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Travelling to, and along, the glyoxylate bypass: a commentary by

Hans L. Kornberg

Department of Biochemistry, University of Cambridge, Cambridge (U.K.)

on 'Synthesis of C_4-dicarboxylic acids from acetate by a 'glyoxylate bypass' of the tricarboxylic acid cycle'
by H.L. Kornberg and N.B. Madsen
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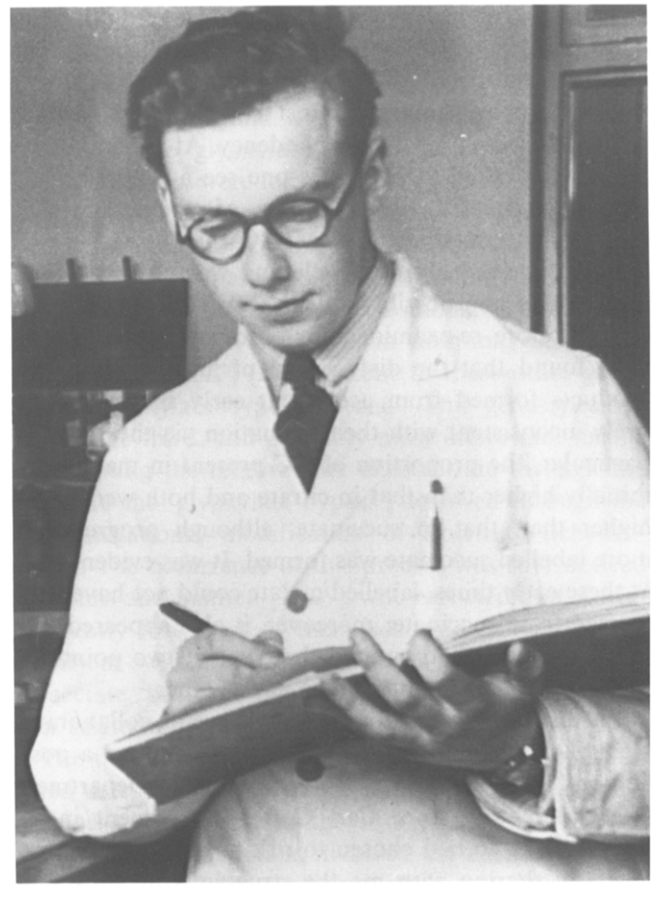
The latest edition of the standard text used by many students of Biochemistry [1] contains 1089 pages; the 'glyoxylate cycle', which arose out of the paper that Neil Madsen and I published in Biochimica et Biophysica Acta in 1957, occupies less than one page of it. It is gratifying to see one's work incorporated into the body of biochemical knowledge, although it is humbling to realize how tiny a fraction of that knowledge it is; it is even more chastening to recollect the many blind alleys we entered before we saw the 'glyoxylate bypass'. I have previously recounted [2] the series of accidents that, despite our initial blindness and stupidity, finally brought us to our goal and I need not repeat that story. But, as I have been asked "to reflect on how the seminal ideas in the fields arose at that time and to outline how they contributed to our present knowledge of the subject", I cannot wholly avoid some repetition.

Success in solving a scientific problem requires at least two ingredients: that the problem be defined in such a manner that it can be tackled, and that the means exist for tackling it. Until the early 1950's, both ingredients were lacking to explain how bacteria were able to synthesize their all from C_2-compounds supplied to them as sole sources of carbon.

It was known that many bacteria readily oxidized acetate to carbon dioxide and water, and that the tricarboxylic acid cycle (TAC) was probably (but not certainly) the route for doing so [3]. It was also known [4] that the TAC supplied the carbon skeletons for many of the components of growing cells. But it had not been possible satisfactorily to explain how acetate could be both totally oxidized and yet give rise to net syntheses: the only mechanism suggested, the 'Thunberg condensation' [5]

$$\underset{\text{2 acetate}}{2CH_3COO^-} \longrightarrow \underset{\text{succinate}}{\begin{matrix} CH_2\cdot COO^- \\ | \\ CH_2\cdot COO^- \end{matrix}} + 2H.... \qquad \text{(i)}$$



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had never been verified experimentally.

