ISOTOPE EQUILIBRATION MEASUREMENTS IN PERFUSED RAT LIVER SYNTHESIZING GLUCOSE FROM L-LACTATE-2-T-2-C^{14}

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1. Introduction

Hydrogen exchange between the NAD/NADH pool and corresponding metabolite couples in the cytosol of rat liver tissue is expected to be very rapid, because of the high activities of NAD-dependent dehydrogenases, i.e. lactate dehydrogenase (EC 1.1.1.8), malate dehydrogenase (EC 1.1.1.37) and glycerol-3-P-dehydrogenase (EC 1.1.1.8) [1].

In perfusion experiments with rat liver, Hoberman [2] has investigated hydrogen transfer from lactate to malate and glycerol-3-P using L-lactate-2-T as substrate. The data were interpreted as indicating a fast equilibration between lactate and malate but not between lactate and glycerol-3-P. This conclusion is in disagreement with the expectations stated above. Objections may be raised, however, concerning the interpretation of the reported data, especially since the data are limited because of missing analyses of tritium distribution in metabolites. In studies of tritium and carbon-14 pathways in gluconeogenesis from L-lactate-2-T-2-C^{14}, we measured the isotope distribution in NAD, lactate, malate and glycerol-3-P between 3 and 6 min after addition of the labeled substrate. The data, presented in this paper indicate the expected rapid hydrogen exchange.

2. Materials and methods

2.1. *Perfusion techniques*

Livers of male albino rats (Wistar strain, 200–300 g) fasted for 48 hr were perfused with Krebs-Ringer bicarbonate buffer, pH 7.4, containing 7 g% dextran-40. The perfusion solution was saturated with 95% O_2 and 5% CO_2 [3]. The temperature was 33°C. The solution was pumped through the liver with an average flow rate of about 25 ml/min. After 15 min of perfusion without exogenous substrate, unlabeled L-lactate (5 mM) was added to the recycling solution (200 ml). At 45 min, the closed perfusion circuit was opened and tracer amounts of L-lactate-2-T-2-C^{14} were added continuously to the solution entering the liver. Freeze stops were made at 3, 5 or 6 min after application of the labeled substrate.

2.2. *Preparation of labeled compounds*

L-lactate-2-T was prepared according to a method described by Hoberman [2,4], and L-lactate-2-C^{14} by enzymatic reduction of pyruvate-2-C^{14} (Amersham) with NADH and lactate-dehydrogenase.

2.3. *Separation of metabolites*

The first separation of the metabolites from the perchloric acid extract of liver tissue was performed by anion exchange chromatography on Dowex-1X8-formate [2]. NAD was purified from contaminating aspartate and glutamate by rechromatography on Dowex-1X8-acetate [5], while malate was purified by two-dimensional chromatography on cellulose thin layer plates [6]. Glycerol-3-P was eluted from the Dowex-1X8-formate together with other sugar-mono-phosphates. Therefore, these compounds were dephosphorylated with acid phosphatase. Subsequently, glycerol was specifically rephosphorylated with glycerolkinase and separated on Dowex-1X8-chloride.

Table 1

Specific tritium and carbon-14 activities of metabolites isolated from liver tissue. The values are expressed in relation to the specific activities of L-lactate-2-T-2-C^{14} in the perfusion solution entering the liver, being: 3 min experiment, 0.216 10^5 cpm T/μmol, 0.068 10^5 cpmC^{14}/μmol; 5 min experiment, 1.118 10^5 cpm T/μmol, 0.117 10^5 cpmC^{14}/μmol; 6 min experiment, 0.480 10^5 cpm T/μmol, 0.075 10^5 cpmC^{14}/μmol.

