[¹³C]Propionate oxidation in wild-type and citrate synthase mutant *Escherichia coli*: evidence for multiple pathways of propionate utilization

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The metabolism of propionate was examined in wild-type *Escherichia coli* and cells lacking citrate synthase by high-resolution ¹³C n.m.r. Spectra of cell extracts from wild-type *E. coli* show that glutamate becomes highly enriched in ¹³C when ¹³C-enriched propionate is the sole carbon source. No glutamate labelling was detected when the tricarboxylic acid cycle was blocked either by deletion of citrate synthase or by inhibition of succinate dehydrogenase by malonate. The ¹³C fractional enrichment in glutamate C-2, C-3 and C-4 in wild-type cells was quantitatively and qualitatively different when [2-¹³C]propionate as opposed to [3-¹³C]propionate was supplied. Approximately equal labelling occurred in the C-2, C-3 and C-4 positions of glutamate when [3-¹³C]propionate was available, and multiplets

due to carbon–carbon spin–spin coupling were observed. However, in cells supplied with [2-¹³C]propionate, very little ¹³C appeared in the glutamate C-4 position, and the remaining glutamate resonances all appeared as singlets. The unequal and non-identical labelling of glutamate in cells supplied with [2-¹³C]- as opposed to [3-¹³C]propionate is consistent with the utilization of propionate by *E. coli* via two pathways, oxidation of propionate to pyruvate and carboxylation of propionate to succinate. These intermediates are further metabolized to glutamate by the action of the tricarboxylic acid cycle. The existence of an organized tricarboxylic acid cycle is discussed as a consequence of the ability to block utilization of propionate in tricarboxylic acid-cycle-defective *E. coli*.

INTRODUCTION

The functional co-ordination of metabolic pathways *in vivo* is central for aerobic energy production and metabolite interconversions in all cells. Thus understanding the relative flow of intermediates through specific pathways *in vivo* has been a basic goal of research in metabolic regulation. Much of our understanding of cellular metabolism, however, is based upon *in vitro* experiments where purified enzymes, substrates and putative regulators are present under non-physiological conditions. As research in cellular metabolism progresses, new methods have become available that enable us to understand these processes in the context of their cellular environment. Current ¹³C n.m.r. technology and molecular biology provide two sensitive and comprehensive methods to study emerging questions in cellular metabolism.

One such question involves the relationships among the tricarboxylic acid cycle, propionate oxidation and propionate carboxylation. All known pathways for the utilization of propionate by *E. coli* use part of the tricarboxylic acid cycle (Scheme 1). Propionate enters the cell via the acetate-transport system and is activated to propionyl-CoA. The fate of propionyl-CoA then may follow one of two pathways that ultimately leads to production of acetyl-CoA or succinyl-CoA, which enter the tricarboxylic acid cycle at the citrate synthase and succinate dehydrogenase steps respectively. Oxidation of propionyl-CoA to pyruvate was inferred from radiorespirometric experiments [1–3], and by detection of enzyme activities involving this pathway in crude extracts of *E. coli* [2,4,5]. In several systems, including higher eukaryotes, the major route of propionate metabolism is via the propionyl-CoA carboxylase pathway. Propionate first is activated to propionyl-CoA, then carboxylated to methyl-

malonyl-CoA, and finally isomerized to succinyl-CoA by the vitamin B₁₂-requiring enzyme methylmalonyl-CoA mutase [6,7]. This pathway is also important in a number of micro-organisms, including *Propionibacterium* [8], *Ochromonas* [9], *Rhizobium* [10] and *E. coli* [2].

Traditional approaches to detecting substrate metabolism, such as release of ¹⁴CO₂ from ¹⁴C-enriched propionate, do not distinguish between propionate oxidation to acetyl-CoA and propionate carboxylation to succinyl-CoA. Analysis of the activity of these pathways *in vivo* is further complicated by the presence of the glyoxylate cycle, which is found in some species, including *E. coli*. When micro-organisms are grown on acetate, the glyoxylate cycle replenishes C₄ acids drained from the TCA cycle for cellular biosynthesis [11].

In the present study we have characterized the relationship between alternative pathways for propionate metabolism in *E. coli*. In order to determine the relative contribution of each pathway, the metabolic products of [2-¹³C]- and [3-¹³C]-propionate were compared in wild-type *E. coli* and a mutant of *E. coli* lacking citrate synthase by ¹³C n.m.r. The activity of the glyoxylate cycle was also included in the analysis. The distribution of label in the 1-, 2-, 3-, 4- and 5-positions of glutamate and the resulting isotopomers were measured by ¹³C n.m.r., and these data were used to calculate flux ratios through the two propionate pathways.

