Compartmentation of Lactate and Glucose Metabolism in C6 Glioma Cells

A 13C AND 1H NMR STUDY*

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Anne-Karine Bouzier, Ruth Goodwin, Florence Macouillard-Pouletier de Gannes, Henri Valeins, Pierre Voisin, Paul Canioni‡, and Michel Merle

From the Laboratoire de Résonance Magnétique des Systèmes Biologiques, UMR 5536, Centre National de la Recherche Scientifique-Université Victor Segalen, 146 rue Léo Saignat, 33076 Bordeaux, France.

13C and 1H NMR spectroscopy was used to investigate the metabolism of L-lactate and D-glucose in C6 glioma cells. The changing of lactate and glucose concentration in the extracellular medium of C6 glioma cells incubated with 5.5 mM glucose and 11 mM lactate indicated a net production of lactate as the consequence of an active aerobic glycolysis. The 13C enrichments of various metabolites were determined after 4-h cell incubation in media containing both substrates, each of them being alternatively labeled in the form of either [3-13C]L-lactate or [1-13C]D-glucose. Using 11 mM [3-13C]L-lactate, the enrichment of glutamate C4, 69%, was found higher than that of alanine C3, 32%, when that of acetyl-CoA C2 was 78%. These results indicated that exogenous lactate was the major substrate for the oxidative metabolism of the cells. Nevertheless, an active glycolysis occurred, leading to a net lactate production. This lactate was, however, metabolically different from the exogenous lactate as both lactate species did not mix into a unique compartment. The results were actually consistent with the concept of the existence of two pools of both lactate and pyruvate, wherein one pool was closely connected with exogenous lactate and was the main fuel for the oxidative metabolism, and the other pool was closely related to aerobic glycolysis.

Neoplastic cells preferentially utilize aerobic glycolysis for their energy needs rather than oxidative phosphorylations, that leads to an important lactate production (1). The reason for this behavior is not yet clearly understood. It has been proposed recently that it would be a means to minimize oxidative stress, in particular during the cell phases linked to biosynthesis and cell division (2). With regard to a tumor, aerobic glycolysis may increase lactate concentration inside the tumor itself and in its close vicinity (3, 4). Consequently, local changes in metabolic activities could be generated as an increased local concentration (5).

To study the possible competition between glucose and lactate metabolism in glioma cells, we investigated the fate of each of these substrates when both were present in cell medium by using either [3-13C]L-lactate or [1-13C]D-glucose. We used the C6 glioma cell clone for which the metabolism of [1-13C]glucose has been investigated already (6, 7). The initial glucose concentration (5.5 mM) was chosen higher than the apparent Km for transport (1.7 mM) (8), whereas the different lactate concentrations used (11, 5.5, and 1.1 mM) were higher or in the same range than the apparent Km for transport (1 mM) (9). The 13C enrichment of extra and intracellular lactate and that of various intracellular metabolites was analyzed after a 4-h cell incubation. The results evidence that when lactate concentration was higher than Km, cells preferentially utilize lactate as carbon source for oxidative phosphorylations notwithstanding a high rate of glycolysis.

EXPERIMENTAL PROCEDURES

L-Lactate (sodium salt) was from Sigma. [3-13C]L-Lactate (sodium salt) (99% enrichment) was from Eurisotop. [1-13C]D-Glucose (99% enrichment) was from the Commissariat à l’Energie Atomique (Saclay, France).

Cell Incubation with Glucose and Lactate—C6 cells were cultured in 10-cm diameter dishes (6). At the end of the growth period, confluent cells taken at least 24 h after the last medium change were washed twice with phosphate-buffered saline (PBS) and then incubated in 10 ml of Dulbecco’s modified Eagle’s medium (DMEM) free of glutamine and pyruvate, but containing 5.5 mM D-glucose and/or 11 mM L-lactate. To determine glucose consumption and lactate production (or consumption), 200 μl of medium were sequentially removed after different incubation times. At the end of the incubation (24 h), cells were rinsed twice with PBS. After addition of 5 ml/dish of 0.9% perchoric acid, the insoluble part of the cellular layer was removed and pelleted by centrifugation. Similar experiments were performed with C6 cells incubated in the presence of 5.5 mM [1-13C]glucose and 11 mM L-lactate. At the indicated time, 200 μl of medium were reserved for glucose and lactate determinations. The remaining medium in each dish was conditioned for 1H NMR analysis (see below).