When, at the suggestion of Professor H.A. (later Sir Hans) Krebs, I began to study this problem in 1955, I was able to bring to bear on it a relatively new technique that had proved successful in elucidating the path of carbon in photosynthesis [6] and that I had been able to learn during a Summer's work in Melvin Calvin's

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laboratory. This technique involves the incubation of growing cells with ^{14}C-labelled substrates for very brief periods and the analysis, by two-dimensional paper chromatography, of the labelled materials formed during such brief exposures to the isotope. Measurement of the fate of ^{14}C-labelled acetate in a strain of *Pseudomonas* growing on acetate as sole carbon source and exposed to the isotopic material for periods from 3 s onwards showed that, even at the earliest time, ^{14}C appeared only in intermediates of the TAC and in amino acids (such as glutamate and aspartate) directly derived therefrom.

Yet the initial premise of our work made it obvious that the Krebs cycle, which from these data appeared to be the sole pathway for acetate metabolism, could not account for growth on acetate. The semantic nature of this paradox (a logical absurdity which G.K. Chesterton [7] described as, "Truth standing on her head in order to attract attention") was hidden from me until a naïve question from my wife (not a biochemist) brought it to my attention. Feeling particularly despondent at having thus apparently come up against a brick wall, I outlined to her the reason for my despondency. At the end of my recital, she asked, "How does one see a Krebs cycle?" This brought me up short: I was, of course, assuming that any intermediate of the TAC that I saw was an intermediate in only that cycle and was not participating in other and possibly novel reactions.

I therefore re-examined my chromatograms and indeed found that the distribution of label amongst the products formed from acetate at early times was entirely inconsistent with their formation via the TAC. In particular, the proportion of ^{14}C present in malate was initially higher than that in citrate and both were much higher than that in succinate, although progressively more labelled succinate was formed. It was evident that, at these early times, labelled malate could not have been formed from succinate; moreover, it also appeared that labelled acetate had entered the cycle at two points, to form citrate at one and malate at the other.

At this time, I was fortunate to have as collaborator Neil Madsen, who had recently come to spend a post-doctoral year in the Oxford Biochemistry Department after graduating from Carl Cori's Department in St. Louis, and who had chosen to risk possible disappointment by sharing with me the struggle with an apparently intractable problem. Neil had already succeeded in showing that acetate per se was not metabolized by extracts of our organisms but that such extracts were rich in acetate thiokinase activity and were thus able rapidly to form acetyl-coenzyme A from acetate, ATP and CoASH. However, despite all manner of tricks, we were unable to persuade such extracts either to oxidise acetate to CO_2 and water, or to catalyse the reductive condensation of 2 mol of acetate to succinate as postulated by the 'Thunberg condensation' (reaction (i)).

Neil was also seized at once with the significance of the anomalous isotope distributions on our chromatograms and, having by chance some fresh extract of acetate-grown cells handy, we set up a simple experiment to look for a second point of entry of acetyl-coenzyme A into the TAC. This experiment was indeed so simple and seemed so much of a 'blunderbuss' approach that it was not reported until a year later [8], when the interpretation to which it pointed so clearly had been adequately substantiated. The experiment consisted of taking some extract of acetate-grown *Pseudomonas* and placing it, together with labelled acetate, ATP, glutathione, CoASH, a magnesium salt and phosphate buffer, into each of eleven test tubes. To these were added, respectively NAD^+, or $NADP^+$, or one of each of the intermediates of the tricarboxylic cycle. It would be expected that the occurrence of the 'Thunberg condensation' (reaction (i)) might lead to the formation of labelled succinate in the presence of either of the electron acceptors: however, such was not found. It would also be expected that the initial reaction of the TAC, the formation of citrate from oxaloacetate and acetyl-coenzyme A, should lead to a major incorporation into acid-stable products of label from acetate and that this label should be found largely if not exclusively as citrate: such was indeed observed. However, there is no reaction in the TAC in which a second molecule of acetyl-coenzyme A enters to form an acid-stable product. It was thus with a mixture of disbelief and awe that Neil and I watched our Geiger counter burst into a veritable paroxysm of activity when the product formed in the tube containing isocitrate as reactant was submitted to it. Chromatographic analysis showed this labelled product to be overwhelmingly one compound: malate.

