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Limitations of detection of anaplerosis and pyruvate cycling from metabolism of [1-¹³C] acetate

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Recently Befroy and colleagues measured TCA cycle flux and anaplerosis in human liver by analyzing ¹³C enrichment in carbon 1 and carbon 5 of glutamate during [1-¹³C]acetate infusion¹. The entry of [1-¹³C]acetyl-CoA into the cycle yields [5-¹³C]glutamate on each turn of the cycle but [1-¹³C]glutamate only on subsequent turns. Anaplerosis, for instance via pyruvate carboxylation, results in the influx of unlabeled oxaloacetate. The subsequent loss of ¹³C-oxaloacetate from the cycle to make phosphoenolpyruvate (PEP) decreases the enrichment in glutamate-C1 but not glutamate-C5. Hence, the steady-state ratio of [1-¹³C] to [5-¹³C]glutamate should reflect anaplerosis relative to TCA cycle activity (i.e. citrate synthase flux = 1). PEP formed from anaplerosis has at least two fates, either in gluconeogenesis (GNG) or to regenerate pyruvate via pyruvate kinase (PK). PK, and other pathways (e.g. malic enzyme), contribute to a futile pyruvate cycle whereby oxaloacetate → pyruvate → oxaloacetate. The relationship among these variables is simple: GNG = anaplerosis – PK flux. This pathway has been included in virtually all models of hepatic GNG²⁻⁹. In humans PK flux, relative to citrate synthase (CS), was 1.5 to 5.8 when determined using a [3-¹⁴C]lactate tracer with radioisotope detection,⁸ [3-¹³C]lactate tracer with mass isotope detection³ or [U-¹³C]propionate tracer with NMR detection^{6,9}. In fact, pyruvate cycling accounted for the majority of anaplerosis in these human studies^{3,6,8,9}, yet was excluded from anaplerosis by Befroy et al.¹ This exclusion complicates the analysis considerably because pyruvate cycling in the presence of H¹³CO₃⁻, as observed in the *in vivo* ¹³C NMR spectra¹, regenerates [1-¹³C]glutamate. Hence, the steady-state ratio of [1-¹³C] to [5-¹³C]glutamate no longer reflects total anaplerosis. Given the implications, it is

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worth examining the effect of pyruvate cycling on ^{13}C in glutamate C1 and C5 using the conditions reported by Befroy et al.

Carbon-13 labeling in glutamate is a complex function of pyruvate cycling, anaplerosis and enrichment in acetyl-CoA and bicarbonate^{4,10}. Consequently, simulations must be used to explore these interactions¹⁰. Using published relationships¹⁰ and the conditions reported by Befroy et al., glutamate $^{13}\text{C}1/^{13}\text{C}5$ is sensitive to total anaplerosis if $^{13}\text{CO}_2 = 0$ and pyruvate cycling = 0 but much less sensitive when $^{13}\text{CO}_2 = 6\%$ ¹ and pyruvate cycling is active²⁻⁹ (Figure 1). The glutamate $^{13}\text{C}1/^{13}\text{C}5$ ratio reported by Befroy et al. is indeed consistent with substantial pyruvate cycling (Figure 1), as previously described in human liver^{3,6,8,9}. However, the presence of pyruvate cycling also means that a 5-fold range in anaplerosis is essentially undetectable by the glutamate $^{13}\text{C}1/^{13}\text{C}5$ ratio. Thus, this method cannot be used to support the conclusion by Befroy et al., that other methods have overestimated anaplerosis¹, since the glutamate $^{13}\text{C}1/^{13}\text{C}5$ ratio cannot detect total anaplerosis under these conditions (Figure 1).

Futile cycles serve a role in the regulation of metabolism. Earlier studies, including those in humans, found that the futile cycle interconverting pyruvate and oxaloacetate is unquestionably active in liver regardless of whether lactate or propionate tracers are used²⁻⁹. Thus, it is best to assume that pyruvate cycling is always a feature of liver metabolism and to incorporate this possibility in data analysis.

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Figure 1.

The effect of pyruvate cycling on the steady-state $^{13}\text{C}_1/^{13}\text{C}_5$ of glutamate. Data was simulated using program tcaSIM (see source data). All simulations were conducted using an $[1-^{13}\text{C}]\text{acetyl-CoA}$ fractional enrichment of 0.22 and $^{13}\text{CO}_2$ fractional enrichment of 0.06 as reported by Befroy et al.¹ Anaplerosis relative to TCA cycle flux (CS) was varied from 1.4, as reported by Befroy et al.¹, to 8. The $^{13}\text{C}_1/^{13}\text{C}_5$ ratio decreases by 40% across this range under the assumption of no pyruvate cycling (e.g. PK) activity. The $^{13}\text{C}_1/^{13}\text{C}_5$ ratio is unresponsive to anaplerosis when pyruvate cycling is concomitantly active because the resulting ^{13}C pyruvate condenses with $^{13}\text{CO}_2$ (i.e. PC) and ultimately re-generates $[1-^{13}\text{C}]\text{glutamate}$. Most notably, the $^{13}\text{C}_1/^{13}\text{C}_5$ method used by Befroy et al.¹ cannot distinguish low anaplerosis from higher values⁹ criticized by Befroy et al.¹