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† To whom correspondence should be addressed: Laboratoire de Résonance Magnétique des Systèmes Biologiques, UMR 5536, CNRS-Université Victor Segalen, 146 rue Léo Saignat, 33076 Bordeaux, France. Tel.: 33-5-57-57-10-19; Fax: 33-5-56-96-13-41; E-mail: canioni@rmsb.u-bordeaux2.fr.

‡ To whom correspondence should be addressed: Laboratoire de Résonance Magnétique des Systèmes Biologiques, UMR 5536, Centre National de la Recherche Scientifique-Université Victor Segalen, 146 rue Léo Saignat, 33076 Bordeaux, France.

The abbreviations used are: PBS, phosphate-buffered saline (0.15 μ NaCl, 5.5 mM KCl, pH 7.4); BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)carboxyfluorescein acetoxymethyl ester; D6O; (3,3′,3′-dihexyloxycarbocyanine iodide; DMEM, Dulbecco’s modified Eagle’s medium; HPLC, high performance liquid chromatography; SE, specific 13C enrichment; Ace-CoA, acetyl-CoA; E4A3, glutamate C4-specific 13C enrichment; alanine C3-specific 13C enrichment ratio; Δ Glu C4, glutamate C4 peak area; Δ Ala C3, alanine C3 peak area; Lac, lactate; Tyr, pyruvate.
For cell incubation with [1-13C]glucose or [3-13C]lactate, approximately 3 x 10^6 cells (10–15 culture dishes) were used for each experiment. After 4-h incubation, 4-mL aliquots of the medium were removed and subsequently freeze-dried. Cells were then resuspended with PBS and their metabolites extracted with 0.9 M perchloric acid (10).

**Sample Preparation for NMR Analyses**—To facilitate the analysis of the lactate methyl signal in the 1H NMR spectra of incubation media and cell extracts, the amino acids contained in the samples were eliminated by fixation on a Dowex 50W-X8 column, and glutamate in cell extracts was further purified (11).

**Determination of Metabolite 13C Enrichments**—The specific enrichment of acetyl-CoA (Ace-CoA) C2 was determined, the statistical significance of value differences was assessed by an initial rate of 265 n mol/h/mg of protein (n = 8). This consumption was accompanied by a large lactate export of 1090 ± 155 n mol/h/mg of the cell (n = 5), which corresponded to a 75% yield of glucose conversion into lactate and a net carbon entry into cell metabolism of around 350 n mol/h/mg of protein (in terms of 3-carbon units). Cells incubated with lactate consumed this substrate at an initial rate of 265 ± 95 n mol/h/mg of protein (n = 4), a value in the same range as the net 3-carbon unit entry obtained with

Using 2,7-bis(2-carboxyethyl)-5(6)carboxyfluorescein acetomethyl ester (BCECF-AM) (17). Cells were suspended in DMEM (10^6 cells/ml) and incubated 30 min at 37 °C with 10 μM BCECF-AM. A 100-μl volume of the cell suspension was then mixed with 900 μl of DMEM containing 5.5 mM glucose and 11 mM lactate before flow cytometry measurement. The standard curve for pH determination was drawn from measurements performed after dilution of 100 μl of the cell suspension in 900 μl of phosphate-buffered saline at different pH values (obtained by mixing in various proportions 135 mM KH₂PO₄ + 20 mM NaCl with 110 mM K₂HPO₄ + 20 mM NaCl) and 10-min incubation with 6 μM nigericin. Mitochondrial membrane potential was investigated with 3,3-dihexyloxacarbocyanine dide (DiOC₃(5)) (18). The suspension of cells in DMEM containing 5.5 mM glucose and 11 mM lactate (10^6 cells/ml) was incubated 10 min at 37 °C with 0.1 μM DiOC₃(3) before measurement.
glucose. When cells were incubated with both glucose and lactate, glucose consumption was lower, 440 ± 70 nmol/h/mg of protein (n = 8), than in the absence of lactate. Despite the high initial lactate concentration, a net lactate production was still observed, 315 ± 195 nmol/h/mg of protein (n = 5). This production corresponded, in terms of net carbon flux, to the conversion into lactate of 35% of the consumed glucose. The net entry of 3-carbon units into cell metabolism appeared slightly higher (around 565 nmol/h/mg of protein) than under the above conditions.

