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How half a century of research was required to understand bacterial growth on C_1 and C_2 compounds; the story of the serine cycle and the ethylmalonyl-CoA pathway

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ABSTRACT

For bacterial growth on substrates with only one or two carbon atoms, special assimilation pathways are required. In 1957, the glyoxylate cycle of Kornberg and Krebs was described for bacterial growth on C_2 compounds such as ethanol and acetate. However this pathway did not operate in some photosynthetic bacteria and in some methylotrophs when they were growing on C_2 compounds, so an alternative pathway must exist. By 1973 Quayle's serine cycle had been described for methylotrophs growing on C_1 compounds such as methanol, but the pathway was incomplete, the unknown part also functioning during growth on C_2 compounds. After more than 35 further years of research, the ethylmalonyl-CoA (EMC) pathway for growth on C_2 compounds, of photosynthetic bacteria has recently been elucidated. This pathway also operates in methylotrophs during growth on C_2 compounds, and on C_1 compounds by way of the serine cycle. This review is a celebration of half a century of research and of the fascinating result of that research.

Keywords: *bacterial metabolism, serine cycle, serine/EMC cycle, ethylmalonyl-CoA (EMC) pathway, EMC pathway, methylotrophs,* Rhodobacter*,* Methylobacterium*, methylaspartate cycle, glyoxylate cycle*

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Dedication: I should like to dedicate this historical review to Hans L. Kornberg and J. Rod Quayle.

General introduction

This review was originally planned to provide a brief summary of the final elucidation of the serine cycle in methylotrophic bacteria which grow on C_1 compounds such as methane, methanol or methylamine. Our understanding of this cycle was thought to be almost complete when I wrote a review for *Science Progress* in 1975[1] but it has taken a further 35 years for its completion. It has depended upon the elucidation of the ethylmalonyl-CoA (EMC) pathway for growth on C_2 compounds in some purple photosynthetic bacteria, almost half a century after their 'problem' was first appreciated. This review has thus expanded to include historical accounts of both of these pathways.

It is usual to give a list of acknowledgements at the end of a publication but I want to emphasise the contributions of many people to the work described here and also to acknowledge their generous encouragement and help in the preparation of this manuscript: these friends include Birgit Alber, Ivan Berg, John Bolbot, Mila Chistoserdova, Tobi Erb, Georg Fuchs, Pat Goodwin, Peter Large, Mary Lidstrom, Hans Kornberg, David Peel and Julia Vorholt.

Bacterial growth on C_1 and C_2 compounds

This review concerns the carbon assimilation pathways of aerobic methylotrophs growing on C_1 compounds and of bacteria able to grow on C_2 compounds such as ethanol and acetate as their sole source of carbon. Energy for growth of methylotrophs is obtained

by oxidation of their C_1 substrates by specific dehydrogenases, and a complete Krebs TCA cycle is not needed[2,3]. By contrast, during growth on C_2 compounds, energy is obtained by oxidation of acetyl-CoA by the TCA cycle or, in photoheterotrophs, from light.

Assimilation of all carbon substrates when provided as the sole source of carbon requires their conversion to the intermediates of central metabolism containing 3 or 4 carbon atoms, which then provide precursors for biosynthesis. During growth of methylotrophs on C_1 compounds every carbon–carbon bond must be created. In this they resemble autotrophic bacteria and photosynthetic organisms which achieve this by the Calvin–Benson–Bassham ribulose bisphosphate pathway for fixation of carbon dioxide. Some methylotrophs do use this pathway but the majority use specific methylotroph carbon assimilation pathways[3].

Similarly, a special pathway for growth on C_2 compounds is needed because there is no route for direct formation of C_3 or C_4 compounds from C_2 compounds such as ethanol or acetate, or during growth on other substrates metabolised exclusively by way of acetyl-CoA such as long chain fatty acids and 3-hydroxybutyrate. Of course the intermediates of the TCA cycle can provide many of the central metabolites required for biosynthesis but they cannot be withdrawn without 'stopping' this oxidative cycle. In the absence of other enzymes the only function of the TCA cycle is to oxidise acetyl-CoA to two molecules of carbon dioxide. During metabolism of substrates with three or more carbon atoms supplementary enzymes are required that replenish the cycle by carboxylation of pyruvate or phophoenolpyruvate [PEP] to oxaloacetate.

During growth of bacteria on C_2 compounds the pathway that replenishes the intermediates of the TCA cycle was described in 1957 by Kornberg and Krebs; this pathway is the glyoxylate cycle which results in production of one molecule of malate from two molecules of acetyl-CoA; oxaloacetate, produced from the malate can then be withdrawn for biosynthesis (Figure 1)[4]. Because, in effect, it bypasses the two decarboxylating enzymes of the TCA cycle, Kornberg and Madsen suggested that the cycle can also appropriately be called the glyoxylate bypass[5]. It requires two key enzymes, isocitrate lyase and malate synthase, in addition to some of the TCA cycle enzymes. Kornberg later suggested that these enzymes, and others involved in 'replenishing' the TCA cycle during extraction of intermediates for biosynthesis, should be named *anaplerotic enzymes*[6].

The operation of the proposed glyoxylate cycle during growth on acetate was confirmed by Kornberg[7] using short-term incubation

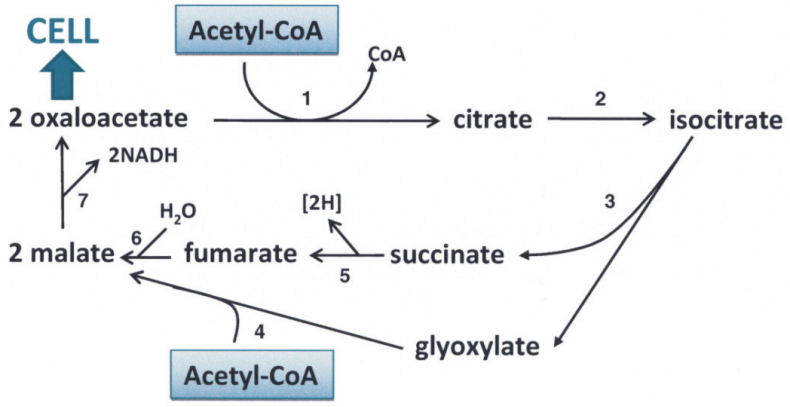

Figure 1 *Kornberg's glyoxylate cycle*[4]*. This achieves the condensation of 2 molecules of acetyl-CoA to malate. The enzymes are: 1, citrate synthase; 2, aconitase; 3, isocitrate lyase; 4, malate synthase; 5, succinate dehydrogenase; 6, fumarase; 7, malate dehydrogenase. In effect this cycle can be summarized as 2 acetyl-CoA →malate. A complete balance equation is given in Table 1 at the end of this review.*

experiments with radioactive ^{14}C acetate, based on those used by Calvin's group to elucidate the path of carbon from carbon dioxide in photosynthesis. His conclusions were further confirmed in experiments with Quayle in which they showed that the labelling pattern within cell constituents was consistent with the pathway but not with alternatives[8]. Such experiments became the key type of experiment that led to the elucidation in methylotrophs of the first part of the serine cycle by Quayle and his colleagues.

The growth of methylotrophs using the serine cycle

There are at least four pathways for assimilation of reduced C_1 compounds in methylotrophs, most of which were proposed, and mainly elucidated, by J.R. (Rod) Quayle and his colleagues[3]. The serine cycle is different from the other pathways in having carboxylic and amino acids as intermediates instead of the usual carbohydrates. This was the first pathway described for growth on methanol but the last to be completed.