METHODS

Materials

Sodium [2-¹³C]- and [3-¹³C]-propionate (98 % ¹³C) was obtained from MSD Isotopes (St. Louis, MO, U.S.A.). All other chemicals were of the highest purity commercially available.

Scheme 1 Flow diagram showing the ^{13}C -isotope distribution during one turn of the citric acid cycle with $[3\text{-}^{13}\text{C}]$ propionate as the supplied substrate

(a) $[3\text{-}^{13}\text{C}]$ propionate is oxidized to $[3\text{-}^{13}\text{C}]$ pyruvate which is transformed to $[2\text{-}^{13}\text{C}]$ acetyl-CoA, before entering the tricarboxylic acid cycle. Note that multiple turns of the cycle will produce glutamate enriched at neighbouring carbon atoms (C-1, C-2, C-3 and C-4), resulting in spin-spin coupling in all resonances. (b) $[3\text{-}^{13}\text{C}]$ propionyl-CoA is carboxylated to form $[2\text{-}^{13}\text{C}]$ succinyl-CoA which enters the tricarboxylic acid cycle. ^{13}C which enters the cycle pools via this route will label either glutamate C-2 or glutamate C-3, but never both carbon atoms. Hence spin-spin coupling is not observed in any intermediates labelled via this pathway. Symbols: ●, ^{13}C ; ○, 50:50 chance of ^{12}C or ^{13}C in this site.

Strains and culture conditions

Wild-type *E. coli* strain 23559 *met* was generously provided by Professor John Guest (Department of Microbiology and Biotechnology, University of Sheffield, Sheffield, U.K.) and was derived from the *E. coli* W strain. *E. coli* strain DEK15 *gltA6 thil pyrD36 galK30 rpsL129 supE44 recA met* (Dr. Kenneth Walsh, Case Western Reserve University, Cleveland, OH, U.S.A.) was derived from the parental strain 23559 and contains a mutation in the *gltA* gene encoding *E. coli* citrate synthase. Cells mutant for citrate synthase did not grow on minimal media containing acetate as the sole carbon source but did grow in minimal media containing glutamate. For labelling experiments the cells were grown in standard Luria-Bertani [12] medium overnight, then

Figure 1 Proton-decoupled ^{13}C n.m.r. spectrum of extracted metabolites following labelling with $[2\text{-}^{13}\text{C}]$ propionate

E. coli cells (1.0 g) were grown in either (a) Luria-Bertani or (b) minimal propionate + vitamin B_{12} medium. Cell pellets were resuspended in 15 ml of M9 medium as described in the Methods section and labelled for 1 h with $[2\text{-}^{13}\text{C}]$ propionate. Peaks: G2-G4, C-2-C-4 of glutamate; P2, C-2 of propionate.

transferred to supplemented M9 medium [12] (containing 0.2% propionate, 2 mM MgSO_4 , 0.1 mM CaCl_2 , 40 $\mu\text{g/ml}$ methionine, 0.01% succinate, 0.5 $\mu\text{g/ml}$ vitamin B_{12} , 10 $\mu\text{g/ml}$ thiamin and 5 mM NaHCO_3) and precultured for 48 h at 37 °C [2]. Propionate precultures (100 ml) were used to inoculate 10 l of supplemented M9, and the cells were grown for an additional 48 h at 37 °C.

Propionate oxidation

A 1 g portion of packed *E. coli* cells was washed in 50 ml of standard M9 medium, then resuspended in 15 ml of M9 medium containing 0.5 $\mu\text{g/ml}$ vitamin B_{12} , 10 $\mu\text{g/ml}$ thiamin, 40 $\mu\text{g/ml}$ methionine, 20 mM KPO_4 , pH 7.5, and 10 mg of either $[2\text{-}^{13}\text{C}]$ - or $[3\text{-}^{13}\text{C}]$ -propionate (sodium salt). The resuspended *E. coli* cells were incubated for 1 h at 37 °C and shaken vigorously to supply the required amount of oxygen. The reaction was stopped by adding HClO_4 to a final concentration of 4% and freezing at -70 °C. The samples were thawed at 25 °C, and the acid-insoluble material was removed by centrifugation. The supernatant solution was neutralized with 10 M KOH, centrifuged to