To analyze more precisely the conversion yield of glucose into lactate, the incubation was carried out starting with 5.5 mM [1-13C]glucose and 11 mM unlabeled lactate (Fig. 1). In this experiment, glucose consumption and lactate production rates were 430 and 470 nmol/h/mg of protein, respectively. [3-13C]Lactate was produced, as demonstrated by medium 1H NMR spectra (Fig. 1). For each incubation time SE Lac C3 was evaluated, and after subtracting the 1.1% natural 13C abundance, the concentration of [3-13C]lactate was calculated knowing the medium lactate concentration: \([3\text{-}13\text{C}]\text{lactate} = (\text{SE Lac C3}) - 1.1/100 \times \text{lactate}\); the concentration values are plotted in Fig. 1. The concentration of [3-13C]lactate was then compared with the amount of consumed glucose, taking into consideration that 1 mol of [3-13C]lactate was generated per mol of consumed [1-13C]glucose. The results indicated a high yield (101 ± 6%) of glucose conversion to lactate. The net lactate production appeared therefore much lower than the amount of lactate produced from glycolysis (470 as compared with 2 × 430 = 860 nmol/h/mg of protein in the experiment shown in Fig. 1). This suggested that concomitantly with lactate production from glycolysis, there was a strong consumption of exogenous lactate.

**Incubation of Cells with Either [1-13C]glucose or [3-13C]lactate**—To investigate more specifically the metabolism of glucose and lactate, cells were incubated for 4 h with 5.5 mM glucose and 11, 5.5, or 1.1 mM lactate using alternatively [1-13C]labeled glucose or lactate.

The 13C NMR spectra of cell extracts are shown in Fig. 2. Their amplitudes were normalized taking as a reference the signal of inositol (not enriched, because of its low turnover). As the cell metabolic status was the same under each couple of incubation conditions (same substrate concentrations), comparing the relative amplitudes of the homologous NMR signals was an indication about the relative metabolism of glucose and lactate, notwithstanding the fact that the 13C enrichment of the pyruvate coming from [3-13C]lactate was higher (at most twice) than that of the pyruvate coming from [1-13C]glucose (1 mol of [1-13C]glucose generates at most 1 mol of [3-13C]pyruvate, depending on the activity of the oxidative branch of the hexose monophosphate shunt).

The spectrum corresponding to the incubation with 11 mM [3-13C]lactate (spectrum A) displayed a strong resonance from lactate C3. This indicated that the exogenous lactate entered cells although a net lactate production occurred. Other major resonances were from Ala and Glu (and also glutathione (20)). Glu resonances appeared as multiplets, indicating multilabeled molecules. In particular, the Glu C3 signal appeared as a quintuplet involving a singlet corresponding to C3-labeled Glu molecules, a doublet corresponding to molecules labeled in C3 and C2 or C4 and a triplet corresponding to C2, C3, and C4 labeling. The high coupling indicated a high yield of 13C incorporation into Glu when starting from [3-13C]lactate. Very unexpectedly, much lower signals were observed in spectrum B corresponding to the use of [1-13C]glucose: Glu C4 and Ala C3 signals were 12 and 9 times smaller than in spectrum A. The examination of spectra C and D corresponding to the incubations with 5.5 mM lactate led to similar observations: a high lactate signal and the appearance of multiplets for Glu resonances (the contributions of the doublet and triplet to Glu C3 resonance were, however, lower than in spectrum A) when using [3-13C]lactate, and a low resonance level when using [1-13C]glucose, although higher than that observed in spectrum B. Glu C4 and Ala C3 signals in spectrum D were 7 and 4.5 times smaller than in spectrum C, respectively. In spectra E and F corresponding to the incubations with 1.1 mM lactate, homologous carbon resonance intensities were in the same range although slightly higher when using [3-13C]lactate than [1-13C]glucose. The degree of coupling for Glu resonances was poor, with a low contribution of the C3 doublet, whereas the triplet was undetectable. Notwithstanding that only 1 mol of [3-13C]pyruvate could be derived from 1 mol of [1-13C]glucose, these results suggested that lactate was a better precursor for...
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Ala and Glu than glucose (at least at 11 or 5.5 mM lactate concentration).

