Orientation-Conserved Transfer of Symmetric Krebs Cycle Intermediates in Mammalian Tissue†

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ABSTRACT: Metabolism of [2-^{13}C]-, [3-^{13}C]-, and [1,2,3-^{13}C]propionate in perfused rat livers and [2-^{13}C]-acetate in perfused rat hearts has been examined in tissue extracts by ^{13}C NMR. Label from [2-^{13}C]-propionate was preferentially incorporated into the C2 carbon of lactate, alanine, and aspartate in liver tissue while label from [3-^{13}C]propionate appeared preferentially in the C3 carbon of those same molecules. These data suggest that ^{13}C may not be completely randomized in the symmetric citric acid cycle intermediates succinate and fumarate as is normally assumed but that some fraction of those intermediates may be transferred between enzymes in this span of the cycle with conservation of spatial orientation, consistent with recent results obtained in yeast [Sumegi et al. (1990) *Biochemistry 29,* 9106–9110]. This was confirmed by performing similar experiments with [1,2,3-^{13}C]propionate. Time-dependent asymmetry was also observed between the intensities of the glutamate C2 and C3 resonances and between the aspartate C2 and C3 resonances in ^{13}C NMR spectra of intact hearts and heart extracts during early perfusion with [2-^{13}C]-acetate. A model is presented which predicts that isotopic asymmetry is observed only during the first 2–3 turns of the cycle pools when isotope enters the cycle via acetyl-CoA even if all symmetric cycle intermediates retain a unique molecular orientation on each pass through the citric acid cycle.

The symmetric Krebs cycle intermediates succinate and fumarate are formed in mitochondrial reactions catalyzed by succinate dehydrogenase (EC 1.3.5.1) and fumarase (EC 4.2.1.2), respectively. Although the two carbon "ends" of these molecules cannot be distinguished chemically (C1 and C4 are equivalent and C2 and C3 are equivalent), early studies using isotopic labeling techniques and purified enzymes have shown that the succinate methylene protons are enantiotopic and prochiral and hence *can* be distinguished enzymatically (Walsh, 1979). Studies of purified succinate dehydrogenase have shown that the enzyme catalyzes trans elimination of dihydrogen (Tchen & van Milligan, 1960). Fumarase also catalyzes addition of water to fumarate stereospecifically to form a single product, L-malate. It has also been demonstrated for purified fumarase that turnover is so rapid that the proton removed from the C3 position of malate remains enzyme-bound for periods longer than that required for fumarate to dissociate into bulk solution and return to the active site (Hansen et al., 1969). The net result is that any L-malate isotopically labeled at one "end" but not the other can enter the active site of fumarase, dehydrate to fumarate, which can dissociate from the active site of the enzyme into bulk solution, undergo an end-to-end reorientation (or exchange with a different isotopically labeled fumarate molecule), and then reenter the active site with 50% probability of having the same active-site orientation as before. This results in "scrambling" of any labeled malate carbon between C2 and C3 or between C1 and C4. Data such as these on isolated enzymes have led to the generally accepted concept that isotopic fractional enrichment of chemically equivalent carbons of these symmetric intermediates must always be identical.

Might there be metabolic situations when this is not the case? It is not difficult to find literature examples showing unequal ^{13}C enrichment of metabolites derived from symmetric cycle intermediates (Cohen et al., 1981; Cohen, 1987; Brand et al., 1992), but in most cases these observations may be ascribed to incomplete back-equilibration of malate through fumarase. Bernhard and Tompa (1990) tested the hypothesis that succinate, isotopically labeled at one "end" via [5-^{13}C]-glutamate → [4-^{13}C]succinyl-CoA in isolated liver mitochondria, could pass through this span of the Krebs cycle without reorienting. This hypothesis was based upon calculations which indicate that these mitochondrial matrix enzymes exist in a milieu similar to that of wet protein crystals (Srere, 1987) even though flux through these reactions in intact mitochondria can be even more rapid than that measured with isolated, water-soluble enzymes (Robinson et al., 1987). However, Bernhard and Tompa observed equal ^{13}C enrichment in aspartate C1 and C4 by ^{13}C NMR, suggesting that symmetric intermediates must leave the enzyme-bound environment and mix with bulk solute molecules. In contrast, recent data from our laboratory using intact yeast cells showed unequal ^{13}C enrichment of three-carbon metabolites derived from Krebs cycle intermediates during oxidation of [^{13}C]-propionate (Sumegi et al., 1990). These data provided the

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first evidence that symmetric intermediates can pass through the succinyl-CoA synthase (EC 6.2.1.4) → succinate dehydrogenase → fumarase span of the cycle in intact cells with *conserved molecular orientation* of symmetric intermediates, i.e., without dissociating from these enzyme active sites and reorienting in bulk solution. Critics of that work (Shulman, 1991; Rognstad, 1991) pointed out that other less well-established pathways for propionate oxidation in yeast may have been responsible for the unequal ^{13}C enrichments observed in that study. This led to the present experiments which were designed to test the hypothesis that conservation of spatial orientation during transfer of metabolites from one enzyme to another might be possible in mammalian tissue, where pathways for propionate metabolism are more firmly established. A recent NMR/mass spectroscopy study of [1-^{13}C]-glucose metabolism in C6 glioma cells also found metabolic channeling of symmetric citric acid cycle intermediates to an extent of about 39% in that cell type (Portais et al., 1993), but with an opposite orientation as we reported previously for propionate metabolism in yeast (Sumegi et al., 1990). This suggests that the molecular orientation by which these intermediates are transferred between enzyme active sites may be cell type dependent.