Figure 2 ^{13}C isotopomer multiplet patterns in the C-1, C-2, C-3, C-4 and C-5 carbon atoms of glutamate (peaks G1–G5)

Cells (1.0 g) were grown in minimal propionate + vitamin B_{12} medium, resuspended in 15 ml of M9 medium, and labelled for 1 h with either (a) 10 mg of $[2\text{-}^{13}\text{C}]$ propionate or (b) 10 mg of $[3\text{-}^{13}\text{C}]$ propionate.

remove insoluble material, and freeze-dried. The resulting powder was redissolved in 0.8 ml of $^2\text{H}_2\text{O}$ for n.m.r. analysis.

N.m.r. spectroscopy

High-resolution ^{13}C n.m.r. spectra were recorded on a GN-500 spectrometer at 11.75 T. All spectra were acquired by using a 45° carbon pulse and a 6 s delay between pulses to ensure non-saturating conditions. All samples were maintained at 25 °C during data acquisition.

Protein and enzyme determination

Samples of whole cell extracts were assayed for protein [13] and citrate synthase activity [14].

RESULTS

To determine the mechanism by which *E. coli* forms glutamate from propionate, we studied the conversion of various labelled propionates under several growth conditions and with different *E. coli* constructs.

Induction of propionate carboxylation

In agreement with previous reports [1,2], exponential growth on propionate was stimulated by inclusion of vitamin B_{12} and HCO_3^- in the media. Little utilization of $[2\text{-}^{13}\text{C}]$ propionate or labelling of glutamate occurred when cells were grown in rich medium lacking in B_{12} (Figure 1a). However, when cells were grown in a minimal propionate medium containing vitamin B_{12} and then exposed to $[2\text{-}^{13}\text{C}]$ propionate, there was a significant increase in the labelling of glutamate C-2 and C-3 (Figure 1b). The labelling pattern was consistent with the entry of ^{13}C into the citric acid cycle via succinyl-CoA derived from propionate, and confirmed previous observations indicating that growth of *E. coli* on propionate involves an adaptive process and the induction of

B_{12} -requiring enzymes [2]. There was no difference between the total protein content and citrate synthase activity of wild-type cells grown in minimal or rich media (1.1 ± 0.1 mg/ml and 1.0 ± 0.1 unit/mg respectively).

Propionate oxidation to acetyl-CoA

Direct oxidation of propionate to acetyl-CoA is also possible. These alternative routes were examined by comparing the ^{13}C spectra of wild-type *E. coli* when presented with either $[2\text{-}^{13}\text{C}]$ - or $[3\text{-}^{13}\text{C}]$ propionate. ^{13}C enrichment in C-2, C-3 and C-4 of glutamate was qualitatively and quantitatively different when $[2\text{-}^{13}\text{C}]$ propionate was the sole substrate compared with $[3\text{-}^{13}\text{C}]$ propionate (Figure 2). When $[2\text{-}^{13}\text{C}]$ propionate was supplied, ^{13}C - ^{13}C coupling was not detected in any of the glutamate resonances, while significant ^{13}C - ^{13}C coupling was observed in all glutamate resonances when $[3\text{-}^{13}\text{C}]$ propionate was presented to the cells. Figure 2 compares the enrichment levels and labelling patterns in glutamate C-3 (27.6 p.p.m.), C-4 (34 p.p.m.), C-2 (55.2 p.p.m.), C-1 (175.5 p.p.m.) and C-5 (182.2 p.p.m.) in extracts of wild-type cells presented with $[2\text{-}^{13}\text{C}]$ propionate versus $[3\text{-}^{13}\text{C}]$ propionate. $[3\text{-}^{13}\text{C}]$ Propionate quantitatively resulted in significantly higher enrichment levels in glutamate C-2, C-3 and C-4, but relatively lower enrichment levels in glutamate C-1 and C-5, than did $[2\text{-}^{13}\text{C}]$ propionate. More importantly, these spectra illustrate the dramatic differences in glutamate enrichment pattern produced by propionate labelled in C-2 compared with C-3. The spin-spin coupling observed in the $[3\text{-}^{13}\text{C}]$ propionate spectra indicate that a significant fraction of propionate also enters the tricarboxylic acid cycle via citrate synthase as methyl-labelled acetyl-CoA.