Two enzymes that, conceivably, might enable acetyl-coenzyme A and isocitrate to give rise to malate had recently been reported to be present in bacterial extracts. Two years previously, Dr Howard Saz, visiting Sidney Elsden's Department at the University of Sheffield, had studied an enzyme then termed 'isocitritase', which catalyses the splitting of isocitrate to glyoxylate and succinate [9]. He found this enzyme, which is an aldolase, in *Pseudomonas* KB1 – the very strain with which we were working. The reaction it catalyses is readily reversible, but its physiological role was obscure. The enzyme is present in many fungi and bacteria [10,11]; it was conceivable that it might provide glyoxylate for the biosynthesis of glycine or, acting in reverse, to provide a cyclic mechanism for the oxidation of compounds more highly oxidised than acetate, such as glycine or glycollate [12]. Another suggestion was that the glyoxylate formed through its action was oxidised via formate to carbon dioxide and water and that this pathway represented a mechanism alternative to the TAC for the total oxidation of acetate. We had known

of the existence of this enzyme, but it was one of several that catalysed peculiar reactions not explicable at the time and we had therefore not paid particular attention to it.

However, by an extraordinary coincidence, a paper appeared at just this time in the 'Journal of the American Chemical Society' which brought the possible metabolism of glyoxylate very sharply to our notice. D.T.O. Wong and S.J. Ajl [13] reported that extracts of *E. coli* could promote the condensation of acetyl-coenzyme A and glyoxylate to form malate: this reaction was, of course, formally analogous to that whereby citrate was formed from acetyl-coenzyme A and oxaloacetate. The authors termed the novel enzyme 'malate synthetase'. The 'blunderbuss' experiment we had done suggested that this enzyme was present also in the *Pseudomonas* extract. Repetition of the experiment with a tube containing sodium glyoxylate instead of isocitrate indeed gave the same result: ^{14}C from acetate was rapidly and massively incorporated into malate. Therefore, it was easy for us to establish the stoichiometry of the process, and to demonstrate that for each mole of isocitrate that reacted with acetyl-coenzyme A there was a net formation of 1 mole of succinate and 1 of malate. It is this work that forms the subject of the paper now reprinted in 'Biochimica et Biophysica Acta'.

The biological significance of the work, which explained how microorganisms could grow on acetate or on substances that give rise to acetate [14], was greatly enhanced by the virtually simultaneous demonstration [15,16] that the 'glyoxylate bypass' played a key role also in the conversion of fatty acids to carbohydrates, which occurs during the germination of fatty seeds, such as those of the castor bean. Subsequently, Harry Beevers and his colleagues identified and elucidated the function of a hitherto unrecognised cellular organelle, the 'glyoxysome'. They showed that the mobilization of lipid in castor bean endosperm during germination depends on the formation of these organelles and on the induction, and incorporation into the 'glyoxysomes', of the enzymes of the 'glyoxylate bypass' [17].

Apart from its important relevance to the physiology of plants, the 'glyoxylate bypass' provides a most useful illustration of the manner in which branched pathways, that do not lead to defined end-products, are regulated. It is evident that the dual role of the TAC, of providing both energy and building blocks for cellular syntheses, imposes also a dual role on isocitrate. In the TAC, isocitrate is oxidised via isocitrate dehydrogenase and $NADP^+$ to α-oxoglutarate and CO_2; in the glyoxylate bypass, it is split to glyoxylate and succinate. How does the cell achieve the required balance between oxidation and cleavage?

Two of the mechanisms involved in the control of the 'glyoxylate bypass' were first discerned by R.A. Smith and I.C. Gunsalus. They observed [18] that isocitrate lyase, purified from *Pseudomonas*, was competitively inhibited by succinate; they also reported [19] that, although isocitrate lyase could be detected in extracts of cells that had been grown on a variety of substrates, it was present in high activity only in cells that had been grown on acetate. The first of these regulatory processes operates at the level of the enzyme; the second, at that of the gene.

Subsequent work [20–22] has elaborated and confirmed these modes of control. In particular, it has been suggested [22] that the intracellular concentrations of phosphoenolpyrurate or pyruvate (depending on the pH of the culture) provide 'feed-back' information that, in effect, acts as a 'fine control' over the operation of the glyoxylate cycle in *E. coli*; these C_3-compounds may also act as triggers for de-repression of isocitrate lyase synthesis. Since the C_3-compounds in question are the starting materials for gluconeogenesis from the TAC, this explanation would be consistent with physiological requirements; however, its validity has been questioned [23] and remains to be rigorously tested.