The cell contents in Glu and Ala (mean values: 26 ± 11 and 15 ± 4 nmol/mg of protein, respectively; n = 17) were used to evaluate the ratio between the specific enrichments of Glu C4 and Ala C3 (E4/A3) (Equation 1) (Table I). At a lactate concentration of 5.5 or 11 mM, the ratio was higher for cells incubated with [3-13C]lactate than [1-13C]glucose. At 1.1 mM lactate, the ratio was restricted to C2: 47.0 ± 6.8 for cells incubated with 5.5 mM lactate and 11 mM lactate, respectively. Each spectrum was obtained after zero filling to 64K, 1-Hz line-broadening, and Fourier transformation on the free induction decay corresponding to at least an overnight acquisition using 0.1-s relaxation delay. Spectra were plotted such as the signal intensity of 13C resonances from [1-13C]glucose or [3-13C]lactate. The intensity decreases with increasing unlabeled signals.

Determination of Cell Glutamate 13C Enrichment—Glutamate was determined from quantitative analysis of spectra using 0.1- or 10-s relaxation delay. These values reflected the functioning of the tricarboxylic acid cycle and its related pathways. According to this pathway, the label enrichment was directly incubated with the labeled substrates. Most of the lactate and glucose to Glu and Ala syntheses was higher than unity at 5.5 or 11 mM lactate.

The evaluation of the specific enrichment of Ace-CoA C2 was restricted to the experiments performed with 11 and 5.5 mM [3-13C]lactate, because the coupling pattern of Glu C4 was too low when using 1.1 mM [3-13C]lactate or [1-13C]glucose. The value, 77.5 ± 60.0% at 11 or 5.5 mM lactate, respectively, revealed the high enrichment of the Ace-CoA entering the tricarboxylic acid cycle (Table I).

Similarly to the spectra shown in Fig. 1, the signal of lactate C3 in the 1H NMR spectra of incubation media and cell extracts involved two satellites enabling the determination of SE Lac C3 (Table I). For the incubations performed with 5.5 or 11 mM lactate, the results indicated different enrichments for intracellular and extracellular lactate whatever the labeled substrate. Moreover, the enrichments of both intracellular and extracellular lactate were different depending on the labeled substrate. These data evidenced the dilution of the external lactate by lactate generated from glycolysis. This dilution was particularly important at 1.1 mM lactate as medium lactate enrichments after 4-h incubation were the same (Table I).

Preincubation of Cells in the Presence of Both Glucose and Lactate—To check whether the data were not biased by some metabolic activity burst following cell rinsing with PBS and addition of the new medium containing the 13C-enriched substrate, the effect of a 3-h preincubation of cells in a medium containing the unlabeled substrates before the addition of the labeled was investigated at 11 mM lactate and 5.5 mM glucose. The NMR spectra were very similar to those obtained from cell directly incubated with the labeled substrates. Most of the parameter values were intermediary between those obtained for the experiments performed without preincubation at 11 and 5.5 mM lactate; in particular, E4/A3 (when using [3-13C]lactate) and I4/I3 had values higher than unity, and SE Ace-CoA C2 was higher than intracellular SE Lac C3 (when using [3-13C]lactate) (Table I).