Propionate utilization during citric acid-cycle inhibition

The experimental results described above indicate that propionate is utilized via an acetyl-CoA pathway and a propionate

Figure 3 Proton-decoupled ^{13}C n.m.r. spectrum of extracted metabolites following labelling with $[3\text{-}^{13}\text{C}]\text{propionate}$

E. coli cells (1.0 g) were grown in minimal propionate + vitamin B_{12} medium, resuspended in 15 ml of M9 medium, and labelled for 1 h with $[3\text{-}^{13}\text{C}]\text{propionate}$. (a) Wild-type *E. coli* 23559; (b) CS^- (citrate synthase-defective) *E. coli* DEK15; and (c) wild-type *E. coli* 23559 labelled in the presence of 65 mM malonate. Peaks: G2–G4, C-2–C-4 of glutamate; P3, C-3 of propionate.

carboxylation pathway in *E. coli*. Propionate metabolism also was examined in (a) cells lacking citrate synthase and (b) in wild-type cells exposed to malonate, a competitive inhibitor of

succinate dehydrogenase, to determine whether a block in the entry of the ^{13}C label into the tricarboxylic acid cycle at either of these steps increases the flux via the opposite pathway. When a citrate synthase-defective *E. coli* strain was cultured in minimal propionate media containing 0.01 % glutamate (to supply substrate), the cells unexpectedly failed to incorporate ^{13}C from $[3\text{-}^{13}\text{C}]\text{propionate}$ into any tricarboxylic acid-cycle intermediates, despite the induction of the propionate carboxylation pathway (Figure 3b). Wild-type *E. coli* was grown as a control in minimal propionate media containing 0.01 % glutamate, and these cells incorporated $[3\text{-}^{13}\text{C}]\text{propionate}$ into C-1–C-5 of glutamate just as did wild-type cells grown in the absence of glutamate (Figure 3a). Labelling in C-1–C-5 of glutamate also was blocked completely when wild-type *E. coli* cells were grown in minimal propionate media in the presence of malonate, a competitive inhibitor of succinate dehydrogenase (Figure 3c). These results not only indicate that both spans of the citric acid cycle, namely the oxalacetate-to-citrate span and the succinate-to-fumarate span, are necessary, but also that propionyl-CoA carboxylation is essential for the conversion of propionate into glutamate by *E. coli*.

DISCUSSION

The ^{13}C enrichment pattern of glutamate and other metabolites has been useful in determining the relative contributions of the pathways which lead to their synthesis from glucose [15,16] and pyruvate or acetate [17–19]. We have used this technique to study propionate metabolism in *E. coli*. The proton-decoupled ^{13}C -n.m.r. spectrum of extracted metabolites was obtained from aerobically grown cultures in the presence of 98 % $[2\text{-}^{13}\text{C}]$ - or 98 % $[3\text{-}^{13}\text{C}]\text{propionate}$ for 1 h. Since glutamate was easily detected in these extracts and its labelling pattern should reflect the ^{13}C isotopic distribution in α -oxoglutarate, we analysed the ^{13}C – ^{13}C splitting pattern and relative peak intensities of all five glutamate resonances to derive information about propionate entry into the tricarboxylic acid cycle.

The observation that the $[2\text{-}^{13}\text{C}]\text{propionate}$ -fed cells yielded glutamate highly ^{13}C enriched in C-5 (Figure 2) indicates that a large amount of ^{13}C entered the citric acid cycle as $[1\text{-}^{13}\text{C}]\text{acetyl-CoA}$, presumably derived via a propionate $\rightarrow$ pyruvate $\rightarrow$ acetate pathway. However, if this were the only entry of ^{13}C into the cycle pools, glutamate C-2 and C-3 would not have become enriched in this experiment. The spectra show that this is not the case. The singlets appearing in these resonance positions clearly indicate that some $[2\text{-}^{13}\text{C}]\text{propionate}$ also enters the cycle pools via the carboxylation pathway, i.e., through succinyl-CoA. The need for vitamin B_{12} and CO_2 with the induction of propionate utilization also indicates that this pathway is operative.