The initial proposal by Quayle, with David Peel and Peter Large[9,10], was based on short term labelling experiments using ^{14}C bicarbonate, ^{14}C formate and ^{14}C methanol in *Methylobacterium extorquens* AM1 (then called *Pseudomonas*

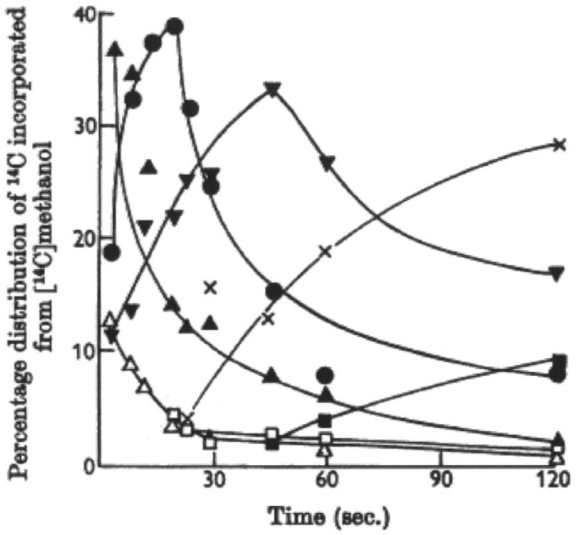

Figure 2 *This is copied with permission from one of the first papers on methanol assimilation by Methylobacterium extorquens; by Large, Peel & Quayle*[9].

AM1). In these experiments whole cells are incubated with the radioactive substrate and samples taken every few seconds up to 2 minutes, inactivated in boiling ethanol and non-volatile ethanol-soluble components separated by 2-way paper chromatography. All radioactive components are eluted, counted, purified and identified. In these experiments it is not the total label in each compound at each time but the percentage in each compound of the total label incorporated that is important (for example see Figure 2). When the percentage labels are plotted against time, those showing a negative slope are likely to be very early intermediates in metabolism of the added substrate. For example, during photosynthesis ^{14}C bicarbonate is first incorporated into phosphoglycerate and the label appears later in phosphorylated sugars. In contrast, the label from methanol in *M. extorquens* was first seen in serine whereas ^{14}C bicarbonate first appeared in malate and aspartate which would be in equilibrium with oxaloacetate which cannot be seen in this sort of experiment; the distribution of ^{14}C label within each compound was subsequently determined. Two possible pathways (one linear, the other cyclic) were proposed; in both the key fixation steps were addition of formaldehyde to glycine to give serine, and carboxylation of a C_3 compound derived from the serine to give oxaloacetate.

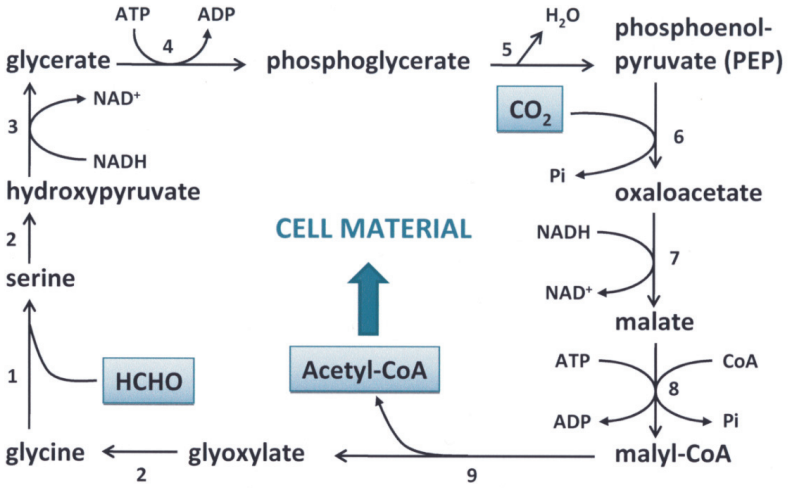

Figure 3 *The serine cycle as proposed by Peel, Large, Salem and Quayle*[9–11]*. The enzymes are 1, serine transhydroxymethylase; 2, serine-glyoxylate aminotransferase; 3, hydroxypyruvate reductase; 4, glycerate kinase; 5, enolase; 6, PEP carboxylase; 7, malate dehydrogenase; 8, malate thiokinase; 9, malyl-CoA lyase. After the initial proposal much further enzymological and mutant evidence was subsequently accumulated to confirm this pathway*[3]*. Note that during biosynthesis of fatty acids and poly 3-hydroxybutyrate which use acetyl-CoA as their biosynthetic starting point, this pathway is sufficient for production of acetyl-CoA from formaldehyde plus carbon dioxide.*

The cyclic pathway was confirmed by Alan Salem's discovery, of a novel malyl-CoA lyase which cleaves malyl-CoA to acetyl-CoA plus glyoxylate[11]. Figure 3 shows the serine cycle as it stood in 1973. In summary, the two carbon atoms of acetyl-CoA are produced from one molecule of formaldehyde plus one carbon dioxide, both derived from methanol oxidation.

The pathway was strongly supported by characterisation of the proposed enzymes, many of them novel, and most of which are induced during methylotrophic growth. Further confirmation of their importance in the pathway was the isolation of mutants lacking them which had lost the ability to grow on C_1 compounds but retained the ability to grow on multicarbon compounds such as succinate. Pat Goodwin showed that some of the serine cycle enzymes are co-ordinately regulated[12], suggesting that, as expected for genes of a well-defined metabolic pathway, the serine cycle genes are encoded on an operon, which has subsequently been amply confirmed by her group[13] and by Mary Lidstrom, Mila Chistoserdova and their colleagues[14,15].

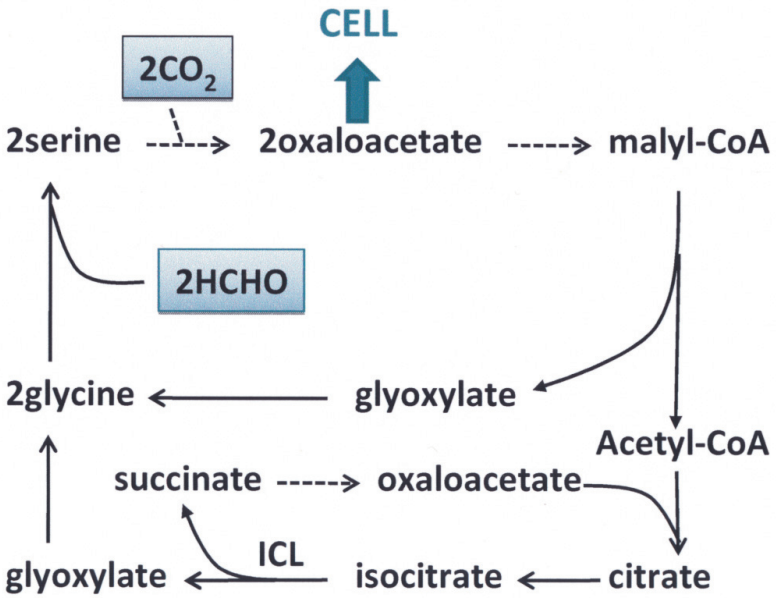

Figure 4 *The serine cycle in methylotrophic bacteria having isocitrate lyase [ICL][3]. The upper part shows the serine cycle as shown on Figure 3. The lower part shows the oxidation of acetyl-CoA to glyoxylate by isocitrate lyase together with the non-decarboxylating enzymes of the TCA cycle. A complete balance equation is given in Table 1 at the end of this review.*

The fate of the acetyl-CoA produced in the serine cycle

The serine cycle as described in Figure 3 produces acetyl-CoA from formaldehyde and carbon dioxide, raising the obvious question of the subsequent route for assimilation of the acetyl-CoA into C_3 and C_4 compounds, and thence into cell material. The first obvious answer is that the glyoxylate cycle must be operating here as it does during growth of other bacteria on acetate (Figure 1). Indeed, some methylotrophs do have the key enzyme (isocitrate lyase) of that pathway and they assimilate C_1 compounds by what is known as the icl^+ serine cycle[3] (Figure 4). The glyoxylate produced by the malyl-CoA lyase reaction is the precursor of one molecule of glycine. The acetyl-CoA is oxidised to a second glyoxylate by a route involving isocitrate lyase (ICL) plus some (non-decarboxylating) TCA cycle enzymes. However, it had been shown previously that there is no isocitrate lyase present during methylotrophic growth of *M. extorquens*, as was later confirmed for the majority of methylotrophs[3].