Flow Cytometry Measurements—The homogeneity of the cell population was investigated by flow cytometry using fluores-
cent probes sensitive to the cell metabolic status. Viable cells represented more than 90% of the cell population. The distribution of the DiOC6(3) fluorescence appeared as a single peak, indicating the same mitochondrial membrane potential for cells (Fig. 4A). The cytoplasmic pH of cells was sharply distributed around a unique value of 7.38, determined using the ratio between the green and red fluorescence of BCECF-AM (Fig. 4, B and C).

**DISCUSSION**

In a previous work it has been shown that incubating C6 cells for 4 h with 5.5 mM [1-13C]glucose led to the following specific enrichments: 36.7% for lactate and Ala C3, 27.3% for Ace-CoA C2, and 22.5% for Glu C4, in agreement with a monocompartmental model of cell carbon metabolism (6). According to its network, the decrease in enrichment from glucose C1 to Glu C4 was the consequence of both the hexose monophosphate shunt activity and several isotopic dilutions occurring at different branch points (pyruvate, Ace-CoA, and 2-oxoglutarate) due to carbon entry from endogenous and exogenous unlabeled sources. In the reported study, E4/A3 was thus less than 1. In contrast, the present study evidenced that when C6 cells were incubated with 5.5 mM glucose and 11 or 5.5 mM [3-13C]lactate, E4/A3 was higher than 1 (Table I). This result was concomitant with a higher enrichment of Ace-CoA C2 than lactate C3 at 11 mM [3-13C]lactate and to similar enrichments at 5.5 mM. These results were unexpected, because [3-13C]lactate was the precursor of [3-13C]pyruvate and thereafter [2-13C]Ace-CoA, keeping in mind the occurrence of isotopic dilutions at the pyruvate and Ace-CoA nodes. Obviously, such an enrichment pattern

| Incubation condition | Labeled substrate | E4/A3 | I_L/G | SE Ace-CoA C2 | SE lactate C3 |
|---------------------|------------------|-------|-------|---------------|--------------|
| 5.5 mM glucose      | [3-13C]Lactate, n = 4 | 2.15 ± 0.35 | 1.75 ± 0.25 | 59.0 ± 8.5 | 90.0 ± 4.0 |
| 11 mM lactate       | [1-13C]Glucose, n = 3 | 1.20 ± 0.30 | ND | 10.0 ± 2.0 | 4.90 ± 1.50 |
| 5.5 mM glucose +    | [3-13C]Lactate, n = 3 | 1.60 ± 0.50 | 60.0 ± 5.5 | 57.5 ± 6.5 | 71.0 ± 4.5 |
| 5.5 mM lactate      | [1-13C]Glucose, n = 3 | 1.20 ± 0.35 | ND | 14.5 ± 2.5 | 11.5 ± 1.5 |
| 5.5 mM glucose      | [3-13C]Lactate, n = 2 | 0.805 ± 0.140 | 1.35 ± 0.20 | 64.5 ± 5.5 | 82.0 ± 4.0 |
| 1.1 mM lactate      | [1-13C]Glucose, n = 2 | 0.805 ± 0.200 | 1.00 ± 0.10 | ND | 25.5 ± 1.5 | 27.0 ± 1.5 |
| 5.5 mM glucose +    | [3-13C]Lactate, n = 3 | 1.60 ± 0.40 | 68.5 ± 1.5 | ND | 19.0 ± 1.0 | 27.0 ± 1.5 |
| 5.5 mM lactate, 3-h preincubation | [1-13C]Glucose, n = 2 | 1.20 ± 0.50 | (p < 0.3) | 89.5 ± 0.70 | 7.90 ± 1.05 |

* Indicates significantly different values by Student's t test between E4/A3 values obtained with labeled glucose and lactate.
* Indicates significantly different values by Student's t test between SE Ace-CoA C2 and cellular SE Lac C3.
* ND, not determined.