In the present study, we provide evidence for asymmetric enrichment of ^{13}C in lactate, aspartate, glutamate, and citrate carbons in perfused rat heart and liver, presented with either a ^{13}C-enriched propionate or [2-^{13}C]acetate. We show that differences in ^{13}C enrichment at aspartate C2 versus C3 and at glutamate C3 versus C2 are time-dependent when ^{13}C enters the citric acid cycle as labeled acetyl-CoA and present a model which may explain why this phenomenon has not been detected previously in numerous NMR and radiolabeling experiments. As in the yeast study (Sumegi et al., 1990), unequal ^{13}C enrichment of aspartate, alanine, and lactate carbons was observed in ^{13}C NMR spectra of extracts of freeze-clamped perfused livers when the tissue was supplied with either [2-^{13}C]- or [3-^{13}C]propionate. We also demonstrate that the ^{13}C isotopomer distribution in lactate, citrate, aspartate, and glucose derived from [1,2,3-^{13}C]propionate provides an alternative method for detecting orientation–conserved transfer of metabolites which does not require measurement of small differences in resonance intensities.

MATERIALS AND METHODS

Materials. Sodium [2-^{13}C]acetate, sodium [3-^{13}C]- and [2-^{13}C]propionate, and [3-^{13}C]pyruvate were obtained from MDS Isotopes (St. Louis, MO). Sodium [1,2,3-^{13}C]propionate was from Cambridge Isotopes (Cambridge, MA). Collagenase (CLS 1, 133 units/mg of protein) was from Worthington Biochemicals (Freehold, NJ). All other chemicals were of the highest purity commercially available. Male Sprague Dawley rats, 200–400 g, were from SASCO.

Perfused Rat Liver Methods. Livers from overnight-fasted rats were perfused via cannulation of the portal vein as described by Seglen (1976). The livers were initially flushed with 500 mL of Krebs–Henseleit bicarbonate buffer (KHB) saturated with 95% O_2/5% CO_2 while the intact livers were isolated *in situ*. The isolated liver was then perfused (40 mL/min) in a recirculating manner (from 15 min to 1 h) with 100 mL of KHB buffer containing 20 mg of ^{13}C-labeled sodium propionate. Oxygenation of the perfusate was maintained by flow over an oxygenating net in an atmosphere of 95% O_2/5% CO_2. The perfusate was sampled at time intervals throughout the experiment, and the entire liver was freeze-clamped at the end of the perfusion period. In some experiments using [1,2,3-^{13}C]propionate (Figure 4 and Table 2, experiments 13–15), the livers were initially flushed with KHB buffer containing propionate (2 mM), lactate (1 mM), pyruvate (0.1 mM), NH_4Cl (2 mM), and a reduced concentration of KCl (2.8 mM) before being perfused in a recirculation manner with this same buffer containing labeled propionate. The livers were extracted with 10 mL of 8% perchloric acid, and the supernatant fraction of the neutralized extract was freeze-dried. The freeze-dried samples were redissolved in 0.7 mL of D_2O for NMR spectroscopy.

Isolation of Glutamate and Aspartate. Neutralized liver extract samples were dissolved in 2 mL of distilled water and applied to a 2.5-mL Dowex 1X8 acetate column. The column was washed with 10 mL of distilled water to remove glucose and all anionic metabolites. Glutamate and aspartate were eluted with 10 mL of 0.5 M acetic acid, freeze-dried to remove excess acetic acid, and redissolved into 0.5 mL of D_2O for subsequent NMR analysis.

Hepatocyte Isolation Techniques. Hepatocytes were prepared from overnight-fasted rats by collagenase perfusion using a slight modification of the procedure described by Seglen (1976). The livers were flushed at a flow rate of 40 mL/min with 500 mL of bicarbonate buffer containing glucose (10 mM), NaCl (122 mM), KCl (6.7 mM), and $NaHCO_3$ (25 mM), pH 7.4, continuously oxygenated with 95% O_2/5% CO_2 (v/v). The liver was then perfused in a recirculating manner for 10 min with 100 mL of buffer supplemented with $CaCl_2$ (3.75 mM) and collagenase (0.5 mg/mL). The liver cells were dispersed in KHB buffer supplemented with glucose (1 mM), lactate (10 mM), pyruvate (1 mM), and BSA (1%, w/v). The resulting liver cell suspension was sieved through nylon mesh and incubated for 30 min at 37 °C with shaking in an atmosphere of 95% O_2/5% CO_2, and the hepatocytes were purified by repeated low-speed centrifugation (100*g*, 4 times) in the KHB buffer just described. The purified hepatocytes (1 g per incubation) were preincubated for 20 min at 37 °C with shaking in the O_2/CO_2 (95:5, v/v) atmosphere in 40 mL of KHB buffer containing propionate (2 mM), lactate (1 mM), pyruvate (0.1 mM), oleate (1 mM), BSA (2.5%, w/v), NH_4Cl (2 mM), and a reduced concentration of KCl (2.8 mM). The hepatocytes were then collected by centrifugation (450*g*, 30 s), resuspended in 40 mL of an identical KHB buffer prepared using [1,2,3-^{13}C]propionate (2 mM), and incubated an additional 30 min. The cells were then separated by rapid centrifugation (450*g*, 30 s), and the supernatant fraction was separated and acidified with perchloric acid (4% final concentration). These samples were then neutralized with KOH and freeze-dried.

Glucose Oxidation. Freeze-dried supernatant samples from the hepatocyte incubations or from 50 mL of each liver perfusate were redissolved into 5 mL of 50 mM sodium acetate buffer, pH 5.5, containing 250–300 units of glucose oxidase (type VII–S, Sigma Chemical Co.). Each solution was bubbled with air at 5–10 mL/min at room temperature for 4–12 h, terminated by the addition of 1 mL of 8% perchloric acid, followed by neutralization, centrifugation, and freeze-drying. The samples were dissolved into D_2O, and the pH was adjusted to 12 with 5 M KOH. This pH was chosen to give the maximum chemical shift between all gluconate carbon resonances (Jones et al., 1994).

Heart Perfusion Methods. Rat hearts were perfused in the Langendorff mode using an all-glass water-jacketed perfusion apparatus. The temperature was maintained at either 37 or 30 °C. A standard Krebs–Henseleit medium containing 1.25 mM Ca^{2+} and the ^{13}C-enriched substrate (no glucose) was

bubbled continuously with a 95% O_2/5% CO_2 mixture at a column height of 70 cm. Approximately 200 mL of perfusate was recirculated with continuous filtration throughout the experiment. Hearts were allowed to beat spontaneously, and the rate was monitored using a water-filled line from the left ventricle to a pressure transducer external to the magnet. At the indicated times, hearts were freeze-clamped, and the resulting tissue was extracted with 7% perchloric acid. After neutralization and freeze-drying, the extract was dissolved into 0.6 mL of D_2O for high-resolution NMR studies.

