

In the presence of 4-methylpyrazole, the rate of tritium transfer as corrected for the detritiation of lactate was determined to 2926 cpm min⁻¹ flask⁻¹ (Fig. 1). Given the specific activity of the *R*- or *S*-tritium atom of ethanol of 140 cpm nmol⁻¹ each and the amount of cells per flask of 92 μ l, the above-mentioned rate of tritium transfer to lactate corresponds to a cytosolic [³H]NADH production of 0.23 μ mol min⁻¹ ml⁻¹.

The rate of NAD³H production in the cytosol will depend partly on the isotope effects on alcohol dehydrogenase and aldehyde dehydrogenase and partly on the degree of inhibition of alcohol dehydrogenase by 4-methylpyrazole. The isotope effect on the reaction catalyzed by aldehyde dehydrogenases is unknown, but assuming a value between 1 and 0.6 (the isotope effect on the alcohol dehydrogenase, see below), the oxidation rate of acetaldehyde in the cytosol will be in the range 0.23–0.38 μ mol min⁻¹ ml⁻¹ if complete inhibition of alcohol dehydrogenase is obtained by 4-methylpyrazole.

The degree of inhibition of alcohol dehydrogenase in the present experiments probably is 95 % or higher.^{2,23}

The rate of ethanol oxidation by alcohol dehydrogenase under the present experimental conditions was determined to 3.18 ± 0.19 μ mol min⁻¹ ml⁻¹ ($n=5$), and thus 95 % inhibition of the enzyme results in a calculated rate of extramitochondrial acetaldehyde oxidation of

about 0.13–0.28 μ mol min⁻¹ ml⁻¹, assuming isotope effects of 0.6 and 1–0.6, respectively, in the reactions catalyzed by alcohol dehydrogenase^{16,37,38} and aldehyde dehydrogenase.

In the absence of 4-methylpyrazole, the rate of tritium transfer as corrected for the detritiation of lactate was determined to 19 366 cpm min⁻¹ ml⁻¹ (Fig. 2), corresponding to a cytosolic [³H]NADH production of 1.5 μ mol min⁻¹ ml⁻¹. The rate of [³H]NADH production from the reaction catalyzed by alcohol dehydrogenase equals the rate of ethanol oxidation (3.2 μ mol min⁻¹ ml⁻¹, see above) corrected for the isotope effect in this reaction (0.5–0.6),^{16,37,38} i.e. 1.6–1.9 μ mol min⁻¹ ml⁻¹. The figures 1.5 and 1.6–1.9 μ mol min⁻¹ ml⁻¹ seems to be different, indicating that some [³H]NADH from the reaction catalyzed by alcohol dehydrogenase escapes equilibrium with the cytosolic NADH pool, a finding which is compatible with the results obtained by Curstedt.²⁹

We therefore conclude, that under the experimental conditions applied the oxidation rate of acetaldehyde in the cytosol is in the range from 0 to 0.4 μ mol min⁻¹ (ml of cells)⁻¹. This result is reconcilable with the distribution studies of Marjanen,⁷ who found that about 20 % of the low- K_m aldehyde dehydrogenase activity was confined to the cytosol in rat liver.

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