The genes *aceA* and *aceB* that specify the two key enzymes of the 'glyoxylate bypass', isocitrate lyase and malate synthase, were located on the linkage map of *E. coli* and were found to be adjacent to a gene *iclR* that specifies a repressor of the synthesis of these two enzymes [24,25]; in partial diploids, the 'inducible' allele $iclR^+$ was demonstrated to be dominant to the 'constitutive' allele *iclR* [26]. But, in addition, there is a further gene (*aceK*) [27] adjacent to these other genes of the 'glyoxylate bypass operon'. This gene specifies what is the third, and probably most important, means of regulating the relative flux of isocitrate through the TAC and the 'glyoxylate bypass', achieved through the post-translational modification of isocitrate dehydrogenase. The occurrence of this process was first noticed by Holms and Bennett [28,29], who reported that *E. coli* apparently lost most of their isocitrate dehydrogenase activity in switching from growth on glucose to growth on acetate, but that this activity was rapidly restored after addition of pyruvate.

The work of a number of investigators [30,31] established that the covalent modification of isocitrate dehydrogenase, which led to the loss of enzymic activity during growth on acetate, involved the ATP-dependent phosphorylation of a serine residue on the protein, catalysed by an isocitrate dehydrogenase/kinase. On addition of pyruvate, enzymic activity was rapidly restored as this phosphate moiety was removed by the action of a phosphatase. It was La Porte and Koshland [32] who made the exciting discovery that these kinase and phosphatase activities resided on the same protein. The nucleotide sequence of *aceK* has been determined [33] and significant sequence similarities have been observed between a run of 25 amino-acid residues in the *E. coli* isocitrate dehydrogenase/kinase and similar

stretches in other (and eukaryotic) protein kinases.

The picture that thus emerges of the control of the 'glyoxylate bypass' and its integration with the supply of energy through the TAC in bacteria is a simple and elegant one. During growth on acetate, nearly three-quarters of the isocitrate dehydrogenase present in the cells is rendered inactive through phosphorylation [27,34], which thereby channels isocitrate into the 'glyoxylate bypass'. Provision of pyruvate – the 'feed-back' signal that indicates that the continued operation of the 'glyoxylate bypass' is no longer required – activates the phosphatase and thus enables isocitrate more readily to be oxidised via the TAC; a fall in ATP concentration (with consequent rise in ADP and AMP levels) has the same effect. An elegant analysis of this inter-relationship has been published [35].

The organisation of the genetic material in eukaryotes is, of course, rather more complex than that in bacteria, and the model described above cannot be applied directly to such organisms, or to fatty seeds. cDNA for the isocitrate lyase from castor bean has been sequenced [36]: the enzyme is specified by a coding region of 1731 bp, which is somewhat larger than the 1654 bp sequence, specifying a protein of 429 amino acids and M_r 47 200, found in *E. coli* [37]. D.H. Northcote and his colleagues have also shown that the increase in activity of isocitrate lyase and malate synthase during germination results from an increase in transcription, and that this is where hormonal and growth factor control are exerted [38,39]. Further details of these important processes remain to be elucidated.

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Correspondence: Sir Hans Kornberg, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW, U.K.

Synthesis of C_4-dicarboxylic acids from acetate by a "glyoxylate bypass" of the tricarboxylic acid cycle

Pseudomonas KB 1[1] grows rapidly on a synthetic medium containing acetate as the sole source of carbon[2]. Washed suspensions of whole cells readily oxidise acetate and all the members of the tricarboxylic acid cycle, indicating that the cycle occurs in this organism when it is grown on acetate[3], as it does when it is grown on succinate[1]. Short-term incubations (3 sec to 15 min) of rapidly growing cultures with ^{14}C-labelled acetate confirmed the occurrence of the cycle. They further indicated that acetate enters the cycle at two sites[3], and that a compound in ready equilibrium with CO_2, which is probably oxaloacetate, lies on the initial stages of the pathway of acetate[2].