NMR Spectroscopy. ^{13}C NMR spectra were recorded on a GN-500 spectrometer at 11.75 T. The spin–lattice relaxation times and nuclear Overhauser enhancements of the aspartate C2 and C3 and glutamate C2, C3, and C4 carbon resonances were determined (25 °C) under solution conditions approximating those of the extracts. Values of 1.54 s (2.83), 1.12 s (2.92), 1.62 s (2.39), 1.15 s (2.62), and 1.16 s (2.67) were measured, respectively (nOe values in parentheses). All extract spectra were acquired using a 45° carbon pulse and a 6-s delay between pulses to ensure nonsaturating conditions. The samples were maintained at 25 °C during data acquisition. Resonance areas in spectra showing largely singlets were quantitated using the General Electric integration software.

Deconvolution of Multiplets. In extract spectra of livers perfused with [1,2,3-^{13}C]propionate, several of the resonances of interest (lactate C2 and gluconate C2) appear as nine-line multiplets, consisting of a singlet (S), a doublet with a spin–spin coupling constant of 34 Hz (D23), another doublet with a coupling constant of 52 Hz (D12), and a doublet of doublets (referred to here as a quartet, Q). These multiplet components have been assigned previously by us and others (Malloy et al., 1988, 1990a; Chance et al., 1983; Jones et al., 1994). Examples of such resonances are shown in Figure 4. The relative multiplet areas (lactate D23/Q and gluconate D12/Q areas) in such spectra were determined by using an automated deconvolution program available in the NMR processing program NMR-286 (Softpulse Software, Guelph, Ontario, Canada). Since the total number of multiplet resonances and the individual chemical shift of each multiplet resonance are known *a priori*, deconvolution of the multiplets into their relative areas was possible with considerable precision. The S, D34, D12, and Q areas of spectra such as those shown in Figure 4 could be evaluated with a precision better than 5%, even with different individuals performing the same deconvolution. Thus, the small variations we observed in lactate D23/Q and gluconate D12/Q areas from animal to animal (see Table 2, for example) do not reflect errors in the deconvolution process but rather small metabolic variations between animals.

EXPERIMENTAL RESULTS AND MODELS

Perfused Liver Experiments. Livers were isolated from eight animals and perfused with either [2-^{13}C]propionate (n = 5) or [3-^{13}C]propionate (n = 3) for periods ranging from 30 min to 1 h before freeze-clamping the tissue and extracting the sample for high-resolution ^{13}C NMR studies. Although the spectra of these extracts were quite complex, the lactate C2 and C3 resonances at 69 and 21 ppm, respectively, were clearly visible and easily identified from other metabolite resonances. In those livers presented with [3-^{13}C]propionate, the ratio of [2-^{13}C]lactate/[3-^{13}C]lactate resonance intensities was 0.82 ± 0.06 while in those livers presented with [2-^{13}C]-propionate, this same ratio was 1.32 ± 0.13 (Table 1). This apparent incomplete randomization of ^{13}C is less dramatic but in the same direction as previously observed in yeast after

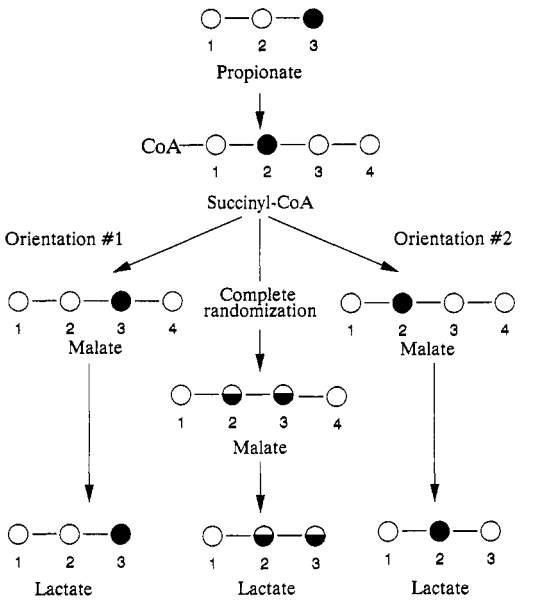

FIGURE 1: Illustration which traces the labeled carbon of [3-^{13}C]-propionate through succinyl-CoA through malate and lactate. Three possibilities are shown for the fate of the ^{13}C label in L-malate derived from [2-^{13}C]succinyl-CoA, depending upon whether complete randomization of label occurs at the symmetric intermediates, succinate and fumarate, or whether these intermediates are passed from one enzyme to another in either of two possible orientations.

Table 1: Lactate C2/C3 Intensity Ratios in Extracts of Rat Livers after Oxidation of either [2-^{13}C]- or [3-^{13}C]Propionate

labeled substrate	expt	lactate C2/C3 resonance intensities
[2-^{13}C]propionate	1	1.41
	2	1.16
	3	1.20
	4	1.50
	5	1.35
		av 1.32 ± 0.13
[3-^{13}C]propionate	6	0.77
	7	0.90
	8	0.79
		av 0.82 ± 0.06

conversion of [2-^{13}C] and [3-^{13}C]propionate to alanine (Sumegi et al., 1990).