**FIG. 3.** 13C NMR and 1H NMR spectra of purified glutamate from cells incubated for 4 h in the presence of 5.5 mM glucose and 11 mM [3-13C]lactate. Glutamate was purified by ion exchange chromatography. The 13C NMR spectrum shows the intense level of homonuclear coupling of carbon signals reflecting the high incorporation of the label. The 1H NMR spectrum displays the heteronuclear coupling making possible the determination of carbon enrichments, particularly Glu C2 enrichment.
could not fit with a metabolic network wherein Ace-CoA derived from a unique pool of pyruvate at equilibrium with alanine and lactate through alanine aminotransferase and lactate dehydrogenase activity, respectively. Data analysis needed therefore to reconsider the route of metabolite enrichment upstream from Ace-CoA. Downstream, the isotopic dilution (from Ace-CoA C2 to Glu C4) was very similar under the two incubation conditions (from 77.5 to 69.5% in the present study using 11 mM [3-13C]lactate and from 27.3 to 22.5% in Ref. 6).

Before proposing an explanation to the experimental data, it was critical to emphasize their reliability. First, from spectrum B in Fig. 2, it appears that using 5.5 mM [1-13C]glucose and 11 mM lactate, metabolite enrichment was very poor. This suggested that despite the high rate of cell glucose consumption, the contribution of this compound as Ace-CoA precursor was very low. It could be proposed that the label was lost through the decarboxylation step of the hexose monophosphate shunt. This proposal did not, however, agree with the data in Fig. 1, which indicated that almost all of the label was recovered as [3-13C]lactate. Moreover, the decarboxylation yield can be estimated from the enrichments of lactate C3 in cell media. Indeed, considering the enrichments obtained after 4-h incubation with 5.5 mM glucose and 11 mM lactate, 90 and 4.9% using either lactate or glucose as labeled substrate (Table I), if \( L_0 \) was the fraction of initial lactate remaining after 4 h and \( L_p \) the fraction of lactate formed from glycolysis, lactate enrichment (%) was expressed by:

\[
90 = 99 \times L_0 + 1.1 \times L_p
\]

when starting from [3-13C]lactate (99 and 1.1% being the initial enrichment and the natural 13C abundance, respectively). As \( L_0 + L_p = 1 \), it came \( L_0 = 0.91 \). Then it was possible to express lactate enrichment when starting from labeled [1-13C]glucose:

\[
4.9 = 1.1 \times L_0 + 1.1 \times L_p/2 + A \times L_p/2,
\]

where \( A \) represents the enrichment of the carbon corresponding to glucose C1 (it would be equal to 99 without decarboxylation). The value found was \( A = 83 \), indicating only 17% of decarboxylation, which could not explain the poor label incorporation into cell metabolites. The same calculation led to 26, 28.5, and 20.5% decarboxylation when using 5.5 and 1.1 mM lactate and for the experiment, including 3-h preincubation, respectively.

Second, during 4-h cell incubation with 5.5 mM glucose and 11 mM lactate, medium glucose decrease and lactate increase were near-linear, indicating that substrate consumption was close to steady state. It has been shown previously that 4-h cell incubation in a medium containing [1-13C]glucose (but no initial lactate) was sufficient to ensure isotopic steady state, particularly for Glu and Ala (6, 7). In the present study, an isotopic steady-state, stricto sensu, could not occur after only 4 h because medium lactate enrichment was time-decreasing (or increasing) due to the release of unlabeled (or labeled) lactate generated from unlabeled (or labeled) glucose. The demonstration of the existence of different pyruvate and lactate pools (as discussed below) was indeed dependent on this condition. From a theoretical point of view, isotopic steady state could only be expected after total glucose consumption, i.e., after 30–40-h incubation. Nevertheless, starting from 11 mM [3-13C]lactate, the decrease in medium lactate enrichment after 4 h was rather moderate (from 99 to 90%), making it possible to consider that the changes in metabolite enrichment were very slow at this time. The value of \( L_{R0} \), the criterion for the relative contributions of lactate and glucose to Glu and Ala syntheses, appeared dependent on lactate concentration. It was higher than 1 only at 11 and 5.5 mM lactate (Table I). This indicated primarily that the relative contributions of lactate and glucose to cell metabolism could be only investigated if their enrichments remained very different. This was obviously not the case using 1.1 mM lactate as the exogenous lactate was strongly diluted by glycolytic lactate (27% enrichment after 4 h). The isotopic dilution effects were also apparent when comparing the enrichments obtained at 11 and 5.5 mM lactate (Table I). In particular, Ace-CoA C2 and lactate C3 enrichments were closer at 5.5 mM lactate. As a consequence, data interpretation was focused on the results obtained at 11 mM lactate.