Cells of acetate-grown *Pseudomonas* KB 1 were crushed in a HUGHES press[4], homogenized with 0.1 *M* potassium phosphate buffer, pH 7.5, and centrifuged for 30 min at 25,000 *g*. When this cell-free extract was incubated with $^{14}CH_3COONa$, ATP*, CoA, glutathione and sodium glyoxylate, malate was the only labelled compound formed in the early stages of incubation. The malate was isolated by two-dimensional paper chromatography, located by autoradiography, and identified by co-chromatography with authentic malic acid. The rate of formation of ^{14}C-malate was linear over one hour, and was of the same order as the rate of acetate activation, as measured by the formation of hydroxamic acid[5] (Table I). When *iso*citrate replaced glyoxylate in the above system, malate was again the first labelled compound formed. The rate of ^{14}C-malate formation from $^{14}CH_3COONa$ and *iso*citrate, which was also linear over the period studied (10 min), was approx. 3.7 μmoles/h/extract from 6 mg dry wt. of cells. This rate was more than doubled by preincubation of the extract with $^{14}CH_3COONa$, ATP, glutathione and CoA; the observed rate was therefore a minimum one, and was limited by the amounts of acetate-activating enzyme present in the 5 months-old extract used. In the absence of glyoxylate or *iso*citrate, no labelled compounds other than traces of acetyl CoA were formed. There was also no incorporation of ^{14}C from $^{14}CH_3COONa$ in the absence of ATP, CoA or glutathione, or with boiled cell extract (Table I).

TABLE I

RATES OF ACETATE ACTIVATION AND OF ^{14}C-MALATE FORMATION FROM $^{14}CH_3COONa$ AND GLYOXYLATE

The rate of acetate activation was measured by the procedure of JONES AND LIPMANN[5]. The incorporation of ^{14}C from acetate was determined by incubating 100 μmoles of K phosphate pH 7.6, 10 μmoles of glutathione, 10 μmoles of $MgCl_2$, 0.08 μmoles of CoA, 2 μmoles of $^{14}CH_3COONa$ (giving $7.4 \cdot 10^5$ counts/min under the conditions used), 10 μmoles of sodium glyoxylate, 0.1 ml of cell-free extract and water to 0.97 ml. At zero time, 6 μmoles of ATP were added. The reaction was stopped by the addition of 3 ml of boiling 95% ethanol. The precipitate was removed, washed with 1 ml of 20% ethanol and discarded. The combined supernatant solutions were evaporated to dryness under a stream of N_2 at 50° C, the dried material redissolved in 0.5 ml of water and portions (0.1–0.25 ml) analysed by two-dimensional chromatography and autoradiography. The radioactivity of the labelled malate was assayed, with a mica end-window β-counter tube, directly on the chromatograms.

Solution	*Time (min)*	*Hydroxamic acid formed (μmoles)*	*^{14}C-malate formed (μmoles)*
Boiled enzyme	60	0	0
No CoA	60	0	0
No ATP	60	0	0
No glutathione	60	0	0
No glyoxylate	20	0.53	0
	40	1.06	0
	60	1.60	0
Complete system	2	—	0.052
	5	—	0.101
	10	—	0.21
	30	—	0.60
	60	—	1.24

* The following abbreviations have been used: ATP = adenosine triphosphate, AMP = adenosine monophosphate, PP = inorganic pyrophosphate, CoA = coenzyme A.

The net formation of malate from acetate and either *iso*citrate or glyoxylate, under anaerobic conditions, is shown in Table II. In the absence of acetate, *iso*citrate forms only succinate and glyoxylate by the action of *iso*citritase[6,7,8]. The presence of an enzyme presumably identical with the malate synthetase of WONG AND AJL[9] is shown by the formation of malate from acetate and glyoxylate.

TABLE II

SYNTHESIS OF MALATE BY THE REACTIONS OF THE "GLYOXYLATE BYPASS"

Each flask contained 300 μmoles of potassium phosphate buffer pH 7.6, 10 μmoles of $MgCl_2$, 5 μmoles of glutathione, 0.16 μmoles of CoA, 40 μmoles of ATP, 0.5 ml of cell-free extract and water to 3.0 ml. Incubation was for one hour at 30° under nitrogen.

Reactants (μmoles)					*Products (μmoles)*			
Potassium acetate	*Sodium glyoxylate*	*d-Isocitrate**			*Succinate***	*Malate****	*Glyoxylate§*	*Sum of malate + glyoxylate*
		initial	*final*	$-\Delta$				
60	40	—				3.2		
—	40	—				1.0		
60	—	—				0.5		
300	—	15.8	2.7	13.1	11.8	8.2	0.9	9.1
—	—	31.6	16.8	14.8	13.4	1.1	9.5	10.6

* Measured with *iso*citric dehydrogenase.
** Measured with succinoxidase.
*** Measured with malic decarboxylase[10].
§ Determined by the method of FRIEDEMAN AND HAUGEN[11]. It was identified by chromatography of its 2,4-dinitrophenylhydrazone and compared with that of authentic glyoxylate as standard.