If complete randomization of label occurred at succinate and fumarate, as normally assumed for these symmetric intermediates, then ^{13}C enrichment at lactate C2 and C3 should have been equal (Figure 1). Hence, one interpretation of these data is that there is incomplete randomization of ^{13}C label in the symmetric citric acid cycle intermediates of mitochondria in intact rat liver and at least some fraction of those symmetric intermediates is transferred between enzyme active sites without a single molecular reorientation. Two conserved molecular orientations are possible; either the C1 carbonyl carbon of succinyl-CoA becomes malate C4 (orientation 1, Figure 1) or it becomes malate C1 (orientation 2, Figure 1). The observed result implies that at least a portion of the succinate and fumarate formed in the succinyl-CoA synthase and succinate dehydrogenase reactions, respectively, was passed between enzyme active sites while retaining information about which carboxyl "end" was originally condensed with CoA, i.e., with a *conserved molecular orientation.* Another plausible interpretation of this result is that the relatively small differences in ^{13}C enrichment observed in the lactate C2 and C3 resonances could reflect a small contribution from another pathway for propionate oxidation which yields pyruvate or lactate directly without entry into

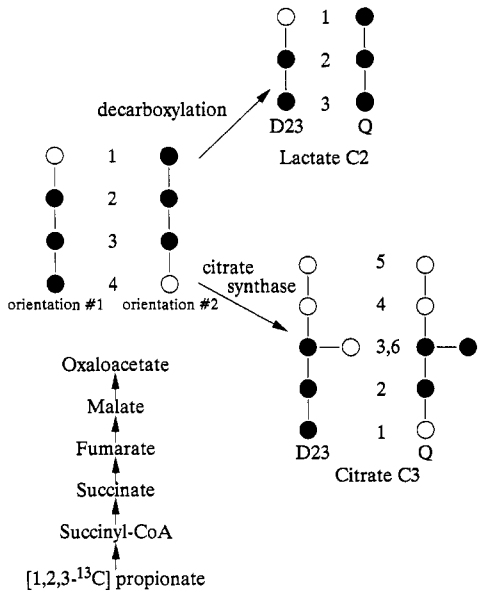

FIGURE 2: Possible ^{13}C labeling patterns in lactate C2 and citrate C3 during the oxidation of [1,2,3-^{13}C]propionate.

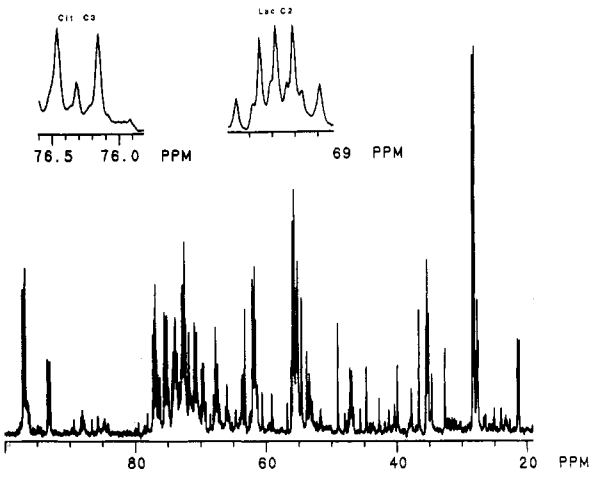

FIGURE 3: Oxidation of [1,2,3-^{13}C]propionate in perfused rat liver. The perfusate contained 20 mg of [1,2,3-^{13}C]propionate in 100 mL of Krebs bicarbonate buffer. Oxygenation of the perfusate was maintained with an atmosphere of 95% O_2 and 5% CO_2. After 30 min, the perfused liver was freeze-clamped, extracted with 10 mL of 8% perchloric acid, neutralized, freeze-dried, and redissolved in 0.7 mL of D_2O for ^{13}C NMR.

the citric acid cycle. Possible routes might include, among others, a methylcitrate pathway (Halarnkar & Blomquist, 1989) or a 3-hydroxypropionate pathway (Ando et al., 1972), both of which have been reported to be active in mammalian tissue.

To help differentiate whether a second pathway might be responsible for the differences in resonance intensities we observed, the same experiments were repeated on four additional intact livers using [1,2,3-^{13}C]propionate. Direct conversion of [1,2,3-^{13}C]propionate + oxaloacetate → methylcitrate → [1,2,3-^{13}C]pyruvate + succinate via the methylcitrate pathway would yield [1,2,3-^{13}C]lactate directly (Halarnkar & Blomquist, 1989). Likewise, conversion of [1,2,3-^{13}C]propionate to [1,2-^{13}C]acetyl-CoA via the 3-hydroxypropionate pathway (Ando et al., 1972) would yield predominantly [1,2,3-^{13}C]lactate after multiple turns of the citric acid cycle (via malate → pyruvate → lactate). Both pathways would yield more [1,2,3-^{13}C]lactate than [2,3-^{13}C]-lactate, and this should be easily detected as a larger quartet (Q) over doublet (D23) in the lactate C2 resonance. In contrast, oxidation of [1,2,3-^{13}C]propionate via the normally accepted route for propionate entry into the citric acid cycle (i.e., via succinyl-CoA) would yield 50% [1,2,3-^{13}C]malate and 50% [2,3,4-^{13}C]malate, if complete randomization of label occurred in the symmetric intermediates (see Figure 2). Hence, any lactate derived from this malate would be 50% [1,2,3-^{13}C]lactate and 50% [2,3-^{13}C]lactate, respectively. If, however, transfer of symmetric intermediates occurred with conserved orientation as suggested by the [2-^{13}C]- and [3-^{13}C]-propionate experimental results (i.e., via orientation 1), then the amount of [2,3-^{13}C]lactate formed should be greater than the amount of [1,2,3-^{13}C]lactate formed [recall that [1,2,3-^{13}C]propionate produces [1,2,3-^{13}C]succinyl-CoA due to rearrangement by methylmalonyl-CoA mutase (Walsh, 1979)]. Notice that this prediction is just opposite that described above for direct pathway conversion of propionate into lactate, so the two possibilities may be distinguished by ^{13}C NMR. This would be easily quantitated in the lactate C2 resonance as a ratio of multiplet areas; if a direct pathway (methylcitrate or 3-hydroxypropionate) was responsible for these observations, one would observe Q/D23 > 1 in the lactate C2 resonance whereas if orientation-conserved transfer of symmetric citric acid cycle intermediates via orientation 1 was responsible, one would observe D23/Q > 1.