So, the question remaining is this: how is acetyl-CoA metabolised to C_3 and C_4 compounds in *M. extorquens* during methylotrophic growth? This question has taken more than 30 years to answer and the search for this answer is the subject of the rest of this review. It has depended on studies of C_2 metabolism by the methylotrophs and on the elucidation of a novel pathway, the ethylmalonyl-CoA (EMC) pathway for photoheterotrophic growth of bacteria on C_2 compounds.

The pathway for acetyl-CoA assimilation during growth of *Methylobacterium extorquens* on ethanol, 3-hydroxybutyrate, malonate, pyruvate, lactate and propane 1,2-diol (1972–1980)

Most of the work I will describe in this slightly self-indulgent section was done by Pat Dunstan (now Goodwin), Ian Taylor and John Bolbot in my lab. Our interest in this topic started, when Pat set out to isolate, using the penicillin enrichment technique, mutants of *M. extorquens* lacking its quinoprotein methanol dehydrogenase [MDH] which is also responsible for oxidation of ethanol during growth on that substrate. We assumed that the pathway for assimilation of ethanol [C_2] must be different from that for methanol [C_1], and that the only common feature of their metabolism would be the initial oxidation step. Therefore, Pat set out to characterise mutants that were able to grow on succinate but not on methanol or ethanol. Remarkably, three different types of mutant were isolated, two of which were unable to oxidise the alcohols. These included MDH mutants as expected, and also mutants lacking cytochrome *c* which had important implications in our study of electron transport during methanol oxidation; we now know that methanol is oxidised by a novel periplasmic electron transport chain in which MDH is coupled directly to a specific *c*-type cytochrome[16]. However, a third type of mutant (*e.g.* mutant PCT48) was able to oxidise both alcohols but was unable to grow on them, suggesting that there might be some step that is common to assimilation of C_1 and C_2 substrates; growth of mutant PCT48 was restored by inclusion of glyoxylate together with methanol or ethanol in the growth medium. We showed that the glyoxylate cycle is unlikely to be involved because *M. extorquens* lacks the key enzyme isocitrate lyase during growth on methanol or ethanol. We therefore set out to elucidate the common part of the pathway for

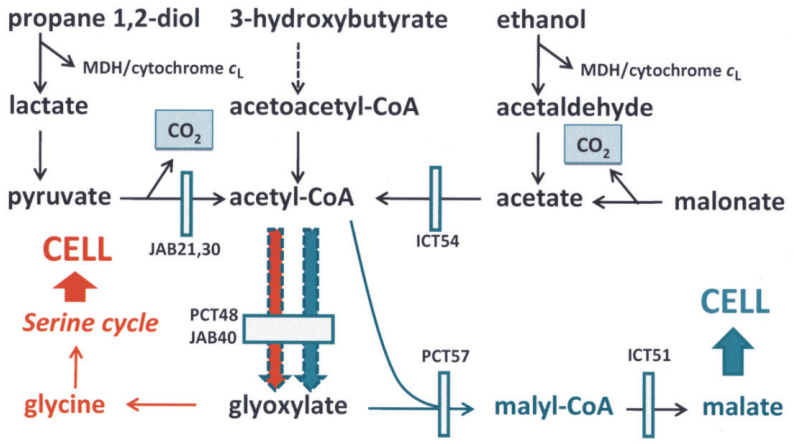

Figure 5 *Pathways for growth of M. extorquens on substrates metabolized by way of acetyl-CoA, based on the work of Pat Dunstan, John Bolbot and Ian Taylor*[12,17–19,21,22]*. NB: only the carbon balance is illustrated. Red indicates pathway on C_1 compounds; blue indicates pathway on C_2 and related compounds. In short-term labelling experiments glycollate would arise by equilibration with glyoxylate. The growth substrates include ethanol, acetate (a poor substrate), 3-hydroxybutyrate, malonate, propanediol, lactate and pyruvate. Propanediol and ethanol are oxidized by methanol dehydrogenase (MDH) whose electron acceptor is cytochrome c_L*[16]*; there is no growth of mutants lacking these proteins. For oxidation of propanediol by MDH an additional modifier protein is required to alter its substrate specificity*[22]*. Note that condensation of glyoxylate and acetyl-CoA to malate requires two enzymes: malyl-CoA lyase and malyl-CoA hydrolase.*

assimilation of C_1 and C_2 compounds that appeared to involve oxidation of acetyl-CoA to glyoxylate[12,17,18].

In short-term labelling experiments, we studied incorporation of ^{14}C acetate in cells grown on either methanol or ethanol using wild type bacteria or mutant PCT48. If the glyoxylate cycle and TCA cycle were operating then early label should be seen in citrate, but it was not. Early label was found in malate and aspartate, and in glycollate (with ethanol-grown cells) or glycine (in methanol grown cells). No glycollate was detected when ^{14}C acetate was used with mutant PCT48 grown on succinate and induced with ethanol plus acetate [note that glycollate reflects the presence of glyoxylate which is not visible in these experiments]. We therefore concluded that there is a novel pathway involving the oxidation of acetyl-CoA to glyoxylate in *M. extorquens* grown on methanol or ethanol; it would appear to be like the serine cycle as shown in Figure 4 but with an alternative route for oxidation of acetyl-CoA to glyoxylate[12,17,18] (Figure 5).

The same unknown pathway was later shown to be involved, as expected, during growth on 3-hydroxybutyrate[18,19] and malonate[19]; and unexpectedly, on pyruvate[20,21], lactate[20,21] and 1,2-propanediol[22]. These C_3 compounds are in effect metabolised as C_2 compounds; they cannot be converted to C_4 compounds by carboxylation because *M. extorquens* lacks pyruvate carboxylase or PEP synthase. Figure 5 summarises the pathways as understood in 1980.

The glyoxylate regeneration cycle (GRC) for oxidation of acetyl-CoA to glyoxylate in the serine cycle

In 1983 at one of the regular International Symposia on Microbial Growth on C_1 Compounds, a paper was presented by Shimizu, Ueda and Sato on the physiological role of vitamin B_{12} in *Protaminobacter ruber* (very similar to *M. extorquens*), which produced relatively large amounts of vitamin B_{12}. Their demonstration that crude extracts could catalyse production of glyoxylate and propionyl-CoA from mesaconyl-CoA (with methylmalyl-CoA as intermediate) led them to propose a pathway for oxidation of acetyl-CoA to glyoxylate that involves mesaconyl-CoA, methylmalyl-CoA, methylmalonyl-CoA, propionyl-CoA and succinyl-CoA (Figure 6)[23]. Part of this sequence of intermediates was subsequently confirmed to be involved in oxidation of acetyl-CoA to glyoxylate in the serine cycle (from mesaconyl-CoA to glyoxylate plus succinate). Their experiments provided the first direct evidence that the key unknown intermediates would be derivatives of Coenzyme A; it should be noted that although such intermediates were often the subject of speculation, they would never have been seen in earlier studies because they are destroyed during preparation and separation of metabolic intermediates.

Remarkably, although the cycle as shown in Figure 6 is not the route for assimilation of acetyl-CoA in *M. extorquens* or *P. ruber*, this cycle has now been shown by Ivan Berg and colleagues to be the route for assimilation of C_2 compounds in haloarchea; this third strategy for assimilation of C_2 compounds is now named the methylaspartate cycle[24].