It follows that *Pseudomonas* KB 1, when growing on acetate as sole carbon source, possesses, in addition to the enzymic reactions of the tricarboxylic acid cycle, an auxiliary mechanism which provides an alternative route from *iso*citrate to malate. This route is nonoxidative and consists of the cleavage of *iso*citrate by *iso*citritase[6,7,8](i) and the condensation of acetyl CoA and glyoxylate by malate synthetase[9](iii). The result of this "glyoxylate bypass" (Fig. 1) is the formation of two C_4-dicarboxylic acids from *iso*citrate and acetate (iv):

*iso*citrate	$\longrightarrow$ succinate + glyoxylate	(i)
acetate + ATP + CoA	$\longrightarrow$ acetyl CoA (+ AMP + PP)	(ii)
acetyl CoA + glyoxylate	$\longrightarrow$ malate (+ CoA)	(iii)
Sum: acetate + *iso*citrate + ATP	$\longrightarrow$ malate + succinate (+ AMP + PP)	(iv)

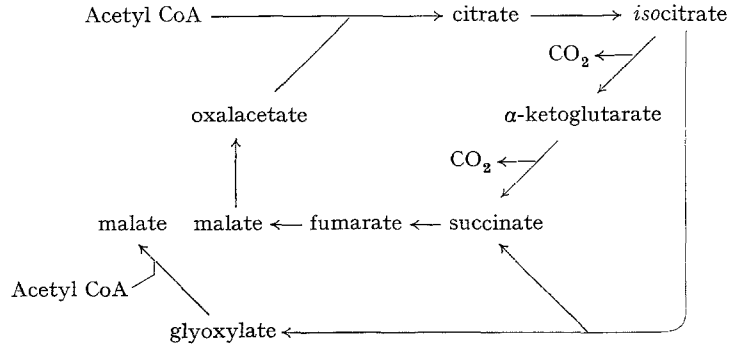

Fig. 1. Metabolic pathways in acetate-grown *Pseudomonas* KB 1: the tricarboxylic acid cycle and the "glyoxylate bypass".

If this bypass is used instead of the oxidative reactions of the tricarboxylic acid cycle, one turn of the cycle results in the net formation of one molecule of C_4-dicarboxylic acid from two molecules of acetate. A reaction of this type, the direct condensation of two molecules of acetate to form one molecule of succinate (v),

$$2 \text{ acetate} - 2H \longrightarrow \text{succinate} \qquad \text{(v)}$$

was first postulated by THUNBERG[12], but the evidence for its occurrence has been disputed. The overall effect of reaction (iv), plus the reactions of the tricarboxylic acid cycle leading to the synthesis of *iso*citrate (vi) and to the regeneration of oxalacetate (vii), is identical with that of the "Thunberg condensation", although the mechanism is entirely different:

	acetate + oxalacetate	$\longrightarrow$ citrate $\longrightarrow$ *iso*citrate	(vi)
	acetate + *iso*citrate	$\longrightarrow$ malate + succinate	(iv)
	malate $- 2H$	$\longrightarrow$ oxalacetate	(vii)
Sum:	2 acetate $- 2H$	$\longrightarrow$ succinate.	

Since both reactions (i) and (iii) seem to be widespread among micro-organisms[13], it is likely that the formation of fumaric acid from ethanol or acetate by *Rhizopus nigricans*, reported by FOSTER *et al.*[14], occurred by the "glyoxylate bypass" rather than the "Thunberg condensation". The labelling patterns observed by FOSTER *et al.*[14,15], support this conclusion.

When micro-organisms grow on two carbon compounds, such as acetate or ethanol, as the sole source of carbon, net synthesis of C_4-dicarboxylic acids must occur from the simple precursors to replace materials drained from the tricarboxylic acid cycle. These conditions apply particularly during rapid growth, when tricarboxylic acid cycle intermediates are used for the synthesis of other cell constituents, and also when incomplete oxidations occur. Examples of the latter are the accumulation of fumaric acid in *Rhizopus nigricans*[14] and of citric acid in *Aspergillus*[16,17]. The operation of the "glyoxylate bypass" would account for all these observations.

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M.R.C. Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford (England)

H. L. KORNBERG
N. B. MADSEN*

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* Post-doctoral Fellow in the Medical Sciences, National Research Council, National Academy of Sciences (U.S.A.).