Table 2: Lactate C2 Multiplet Areas (D23/Q) and Citrate C3 Multiplet Areas (D23/Q) from Spectra of Intact Liver Extracts, Perfusates Collected from Perfused Livers, or Isolated Hepatocytes after Exposure to [1,2,3-^{13}C]Propionate[a]

		D/Q multiplet area ratios	
sample	expt	lactate C2 (D23/Q)	citrate C3 (D23/Q)
spectra from perfused liver extracts	9	1.92	5.23
	10	3.67	n.d.
	11	1.41	1.85
	12	1.15	4.00
		D/Q multiplet area ratios	
sample	expt	lactate C2 (D23/Q)	gluconate C2 (D12/Q)
spectra of perfusates collected from perfused livers	13	1.35	1.55
	14	1.18	1.38
	15	1.15	1.43
		D/Q multiplet area ratios	
sample	expt	lactate C2 (D23/Q)	gluconate C2 (D12/Q)
spectra of medium collected from isolated hepatocytes	16	1.42	1.96
	17	2.43	1.32
	18	1.30	1.41

[a] Glucose in the perfusate samples was oxidized to gluconate before the spectra were collected.

A typical ^{13}C NMR spectrum from a neutralized, perchloric acid extract of a rat liver perfused with [1,2,3-^{13}C]propionate is shown in Figure 3. The glutamate C2 and C3 resonances (55 and 27 ppm), lactate C2 and C3 resonances (69.6 and 21.2 ppm), citrate C2 and C3 resonances (47 and 76.3 ppm), and glucose resonances (from 97 to 62 ppm) now appeared as multiplets due to nearest-neighbor ^{13}C–^{13}C couplings. Although there was some overlap of the individual multiplet resonances (the glutamate resonances overlap with those of glutamine, for example), the lactate C2 and citrate C3 resonances could be identified on the basis of their chemical shift values. Expanded plots of these resonances are shown as insets in Figure 3, and a summary of the D23/Q ratio observed in lactate C2 in four spectra (experiments 9–12) and in citrate C3 in three spectra is in Table 2. Although there

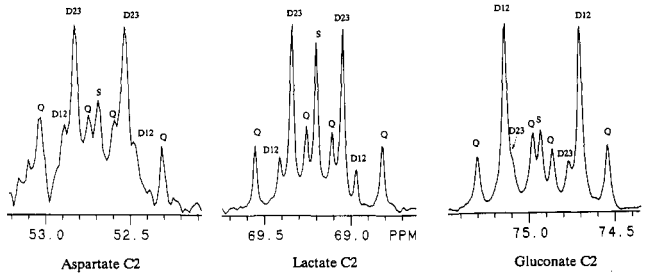

FIGURE 4: ^{13}C NMR spectra of aspartate (left) isolated from a liver extract, lactate (center) from the perfusate, and gluconate (right) from the same perfusate after oxidation of the glucose. The perfusate contained unlabeled lactate (1 mM) and pyruvate (0.1 mM) in addition to 2 mM [1,2,3-^{13}C]propionate. The D23/Q ratios in aspartate C2 and lactate C2 were 1.51 and 1.35, respectively, while the D12/Q ratio in gluconate C2 was 1.55.

appeared to be considerable variation in the ratio of D23/Q from sample to sample for reasons that are not clear at this point, the observation that D23/Q > 1 in *every* spectrum suggests that orientation-conserved transfer of symmetrical citric acid cycle intermediates must have occurred to some extent in each perfused liver. This result does not discount the possibility that some of the propionate may be converted directly into lactate via one of the pathways described above, but the fact that the [2,3-^{13}C]lactate isotopomer predominates over the [1,2,3-^{13}C]lactate isotopomer indicates that the contribution of any direct pathway must be relatively minor. The citrate C3 resonance which appeared at 76.3 ppm in three of the four extract spectra reported similar but quantitatively different results from that reported by lactate C2. As there was little labeling of glutamate C4 in these spectra (due to entry of largely unenriched acetyl-CoA in the citrate synthase reaction), the coupling observed in citrate C3 must have reflected the ^{13}C enrichment in the neighboring C2 and C6 positions only (see Figure 2 for numbering sequence). Since citrate C3 is derived from oxaloacetate C2, and D23/Q multiplet ratio in this resonance should report the same information as found in lactate C2. This trend was indeed maintained in citrate C3 where the D23/Q ratio ranged from a low of 1.85 to a high of 5.23 (Table 2).

Although the data obtained from the lactate C2 and citrate C3 resonances in spectra of liver extracts were internally consistent, it was of some concern that these resonances may have had contributions from underlying resonances of other metabolites, thereby distorting the multiplet areas. To eliminate this as a possible source of error, three additional livers were perfused with [1,2,3-^{13}C]propionate; in addition to freeze-clamping these livers, the entire perfusate which had recirculated through each liver during the 30-min perfusion period was collected and freeze-dried. One tissue extract sample was placed on an ion-exchange column as described under Materials and Methods, and the samples containing purified glutamate and aspartate were combined and scanned by NMR. All three perfusate samples containing enriched glucose, lactate, and excess [1,2,3-^{13}C]propionate were oxidized to convert the glucose to gluconate. The aspartate C2 resonance from the sample obtained by ion-exchange chromatography and gluconate and lactate C2 resonances from the perfusate sample from this same liver are shown in Figure 4. Qualitatively, these resonances look very similar, except for the larger lactate singlet due to 1 mM unenriched lactate added to the perfusate in these samples. Quantitatively, the D23/Q ratio in aspartate C2 was 1.51, and D23/Q ratio in lactate was 1.35, and the D12/Q ratio in gluconate C2 was 1.55. Data for the remaining samples are summarized in Table 2 (experiments 13–15).

Hepatocyte Experiments. Since liver tissue is heterogeneous and metabolism from differing cell types could also complicate the data analysis, similar experiments were performed with isolated hepatocytes. Hepatocytes were isolated from adult rat livers, incubated with [1,2,3-^{13}C]propionate for 30 min, and rapidly centrifuged to collect the supernatant fraction. BSA was removed from the supernatant fraction by acid precipitation, and, after freeze-drying, the glucose in the sample was oxidized to gluconate. ^{13}C spectra of the resulting perfusates were recorded, and a summary of the lactate and gluconate multiplets was added to Table 2. The average lactate D23/Q ratio was 1.72 ± 0.5, and the average gluconate D12/Q ratio was 1.56 ± 0.28 in spectra acquired from three different hepatocyte preparations. Both ratios were clearly different from unity.