From the Glu C4 enrichment (69.5%) and the ratio E4/A3 (2.15, Table I), it follows that Ala C3 enrichment was around 32% when using [3-13C]lactate. Therefore, the fact that the enrichment of Ace-CoA C2 was higher than both that of Ala C3 and intracellular lactate C3 dictated to consider at least two pools of pyruvate. Moreover, the pool corresponding to the Ace-CoA precursor had to display an enrichment of at least 77.5% and thus to derive from an intracellular pool of lactate at least at the same enrichment. As intracellular lactate enrichment was measured to be less (59%), this implied also the existence of two intracellular lactate pools. This situation led to consider that the exogenous lactate was the main mitochondrial substrate (via pyruvate). Therefore, the metabolic pathway including the different steps on the conversion of extracellular lactate into mitochondrial Ace-CoA had to be compartmentalized toward glycolysis. This phenomenological compartmentalization is depicted in Fig. 5, wherein compartments...
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FIG. 5. Schematic representation of glucose and lactate metabolic compartmentation as deduced from the fate of 13C in cell metabolites. Medium lactate and glucose enter the cell and generate pyruvate in two different compartments (1 and 2). The major part of pyruvate derived from lactate enters mitochondria and generates acetyl-CoA, whereas pyruvate generated from glycolysis is mostly converted into lactate, which is released into the medium. Ala1 and Glu1 correspond to the fractions of alanine and glutamate synthesized from exogenous lactate, Ala2 and Glu2 correspond to the fractions synthesized from exogenous glucose and Ala3 and Glu3 correspond to the fractions synthesized from other endogenous or exogenous carbon sources (compartment 3). The thickness of the arrows illustrates the main pathway for lactate and glucose metabolism.

1 and 2 correspond to lactate and glucose metabolism, respectively. However, the fact that E4/A3 remained close to 1 (Table 1) when using [1-13C]glucose also needed to be explained. Indeed, if lactate and glucose were the sole carbon sources for glu and Ala syntheses, the expected value for E4/A3 would be less than 1 when using [1-13C]glucose. The means to explain why the value remained close to 1 was to consider the contribution of unenriched endogenous and exogenous carbon sources to amino acid syntheses. This led to include a third compartment in Fig. 5 containing Glu and Ala pools not originating from glucose or lactate metabolism. It was thus interesting to test the relevance of the model by estimating Glu1, Glu2, and Glu3 or Ala1, Ala2, and Ala3, the fractional amounts of Glu or Ala synthesized from lactate, glucose, and the other sources, respectively. The procedure involved Equations 4–6 (see “Experimental Procedures”). In Equation 4 (or 5), lactate C3 enrichment was 95% (or 85%), corresponding to the mean medium lactate enrichment between 0 and 4 h, starting from 11 (or 5.5) mM [3-13C]lactate, respectively. In Equation 6 (or 7), glycolytic pyruvate C3 enrichment was 42.5% (or 37.7%), corresponding to 17% (or 26%) glucose C1 decarboxylation (as discussed above), and the mean enrichment of medium lactate C3 was 3% (or 6.3%), starting from 11 (or 5.5) mM lactate, respectively. The best set of parameter values was: Glu1 = 0.72, Glu2 = 0.09, Glu3 = 0.19, Ala1 = 0.42, Ala2 = 0.10, and Ala3 = 0.48. These parameters were determined with a mean relative error of 13.5 ± 5.8% for the variables used in the iterative procedure, indicating a rather good fitting.