The involvement of derivatives of Coenzyme A in oxidation of acetyl-CoA to glyoxylate was confirmed 12 years later by Pat Goodwin who described the isolation and sequencing of a gene that complemented the PCT48 mutation which had led to inability to oxidise acetyl-CoA to glyoxylate; this gene (*meaA*) encoded an

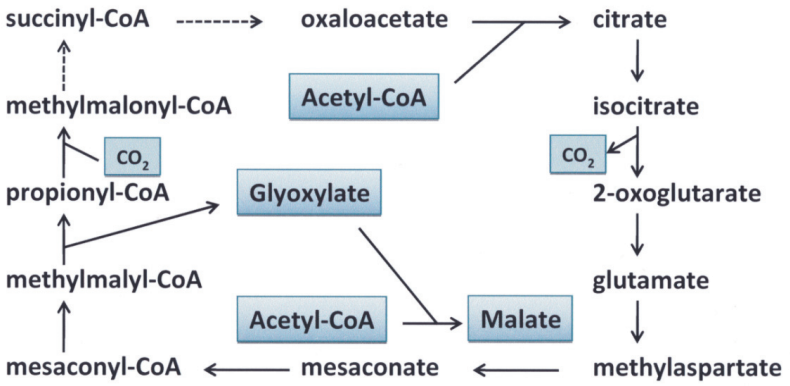

Figure 6 *The methylaspartate cycle*[24]*. This pathway for oxidation of acetyl-CoA to glyoxylate in methylotrophs was proposed in 1984 by Shimizu, Ueda and Sato*[23]*. Only the carbon skeletons have been included. The left hand side from mesaconyl-CoA to succinyl-CoA remains an essential part of the serine pathway as it is now understood. This cycle has recently been shown by Ivan Berg and colleagues to operate in haloarchaea for assimilation of* C_2 *compounds*[24]*. In the complete methylaspartate cycle the glyoxylate condenses with a second molecule of acetyl-CoA to give malate, the overall carbon balance being the same as the glyoxylate cycle (Figure 1). A complete balance equation is given in Table 1 at the end of this review.*

unknown type of B_{12}-dependent mutase[25], now known to be ethylmalonyl-CoA mutase (see below). At the same time, and using a similar approach, Chistoserdova and Lidstrom described this gene and also concluded that propionyl-CoA carboxylase (encoded by *pccA*) and an unknown dehydrogenase (encoded by *adhA*) are also involved[26]; the *adhA* designation was later changed to *ccr*, encoding crotonyl-CoA reductase[27].

With the exception of the contribution of Shimizu and colleagues, advances in our understanding of the serine cycle in the two decades after 1980 depended on the development of methylotroph genetics and mapping of *M. extorquens* genes (see above). In particular, from about 1991 Mary Lidstrom and Mila Chistoserdova started to publish a lengthy series of papers on the enzymes and genes of the serine pathway, and of methanol oxidation, in *M. extorquens*, establishing an important centre for future genetic studies of methylotrophs[15,28–29], leading to publication in 2003 of a preliminary version[29] and, in 2009, the complete genome of *M. extorquens*[30]. Their experimental approach to solving the 'missing' part of the serine cycle was to use genome analysis to select candidate genes for enzymes that might be involved. Candidate genes were tested by mutation, and potential intermedi-

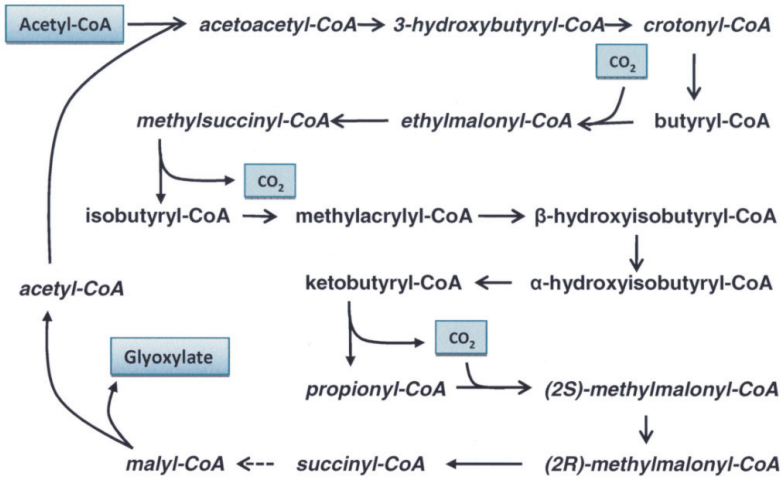

Figure 7 *The glyoxylate regeneration cycle (GRC) for oxidation of acetyl-CoA in M. extorquens as proposed by Lidstrom, Chistoserdova and colleagues*[27,31–33]*. Their papers should be consulted for details of the extensive experimental work, mainly using mutants and radioactive substrates that led to this [necessarily] speculative proposal. The compounds in italics were later shown to be intermediates in the ethylmalonyl-CoA (EMC) pathway. The solid arrows merely indicate proposed reactions (or series of reactions); they do not necessarily indicate that such reactions are known reactions.*

ates identified by monitoring the fates of labelled acetate, butyrate and bicarbonate in these mutants and wild type bacteria[27,31–33]. They showed that the most likely start of the route for oxidation of acetyl-coA to glyoxylate (the glyoxylate regeneration cycle; GRC; Figure 7), would be conversion of acetyl-CoA to crotonyl-CoA by enzymes similar (or identical) to those involved in biosynthesis of poly-3-hydroxybutyrate or oxidation of fatty acids, and that a later part of the GRC would involve carboxylation of propionyl-coA to methylmalonyl-CoA followed by conversion to succinyl-CoA. Their (unavoidably) speculative GRC had 18 intermediates between acetyl-CoA and glyoxylate[27]. Although many of these were later shown not to be involved, one key intermediate, ethylmalonyl-CoA, was later shown to be essential in what has become known as the ethylmalonyl-CoA (EMC) pathway.

The eventual elucidation by Fuchs' group in Freiburg of this pathway for glyoxylate formation from acetyl-CoA in the serine cycle came from definitive enzymological work on the pathway for growth on C_2 compounds of photosynthetic bacteria lacking the glyoxylate cycle.

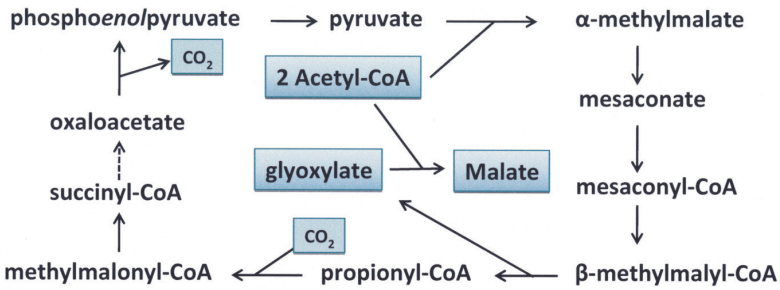

Figure 8 *The citramalate cycle proposed in 1977 for oxidation of acetyl-CoA to glyoxylate in Rhodospirillum rubrum by Ivanovsky's group in Moscow (note: citramalate is α-methylmalate)*[39,40]*. The pathway is completed by the condensation of the glyoxylate with a second acetyl-CoA to give malate.*

The pathway for assimilation of C_2 compounds in some purple photosynthetic bacteria lacking the glyoxylate cycle

After the glyoxylate cycle was established for bacterial growth on C_2 compounds (Figure 1) it was very soon demonstrated that this pathway is not universal; it does not operate in many methylotrophs[3], or in Streptomyces[34], or in some photosynthetic bacteria such as *Rhodospirillum rubrum* and *Rhodobacter sphaeroides* which are unable to synthesise the key enzyme of the glyoxylate cycle, isocitrate lyase[35]. These photoheterotrophic bacteria use acetate as a carbon and energy source in the dark or as a carbon source when grown anaerobically with light as their source of energy. As early as 1951 it was shown by Cutinelli and colleagues[36], using $^{13}CH_3.^{14}COONa$, that acetate was incorporated into protein amino acids without its prior oxidation to CO_2, indicating the presence of a metabolic pathway for direct assimilation of acetate; there was also significant incorporation into protein amino acids from labelled bicarbonate. The existence of such a novel pathway in *Rhodospirillum rubrum* was confirmed by Benedict[37] and Hoare[38] who showed that ^{14}C acetate was accumulated in citramalate (α-methylmalate) and this led Ivanovsky and colleagues[39,40] to propose, in 1997, a citramalate cycle for acetate assimilation (Figure 8), based in part on work by Osumi and Katsuki who had demonstrated 20 years previously that these bacteria contain an enzyme catalysing the reversible condensation of glyoxylate and propionyl-CoA to C_5 compounds[41].