Perfused Rat Heart Experiments. Data from the early 1950's using radiolabeled acetate in yeast (Ehrensvard et al., 1951) supported the idea that label is fully randomized in the symmetric citric acid cycle intermediates and continues to be supported by very recent data using [2-^{14}C]acetate in humans (Schuman et al., 1991). In both studies, equal labeling of glutamate C2 and C3 was observed. Similarly, we (Malloy et al., 1987, 1988) and numerous others (Chance et al., 1983; Lewandowski & Johnston, 1990; Weiss et al., 1992) have examined acetate metabolism in perfused hearts by ^{13}C NMR, and none of these independent investigators have noted differences between ^{13}C fractional enrichments at glutamate C2 versus C3 or at aspartate C2 versus C3. However, upon examination of the spectra reported in a ^{13}C NMR study of propionate metabolism in perfused rat hearts (Sherry et al., 1988), one finds clear asymmetry in the aspartate carbon resonances, with aspartate C4/C1 ≈ 1.3 in those hearts perfused with [1-^{13}C]propionate and aspartate C3/C2 ≈ 1.3–1.5 in those perfused with [3-^{13}C]propionate. These data agree with the liver data reported here and with the yeast data reported elsewhere (Sumegi et al., 1990). What is the origin of the apparent dichotomy that asymmetry seems to be rather easily detected when asymmetrically ^{13}C-enriched succinyl-CoA is derived from [^{13}C]propionate but not when it is derived from [^{13}C]acetyl-CoA?

The pathway for forming asymmetrically enriched succinyl-CoA from [2-^{13}C]acetyl-CoA (enriched in the methyl carbon) is summarized in Figure 5. If succinate and fumarate are transferred between enzyme sites with conserved molecular orientation via orientation 1 as the liver and yeast data suggest, then [3-^{13}C]succinyl-CoA would only yield [2-^{13}C]oxaloacetate on the first pass through the cycle pools. This ^{13}C would appear at glutamate C3 on the second pass (glutamate C2 would not be enriched at this point) and at glutamate C2 on the third pass. The net result is that glutamate C2 and C3 would have equal enrichments after three or more turns through the cycle pools *even if* 100% of the symmetric intermediates were passed between enzymes with a conserved molecular orientation via direction 1. This means that one cannot differentiate between transfer of symmetric intermediates via orientation 1 *versus* complete randomization of symmetric intermediates in any sample exposed to isotope long enough for the cycle to turn over a minimum of 3 times. Thus, it may be extremely difficult to find experimental conditions which would unequivocally differentiate between these two alternatives.

A high-resolution ^{13}C NMR spectrum of a perchloric acid extract of a heart perfused to isotopic and metabolic steady-state with [2-^{13}C]acetate is shown in Figure 6A. [2-^{13}C]-Acetate provides about 95% of the acetyl-CoA entering the

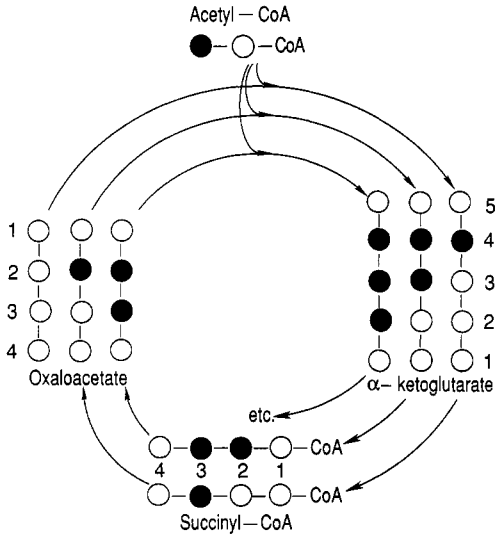

FIGURE 5: Schematic diagram which illustrates that the fractional ^{13}C enrichment of glutamate C2 and C3 becomes equal after three turns through the cycle pools if the symmetric intermediates are passed from one enzyme to another via orientation 1. Note, the number of turns required to achieve equal enrichment would increase if the ^{13}C- enrichment of the acetyl-CoA methyl carbon was not 100% and the pool sized were not infinitesimally small. The figure illustrates that the glutamate C3/C2 ratio would be >1 only very early during isotopic turnover.

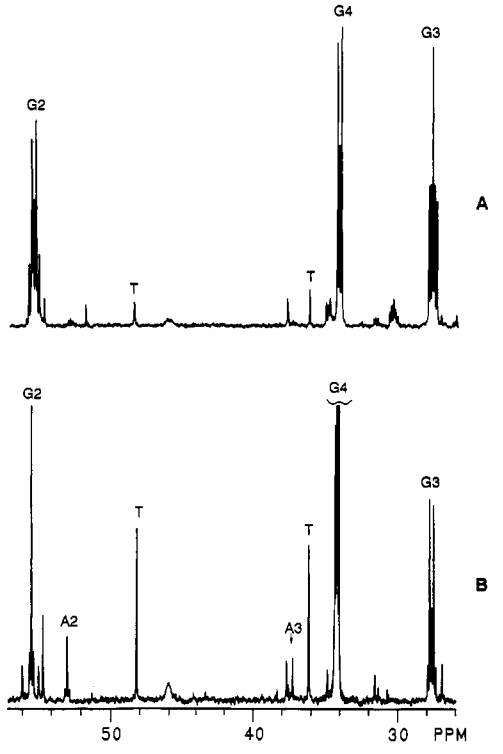

FIGURE 6: (A) ^{13}C NMR spectrum of an extract of a rat heart which had been perfused to steady-state with 10 mM [2-^{13}C]acetate. (B) ^{13}C NMR spectrum of a rat heart perfused with 5 mM [2-^{13}C]acetate for 3 min before freeze-clamping. The resonances labeled G2, G3, and G4 correspond to the glutamate carbons, those labeled A2 and A3 correspond to aspartate carbons, and those labeled T correspond to natural-abundance taurine resonances.

