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)

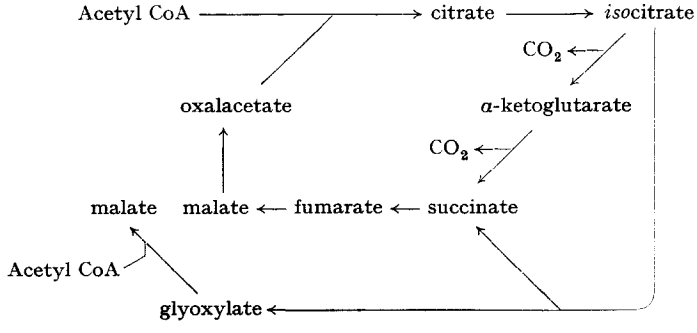

Fig. 1. Metabolic pathways in acetate-grown *Pseudomonas* KB 1: the tricarboxylic acid cycle and the "glyoxylate bypass".

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),

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

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.

We wish to thank Professor H. A. KREBS, F.R.S., for his interest and encouragement, Dr. D. E. HUGHES for his advice on microbiological procedures, and Drs. D. B. SPRINSON AND I. ZELITCH for gifts of sodium glyoxylate. This work has been supported in part by the Rockefeller Foundation, and by the Office of Scientific Research of the Air Research and Development Command, United States Air Force, through its European Office, under contract no. AF 61-(514)-1180.

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Received March 20th, 1957

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