Key steps of the proposed citramalate cycle (Figure 8) are the condensation of acetyl-CoA and pyruvate to citramalate (α-methylmalate); this is converted to β-methylmalyl-CoA, which is cleaved to glyoxylate plus propionyl-CoA. Pyruvate is then regenerated from propionyl-CoA by way of methylmalonyl-CoA and phospho-*enol*pyruvate. The glyoxylate condenses with a second molecule of acetyl-CoA to form malate. Work on these photosynthetic bacteria eventually led to the elucidation of the ethylmalonyl-CoA (EMC) pathway, operating during growth in bacteria growing on C_2 compounds and also during methylotrophic growth.

The elucidation of the ethylmalonyl-CoA (EMC) pathway for bacterial growth on C_2 compounds

Recent work on this pathway was initiated by Georg Fuchs, with Birgit Alber, Tobi Erb, Ivan Berg and their colleagues in Freiburg; the success of this work depended on a multitude of experimental approaches including genetic analysis, proteomics, analysis of metabolites (linked to Coenzyme A) and excellent conventional enzymology. They showed in 2005 that malyl-CoA lyase was induced during growth on acetate of *Rhodobacter capsulatus*; its gene (*mcl1*) was also present in *Rhodospirillum rubrum*, *Rhodobacter sphaeroides* and *Streptomyces coelicolor*, and was 51% identical to the malyl-CoA lyase gene in the methylotroph *M. extorquens*[42]. A key observation was that the purified enzyme was also able to catalyse cleavage of β-methylmalyl-CoA to propionyl-CoA plus glyoxylate. This led to their conclusion that this enzyme functions twice in the pathway (Figure 9). It catalyses cleavage of methylmalyl-CoA to propionyl-CoA plus glyoxylate; it subsequently catalyses the condensation of glyoxylate with acetyl-CoA to give malyl-CoA and thence malate. The propionyl-CoA from the cleavage reaction is carboxylated to methylmalonyl-CoA, eventually to give succinate. This left the question of the origin of the β-methylmalyl-CoA; this was investigated using mutagenesis, genome analysis, proteomics, and HPLC and mass spectrometry of metabolites produced by cell free extracts of wild type and mutant bacteria[43]. In their proposed pathway (Figure 9) two molecules of acetyl-CoA are condensed to acetoacetyl-CoA which is then reduced to 3-hydroxybutyryl-CoA. In an unknown reaction a C_4 derivative of this is carboxylated to an unknown C_5 compound. This gives rise to mesaconyl-CoA, which is hydrated to give the β-methylmalyl-CoA. They showed that a gene cluster present in the genomes of all bacteria proposed to use this pathway contained *ccr*-

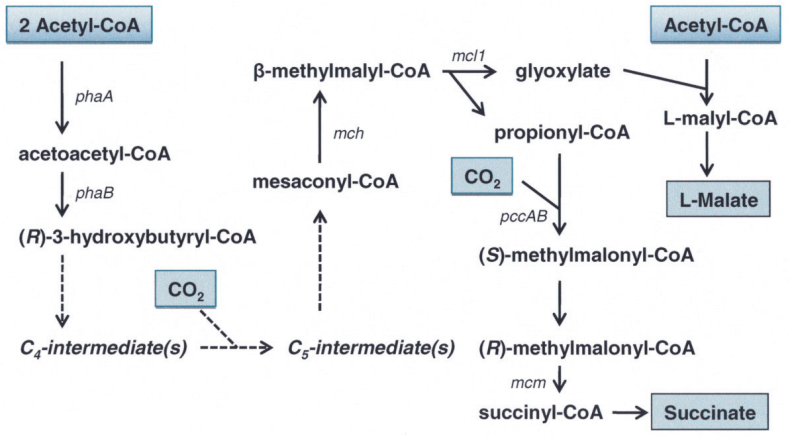

Figure 9 *Proposed pathway for acetyl-CoA assimilation by Rhodobacter sphaeroides. This is redrawn from the 2006 paper by Alber, Spanheimer, Ebenau-Jehle and Fuchs*[43]*. The gene phaA encodes* β*-ketothiolase; phaB, acetoacetyl-CoA reductase; mch, mesaconyl-CoA hydratase; mcl1, L-malyl-CoA/*β*-methylmalyl-CoA lyase; pccAB, propionyl-CoA carboxylase and mcm encodes (R)-methylmalonyl-CoA mutase. Although the enzymes catalyzing the conversion of the* C_4 *compound 3-hydroxybutyryl-CoA to the* C_5 *intermediate mesaconyl-CoA were not known at the time, it was suggested that this process probably involves a carboxylation step, as was subsequently demonstrated when the ethylmalonyl-CoA pathway was finally elucidated (Figures 10–12).*

and *meaA*-like genes, suggesting that a crotonyl-CoA reductase and a B_{12}-dependent mutase (unknown specificity) may be involved in the 'missing' part of the pathway.

The discovery of three novel enzymes in *Rhodobacter sphaeroides* to complete the EMC pathway

The solution of the 'missing' part of the pathway depended on the discovery of three novel enzymes in *R. sphaeroides*. These are crotonyl-CoA carboxylase/reductase, ethylmalonyl-CoA mutase and methylsuccinyl-CoA dehydrogenase (Figure 10).

Crotonyl-CoA carboxylase/reductase[44]

This novel enzyme was described in Freiburg in the year marking the 50th anniversary of the publication in 1957 of the glyoxylate cycle by Kornberg and Krebs (1957). Because a key part of the

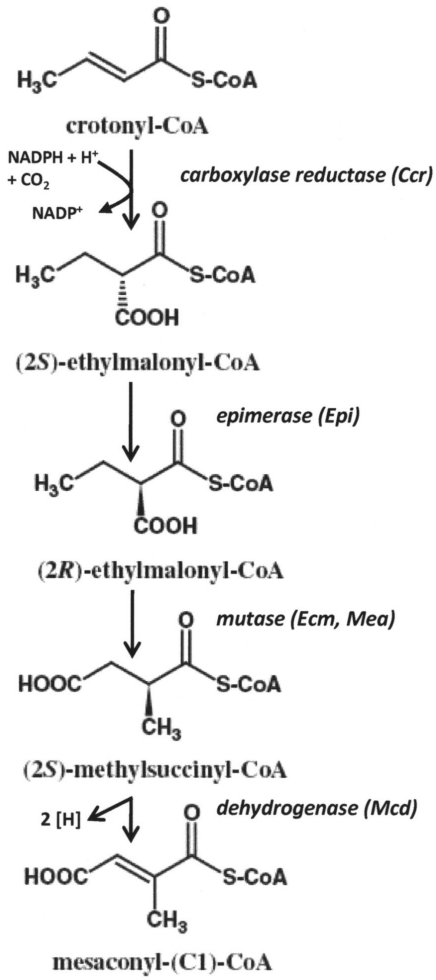

Figure 10 *The 'missing' part of the ethylmalonyl-CoA (EMC) pathway. The conversion of crotonyl-CoA to to mesaconyl-CoA depends on three novel enzymes: crotonyl-CoA carboxylase/reductase*[44]*, (2R)-ethylmalonyl-CoA mutase*[46] *and (2)-methylsuccinyl-CoA dehydrogenase*[47]*. The two forms of ethylmalonyl-CoA are interconverted by ethylmalonyl-CoA/methylmalonyl-CoA epimerase.*

'missing' part of the pathway from acetyl-CoA involved carboxylation and reduction (Figure 9), this work was initiated by investigating products of cell free extracts incubated with NADPH, ^{14}C bicarbonate and various thioesters. The best substrate was crotonyl-CoA and the product was shown by HPLS-MS to be ethylmalonyl-CoA; this was then confirmed by 2D NMR spectroscopy. It had been shown previously that crotonyl-CoA reductase (from the *ccr*

gene) is essential for growth on C_2 compounds by *Streptomyces* and by *M. extorquens*, and it was thought that this reductase might also catalyse carboxylation.