heart mitochondria under these conditions, and consequently glutamate was highly enriched. The relative ^{13}C fractional enrichment in glutamate C2, C3, and C4 in this sample was 1:1:1, exactly as one would predict if there was random scrambling of ^{13}C in the symmetric four-carbon intermediates in the citric acid cycle and as observed in most other labeling studies. However, the model presented in Figure 5 indicates

Table 3: Time Course of ^{13}C Incorporation into Glutamate C2, C3, and C4 during Oxidation of [2-^{13}C]Acetate in an Intact, Perfused Rat Heart in Situ[a]

length of perfusion (min)	^{13}C fractional enrichments	
	glutamate C3/C2	glutamate C4/C3
3	2.0	2.1
6	1.7	2.0
9	1.5	1.8
12	1.2	1.4
15	1.2	1.4
18	1.06	1.3

[a] 2 mM [2-^{13}C]acetate was added to the perfusate at $t = 0$, and spectra were acquired every 3 min in an 11.75-T magnet.

the same result would be observed if the cycle pools turned over >3 times *and* the succinate → fumarate → malate transfers occurred via orientation 1 with 100% efficiency. Thus, a single steady-state spectrum of the protonated glutamate carbons alone cannot distinguish between orientation-conserved transfer of symmetric intermediates versus complete randomization of label in these metabolites.

In an attempt to test the hypothesis that complete randomization of metabolites may not be occurring in heart mitochondria, we reexamined some data reported previously (Malloy et al., 1990b) on intact Langendorff perfused rat hearts where ^{13}C spectra were recorded at 3-min time intervals in an 11.75-T magnet. The relative ^{13}C fractional enrichments in glutamate C2, C3, and C4 measured in those spectra and summarized in Table 3 clearly indicate there is a significant difference in the glutamate C3/C2 resonance areas early after the addition of [2-^{13}C]acetate, while after 12 min there is less than 20% difference between these resonance areas. The data also show that the C3/C2 ratio approaches 1 somewhat more quickly than the C4/C3 ratio approaches its steady-state value. One could argue that our model would predict this trend since the C3/C2 ratio should approach unity after the intermediate pools have turned over approximately 3 times while the C4/C3 ratio reflects an approach to isotopic steady-state, which requires more like six to seven turns through the cycle pools (Malloy et al., 1990a). These data suggest that detection of orientation-conserved transfer of intermediates is temporal and that NMR methods or radioisotope methods may only detect differences in fractional enrichment during the first few minutes after presentation of an isotope which enters the cycle as acetyl-CoA.

As the signal-to-noise ratio obtainable on an intact, perfused rat heart over a 3-min interval may be too low to accurately quantitate resonance areas [see spectra in Malloy et al. (1990b), Figures 3 and 4], we perfused another group of hearts with 5 mM [2-^{13}C]acetate for 3 min and then freeze-clamped the tissue. The ^{13}C NMR spectrum ot an extract of one heart is given in Figure 6B. This spectrum is different from that shown in Figure 6A because the cycle pools had not reached isotopic steady-state after the 3-min perfusion period. A comparison of glutamate C3/C2 and aspartate C2/C3 resonance areas (in this case, the total resonance areas were measured, not the relative multiplet areas as in the propionate studies) in spectra from three different hearts freeze-clamped after 3 min gave values of 1.25 ± 0.11 and 1.84 ± 0.10, respectively. The absolute value of glutamate C3/C2 measured in the extract spectra was different from that reported in Table 3 because the intact heart spectra were collected under partial saturation conditions. Nevertheless, the glutamate C3 intensity was significantly greater than the glutamate C2 intensity in both experiments. It is also noteworthy that the aspartate C2/C3 ratio was also greater

than unity in these 3-min spectra. This is expected since [2-^{13}C]oxaloacetate (equal to [2-^{13}C]aspartate) becomes [3-^{13}C]glutamate on each cycle turn (see Figure 5); hence, glutamate C3/C2 and aspartate C2/C3 should provide similar information.

We have also perfused a heart with 5 mM [2-^{13}C]acetate at 30 °C in an attempt to slow isotopic turnover through the cycle pools. ^{13}C NMR spectra of an intact heart signal-averaged over 6 min in our 11.75-T magnet gave a glutamate C3/C2 ratio of 1.46 (spectra not shown) in the first 6-min spectrum. This ratio gradually approached unity after about 48–50 min, consistent with the observations presented in Table 3 (total citric acid cycle flux was slower in this heart because of the temperature differential).

DISCUSSION

When asymmetrically labeled succinyl-CoA is converted to malate in the Krebs citric acid cycle, one normally assumes that the label will be equally distributed in malate (either between C1 and C4 or between C2 and C3) due to randomization of label at the two symmetric intermediates, succinate and fumarate. Data from the early 1950's using radiolabeled acetate in yeast (Ehrensvard et al., 1951) and very recent data using [2-^{14}C]acetate in humans (Schumann et al., 1991) continue to support the hypothesis that isotopes in symmetric citric acid cycle intermediates are fully randomized. Since a large amount of experimental data exists concerning the organization of functionally related enzymes, we recently reexamined this question in cultured yeast cells during oxidation of ^{13}C-labeled propionate (Sumegi et al., 1990) using ^{13}C NMR techniques. In that system, we observed asymmetric labeling of intermediates derived from L-malate by ^{13}C NMR and concluded that a significant fraction of [2-^{13}C]succinyl-CoA (derived from [3-^{13}C]propionate) appears as [3-^{13}C]malate after passing through the succinate and fumarate pools in this span of the citric acid cycle. That study also showed that it is quite difficult to find experimental conditions where asymmetric labeling is detectable; both a low respiratory rate resulting from a poor O_2 supply and added inhibitors which decreased flux through malate dehydrogenase and/or fumarase altered those observations. Bernhard and Tompa (1990) also investigated this issue in isolated rat liver mitochondria using [5-^{13}C]glutamate to generate asymmetrically labeled succinyl-CoA and were unable to detect any differences in ^{13}C enrichment in aspartate C1 versus aspartate C4. These apparent disparate observations have been recently debated in journal club articles (Shulman, 1991; Rognstad, 1991; Srere, 1990, 1991), with two principal arguments against transfer of symmetric intermediates with conserved orientation: (1) the simple fact that this phenomenon has not been detected in numerous radiolabeling and NMR experiments involving substrates oxidized via acetyl-CoA; and (2) the results observed in yeast with ^{13}C-labeled propionate might be explained by small contributions from propionate pathways which do not include succinyl-CoA.