Metabolite	3 min		5 min		6 min	
	Relative specific activities					
	T	C^{14}	T	C^{14}	T	C^{14}
NAD	0.25	–	0.26	–	0.18	–
Lactate	0.75	0.84	0.72	0.80	0.80	0.77
Malate						
total	0.66	0.52	0.47	0.66	0.38	0.48
2 position	0.33		0.23		0.19	
3 position	–		0.18		–	
Glycerol-3-P						
total	0.25	0.13	0.33	0.18	0.25	0.13
2 position	0.25		0.20		0.18	
1 position	–		0.05		0.05	
Lactate in perfusion solution leaving liver						
total			0.73	0.94		
3 position			0.03	–		

2.4. *Analysis of tritium distribution in metabolites*

Tritium in the 2 and 3 position of malate was analysed by enzymatic conversion of malate to citrate and NADH. The A-side hydrogen of NADH was analysed in the form of ethanol, formed by adding acetaldehyde and alcohol dehydrogenase to the incubation mixture. The specific activity of ethanol represents tritium in the 2 position of malate, whereas the specific activity of citrate represents tritium in the 3 position.

The different positions in glycerol-3-P were analysed by incubation with NAD, glycerol-3-P-dehydrogenase, aldolase and triose-P-isomerase. Tritium in position 1 of glycerol-3-P was exchanged with water [7]. The B-side hydrogen of the NADH formed was transferred to glutamate by adding α-ketoglutarate, ammonium chloride and glutamatedehydrogenase to the incubation mixture. Water was lyophilized and glutamate was separated on Dowex-50-H^+ to calculate the specific tritium activities in the 1 and 2 position of glycerol-3-P.

Tritium and carbon-14 activities were determined simultaneously with a Packard scintillation spectrometer, as described by Hempel [8]. The metabolite concentrations were measured by standard enzymatic assays [9].

3. Results and discussion

The rate of gluconeogenesis from lactate was constant after 30 min of perfusion (average value 22 μmoles/h/g fresh wt). Tritium incorporation into water corresponded to a rate of L-lactate-2-T-detritiation of about 50 μmoles/h/g fresh wt. The specific activities of metabolites are shown in table 1. The values refer to the specific activity of L-lactate-2-T-2-C^{14} in the perfusion solution entering the liver. Since significant changes in specific activities were not observed between 3 and 6 min after addition of labeled lactate, these data clearly demonstrate that isotopic steady state conditions were already established after 3 min. Thus, rates of hydrogen transfer cannot be calculated from these data.

Tritium in the 2 position of malate and glycerol-3-P is equilibrated with the NAD/NADH pool, as indicated

by the identical specific activities of hydrogen in these positions and in NAD. However, the estimated specific activity of intracellular lactate is at least two times higher than that of NAD, malate or glycerol-3-P. This may indicate that tritium exchange between the NADH/NAD system and water, directly or indirectly, occurs with a higher rate than that between the NADH/NAD system and lactate. The specific activity of extracellular lactate is considered as constant due to the high flow rate of lactate through the liver tissue (425 μmoles/h/g fresh wt). Furthermore, it is concluded, that an equilibration between the 2 and 3 positions of malate was achieved by the fumarase reaction.

Theoretically, all three positions in glycerol-3-P may incorporate tritium via NADH (position 1 from the glyceraldehyde-3-P-dehydrogenase-, position 2 from the glycerol-3-P-dehydrogenase- and position 3 from the malate dehydrogenase- and fumarase-reaction). Our results show, however, that tritium is located predominantly in the 2 position. A possible explanation for a low activity of the 3 position is a dilution of labeled with unlabeled glycerol-3-P originating from lipolysis, being in agreement with the low carbon-14 activity. On the other hand tritium in position 1 may be exchanged with water on the dihydroxyacetone-3-P level by aldolase [7].

These conclusions differ largely from those based on results previously reported [2]. In those experiments, equilibration between lactate and malate, but non-equilibration between lactate and glycerol-3-P were postulated because of identical and different *total* specific activities, respectively. However, these results have to be interpreted differently on the basis of detailed analyses of tritium distribution in the metabolites, as presented in this paper. Furthermore, the doubtful time dependence of tritium incorporation into glycerol-3-P does not allow the calculation of a hydrogen transfer rate from lactate to glycerol-3-P. Moreover, tritium incorporation into water is not the only result of oxidation of tritium-labeled mitochondrial NADH, as assumed previously [2]. Tritium exchange with flavoproteins [10], from the methylen-group of oxaloacetate [11] or from dihydroxyacetone-3-P by aldolase will give a considerable contribution.

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