The *ccr* gene from *R. sphaeroides* was therefore cloned into *E. coli*, and extracts shown to catalyse the carboxylation of crotonyl-CoA in the presence of NADPH. The enzyme catalysing this reaction was purified, thoroughly characterised and named crotonyl-CoA carboxylase/reductase:

$$\text{crotonyl-CoA} + \text{NADPH} + \text{H}^+ + \text{CO}_2 \rightarrow \text{ethylmalonyl-CoA} + \text{NADP}^+$$

The NADPH donates a hydride to the β-carbon of crotonyl-CoA whose α-carbon is then carboxylated to yield the product ethylmalonyl-CoA[44,45]. This inducible enzyme, catalysing the reductive carboxylation of an enoyl-CoA ester, is a reaction unprecedented in biology and is one of the key reactions of the new proposed ethylmalonyl-CoA pathway; its presence can therefore be used as a good indicator of the operation of the pathway in other organisms. For example it is also present in *Streptomyces* growing on butyrate and also on *M. extorquens* during growth on methanol[44].

Ethylmalonyl-CoA mutase; a new category of coenzyme B_{12}-dependent acyl-CoA mutases[46]

In Fuchs' lab, using NMR spectroscopy it was shown that, in the presence of coenzyme B_{12}, [3-carboxy-^{14}C]ethylmalony-CoA was rapidly converted by crude cell extracts of *R. sphaeroides* to [*4-carboxy*-^{14}C]methylsuccinyl-CoA, demonstrating that a rearrangement of the carbon skeleton had taken place. The mutase is encoded by the gene *ecm* which is equivalent to *meaA* in *M. extorquens*, encoding the unknown mutase involved in the proposed glyoxylate regeneration cycle (GRC)[25,26]. The ethylmalonyl-CoA mutase was expressed from *ecm* in *E. coli* and the novel enzyme purified and characterised. It is specific for ethylmalonyl-CoA and completely distinct from the methylmalonyl-CoA mutase that catalyses the conversion of methylmalonyl-CoA to succinyl-CoA later in the pathway.

The substrate for the mutase is (*2R*)-ethylmalonyl-CoA whereas the product of the carboxylase/reductase reaction (above) is (*2S*)-ethylmalonyl-CoA; an epimerase to catalyse their interconversion is therefore required. This 'promiscuous' enzyme, also responsible for the epimerisation of methylmalonyl-CoA later in the pathway, is

called ethylmalonyl-CoA/methylmalonyl-CoA epimerase and is encoded by the *epi* gene.

Methylsuccinyl-CoA dehydrogenase[47]

This third enzyme, recently described by Erb, Fuchs and Alber, 'closes' the ethylmalonyl-CoA pathway for acetate assimilation[47]. This FAD-linked dehydrogenase (encoded by *mcd*) is highly specific for (*2S*)-methylsuccinyl-CoA, oxidising it to mesaconyl-CoA.

The discovery of these three novel enzymes enabled the description of the ethylmalonyl-CoA (EMC) pathway for assimilation of acetate in these photosynthetic bacteria.

Summary of the EMC pathway for growth on acetate in *Rhodobacter sphaeroides*

Figures 11 and 12 summarise this pathway for growth on C_2 compounds as described by Fuchs' group as a result of their work, culminating in the description of the three key enzymes that convert crotonyl-CoA to methylsuccinyl-CoA (Figure 10). The genes encoding these novel enzymes, *ccr*, *ecm* (*meaA*) and (sometimes) *mcd*, are clustered together on the same genetic locus on the chromosome of those bacteria that grow on acetate (or other C_2 compounds) but not having the classic glyoxylate cycle, including *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, *Streptomyces coelicolor* and *M. extorquens*[44]. It is remarkable that two of the enzymes function twice in the pathway. A single epimerase is responsible for conversion of (*2S*)-ethylmalonyl-CoA and (*2S*)-methylmalonyl-CoA. Similarly a single lyase (encoded by *mcl1*) is responsible for cleavage of methylmalyl-CoA to propionyl CoA plus glyoxylate, and also for condensation of glyoxylate and acetyl-CoA to malyl-CoA. A homologue of this lyase (encoded by *mcl2*) was shown to be upregulated during growth on acetate. Gene activation and biochemical studies of the two enzymes confirmed the dual role of the Mcl1 lyase and showed that *mcl2* encodes a malyl-CoA thioesterase[48]. The two enzymes together thus catalyse the condensation of glyoxylate and acetyl-CoA to malate, a reaction which is usually catalysed by the single enzyme malate synthase

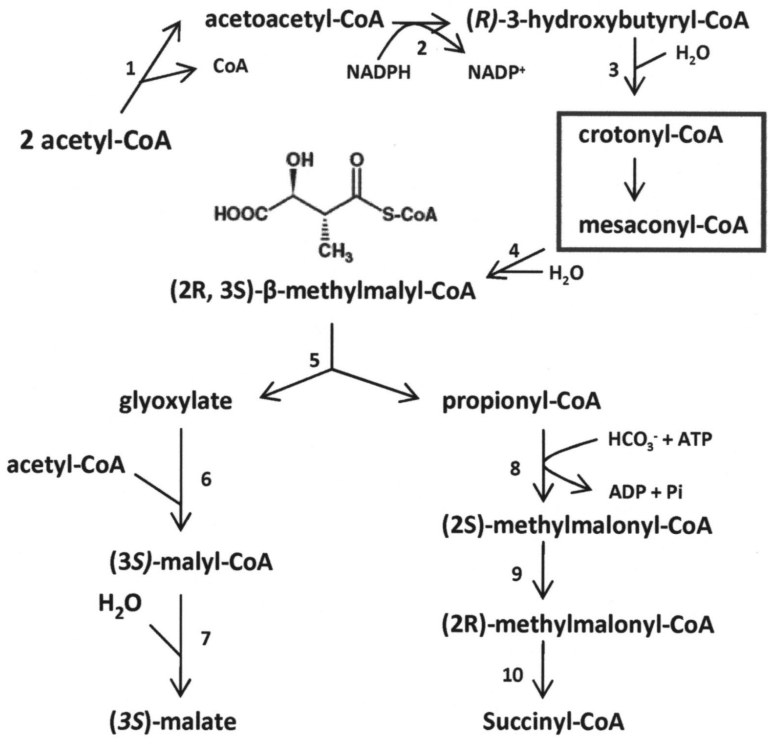

Figure 11 *The ethylmalonyl-CoA (EMC) pathway for acetyl-CoA assimilation in Rhodobacter sphaeroides growing on acetate. This was based on the work of Alber, Berg, Brecht, Ebenau-Jehle, Erb, Muller, Retey, Spanheimer, and Fuchs, published in 2007–2010*[42–48]*. This Figure is redrawn from reference no. 47. The conversion of crotonyl-CoA to mesaconyl-CoA is described in more detail in Figure 10. The enzymes are as follows: 1,* β*-ketothiolase (PhaA); 2, acetoacetyl-CoA reductase (PhaB); 3, crotonyl-CoA hydratase; 4, mesaconyl-CoA hydratase; 5,* β*-methylmalyl-CoA/(3S)-malyl-CoA lyase (Mcl1); 6,7, (3S)-malyl-CoA thioesterase; 8, propionyl-CoA carboxylase (PccAB); 9, ethylmalonyl-CoA/methylmalonyl-CoA epimerase (Epi); 10, (2R)-methylmalonyl-CoA mutase (Mcm). The carbon balance of the pathway as shown here is:*

$$3\text{acetyl-CoA} + 2CO_2 \rightarrow \text{malate} + \text{succinate}$$

A complete balance equation is given in Table 1 at the end of this review.