An analysis of the ^{13}C spectral data from cells presented with $[2\text{-}^{13}\text{C}]\text{propionate}$ is complicated by the fact that the glyoxylate cycle might also be operative in these cells. Entry of $[1\text{-}^{13}\text{C}]\text{acetate}$ into the tricarboxylic acid cycle as $[1\text{-}^{13}\text{C}]\text{acetyl-CoA}$ would yield $[5\text{-}^{13}\text{C}]\text{glutamate}$ on each turn of the cycle, providing that the flux through isocitrate dehydrogenase predominates over flux through the glyoxylate pathway. Any $[5\text{-}^{13}\text{C}]\text{glutamate}$ formed in this reaction pathway will subsequently produce equal amounts of $[1\text{-}^{13}\text{C}]\text{malate}$ and $[4\text{-}^{13}\text{C}]\text{malate}$ when re-entering the tricarboxylic acid cycle, assuming that passage through fumarase produces a statistical averaging. Mixing of these isotopomers into the oxaloacetate pool which feeds citrate synthase would place 50 % of the total ^{13}C , which originally entered the cycle as $[1\text{-}^{13}\text{C}]\text{acetyl-CoA}$, at glutamate C-1 on the second turn of the cycle (only ^{13}C in $[4\text{-}^{13}\text{C}]\text{malate}$ would survive passage through citrate, isocitrate and α -oxoglutarate). Since label at glutamate C-1 would be lost

as CO_2 on subsequent turns, one could conclude that the observed glutamate C-1/C-5 ratio at steady-state would be exactly 0.5 if all isocitrate formed from $[1-^{13}\text{C}]\text{acetyl-CoA}$ was processed via the tricarboxylic acid-cycle reactions. Alternatively, any $[5-^{13}\text{C}]\text{isocitrate}$ formed from $[1-^{13}\text{C}]\text{acetyl-CoA}$ which bypasses the tricarboxylic acid cycle and enters the glyoxylate cycle would initially produce $[1-^{13}\text{C}]$ - or $[4-^{13}\text{C}]\text{-succinate}$ plus unlabelled glyoxylate. This labelled succinate would contribute equally to the pools of $[1-^{13}\text{C}]$ - and $[4-^{13}\text{C}]\text{-oxaloacetate}$. The unlabelled glyoxylate could condense with another $[1-^{13}\text{C}]\text{acetyl-CoA}$ to produce $[4-^{13}\text{C}]\text{malate}$ which, after equilibration through fumarase, would also yield equal amounts of $[1-^{13}\text{C}]$ - and $[4-^{13}\text{C}]\text{-oxaloacetate}$. Thus, even though half of this label is lost as CO_2 during subsequent formation of glutamate, twice the amount of $[4-^{13}\text{C}]\text{oxaloacetate}$ is formed on each turn through the glyoxylate cycle, so one can readily conclude that the glutamate C-1/C-5 ratio should approach unity as flux through the glyoxylate cycle approaches tricarboxylic acid-cycle flux. The same ratios of glutamate C-1/C-5 were previously predicted by Walsh and Koshland [20] for these two pathways. The experimental value of glutamate C-1/C-5 ratio in the spectrum of cell extracts was 0.57 ± 0.07 ($n = 3$), which, after correction for small differences in spin lattice relaxation time (T_1) and nuclear-Overhauser-enhancement (n.O.e.) values, does not differ significantly from 0.5. Thus, it may be concluded from these data that flux through the glyoxylate cycle is relatively minor under these conditions.

The model presented above assumes that flux of anaplerotic molecules through the citric acid-cycle pools is insignificant compared with total tricarboxylic acid-cycle and glyoxylate-cycle flux (this same assumption was made by Walsh and Koshland [20]). However, this is obviously not true in these experiments, since entry of labelled propionate via propionyl carboxylase is quite apparent from the ^{13}C spectra, and this is by definition an anaplerotic reaction. Although entry of $[2-^{13}\text{C}]\text{propionate}$ via succinyl-CoA cannot place a labelled carbon at either glutamate C-1 or C-5, flux through this pathway can dilute the glutamate C-1 pool such that the C-1/C-5 ratio could decrease to below 0.5 in the absence of flux through the glyoxylate cycle. Thus one cannot distinguish between a high anaplerotic flux offset by a high glyoxylate flux versus low anaplerotic flux and near zero glyoxylate flux from the $[2-^{13}\text{C}]\text{propionate}$ data alone.