In this work, we have used several ^{13}C-labeled substrates catabolized by well-established metabolic pathways in two different mammalian tissues. The ^{13}C spectra of rat hearts freeze-clamped shortly after addition of [2-^{13}C]acetate show clear differences between the intensity of aspartate C2 versus C3 and between glutamate C2 versus C3. Both observations (aspartate C2/C3 > 1 and glutamate C3/C2 > 1) are consistent with partial orientation-conserved transfer of intermediates between succinyl-CoA synthase → succinate dehydrogenase → fumarase via orientation 1 (Figures 1, 2, and 5), the same orientation as detected previously in yeast. We cannot exclude the possibility that an unknown pathway is responsible for these observations, but it is difficult to imagine that in one of the most widely studied tissues, the rat heart, a significant portion of acetyl-CoA is oxidized via an unknown pathway. The observation that this asymmetry exists only during the first 10–15 min of perfusion (at 37 °C) and during the first 45–50 min of perfusion (at 30 °C) and then disappears after longer periods makes our model for orientation-conserved transfer even more compelling. The simple model presented in Figure 5 suggests that previous labeling studies (either by NMR or by radioisotope methods) may not have detected this phenomenon because it is impossible to differentiate between orientation-conserved transfer of substrates via orientation 1 versus complete randomization of labeled intermediates in any experiment at steady-state.

The question of alternate propionate pathways is also less ambiguous in mammalian tissue than in bacterial or yeast systems. Previously reported ^{13}C NMR spectra of extracts of rat hearts perfused with ^{13}C-enriched propionates (Sherry et al., 1988) show clear asymmetry in the aspartate carbon resonances, with aspartate C4/C1 > 1 in those perfused with [1-^{13}C]propionate and aspartate C3/C2 > 1 in those perfused with [3-^{13}C]propionate. This is exactly the same asymmetry as observed here in the lactate resonances seen in extracts of freeze-clamped livers after exposure to either [2-^{13}C]- or [3-^{13}C]propionate. An important concern is that the small differences in enrichment at C2 and C3 of lactate could simply be an artifact related to minor differences in nOe or T_1 values. However, the observation that the opposite preferential enrichment appeared in these same molecules when we used [2-^{13}C]- versus [3-^{13}C]propionate argues that our observations are not an experimental artifact. Furthermore, the ^{13}C multiplet ratios observed in the citrate C3, aspartate C2, lactate C2, and gluconate C2 resonances of spectra of intact liver extracts and of perfusates from isolated hepatocytes presented with [1,2,3-^{13}C]propionate are *all* consistent with orientation-conserved transfer of citric acid cycle intermediates via orientation 1, and this analysis does not require a comparison of ^{13}C enrichments at different carbon sites within these molecules. The variations we observed in the multiplet ratios from animal to animal (see Table 2) appear to be real, perhaps reflecting small metabolic variations between animals involving relatively minor differences in restricted metabolite diffusion within their mitochondria. A recent fluorescence anisotropy study (Scalettar et al., 1991) was shown that diffusion of a small, fluorescent metabolite probe located within the inner matrix of isolated liver mitochondria is quite sensitive to the mitochondrial respiratory state. This interesting study suggests that orientation-conserved transfer of fumarate and succinate of the type we are proposing here may be sensitive to volume-dependent regulation of protein packing within the mitochondrial matrix.

Experiments with [1,2,3-^{13}C]propionate support our contention that a pathway which might allow some direct conversion of propionate to lactate is not the origin of these observations; if that situation should arise, [1,2,3-^{13}C]lactate would be formed in preference to [2,3-^{13}C]lactate. Our observation that [2,3-^{13}C]lactate is formed in excess over [1,2,3-^{13}C]lactate in all cases (ratio of 2 to 1, average from four livers) provides further evidence that orientation-conserved transfer of symmetric intermediates between citric acid cycle enzymes in liver mitochondria does indeed occur. The apparent numerical differences between the average D23/Q ratios in lactate C2 versus citrate C3 may reflect the

fact that a portion of the malate which leaves the mitochondria could become partially "scrambled" through cytosolic fumarase before being converted to lactate (oxaloacetate → PEP → pyruvate → lactate). Since citrate is formed in liver mitochondria by direct condensation of oxaloacetate with acetyl-CoA, the citrate isotopomer distribution may more accurately reflect the oxaloacetate isotopomers present within the mitochondrial space.

The rotational correlation time of succinate and fumarate in water is under 100 ps (Akitt, 1987) while the average lifetime of one succinate molecule is about 1 s (calculated from the succinate concentration and SDH activity) (Sumegi & Alkonyi, 1983). This calculation and the data presented here suggest that a significant portion of succinyl-CoA is converted to malate without release of either succinate or fumarate from enzyme active sites. It is also quite clear from our data that this process is *not* 100% efficient. Those succinate or fumarate molecules released from catalytic surfaces into the matrix might rotate enough to randomize the label in malate upon reentering the pathway. Another possibility is that transfer of intermediates in this span of the cycle with conserved orientation is indeed quite efficient but that a portion of asymmetrically labeled malate is tranpsorted out of the mitochondria where it equilibrates with fumarate in the cytosol before reentering the cycle. In the cytoplasmic environment, these intermediates may more freely rotate (Scalettar et al., 1991) and hence contribute to that portion of the observed symmetrically labeled metabolites. If this is the case, the asymmetry measured in aspartate C2/C3 and in glutamate C3/C2 would report only the lower limit for orientation–conserved transfer of intermediates in this span of the cycle.

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