in bacteria having the glyoxylate pathway. The overall carbon balance[47] of the EMC pathway is:

$$3\text{acetyl-CoA} + 2CO_2 \rightarrow \text{malate} + \text{succinate}$$

It is worth noting that the elucidation of the EMC pathway for oxidation of acetyl-CoA to glyoxylate has not only solved long-

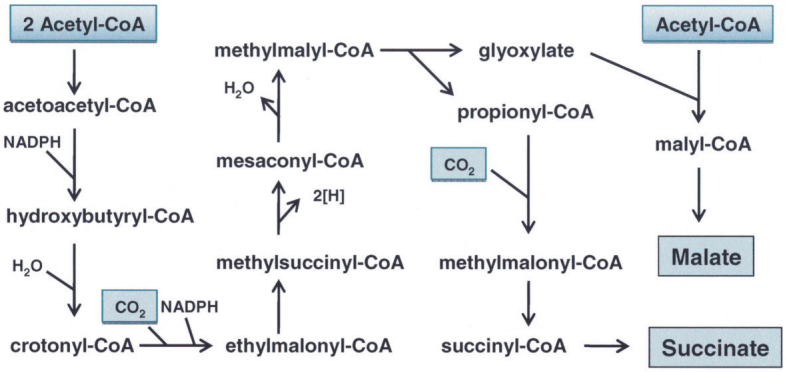

Figure 12 *The ethylmalonyl-CoA (EMC) pathway for acetyl-CoA assimilation in Rhodobacter sphaeroides, presented in a convenient form and similar layout to Figure 9. Note that there are two forms (R and S) of ethylmalonyl-CoA and two forms (R and S) of methylmalonyl-CoA (see Figure 11) which are interconverted by the same epimerase. The carbon balance of the pathway as shown here is:*

$$3\text{acetyl-CoA} + 2CO_2 \rightarrow \text{malate} + \text{succinate}$$

A complete balance equation is given in Table 1 at the end of this review.

standing problems in the photosynthetic bacteria, and the methylotrophs [see below] but, as pointed out in a valuable recent summary of the pathway by Birgit Alber, it also has biotechnological implications, providing the opportunity of tapping into the potential of using the new intermediates and enzymes to produce value-added products[49].

The ethylmalonyl-CoA (EMC) pathway in methylotrophs during their growth on C_2 compounds

All the previously accumulated evidence relating to growth on C_2 compounds of methylotrophs like *M. extorquens* is consistent with the operation of the EMC pathway, but one problem requires some discussion. Malate synthase catalyses the condensation of glyoxylate and acetyl-CoA to malate and is essential in all bacteria that use the glyoxylate cycle; the proposed alternative EMC pathway in *M. extorquens* also requires this enzyme, and we showed in 1976 that mutant ICT51 (Figure 5) which had lost malate synthase activity had also lost the ability to grow on C_2 compounds, although able to grow on C_1 compounds or succinate[19]. It was then shown that this 'malate synthase' activity is due to a combination of the

reversible malyl-CoA lyase plus a malyl-CoA hydrolase[50]. The 'malate synthase' activity was also lost from mutant PCT57 which lacked malyl-CoA lyase (Figure 5). About 30 years later similar conclusions have been drawn for *R. sphaeroides* during growth on acetate by the EMC pathway[42,48]; the enzyme that catalyses the cleavage of methylmalyl-CoA in the first part of the pathway also catalyses condensation of glyoxylate and acetyl-CoA in the later part of the pathway [hence its name malyl-CoA/methylmalyl-CoA lyase].

In *M. extorquens* there remain unanswered questions relating to the properties of some mutants lacking the ability to grow on C_2 compounds. The more recent experiments on this topic are relatively complex; furthermore it is, of course, extraordinarily difficult to attempt to reconcile some growth properties with levels of enzymes required in wild type and mutant in experiments spread over almost 40 years. For further discussion of this the reader is referred to the original literature[3,11,12,18–20,50–53].

The role of the ethylmalonyl-CoA (EMC) pathway in the serine cycle for growth on C_1 compounds by methylotrophs

After elucidation of the EMC pathway for oxidation of acetyl-CoA to glyoxylate in *Rhodobacter sphaeroides*, it was immediately apparent that this pathway could also provide the answer to the related problem in methylotrophs growing by the serine cycle, and this solution was published in the same paper as the first proposal of the EMC pathway by Fuchs and his colleagues[44]. Figure 13 summarises their EMC pathway as it links to the serine cycle during growth of *M. extorquens* on C_1 compounds. It is the same as when it operates during growth on C_2 compounds, the only difference being the fate of the glyoxylate produced from acetyl-CoA. During growth on C_1 compounds the glyoxylate is transaminated to glycine which condenses with formaldehyde to give serine; whereas during growth on C_2 compounds the glyoxylate condenses with acetyl-CoA, eventually producing malate. In the depiction of the complete serine cycle as shown in Figure 13 the carbon balance is:

$$3\,C_1 + 4\,CO_2 \rightarrow 1C_3 + 1C_4$$

It should be noted that this pathway is consistent with all major previous studies (described above) of metabolism in wild type and mutants. Such studies have been extended by Fuchs' group who

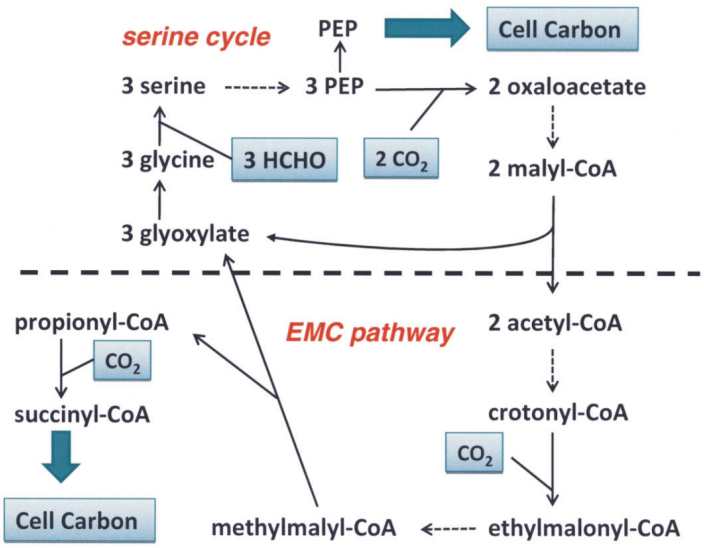

Figure 13 *The serine/EMC cycle for assimilation of C_1 compounds by methylotrophs*[44]*. The ethylmalonyl-CoA (EMC) pathway for oxidation of acetyl-CoA to glyoxylate (lower half) (Figure 12) is coupled to the serine cycle as shown on Figure 3 (upper half). This is taken from the 2007 paper of Erb et al.*[44] *but for convenience only the carbon skeletons are shown. Dotted lines indicate that more than one reaction step is involved. Note that if acetyl-CoA is required as the biosynthetic precursor of membrane fatty acids or the storage compound poly 3-hydroxybutyrate then the EMC pathway is not required for oxidation of acetyl-CoA to glyoxylate. The carbon balance of the pathway as shown here is:*

$$3C_1 + 4CO_2 \rightarrow 1C_3 + 1C_4$$

A complete balance equation is given in Table 1 at the end of this review.

have demonstrated all the reactions of the ethylmalonyl-CoA pathway in methanol-grown *M. extorquens*[52]. They showed regulation of all the enzymes involved in response to C_1, C_2 and C_4 substrates, with (as would be expected) significantly higher activities of EMC pathway enzymes in methanol- and acetate-grown cells compared with succinate-grown cells. Similar conclusions were drawn in an extensive study by Lidstrom and colleagues in which they added methanol to a succinate-limited chemostat culture of *M. extorquens* and followed the transition of the cells from succinate to methanol growth, measuring transcription, enzyme activity, metabolites and fluxes[53]. This integrated analysis provided information about the transient imbalance and the response to carbon starvation when they are shifted out of the multi-carbon metabolic mode, the flux redistribution that occurs while the cells are adapting to the new

C_1 metabolic mode, and the relationships between transcript, enzyme activity, metabolite and flux during this transition.