Instead of intracellular compartmentation of lactate and glucose metabolism, it could be proposed that the different metabolite labeling pattern as a function of the labeled substrate was the consequence of the presence of a mixed population of cells, one that was primarily oxidative in nature and principally oxidizing lactate and some glucose, with no net lactate production from glucose, and the other glycolytic and thus producing lactate, with no oxidation of lactate, i.e., intercellular compartmentation. Such markedly different metabolic behavior could not be the consequence of a lack in oxygen supply to the glycolytic cells, because all cells were incubated in the same conditions, but rather by impairment of their mitochondria. Therefore, a difference in membrane potential between functional and impaired mitochondria would be expected. Furthermore, the fact that one cell population produced lactate when the other oxidized this compound would generate a difference in cytosolic pH. Such differences were not, however, evidenced by the flow cytometry experiments carried out with the specific probes. There is no particular reason therefore, to privilege the occurrence of two different cell populations, all the more viable cells from the C6 clone are known to produce and oxidize lactate (5).

The existence of two functional cytosolic pyruvate pools, one closely connected with glycolysis and the other communicating with mitochondria, has been described already in rat heart cells from a study with radiolabeled substrates (21). One pool was identified as “glycolytic,” being associated more closely with glycolysis and tissue lactate, while the other “peripheral” pool communicated more closely with extracellular than with cellular lactate. Importantly, this latter pool was in close connection with mitochondrial pyruvate (21). The same conclusion was drawn from a study on the metabolism of [3-13C]pyruvate in rabbit heart (22). Using 99% enriched [3-13C]pyruvate, metabolite enrichments were as follows: around 80% for Ace-CoA C2, 70% for Glu C4, 60% for Ala C3, and 20% for lactate C3. These results, recently confirmed (23), indicated a metabolic heterogeneity of pyruvate pools within the myocyte. Pyruvate compartmentation was also proposed in the case of mouse fibroblasts and rat astrocytes (24, 25).

Our data were also consistent with the existence of two metabolically compartmentalized pools of intracellular lactate (Fig. 5). One pool was closely connected with exogenous lactate and was the main fuel for the oxidative metabolism. The other pool was closely related to glycolysis, but was metabolically disconnected from oxidative metabolism. Lactate uptake by C6 cells was demonstrated to be dependent on a saturable component at low concentration (Km in the millimolar range) and on a nonsaturating process at high concentration (9). Therefore, at a concentration of 11 or 5.5 mM, lactate exchange between intracellular and extracellular space would result mainly from free diffusion. The existence of two metabolically different pools of lactate asked therefore the question of their subcellular location in connection with that of the enzymes involved in their metabolism. In our experiments, there was both a net consumption of exogenous lactate and a net lactate production from glycolysis, suggesting the activities in the opposite directions of lactate dehydrogenase isoenzymes. Assuming that the different isoenzymes were present in C6 cells as they were demonstrated to be in glial cells (26), one may think that they have different subcellular locations. Preliminary immunochromal observations effectively suggested that this was the case. The interpretation of our results appears therefore in close connection with the idea of microcompartmentation and enzyme association inside the cytosol (27).

In this study, the major part of glycolytic pyruvate produced in C6 cells was not oxidized but reduced to lactate, in agree-

**Footnotes:**

1 A.-K. Bouzier, R. Goodwin, P. Voisin, P. Canioni, and M. Merle, unpublished results.
ment with Warburg’s theory (1). However, the fuel for their oxidative metabolism was primarily lactate provided in the medium. Extrapolating this behavior to a brain tumor, it could be tempting to propose that lactate produced by anaerobic glycolysis within an hypoxic tumor region could be oxidized in another region where oxygen supply was better. Such an adaptation would contribute to regulate lactate level inside the tumor, in addition to the loss by perfusion or diffusion (28). On the other hand, although C6 cells could not be retained as the best model of normal astrocytes, one may think that the occurrence in astrocytes of a metabolic pathway for lactate synthesis disconnected from the oxidative route would be of major interest for the understanding of brain carbon metabolism, in particular, the role of astrocytes in the supply of lactate to neurons (29).

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