Propionate entry via acetyl-CoA versus succinyl-CoA

The spectrum of wild-type *E. coli* which had been fed $[3-^{13}\text{C}]\text{propionate}$ is considerably more complex (Figure 2b). The observation that the glutamate resonances are multiplets in this case indicates that a large influx of $[2-^{13}\text{C}]\text{acetyl-CoA}$ had indeed occurred (corresponding to the acetate pathway). Because of these multiplets, this spectrum contains considerably more metabolic information which may be extracted using a ^{13}C isotopomer analysis of the glutamate multiplets [21]. The relative singlet, doublet, and other multiplet intensities were measured for glutamate C-2, C-3 and C-4 and analysed using published techniques [21]. The analysis of five different samples indicated that $83 \pm 9\%$ of the total acetyl-CoA pool was enriched in the methyl carbon (i.e., was derived from $[3-^{13}\text{C}]\text{propionate}$), the activity of the combined anaplerotic reactions relative to the citrate synthase activity was $24 \pm 10\%$, the fraction of anaplerotic substrate derived from $[3-^{13}\text{C}]\text{propionate}$ was $59 \pm 14\%$ (i.e., carboxylation of propionate to succinyl-CoA), and the activity of the glyoxylate cycle was zero. This indicates that γ , the relative anaplerotic flux versus tricarboxylic acid-cycle flux [22], is 0.24, with 83% of the acetyl-CoA derived from propionate. Thus

entry of $[3-^{13}\text{C}]\text{propionate}$ into the tricarboxylic acid cycle as acetyl-CoA is about 6 times greater [$0.83/(0.24 \times 0.59)$] than entry as succinyl-CoA at steady state.

Given that flux through the glyoxylate cycle appears to be near zero in *E. coli* when presented with propionate as a sole carbon source, one can analyse further the spectra derived from the $[2-^{13}\text{C}]\text{propionate}$ -fed cells to see if they yield consistent results. Again, if $[1-^{13}\text{C}]\text{acetyl-CoA}$ can only enrich glutamate C-5 or C-1 while $[3-^{13}\text{C}]\text{succinyl-CoA}$ (derived from $[2-^{13}\text{C}]\text{propionate}$) can only enrich glutamate C-2 or C-3, then the flux ratio between the acetyl-CoA versus succinyl-CoA pathways can be related by:

$$\frac{\text{Acetate pathway flux}}{\text{Propionate pathway flux}} = \frac{\text{C-5} + 2 \times \text{C-1}}{\text{C-2} + \text{C-3}} \times \text{correction factor for } T_1 \text{ and n.O.e. differences}$$

The intensity of glutamate C-1 was corrected by a factor of 2 to account for the loss of $^{13}\text{CO}_2$ on the second and subsequent turns. The areas of the resonances taken directly from three different cell extract spectra multiplied by a correction factor of 2.66 (measured on a natural-abundance glutamate sample using these same experimental conditions) gave an acetyl-CoA/succinyl-CoA flux ratio of 7.1 ± 2.6 , a result that is entirely consistent with the isotopomer analysis of the ^{13}C spectrum of the cells presented with $[3-^{13}\text{C}]\text{propionate}$.

Possible organization of the TCA-cycle enzymes

We and others have now published evidence for the physical and functional interaction between sequentially related tricarboxylic acid-cycle enzymes in higher eukaryotes [23–27]. Recently, significant asymmetry was observed between the C-2 and C-3 of alanine resonances in yeast extracts after oxidation of $[2-^{13}\text{C}]$ - or $[3-^{13}\text{C}]\text{propionate}$ [26], indicating that tight substrate channelling occurs in the succinyl thiokinase $\rightarrow$ succinate dehydrogenase $\rightarrow$ fumarase span of the citric acid cycle in actively respiring yeast *in vivo*. Although there was asymmetry between the glutamate C-2 and C-3 resonance intensities in some of the spectra in the present study (Figures 1b and 2), this ratio was not statistically different from unity when all of the spectra were compared. However, two other observations made in the present study suggest that metabolic channelling may be occurring in *E. coli*. Mutation of citrate synthase or inhibition of succinate dehydrogenase had similar consequences in terms of propionate utilization by *E. coli*; each completely blocked utilization of propionate by both the acetyl-CoA and propionyl-CoA pathways, and no accumulation of other tricarboxylic acid-cycle intermediates was observed. This result is consistent with the hypothesis of a highly organized tricarboxylic acid-cycle multi-enzyme cluster in *E. coli*, since a freely diffusing tricarboxylic acid cycle in the presence of a high anaplerotic flux, as determined in these experiments, would predict the labelling of intermediates from propionate, even in the absence of a complete cycle or when only part of the tricarboxylic acid cycle is active. Currently we are further investigating the hypothesis of an organized tricarboxylic acid cycle in *E. coli* by use of various chimaeric tricarboxylic acid-cycle enzymes.

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