Further confirmation of the operation of the EMC pathway during methanol assimilation by *M. extorquens* has been provided by an investigation by Julia Vorholt and colleagues in Zurich, using a remarkably powerful set of experimental approaches[54]. The development of an original method of liquid chromatography high-resolution mass spectrometry (LC-HRMS) allowed for the identification of almost all intermediates of the ethylmalonyl-CoA pathway. Detailed and conclusive information on its role as the predominant process for glyoxylate formation was obtained from experiments in which kinetic isotopomer profiles collected by LC-HRMS during short-term experiments with [1-^{13}C] acetate were combined with steady-state isotopomer distributions measured by NMR (^{13}C metabolomics!). The steady-state distribution of [^{13}C] isotopomers in glycine was subsequently used to determine the metabolic origin of glyoxylate in *M. extorquens* under purely methylotrophic conditions. Their results suggested that the propionyl-CoA produced in the EMC pathway 'recycles' by way of methylmalonyl-CoA, and succinyl-CoA to malyl-CoA which is then cleaved to give a second molecule of glyoxylate. This is incorporated into their proposed depiction of the serine cycle shown in Figure 14. The overall carbon balance of this cycle is then:

$$3\mathrm{MeOH} + 3\mathrm{CO_2} \rightarrow 2\mathrm{C_3}$$

Exactly the same enzymes and intermediates are involved in the cycle operating to produce two molecules of PEP as are involved in the depiction in Figure 13 in which the balance is:

$$3\mathrm{HCHO} + 4\mathrm{CO_2} \rightarrow 1\mathrm{C_3} + 1\mathrm{C_4}$$

The difference in the two representations is the fate of some of the propionyl-CoA. It is obvious that oxaloacetate, PEP, succinyl-CoA and acetyl-CoA must always be produced for biosynthesis of amino acids, carbohydrates, haem, and fatty acids and poly-β-hydroxybutyrate. It is equally obvious therefore that no single representation can summarise the operation of the serine/EMC cycle in all growth conditions. For convenience in comparing the flow of carbon from substrate to cell material in all the pathways discussed in this review, balances taken from those pathways, but normalised to produce oxaloacetate, have been put together in Table 1.

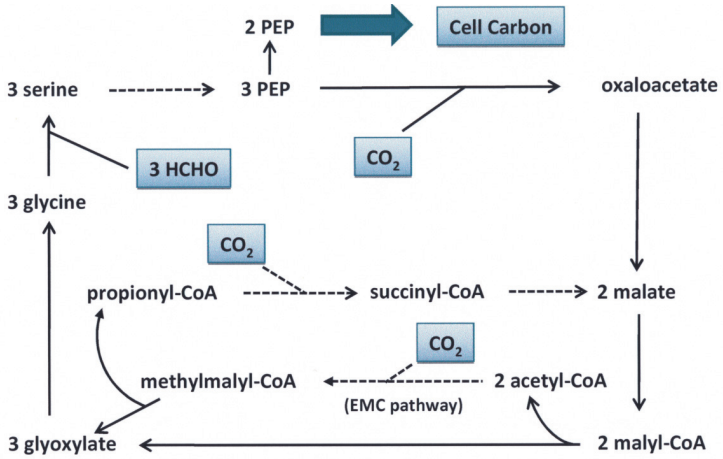

Figure 14 *The serine/EMC cycle for assimilation of C_1 compounds as it occurred during experiments described by Vorholt and colleagues[54] (re-drawn for ease of comparison with Figures 3 and 13). This depiction of the pathway shows the succinyl-CoA, derived from propionyl-CoA, being 'recycled' to produce a third glyoxylate. The carbon balance of the pathway as shown here is:*

$$3C_1 + 3CO_2 \rightarrow 2C_3$$

A complete balance equation is given in Table 1 at the end of this review.

Conclusion

I should like to record that the ethylmalonyl-CoA (EMC) pathway was, appropriately, first presented in 2006 by Tobi Erb, in Magdalen College, Oxford during the same Gordon Conference on Microbial Metabolism of C_1 Compounds at which I gave a lecture commemorating the work of the late Rod Quayle who would have been so excited to have seen the work he had started more than half a century ago on the serine cycle in methylotrophs to be so magnificently concluded.

Table 1 *Summary of the carbon and energy balances of the assimilation pathways. Growth substrates must provide acetyl-CoA, for synthesis of fatty acids and 3-hydroxybutyrate (for carbon storage as poly 3-hydroxybutyrate, and C3 and C4 compounds for synthesis of carbohydrates, amino acids and nucleic acids. The relative requirements for these metabolic precursors will depend on the growth conditions. The pathways and carbon balances in this review have been presented in the Figures as originally described but the balances in this Table have been normalised for production of oxaloacetate (OAA) so that they may be compared more readily. It has been assumed that production of thioesters requires ATP and the production of AMP plus pyrophosphate, and that conversion of succinyl-CoA to succinate produces ATP (although the energetically equivalent GTP may be produced). Similarly NAD^+ and $NADP^+$ are treated as being energetically equivalent and written as $NAD(P)^+$. Water, and protons involved in $NAD(P)^+$-linked reactions, are omitted. It should be noted that during growth on C_2 compounds by way of acetyl-CoA growth yields (g of cells/mole substrate consumed) will be limited, as is usual with bacteria, by ATP. By contrast, during growth by way of the serine cycles growth yields will be limited by NAD(P)H (reductant). This is because reductant is required for [in effect] reduction of CO_2, and because oxidation of the C_1 substrates to HCHO provides no NAD(P)H*[3,53,55]*

Reactants	Products
The glyoxylate cycle for assimilation from acetyl-CoA (Fig. 1)	
$2\text{acetyl-CoA} + 2NAD^+ + FAD$	$OAA + 2NADH + FADH_2 + 2CoA$
The methylaspartate pathway for assimilation of acetyl-CoA (Fig. 6)	
$2\text{acetyl-CoA} + 2NAD(P)^+ + FAD + ATP$	$OAA + 2NAD(P)H + FADH_2 + AMP + PP_i + 2CoA$
The ethylmalonyl-CoA pathway for assimilation of acetyl-CoA (Fig. 11 and 12)	
$3\text{acetyl-CoA} + 2CO_2 + 2FAD$	$2OAA + 2FADH_2 + 3CoA$
The serine cycle [ICL variant] for assimilation of formaldehyde (Fig. 4)	
$2HCHO + 2\ CO_2 + 2NAD(P)H + FAD + 3ATP$	$OAA + 2NAD(P)^+ + FADH_2 + 2ADP\ + 2P_i + AMP + PP_i$
The serine cycle [EMC variant] for assimilation of formaldehyde (Figs. 13 and 14)	
$3HCHO + 5CO_2 + 6NAD(P)H + 2\ FAD + 5ATP$	$2OAA + 6NAD(P)^+ + 2FADH_2 + 3ADP\ + 2AMP + 2\ PP_i + 3P_i$

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